PROPERTIES AND STRUCTURE OF PROTEIN MONOLAYERS[1]

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Many proteins, although soluble in water, may be spread on a cleaned water surface to form insoluble monomolecular films. Such films can then be studied in a number of different ways, either while they are still on the water surface or after they have been removed from the water by depositing them on a metal or glass plate.

Some of the properties, such as thickness, reversible compressibility, maximum ease of spreading at the isoelectric point, and insolubility, are quite similar for many proteins of widely different origins, molecular size, and chemical composition. On the other hand, monolayer viscosity and plasticity, anisotropy after shear or linear compression, specific reactivities, expansion patterns, contact angles, and sensitivity to the effect of pH are usually highly specific for particular proteins.

METHODS OF FORMING MONOLAYERS

Proteins may be spread as monolayers in several different ways. When the protein is dissolved in water, a surface monolayer forms spontaneously in less than a second if the solution contains about 0.1 per cent by weight of a protein such as insulin in distilled water at pH 5.8. The film thus formed has a thickness of about 8.5 Å. after 1 min., since the process of spreading at an air–water interface proceeds with enough energy to build up a pressure of 8 dynes per centimeter in 1 min., 10 dynes in 5 min., and 15 dynes in 15 min. Devaux has used this method (5) to study the concentration of a protein solution as a function of the rate of formation of the monolayer.

If the quantity of protein in solution is so small (about 0.1 mg. of protein in a tray with surface area of 200 $cm.^2$) that all of it spread on the water surface would produce five successive monolayers, then, in order to obtain

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a monolayer of the protein, it is necessary either to wait for a long period of time or to increase the rate of diffusion by thoroughly stirring (14) the substrate. While this technique is quite convenient for reaction studies, the extreme care which must be exercised to prevent surface contamination from diluting the monolayer makes it a less desirable method for general studies of protein monolayers than other methods to be described.

Gorter and Grendel (7) have shown that a dissolved protein may be spread as a monolayer by placing a micropipet in contact with a cleaned water surface and expelling about 50 $mm.^3$ of a 0.1 per cent protein solution from the pipet. If the protein is pure and the water adjusted to the isoelectric point, this amount of protein will produce a monolayer about 500 $cm.^2$ in area. We have modified this method by using a nickel sheet about 0.010 in. thick, as long as the width of the tray and as wide as its depth. The protein to be used is spread on this band from a micropipet and the band is lowered at a uniform rate through the cleaned water surface. We believe that this method is particularly efficient when the protein used is of the egg albumin type, which forms a gel-like monolayer. The pressure which is built up during formation of such a monolayer retards the rate of formation somewhat, so that it is quite possible for some of the solution to be dispersed into the substrate. When the protein solution is introduced as a uniform strip across the tray, the spreading proceeds very efficiently on the surface of the upflow of water which follows the lowering of the band.

Still another method of producing good protein monolayers is that developed by Hughes and Rideal (8), who placed a weighed amount of dried protein on the water surface. This method is limited, however, to those proteins which readily spread while in the dried form. Many proteins, such as the globulins, casein, zein, and lactalbumin, to mention a few, spread poorly or not at all when applied in this manner.

Water-soluble proteins produce thick layers, which are visible by interference colors, on the surface of a salt solution (16). A portion of this compact film can then be transferred as a B-layer on a clean nickel plate of known dimensions to another trough containing water at the isoelectric point of the protein, and a monolayer can be formed. This method should be particularly useful as a micromethod for purifying and studying proteins which are not available in quantity.

THICKNESS OF PROTEIN MONOLAYERS

Many investigators have shown (6, 19) that the thickness of a protein monolayer, as calculated from the area covered by a weighed amount of protein, is of the order of 10 Å. This calculation can only be approximate, since it assumes that all the protein used has spread to produce the monolayer.

By depositing the protein monolayer on a barium stearate step-plate and measuring its optical thickness (4) it is possible to check the calculated thickness derived from its force–area curve and thus find the efficiency of the spreading technique.

Since different values of pH affect the area of a protein monolayer when identical amounts and methods of spreading are used, changes in observed area as large as ten to one have been interpreted by some investigators as indicating that the protein monolayer becomes thicker as the area decreases. That this is not the case is easily shown by optical measurements of the thickness of deposited monolayers.

Experiments with pepsin (Northrup's crystalline) spread on water having its pH adjusted to a series of different values have given the results shown in table 1. The data of the third column, which give areas per milligram of applied protein (calculated by linear extrapolation to $F = 0$)

TABLE 1

Effect of pH on the area of a pepsin monolayer spread on a water surface

pH	THICKNESS AT $F = 16.5$ DYNES CM.$^{-1}$	AREA PER MILLIGRAM EXTRAPOLATED TO $F = 0$	AMOUNT USED	AMOUNT CALCULATED ($d = 1.3$)	RECOVERY
	Å.	*square meters*	*milligrams*	*milligrams*	*per cent*
2.0	16.	0.620	0.0416	0.0382	92
2.6	15.	0.640	0.040	0.0376	94
3.0	16.	0.630	0.0118	0.0110	93
4.2	15.	0.570	0.040	0.033	82
5.8	16.	0.095	0.034	0.007	20
7.0	15.	0.001	0.200	0.003	1

show, in accord with Gorter's findings, that the maximum spreading occurs near the isoelectric point, pH 2.6. At high pH the area becomes very small. The thickness of the monolayer, as determined after deposition on to a barium stearate plate, remains constant within the accuracy of the measurements.

The total amount of protein in the monolayer (column 5) has been calculated by multiplying the thickness of the dry deposited protein film by its density, assumed to be 1.30, and multiplying this product by the total area of the monolayer on the water. By comparing this with the amount applied to the surface (column 4) we have obtained the percentage recovery as given in the last column.

FORCE–AREA CURVES

The force–area curves for monolayers of proteins obtained by Gorter and others (6, 18, 19) have shown high compressibilities, but, in general,

attention has not been directed to the differences between particular proteins.

