Some Remarks on the Spreading of Protein Monolayers (Dynamic Studies on Monolayer. I)

By Maresuke KASHIWAGI

(Received October 16, 1957)

The monolayer of low molecular compounds such as fatty acids has been extensively studied[1]. These molecules dissolve often in benzene and usually they can be spread from the benzene solution, leaving a stable monolayer in the air-water interface after the evaporation of the solvent. In such a case, the spreading process itself does not present any difficulty and much more important are the physico-chemical investigations on the spread monolayer.

Proteins, on the other hand, are quite different from these compounds in respect to surface chemical behavior owing to their unique mloecular structure. Water-soluble protein can not be expected to spread on water substrate. In fact, most proteins fail to spread on pure water.

1) W. D. Harkins, "The Physical Chemistry of Surface Films", Reinhold, New York (1954).

Thus the spreading process becomes important and this spreading is known to be dependent largely on sub strate and other experimental conditions. Many pioneer chemists have emphasized the importance of those experimental conditions since those are rather fundamental for the protein monolayer. Certain proteins may spread immediately on some good substrate, but may take a considerable time to reach equilibrium state on the other substrate. This time effect was attributed mainly to the unfolding of a large complex structure of protein, by Bull and Neurath[2], and also by Jonxis[3].

The author also demonstrated[4] this effect, using one of the largest proteins, myosin. A theoretical equation was derived which was found to fit the experiments quite well. Attempts were made to examine other types of protein (i. e., relatively low molecular weight) in this respect. The spreading of ovalbumin is found to be complete within the first 5 minutes on 20% ammonium sulfate substrate at its isoelectric point and the spreading was not disturbed by different rates of compression. But a partial collapse is observed on 0.1 N hydrochloric acid substrate. Hemoglobin appears to take considerable time to come to equilibrium on account of physical or chemical changes in molecular structure. These results will be discussed on the basis of protein structure in the surface film.

Experimental

Apparatus.—The surface balance used for the present investigation is a vertical plate type of the size $60\times15\times2$ cm. The sensitivity is 0.02 dyn./cm. The whole apparatus is installed in a glass cabinet to avoid accidental contamination during the measurement. The use of such an apparatus is made possible by the courtesy of Professor T. Tachibana of Ochanomizu Women's University. All normal precautions, namely those described by Adam[5] were taken.

Protein.—Ovalbumin is extracted from fresh egg by the usual method[6] and recrystallized three times from saturated sodium sulfate solution. Bovine serum Hemoglobin is purchased from Pentex Corp., Kankakee, Illinois and used without further purification.

To examine the extent of spreading of protein monolayer, surface pressure change is observed with time and the constant value of surface pressure is considered to be that at equilibrium. The measurement is carried out at room temperature, but the temperature remained practically constant during each time of operation.

Experimental procedure.—Protein is spread in the air-water interface from its aqueous solution and the concentration is determined by drying a solution to constant weight. General spreading conditions are as follows: a known amount of protein solution is spread by means of a calibrated micropipet over a very large area (2.5 m^2/mg) and the film is kept there for exactly 5 minutes before the start of compression. The moving barrier is compressed gently to the desired area. The measurement is begun immediately when the barrier is adjusted to the area.

Theoretical Consideration

When a stable monolayer formation is not completed, the film is still in an unstable state. The system therefore will endeavor to reach lower free energy state and such a transitional process may involve change in surface pressure at a constant area, if the area is smaller than that in the gaseous state. Absence of such phenomena would indicate that the monolayer is at equilibrium under that condition. This equilibrium will be achieved quickly only on a good substrate. These structural changes in the film may be grouped, excluding surface interaction phenomena, as follows;

a) *Surface pressure increase with time* ($\Delta F>0$): This increase would mean that a configurational change of molecules in the film is occurring to reach a state of lower free energy. It may include, in a special case, dissociation of molecules.

b) *Surface pressure decrease with time* ($\Delta F<0$): Owing to a strong hydrophilic attraction between protein and water, some molecules in the film are dissolving into the water underneath them. Or else, some proteins are aggregating in the film.

c) *Constant surface pressure* ($\Delta F=0$): This means that all molecules in the film are in a state of the lowest free energy under that experimental condition. The film is thermodynamically in a stable equilibrium.

Results

Table I is a summary of measured values of surface pressure change ΔF as a function of time t at various areas under an identical condition.

2) H. B. Bull and H. Neurath, *Chem. Revs.*, **23**, 391 (1938); *J. Biol. Chem.*, **118**, 163 (1937).
3) J. H. P. Jonxis, *Biochem. J.*, **33**, (1943).
4) K. Kashiwagi and B. Rabinowitch, *J. Phys. Chem.*, **59**, 498 (1955).
5) N. K. Adam, "The Physics and Chemistry of Surfaces", 3rd ed. p. 27-31, Oxford Univ. Press, London (1944).
6) R. A. Kekwick and R. K. Cannon, *Biochem.*, **30**, 227 (1936).