Using a surface balance we have determined force-area curves for several proteins as shown in figure 1. The films of insulin, pepsin, and ovalbumin were spread from 0.1 per cent aqueous solutions by our band method, while the wheat gliadin was spread from a 0.1 per cent solution in 70 per cent alcohol using Gorter's method. The pepsin monolayer was

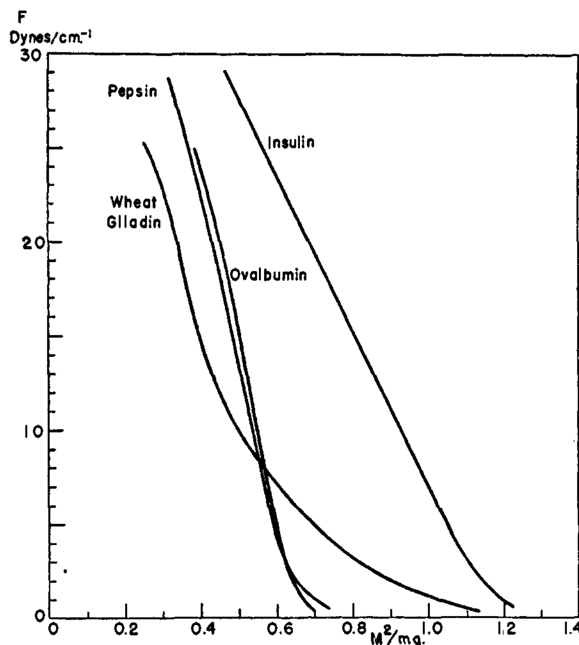

FIG. 1. Typical force-area curves obtained with protein monolayers. Abscissas calculated from measured thickness of deposited monolayers.

formed on water having its pH adjusted to 2.0 by hydrochloric acid, but the others were on distilled water, pH 5.8.

The weight of protein in each monolayer (used in calculating the abscissas in figure 1) was obtained from a measurement of the optical thickness of a pair of layers deposited at F = 16.5 dynes per centimeter (dehydrous AB-film), taking the density to be 1.30. With pepsin, ovalbumin, and insulin the weights of the monolayers found in this way were about 95 per cent of the amounts applied to the surface, but with wheat gliadin only 70 per cent was found on the surface.

The curves for pepsin and ovalbumin resemble one another closely but differ widely from those of insulin and wheat gliadin.

REVERSIBLE COMPRESSIBILITY OF PROTEIN MONOLAYERS

One of the outstanding similarities between protein monolayers spread on a water surface is the high reversible compressibility of the films. Even after being subjected to a momentary decrease in area of 80 per cent, involving a surface pressure of more than 35 dynes per centimeter, an insulin monolayer reëxpands to show a complete recovery of its original

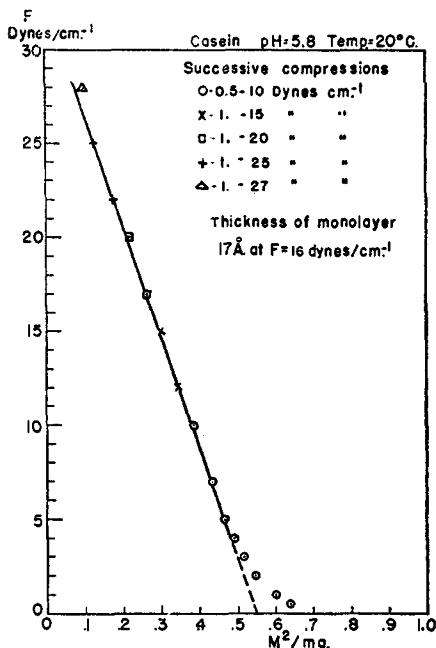

FIG. 2. Reversible compressibility of a casein monolayer. The abscissas represent areas per milligram of protein applied to the surface.

area at $F = 4$ dynes per centimeter. When a film is exposed to this high pressure for several minutes, the recovery is not complete, showing that a permanent change or collapse has occurred.

The force–area curve of a casein monolayer given in figure 2 was obtained from a series of five successive runs in each of which the surface pressure, F, was increased from 1 dyne per centimeter up to a limit indicated in figure 2. The lower portion of each of the curves obtained in this way coincided with the measurements in the previous runs. Thus within the time needed

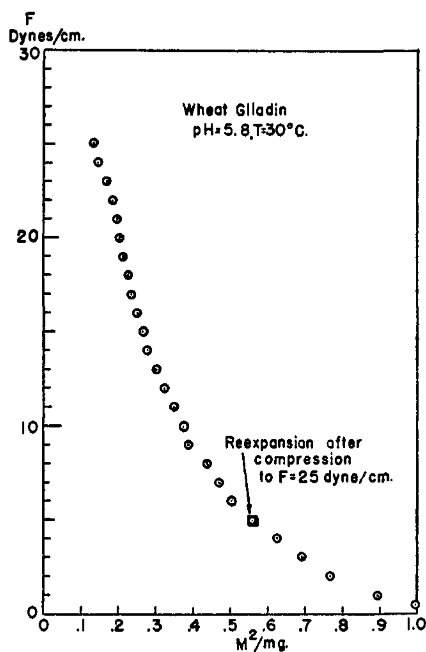

FIG. 3. Reversibility of the compression of a wheat gliadin monolayer between 5 and 25 dynes per centimeter.

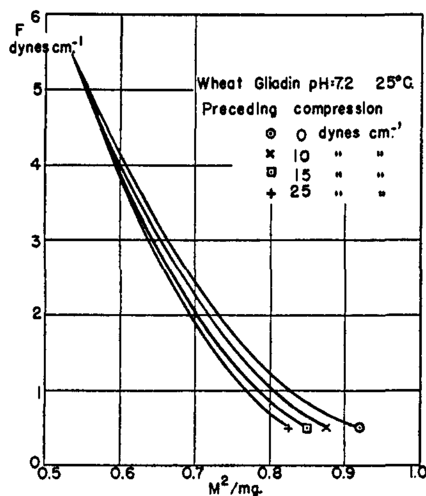

FIG. 4. Irreversible effects observed with a wheat gliadin monolayer at low surface pressures.

for these measurements there was no appreciable collapse or solution of the monolayer.

The abscissas for the curve in figure 2 are expressed in terms of the weights of protein actually applied to the surface. Optical measurements of the thickness of a monolayer deposited at $F = 16$ dynes per centimeter gave 17Å., which corresponds to 0.45 square meter per milligram, while the curve shown in the figure gives for this value of F an abscissa of 0.28. Thus to obtain a force-area curve comparable to figure 1 expressed in terms of area per milligram of protein actually present in the monolayer, all the abscissas in figure 2 should be multiplied by 1.60.

Figures 3 and 4 give the results of similar experiments with monolayers of wheat gliadin. The abscissas represent areas per milligram of applied protein. The points marked by circles in figure 3 were measured at successively increasing values of F up to 25 dynes per centimeter. Upon lowering the pressure to $F = 5$, the film expanded to the same area as was previously observed at this pressure, showing complete reversibility over this range. Some later experiments were made to test an alleged anomalous behavior of gliadin films at very low pressures (18). The force–area curves of figure 4, which represent measurements at intervals of 1 dyne per centimeter, show that the lower part of the curve is distinctly modified by previous compression of the film to pressures even as low as 10 or 15 dynes per centimeter. Since this effect is limited to the portion of the curve below 5 dynes per centimeter, this result is not incompatible with the reversibility indicated by the data of figure 3.

INSOLUBILITY OF PROTEIN MONOLAYERS

It is remarkable that, although many proteins are very soluble in water, the monolayers formed from them are extraordinarily insoluble. Thus a radical change in structure must occur during the spreading. Substances form insoluble monolayers on a water surface only if the surfaces of their molecules have both hydrophilic and hydrophobic parts (9).

Traube (23) showed that the solubilities of adsorbed films of substances containing hydrophobic groups increased by a factor of 3 upon the removal of each successive CH_2 group from the molecule. This was interpreted (9) as an effect of the decrease of surface energy that occurs when CH_2 groups occupy positions in the air–water interface.

The high solubilities in water of such proteins as ovalbumin and pepsin prove that the surfaces of the molecules of these substances contain very few hydrophobic groups. The hydrophobic groups that are responsible for the insolubility of the monolayers must therefore be buried in the interior of the globular molecule of the soluble protein.

The solubility of any adsorbed film, such as a monolayer on a water surface, must theoretically increase if the film is subjected to a surface pressure. The rate of increase can be calculated by the Gibbs equation

$$\frac{\mathrm{d}\,F}{\mathrm{d}\ln c} = \sigma k T \tag{1}$$

where c is the concentration of the dissolved substance in the solution, σ represents the number of molecules in the adsorbed film per square centimeter, T is the absolute temperature, and k is the Boltzmann constant, 1.37×10^{-16} ergs per degree.

In the case of condensed films of fatty acids for which $\sigma = 5 \times 10^{14}$ molecules per square centimeter, the solubility should thus increase about fivefold for an increase of 30 dynes per centimeter in the value of F. On the other hand, with a protein of molecular weight of 35,000, the number of molecules per unit area is only about one three-hundredth as great as for the fatty acid, so that there should be a millionfold increase in solubility for each increment of pressure of 1 dyne per centimeter (13a). According to this theory the surface pressure of 25 dynes per centimeter, which was applied to the protein monolayers in the experiments illustrated in figures 2 and 3, should have caused an increase in solubility by a factor of 10^{150}. Actually, however, the complete recovery of the area upon removal of the pressure showed that there was no measurable solubility even at these high pressures. We must therefore conclude that the formation of the monolayer by the unfolding of the globular molecules is an irreversible process to which Gibbs' equation would not apply.

On the basis of studies (10, 11, 12) of the forces that determine solubilities, volatilities, and surface tensions of liquids, it has been calculated (13a) that the presence of hydrophobic groups equivalent to about three hundred CH_2 radicals in each protein molecule would be sufficient to account for the observed insolubility of protein monolayers.

VISCOSITY OF PROTEIN MONOLAYERS

Among the measurable properties of a protein monolayer is its viscosity. This property may be expressed in qualitative terms or in absolute C.G.S. units according to the procedure followed.

A very simple method which serves to classify the monolayer into one of three general classes has been described recently (20). It involves the formation of the protein monolayer, followed by the expansion of its central region with a spreading oil which reveals the degree of cohesion by which the units in the monolayer are joined to each other. The greatest difference found among monolayers of different proteins occurs when they are

under low surface compression of the order of 0.5 to 1 dyne per centimeter. The three general patterns observed have been termed star-like, rough circular, and smooth circular. These types are illustrated in figure 5.

The star-like pattern expands to form geometrical figures, sometimes as

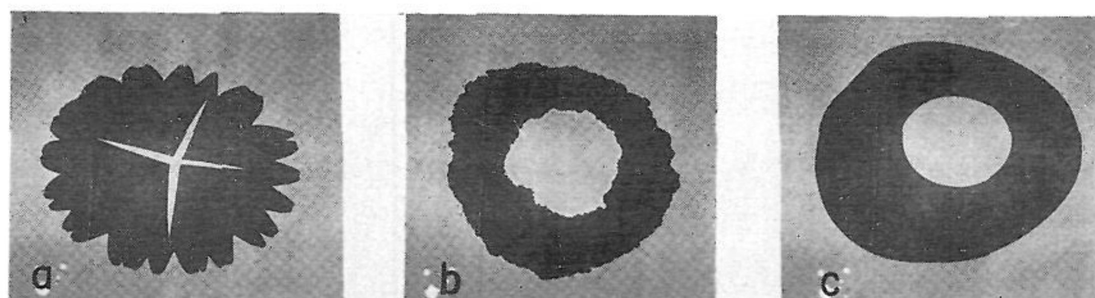

FIG. 5. General types of expansion patterns: a, star-like (egg albumin); b, rough circular (papain); c, smooth circular (insulin).