TABLE I

SURFACE PRESSURE CHANGE OF OVALBUMIN AT CONSTANT AREA

Time Allowed For After Spreading, 5 min.
Substrate is 20% Ammonium Sulfate at pH 4.8.

Const. Area $m^2/mg.$	Temp. °C	Rate of compression cm./min.	ΔF dyn./cm.				
			1 min.	5	15	30	60
0.75	26	15	0	−0.12	−0.12	−0.12	0
0.76	26	100	0	0	0	0	0
0.81	26	12	0	0	0	0	0
0.82	26	90	0	0	0	0	0
0.85	26	80	0	0	0	0.12	0.12
0.90	28	76	0	0	0	0.12	0.12
0.99	30	92	0	0	0	0	−0.12
1.10	26	12	0	0	0	0	0

It is clear that surface pressure is constant over the whole period of experiment and that it is independent of the rate of compression. These facts can be taken as a direct indication that albumin spreading is complete within the first 5 minutes. Concentrated ammonium sulfate solution at isoelectric point is therefore a good substrate. Some proteins are known to spread on strong acid substrate. Whether this is true or not may be tested in the same way.

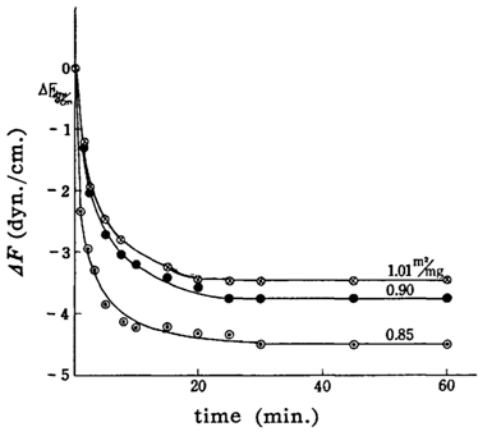

Fig. 1. Surface pressure change of ovalbumin (substrate 0.1 N HCl, 31°C).

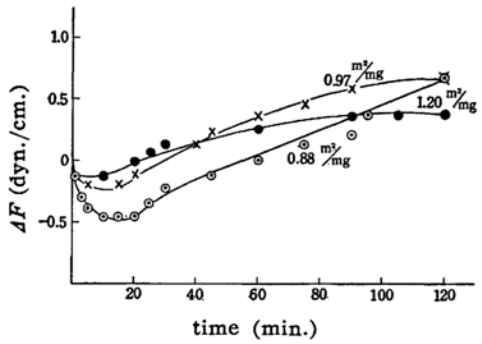

Fig. 2. Surface pressure change of Hemoglobin (substrate 20 % $(NH_4)_2SO_4$, pH 4.2, temp. 31°C).

Fig. 1 shows surface pressure change of ovalbumin on 0.1 N hydrochloric acid at various areas. ΔF decreases with time, in all cases, on 0.1 N hydrochloric acid. It reaches, however, a final pressure after a certain lapse of time. Fig. 2 shows ΔF–t curves of Hemoglobin on 20 % ammonium sulfate at pH 4.2. ΔF decreases with time in the initial stage and, after reaching a definite minimum, increases again. If the main cause for pressure change is attributed to molecular movements in very limited space in the film, those time effects and the values of final pressure will be related to the compressibility, which is expressed as $-I\,dA/A\,dF$.

One convenient way of calculation may be an adoptation of the following approximation.

$$-\frac{I}{A}\frac{dA}{dF} \doteqdot -\frac{I}{A}\frac{\Delta A}{\Delta F} \doteqdot \frac{I}{\frac{A_1+A_2}{2}}\frac{A_2-A_1}{F_1-F_2}, \quad (1)$$

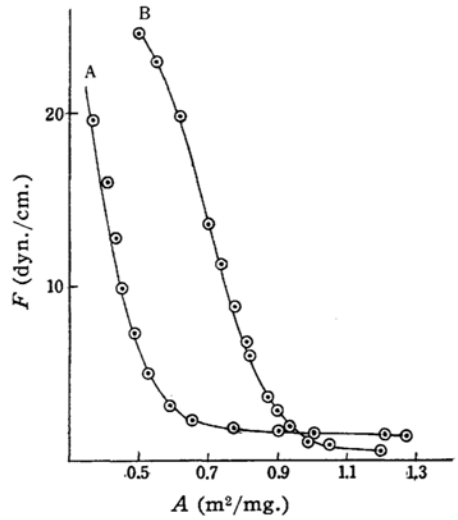

Fig. 3. Surface pressure of protein (time for spreading 20 min.).
A Albumin on 0.1 N HCl, 22°C
B Hemoglobin on 20 % $(NH_4)_2SO_4$, 31°C

where subscripts 1 and 2 refer to specified points close to each other on F–A curve. The F–A curves of albumin on 0.1 N hydrochloric acid and Hemoglobin on 20% ammonium sulfate are shown in Fig. 3.