TABLE 2

Absolute viscosities of protein monolayers

$M = 131.8$; $D = 4.5$; pH $= 5.8$; $T = 25°C.$; $t =$ about 34 sec.

PROTEIN	μ_s IN GRAMS PER SECOND					
	$F = 2$ dynes	$F = 6$ dynes	$F = 10$ dynes	$F = 16.5$ dynes	$F = 19.$ dynes	$F = 29.5$ dynes
Casein	0.0036	0.010	0.015	0.4	12.	
Edestin	5.	26.	52.			
Egg albumin	0.15	0.28				
Gliadin*	0.001	0.003	0.01	0.8		54.
Gliadin acetate	0.001	0.007	0.016			
Hemoglobin	0.024	0.12	0.37			
Horse globulin	120.	210.	340.			
Insulin	0.004	0.028	0.10			
Papain	0.08	0.18	0.38			
Pepsin	0.68	0.75	1.5	150.		
Pepsinogen	0.110	0.17	0.46			
Tobacco seed globulin	0.048	0.20	0.36	140.		340.
Trypsin	0.009	0.23	0.97			
Trypsinogen	0.40	1.0	5.5			
Zein	0.003	0.003	0.003			

* At pH = 7.2.

a conventional five-pointed star but at times with points ranging in number from three to six or more. The star-like form at $F = 2$ dynes ranges in viscosity from 0.024 to 120 C.G.S. units. Proteins having this form are egg albumin, pepsin, or tobacco seed globulin.

The rough circular pattern shows a circular internal outline which upon

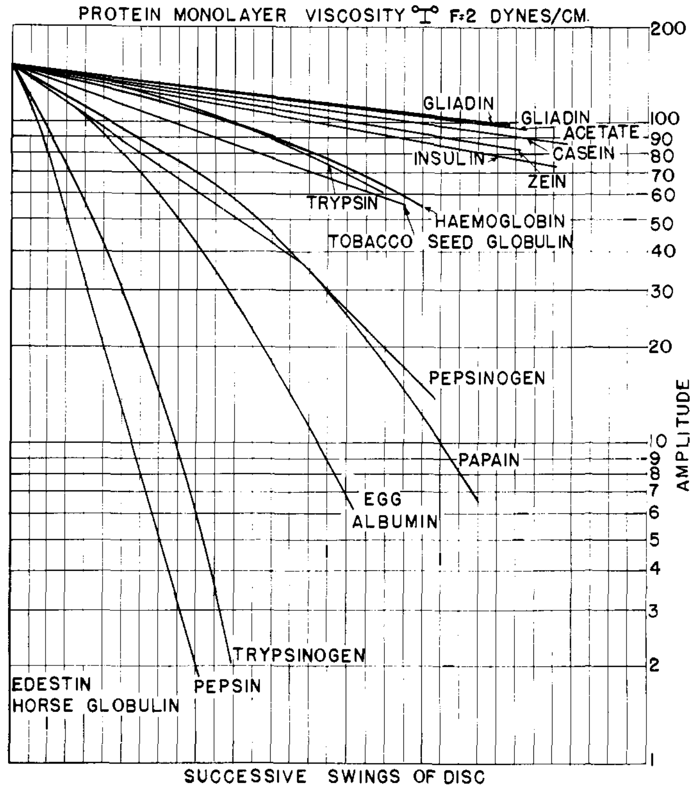

FIG. 6. Viscosities of protein monolayers. $F = 2$ dynes per centimeter. Oscillation method. pH = 5.8; $T = 22°C.$; $M = 131.8$; $D = 4.5$; t = about 34 sec.

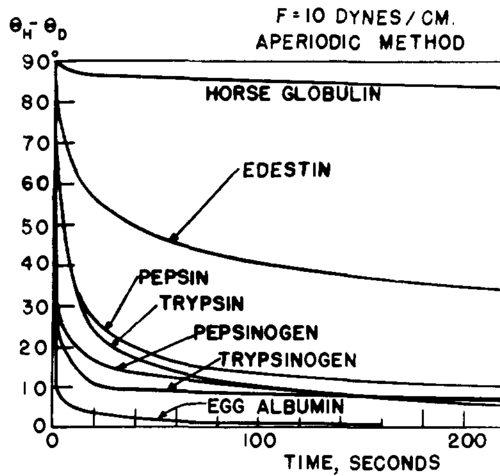

FIG. 7. Viscosities of protein monolayers. $F = 10$ dynes per centimeter. Aperiodic method. pH = 5.8; $T = 22°C.$; $D = 4.5$.

close examination discloses an irregular or roughened edge at the boundary of the oil and the protein. The viscosity range of this form is from 0.009 to 0.081 C.G.S. units at $F = 2$ dynes for those investigated. Typical proteins of this type are trypsin, papain, and wheat gliadin (when the latter is spread in its dry form).

The smooth circular type shows a circular internal pattern with the oil-protein boundary perfectly regular and smooth. Such patterns are found with insulin, casein, and zein, and denatured proteins and have a viscosity range at $F = 2$ dynes of 0.001 to 0.004 C.G.S. units.

The viscosity of monolayers of all types can be determined quantitatively by a relatively simple method previously described (14). Table 2 gives a summary of viscosities of a number of proteins measured under definite degrees of compression. These data are presented primarily to indicate the interesting ranges existing in protein monolayers. More detailed work is planned to investigate the importance of pH, temperature, method of spreading monolayers, and the effect of various modifications to the native protein which occur when it is subjected to heat, acid, ultraviolet irradiation, shaking, and other methods of "denaturation". We have shown qualitatively (20) that a large change in viscosity occurs when a protein such as pepsin is subjected to any of these agents.

In general, proteins producing monolayers having smooth and rough circular expansion patterns may be investigated by the oscillation method. This involves the measurement of the decrease in amplitude of the successive swings of a disk lying in the surface of the water in contact with the monolayer and suspended at the end of a calibrated fiber. The disk is provided with a bracket upon which is placed a rod having a known moment of inertia. The fiber is given an initial displacement by the torsion head, and the decrease in amplitude of the disk is noted. Figure 6 shows typical curves obtained by this method.

While the viscosity of a few of the proteins showing the star-like expansion pattern may be measured by the method just referred to, most of them have such high viscosities as to necessitate the use of the aperiodic method. This uses the same viscosimeter but involves the comparison of the relative movement of the suspended disk in contact with the monolayer with the initial displacement of the torsion head as a function of elapsed time. In this method the rod used to give a known moment of inertia to the system is removed. Figure 7 shows a series of curves obtained in this way at $F = 10$ dynes per centimeter.

THE VISCOSITIES OF MONOLAYERS OF LIPO-PROTEIN COMBINATIONS

Schulman and Rideal (21) have described experiments which show a very interesting interaction between a protein and a lipoid substance.

Using a mixture of cholesterol and wheat gliadin on water at pH 7.2 in a ratio of one part of cholesterol to four parts of protein, they demonstrated that at a critical pressure the protein was apparently squeezed out of the monolayer on the surface into the substrate and that certain properties of the surface layer were altered if this critical pressure were exceeded.

A wheat gliadin monolayer at low pressures is liquid, but as it is compressed it changes from a liquid to a gel-like solid. Cholesterol, on the other hand, is a liquid film under all compressions up to 30 dynes per centimeter. Schulman and Rideal showed that as the complex film of lipo-protein was compressed it resembled the protein monolayer, but at a critical pressure of about 22 dynes per centimeter the film suddenly liquefied and on further compression resembled the cholesterol film. This

TABLE 3

Absolute viscosities of protein, lipo-protein, and lipoid films

M = 131.8; D = 4.5; pH = 7.2; T = 25°C.; t = about 34 sec.

CHOLESTEROL	GLIADIN	μ_s IN GRAMS PER SECOND							
		$F = 2$	$F = 10$	$F = 16.5$	$F = 19$	$F = 29.5$	$F = 16.5$	$F = 17.5$	$F = 29.5$
parts by weight	*parts by weight*								
1	0	0.002*	0.002	0.019	0.002	0.002			
1	2	0.002	0.02	0.04	0.04	0.002			
1	4	0.001	0.001	0.76	0.16	0.05		0.49	0.04
1	8	0.002	0.002	0.36	0.06	0.02	0.36		
1	16	0.006	0.006	0.18	0.85	1.25			
0	1	0.001	0.01	0.83		54.			

Measurements of viscosities were made in successive order as shown.

* The low values of μ_s are uncertain within about ±0.005.

sequence was shown to be reversible; if the surface pressure were lowered, the liquid film showed a re-gelation at the same critical pressure.

We have repeated these experiments with rough quantitative determinations of viscosity, varying the ratio of gliadin to cholesterol over a wide range, and have thus obtained the data given in table 3.

A monolayer of pure cholesterol, which is relatively incompressible, has a low viscosity even at a high surface pressure, but the viscosity of a gliadin monolayer undergoes a very rapid increase at pressures above 10 dynes per centimeter. The viscosities of the mixed films rise to a broad maximum between 15 and 20 dynes per centimeter and fall to low values at F = 30. This effect is most marked with the 1:4 mixture, but even as little as one part of cholesterol to sixteen parts of gliadin produces a very

great decrease in viscosity at high pressures, the cholesterol apparently acting as a lubricant within the gliadin monolayer.

ANISOTROPY IN PROTEIN MONOLAYERS

When a monolayer of a protein such as egg albumin, urease, casein or edestin is compressed for a few minutes by a motion of a barrier which raises the pressure to about 35 dynes per centimeter, so that some collapse occurs, a permanent anisotropic condition is produced which can be demonstrated in several ways.

Thus if the pressure is lowered to about 20 dynes per centimeter, the monolayer can be split into parallel sheets by using a concentrated oxidized oil. These molecular strips can be lifted from the surface of the water by placing a glass rod or similar article underneath one end and slowly raising it through the water surface. The strips come off as fibers which are almost invisible. Fibers can also be removed by simply inserting the rod into a compressed film of these proteins and slowly raising it through the surface. When this is done, the fiber always comes off parallel to the barrier which has compressed the monolayer. Not all protein monolayers under compression show this property. Insulin, for example, when cracked with indicator oil breaks into small segments which will not produce fibers.

The fact that the insulin expansion pattern is reversible and shows a smooth circular pattern at $F = 2$, a star-like pattern at $F = 25$, and a smooth circular pattern again when the pressure is lowered suggests that its structure differs in important features from that of the fiber-forming proteins. Casein, for example, which is characterized by a circular pattern and a viscosity lower than that of insulin at $F = 2$, has a fibrous structure at $F = 30$. When the pressure is decreased, the expansion pattern shows that the casein now has a coherent gel-like structure, even at $F = 2$.

By cutting a portion of the spread monolayer into various shapes, such as a square, rectangle, circle, etc., we can study the effect of the presence or absence of anisotropy under compression. The fragments are made with a wire about 0.015 in. in diameter which, after being cleaned in a Bunsen flame, is coated with indicator oil. The wire is lowered through the monolayer, and the compression is decreased. As the oil spreads, the monolayer is sharply outlined. Any inequality produced by the initial compression is immediately indicated by a distortion of the original configuration. These experiments are best performed with a suitably illuminated trough having a black bottom (20).

A protein monolayer on water when compressed equally in all directions by an indicator oil or piston oil shrinks uniformly toward its geometrical center and is isotropic. Thus, the piston oil applied at one end of the monolayer is found not only at this end but also along both sides of the

tray. Because of this phenomenon, we make a practice of applying compression by placing the piston or indicator oil in one corner of the tray. This produces a uniformly compressed, rectangular monolayer having oil on only two sides.