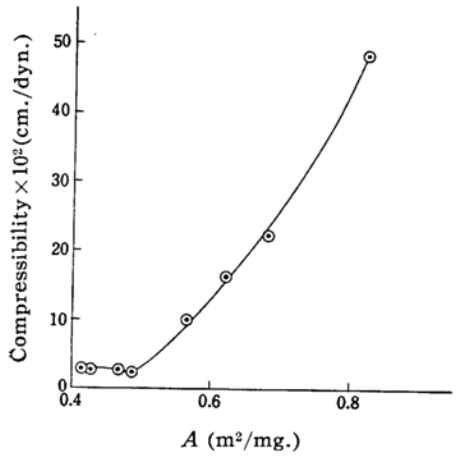

Fig. 4A. Compressibility of Albumin.

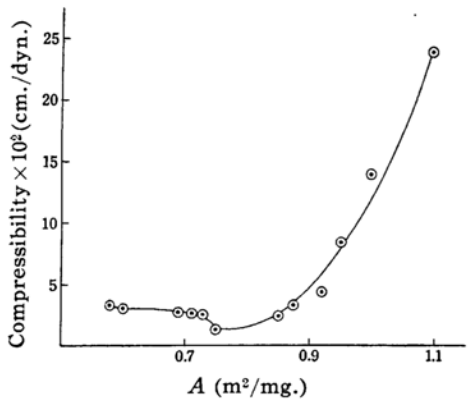

Fig. 4B. Compressibility of Hemoglobin.

Calculated values of compressibility are indicated in Fig. 4. In the region of low compressibility partially unfolded protein will be pushed downward more strongly by the surrounding molecules or will take more time to reach equilibrium. Both surface pressure change and the time needed for complete spreading are thus connected with compressibility.

Discussion

Neurath[7] considered that the unfolding of the protein molecule is a main cause for the time dependence of spreading. In fact, it will readily be thought that polypeptide chains in protein would need considerable time to orient and unfold themselves in the very viscous surface film into positions which yield minimum free energy. The author finds close analogy between behaviors of protein in bulk and in surface film. There seem to be plentiful evidences to prove that protein can take different configurations under different conditions[8,9].

According to Kauzmann et al.[10], the denaturation of protein in bulk may be illustrated as follows (Fig. 5). Stage 1

Fig. 5. Possible stages in the denaturation of a protein according to Kauzmann.

represents the initial unfolding reaction caused by rupture of many weak linkages and stage 2 the subsequent loop opening caused by rupture of relatively strong few intramolecular cross links. These ideas lead Kashiwagi and Rabinowitch[3] to the following expression

$$\frac{I}{(F_e+F^0)^2}\ln\frac{(F_e-F_0)(F+F^0)}{(F_e-F)(F_0+F^0)}+\frac{I}{(F_e+F^0)}\left\{\frac{I}{(F_0+F^0)}-\frac{I}{(F+F^0)}\right\}=kt/c, \qquad (2)$$

where F^0, a parameter, F_0, the surface pressure at zero time, F_e, the final equilibrium pressure, and F, the measured surface pressure at time t.

The derivation of this equation involves one assumption that the rate of increase in area occupied by the unfolding molecules at time t is proportional to the pressure under which the film finds itself, that is,

$$dA_0/dt = k(F_e-F) \qquad (3)$$

Although equation (2) fits myosin very well[4], it appears to fail for albumin. The reason for this seems clear, because on 20% ammonium sulfate at pH 4.8 spreading was complete in the first 5 minutes. There should be no increase in surface pressure after 5 minutes. Decrease in surface pressure on 0.1 N hydrochloric acid may indicate that some molecules

7) H. Neurath, *J. Phys. Chem.*, **40**, 361 (1936).

8) J. T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**. 761 (1957).

9) A. Elliott and B. R. Malcolm, *Biochim. et Biophys. Acta*, **21**, 466 (1956).

10) H. K. Frensdorff, M. T. Watson and W. Kauzmann, *J. Am. Chem., Soc.*, **75**, 5157 (1953).

which failed to unfold completely at the time of delivery tend to dissolve slowly into substrate. A once-unfolded molecule in low free-energy state will not dissolve again. These become evident when consideration is given to the difference in molecular structure as shown in Table II.

TABLE II
DIFFERENCE IN MOLECULAR STRUCTURE

	Myosin[11]	Albumin
Mol. Wt.	1,000,000	45,000
Solubility	insoluble	very soluble
Shape	flexible rod-like	globular

Concerning the complicated surface behavior of Hemoglobin, it is difficult to distinguish whether the pressure rise after the initial decrease with time is due to dissociation of molecule and/or chemisorption of oxygen and carbon dioxide from the air.

The author wishes to express deep thanks to Professor T. Tachibana without whom this research would not have been possible. Thanks are also due to Miss E. Hirano and Miss N. Hayashi for their assistance.

Mitsuwa Chemical Laboratory
Marumiya Co. Ltd.
Terajima-machi, Sumida-ku, Tokyo

11) A. Szent-Ggörgyi, "Chemistry of Muscular Contraction," Academic Press, New York (1951).