INDICATOR AND PISTON OILS USED FOR STUDYING MONOLAYERS

We have often referred to indicator and piston oils as convenient tools in the study of monolayers. Dr. K. B. Blodgett (1) has described the method for preparing and using highly oxidized oil. For most experiments it is quite practical to use old automobile crank-case oil. Fresh oil is not satisfactory. The sludge in the oil may be removed by filtering or settling. The oxidized oil when diluted with a non-spreading oil such as Petrolatum or Nujol will produce stable films of uniform thickness, which in certain mixtures will show interference colors.

A sample of used oil was placed on a cleaned water surface and spread at $F = 0$ to produce a film which, viewed at approximately 45°, showed a faint silver-grey color. The addition of an equal amount of Petrolatum produced a film having a faint yellow color of the first order. When two parts of Petrolatum to one of oxidized oil were blended, the resulting color was a dark yellow of the first order; three parts of Petrolatum and one of oxidized oil produced a first-order blue. Four parts of Petrolatum to one of oxidized oil resulted in a yellow again, and with a five-to-one ratio red was produced, these colors being of the second order.

When an indicator oil, which will spread on clean water if unrestricted to give a dark yellow, first-order color, is limited in area, the color produced by the increase in thickness will be related to the decrease in area. In order to decrease the area, it is necessary to exert a definite amount of pressure on the oil film. If a trough equipped with a surface balance is used, it is quite easy to calibrate a given oil sample in terms of color observed for pressure exerted on the floating barrier. This has been done with various samples of oils which we use to illustrate the possible ranges of pressure. These are given in table 4. Although such oils are fairly stable if protected from light, they should be checked occasionally for changes which might occur.

With a series of oils such as those indicated in table 4, we have a very convenient and simple method for producing compressions up to 30 dynes per centimeter upon a monolayer spread on water.

To complete the series a few piston oils, such as those given in table 5, may be used. These are purified by shaking a good grade of commercial oil with activated fuller's earth or similar substance at about 100°C. The oils may then be clarified by centrifuging or allowing the fuller's earth to settle. These oils have the property of spreading as liquid monolayers,

the excess oil forming lenses which serve as reservoirs and produce a constant, known pressure upon anything floating on the water surface.

Indicator oil used in conjunction with piston oil can be used in many ways other than those suggested.

TABLE 4

The relation of the color of oxidized oil to the pressure exerted against a monolayer on water

ORDER	COLOR $i = 45°$	F IN DYNES PER CENTIMETER						
		Oxidized oil	Oxidized lubricating oil diluted with petrolatum					Crank-case oil
1	Invisible	0–5						
1	Silver-grey	11.	0.					2–5
1	Light yellow	14.	3.					7.
1	Dark yellow	15.	5.	0.				8.5
1	Red-brown	15.5	7.	2.5				9.5
1	Blue	16.0	8.	4.	0.			10.5
1	Blue-green		9.	5.	2.	0.		11.2
2	Yellow	17.0	9.5	6.	3.5		0.	11.8
2	Red	18.	10.5	7.	5.	2.	1.	12.5
2	Blue	18.		8.	6.	3.	2.	13.0
2	Green	18.	11.	8.2	6.5	3.3	3.0	13.5
3	Red	18.	12.5	9.5	7.	5.	4.5	14.5
3	Green		13.5	10.0	8.5	6.0	5.2	15.0
4	Red		13.7	10.5	9.0	7.0		15.5
4	Green			11.2	9.5			16.
	Visible lens	20–30	20–30	15.	13.5	12.	12.	20–30

TABLE 5

Force exerted by piston oils spread on a water surface

PISTON OIL	F IN DYNES PER CENTIMETER
Tricresyl phosphate	9.5
Rape seed oil	10.5
Castor oil	16.5
Neatsfoot oil	19.0
Oleic acid	29.5

DEPOSITION OF MONOLAYERS

In determining the thickness of protein monolayers several precautions must be observed. Since most proteins undergoing compression develop considerable rigidity, the monolayer does not flow at a uniform rate toward the plate upon which it is to be deposited. This difficulty can be overcome by using a plate of the same width as the monolayer. Since it is not

convenient to produce a step-plate of this size, we use chromium-plated steel plates of two sizes. One plate is made as wide as the tray (allowing 1-mm. clearance on either side), and the other plate is of a convenient size (5 cm. x 2 cm. x 1 mm.). The former is held by a small permanent magnet of sufficient strength to cause the prepared plate to adhere firmly to the side of the larger plate. Glass or a non-magnetic metal could also be used for the larger plate.

We prefer to produce the initial compression by using a calibrated indicator oil. This spreads and outlines the boundary of the protein monolayer; it also prevents the invisible piston oil from contaminating the monolayer. The indicator oil is then compressed to a force approaching that of the piston oil to be used. The piston oil is added and drives the protein monolayer on to the prepared plate (2, 3, 4), where it is deposited as a hydrous AB-film (17). When monolayers are to be deposited at pressures higher than $F = 16$ dynes, concentrated oxidized lubricating oil may be used as the indicator oil. This, no matter how highly compressed, can be seen either as a colored film or as a line of visible oil.

A monolayer in contact with a film spread from a specific sample of calibrated indicator oil will be under a definite compression directly related to the color of the oil film (see table 4). If the color is held constant, the monolayer may be deposited as a hydrous AB-film. To do this, the synchronous movement of the surface barrier compressing the film and the dipping plate is necessary.

A monolayer may also be deposited by lowering the prepared plate face downward upon the top surface of the protein monolayer. A film so deposited has been termed a lifted film, symbolized by A_L. Best results are obtained if one corner of the prepared plate, held almost parallel to and about 1 mm. from the film surface, is touched to the monolayer. In this way the formation of an air bubble between the prepared plate and the monolayer is prevented. After the monolayer is deposited, the surrounding film is scraped away, and the face of the plate, now bearing the deposited A-layer, is raised through the cleaned surface. Care must be exercised that the edges of the plate are free from surface-active materials. This is easily checked by noting any change in color of the indicator oil before and after the film is deposited. The deposition of an A_L-film is particularly applicable to the deposition of films under low compression, since no surface movement of the monolayer is necessary.

THE SURFACES OF PROTEIN MONOLAYERS

In a previous paper (17) data were given which showed certain relationships found when successive protein monolayers were deposited on a prepared plate. Using our improved optical method of measuring incre-

ments of thickness, we have repeated these experiments with monolayers of insulin (Lilly).

PRAAA-layers were deposited by spreading a monolayer on the water surface, applying pressure, and then lowering a prepared step-plate bearing a multilayer of barium stearate into the water. The protein monolayer was deposited on the down trip. Before the plate bearing the deposited monolayer was withdrawn through the cleaned water surface, the remaining film on the water was scraped off. This process was repeated for each succeeding layer added.

Successive PRBBB-layers are deposited by a reversal of the above-described process. The plate is lowered through a cleaned water surface, a protein monolayer spread on the surface, compressed, and deposited as the plate is withdrawn from the water. PRABAB-layers are deposited in a manner similar to that used in building ordinary barium stearate multilayers. However, the water between the two layers must be allowed to evaporate before the next pair is deposited, otherwise the B-layer returns to the water surface.

We first thought it necessary to condition the monolayer with divalent salts in order to prevent the return of the B-layer to the water as successive AB-layers were deposited. We have since found that protein monolayers can be built without this conditioning step by increasing the speed of the down trip to about 30 cm. per second.

The observed thicknesses show that with a surface pressure of $F = 16.5$ dynes per centimeter the PRABAB-layers increased 22 Å. each trip, an average increment of 11 Å. per layer. The PRAA combination showed an increase of 10 Å. per layer for the first three trips, with no further increase thereafter. Each layer of the PRBB combination gained 10 Å. for each trip made; there appeared no limit in this case to the number which could be deposited.

When a multilayer of insulin consisting of successive dehydrous AB-layers is slowly lowered into the water, the outer B-layer returns to the water surface. If this layer is scraped off, the plate withdrawn and dried, the multilayer shows the loss of a single layer. If this process is repeated the second time, the multilayer when dried shows the loss of two additional layers. This may be continued until all the pairs of BA-layers are stripped off successively down to the PRA protein monolayer. This layer cannot be removed in the manner described. Each of these BA-layers consists of two monolayers which are held together by contact between their hydrophobic surfaces. On the other hand a pair of monolayers that form an AB-layer are joined by their hydrophilic surfaces. Apparently the penetration of water between the hydrophilic surfaces within the AB-layers accounts for the manner in which the multilayer was stripped from

the plate as BA-layers and not in the AB combination in which they were applied.

"Breath figures" produced when moist air is brought into contact with a slightly chilled slide bearing deposited monolayers show certain striking differences between protein layers deposited in various ways.

If we breathe upon a chilled chromium-plated surface, which has previously been polished with Shamva and is therefore hydrophilic, we find that moisture condenses as a fine fog-like deposit consisting of minute drops which scatter light strongly but give no interference colors. A quite similar fog-like deposit is produced when one breathes upon deposited films of insulin of the following types: PRA, PRB, PRAAA, and PRABABA. It appears, therefore, that these films act as if the outside surface were hydrophilic.

From the orientation of the hydrophobic and hydrophilic groups in the protein monolayer on the water surface before deposition, one would expect an A-layer to be exotropic (13), that is, it should have its hydrophilic groups turned outward (away from the plate). Similar considerations would indicate that B-layers should be endotropic, having their hydrophilic groups turned inwards (toward the plate).

The fact that the PRB-films are found to act as though they have hydrophilic surfaces is presumably accounted for (13) by the overturning of the layer which occurs because the underlying hydrophobic R-layer tends to anchor the hydrophobic parts of the B-layer and so makes it easy for the hydrophilic parts to come into contact with water when this is condensed on the surface.

Films of the type PRABAB and PRBBB, when breathed upon, give an entirely different kind of breath figure. At first sight there appears to be no breath figure at all, since there is no fog-like deposit which scatters light. Closer examination, however, shows that the surface gives bright interference colors which indicate the presence of a uniform film of water of thickness even greater than the wave length of light. Evidently the water condenses as a thick film under the B-layer that forms the surface of the film, the phenomenon being very similar to that observed when the outside B-layer is stripped off of a PRABAB-film by dipping this slowly into water. The formation of these thick uniform condensates is therefore not incompatible with an endotropic orientation of the B-layer.

These experiments seem to indicate definitely that B-layers and A-layers normally retain the dorsiventral character of the protein monolayer on water, so that the outer surface of an A-layer tends to be hydrophilic while that of a B-layer is hydrophobic. In the case of the PRB-films,

however, the phenomenon is complicated by the overturning which may occur if the films are not suitably anchored by the underlying layers.

REACTIVITY OF PROTEIN MONOLAYERS

A previous paper (15) has discussed the reactivity of certain proteins when spread as monolayers. We concluded that in the case of pepsin the process of spreading the protein as a monolayer and depositing it on a plate did not seriously interfere with its ability to clot milk. We found, however, that the monolayer left the plate, being removed presumably by something in the milk, since washing with distilled water or a buffered solution failed to remove it. A resynthesis of the pepsin, as the protein leaves the slide, is suggested as having occurred.

TABLE 6

Activities of urease monolayers

T = 25°C.; citrate buffer; pH = 6.5

BASE	TYPE	F	THICKNESS	UREASE UNITS† PER GRAM	UREASE UNITS PER SQUARE CENTIMETER	UREASE UNITS PER GRAM IN SOLUTION
		dynes	Å.			
PR	A_L	1.	16	11,100	0.0023	0
PR	A_L	10.5	17	9,100	0.0020	800
PR	A_L	16.5	31	2,400	0.0010	0
PR	A_L	29.5	49	2,700	0.0016	
PR	A_L	1.	22	10,100	0.0023	
PR	A_L	1.	21	7,330	0.0016	800
PRC (thorium desoxycholate)	A_L	1.	18	5,200	0.0013	0
PRC (thorium desoxycholate)	A_L	29	21*	16,800	0.0041	0
(same film, second dip)	A_L	29		16,800	0.0041	16,800
PRC (ThO_2)	S		15	2,000	0.0004	

* This observed thickness is 28 Å. less than that observed on a PR barium stearate film.

† A urease unit is defined as that amount of urease which will form from urea phosphate 1 mg. of nitrogen at 20°C. and at pH 7 in 5 min.

Nitrogen was determined with Nessler's reagent.

A few preliminary qualitative results were also announced concerning the enzyme urease. Further quantitative work (13a) has indicated that although the monolayer shows the ability to convert urea into ammonia it has an efficiency in urease units (22) of only 5 per cent when compared to that of a similar amount added in bulk to a urea solution. Examination

of the monolayer shows that considerably more light is scattered from the surface of a deposited urease monolayer than from one of insulin or pepsin applied under the same conditions. Thus it is possible that the observed activity is due primarily to entrapped globular molecules of the urease which have not been broken down into a monolayer. This possibility is further strengthened by the fact that a monolayer left on water for 15 min. (a period which should produce greater spreading if there were entrapped unspread globular molecules) shows less than 2 per cent activity. Table 6 contains some of the quantitative data obtained.

It will be noted that as a result of the quantitative measurements which we have made with the urease monolayers our previous conclusions must be modified, particularly those concerning the effect of conditioned surfaces on the activity of urease. These data indicate that the monolayer is less firmly held by the thorium desoxycholate surface than by the barium stearate surface and therefore becomes detached from the slide and diffuses into the urea solution. This is shown by the observed decrease in thickness of the monolayer as well as by the continuation of urea conversion after the monolayer is removed. Our previous technique would not have disclosed this condition.

Further work is planned with particular attention to the amount of light scattered by single monolayers and the loss of activity with aging of the monolayer on the water surface.

When a monolayer of catalase is spread on a water surface, transferred to a plate and immersed into a solution of hydrogen peroxide, oxygen accumulates on the surface of the monolayer as visible bubbles of gas 1 mm. or more in diameter. When the slide is withdrawn from the solution, both the bubbles and the catalase monolayer beneath each bubble disappear.

A monolayer of catalase deposited as an A_L-layer at $F = 1$ dyne per centimeter was quite hydrophilic and after a thorough washing was found to be about 23 Å. thick. After rewetting, by dipping through a cleaned water surface, the plate bearing the monolayer was dipped into a 3 per cent hydrogen peroxide solution. After the bubbles of oxygen had become about 1 mm. in diameter the plate was agitated to remove the bubbles and permit new ones to form. After this was done four times the bubbles stopped forming. The plate after removal from the solution was found to be relatively hydrophobic, with the bubble locations visible as regions of decreased thickness.

When a catalase monolayer was deposited on top of a barium stearate step-plate conditioned with thorium desoxycholate and treated in the manner just described, an interesting difference was observed. As the bubbles started forming, they left the surface of the monolayer before reaching

one-quarter the size of those formed on a catalase monolayer deposited on barium stearate. As far as could be determined, the total activity of the film before the evolution of oxygen stopped (a period of about 15 min.) was of the same order of magnitude,—roughly 700 cc. of oxygen per milligram or 0.2 cc. of oxygen per square centimeter of monolayer.

Further work is planned to determine whether in the case of catalase as with urease the activity of the monolayer is possibly due to unspread molecules enmeshed in the fabric of the spread monolayer.

STRUCTURE OF PROTEIN MONOLAYERS

The solubility and other properties of the globular proteins, together with the insolubility and irreversible formation of the monolayers, indicate that the globular proteins have a symmetrical, highly organized cage-like structure which breaks down or unfolds during this spreading process, permitting the hydrophobic groups to come into contact with the air–water interface.

A study of the properties of several long-chain polymers, such as methyl methacrylate and polyvinyl acetate, has shown that the force–area curves, the expansion patterns, and the viscosities of these monolayers resemble closely those given by proteins. The monolayers of some of these polymers when compressed become anisotropic and yield fibers much like those observed with egg albumin.

Protein monolayers thus consist mainly of polypeptide chains which are anchored to the air–water interface at intervals along their length by hydrophobic groups contained in the side chains (13a). The structure is like that of a net made to float on the surface of water by corks distributed over the surface of the net. The hydrophilic parts of the polypeptide chains remain in contact with and are surrounded by water but are not free to go far from the surface because they form parts of chains attached to the surface. The hydrophobic groups by their Brownian movement tend to distribute themselves over the surface like the molecules of a two-dimensional gas, but this expansion is constrained by the elastic properties of the long chains which result from the flexibility of the chain (17a).

The marked insolubility of the protein monolayers even under high compression results from the great length of the polypeptide chains in the monolayers. The incomplete reversibility shown in the force–area curve of a wheat gliadin monolayer at low compressions, figure 4, may be explained by the presence of a small proportion of relatively short-chain degradation products produced during the spreading of the protein, which are driven into solution by subjecting the film to a high pressure. We plan to study this phenomenon with films given by more strongly denatured proteins.

The dorsiventral properties of protein monolayers shown in our studies of deposited films indicate that the deposited monolayers resemble a fabric. Cross-linkages which give to the monolayer this fabric-like character may be due to the adherence between hydrophobic groups or may involve the cyclol bonds which have been postulated in the structure of the globular protein (25).

Quantitative studies of viscosities, compressibilities, elasticities, etc. of protein monolayers should help greatly to throw more light not only on the structure of monolayers themselves but also on that of the globular proteins from which they are derived.

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