lization very rapidly at 60° and decomposed at 110°. Optical-crystallographic properties are given in Table I.

Anal. Calcd. for $ZnHAcon \cdot 4H_2O$: Zn, 21.14; H_3Acon, 56.2; neutralization equivalent, 309.5. Found: Zn, 21.13, 21.10; H_3Acon isolated, 55.1; neutralization equivalent, 324.

Tricadmium Aconitate Hexahydrate, $Cd_3Acon_2 \cdot 6H_2O$.[7]—This salt, described by Guinochet,[7] was prepared by double decomposition from hot solutions of sodium aconitate and cadmium chloride or acetate. The optical-crystallographic properties are given in Table I.

Anal. Calcd. for $Cd_3Acon_2 \cdot 6H_2O$: Cd, 42.72; H_3Acon, 44.2. Found: Cd, 42.43, 42.63; H_3Acon isolated, 43.9, 41.8.

Monopotassium Aconitate, KH_2Acon.[7]—The optical-crystallographic properties of this salt, described by Guinochet,[7] are given in Table I.

Anal. Calcd. for KH_2Acon: K, 18.43; H_3Acon, 82.1; neutralization equivalent, 106.1. Found: K, 18.23, 18.22; H_3Acon isolated, 81.3; neutralization equivalent, 106.3, 106.4.

Acknowedgment.—Thanks are due to A. L. Curl, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture, for his aid in making the isolations and determinations of aconitic acid.

Summary

1. The insoluble aconitates which separate from sugar cane and sorgo sirups have been identified as calcium magnesium aconitates having the optical-crystallographic properties of dicalcium magnesium aconitate hexahydrate, although they generally contain less than the theoretical proportion of magnesium. It is suggested that they are solid solutions of tricalcium aconitate hexahydrate with either trimagnesium aconitate or dicalcium magnesium aconitate hexahydrate.

2. The preparation and properties of crystalline tricalcium aconitate hexahydrate, tricalcium aconitate trihydrate, calcium sodium aconitate dihydrate, dicalcium magnesium aconitate hexahydrate, magnesium acid aconitate tetrahydrate, and zinc acid aconitate tetrahydrate are described.

3. Optical-crystallographic properties and indices of refraction are given for the above salts and for tricadmium aconitate hexahydrate and monopotassium aconitate.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

Monolayers of Egg Albumin on Concentrated Salt Solutions[1]

BY HENRY B. BULL

An examination of the literature[2,3,4,5] reveals a disconcerting lack of agreement between the results of various workers on the force area curves of spread films of such well defined proteins as egg albumin. To anyone who has done experiments on spread films of proteins this lack of agreement is not surprising. It is by no means an easy task to obtain complete spreading of a protein on a dilute buffer solution; some protein is very apt to be lost in the underlying buffer solution. In addition to the difficulty of obtaining complete spreading is the complication due to surface active contaminants to which sufficient attention has not always been paid. There is another and possibly more important reason for such divergent results as have been obtained; the peptide chains in the protein molecules may spread apart on the surface and the extent to which this spreading occurs would be expected to be a function of a number of factors. This point will be discussed later.

The author feels that he has improved the technique of protein spreading and of the measurement of force area curves in two respects. First, a Wilhelmy balance employing a good analytical balance has been substituted for the conventional mica float thus avoiding all question of leakage around the mica float. The use of the Wilhelmy balance was suggested by the work of Harkins and Anderson.[6] Second, a concentrated solution of ammonium sulfate has been used as the underlying solution upon which the protein film is spread. It had been noted by Gorter[7] that the spreading of proteins into surface monolayers appeared to be much more rapid and complete as the electrolyte concentration of the underlying solution was increased. The results of our investigations along this line were so encouraging that 35% ammonium sulfate solution has been used as the underlying solution in all the experiments reported in this paper. It was found very helpful to enclose the entire apparatus in a cabinet which protected the surface of the ammonium sulfate solution from accidental contaminants from the air.

Experimental

Egg albumin was prepared from fresh hen's eggs by the method of Kekwick and Cannan.[8] It was recrystallized three times and dialyzed against distilled water until

(1) Presented at the Symposium on Surface Active Agents and their Application to Biological Systems held by the Division of Physical Chemistry of the American Chemical Society at Cleveland, Ohio, April 4, 1944.

(2) Gorter and Philippi, *Proc. Acad. Sci. Amsterdam*, **37**, 788 (1934).

(3) Gorter, Van Ormondt and Dom, *ibid.*, **35**, 838 (1932).

(4) Philippi, On the Nature of Proteins. Thesis, University of Leyden, 1936.

(5) Bull, *J. Biol. Chem.*, **125**, 585 (1938).

(6) Harkins and Anderson, THIS JOURNAL, **59**, 2189 (1937).

(7) Gorter, *Proc. Acad. Sci. Amsterdam*, **37**, 20 (1934).

(8) Kekwick and Cannan, *Biochem. J.*, **30**, 227 (1936).

sulfate free. The concentration of the solution was obtained by drying samples at 105° in a vacuum oven for twenty-four hours. The ammonium sulfate was C. P. grade and was recrystallized twice. This treatment was not, however, sufficient to remove all the surface impurities and it was found necessary to treat the ammonium sulfate solutions with activated carbon black (one gram per liter of solution). The carbon black was filtered off. The resulting ammonium sulfate solution was almost completely free of surface active impurities. Complete compression of the "clean" ammonium sulfate solution surface with a movable barrier gave a film pressure of about 0.02 dyne. It appeared impossible to reduce the surface impurities below this point. The treatment of the ammonium sulfate solutions with carbon black is absolutely essential for unambiguous studies on films spread on ammonium sulfate solutions. The Wilhelmy balance consisted of a good analytical chainomatic balance from which the pans along with the glass case were removed. A thin microscope cover glass whose dimensions were 6 $\times$ 4.5 centimeters was suspended from one arm of the balance. The slide dipped about one centimeter into the underlying ammonium sulfate solution. This cover glass slide was counterbalanced by a corresponding glass slide on the other arm of the balance. One milligram weight was equivalent to a surface tension of 0.0818 dyne per centimeter. The ammonium sulfate solution was held in a well-paraffined aluminum trough which was 65 cm. long and 14 cm. wide.

The egg albumin solution was applied to the surface of the ammonium sulfate solution from a Blodgett pipet[9] whose delivery volume was 0.101 cc. The concentration of the protein solution used in spreading was 0.0330 per cent. As the drops formed at the tip of the Blodgett pipet in response to pressure of the rubber bulb, they were touched to the ammonium sulfate solution. Spreading was rapid and complete.

Results

Figure 1 shows the plot of the film areas in square meters per milligram of protein against the time which was allowed to elapse between spreading and the start of compression. Areas are reported for film pressures of 2.5 dynes per centimeter and for 15 dynes per centimeter. The points shown in Fig. 1 were obtained by interpolation of the force–area curves at 2.5 and at 15 dynes per centimeter for the indicated elapsed times.

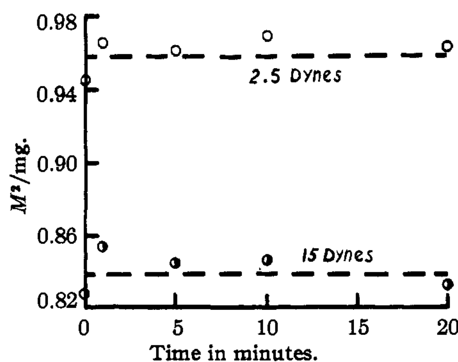

Fig. 1.—Film area in square meters per milligram of protein at 2.5 and 15 dynes per centimeter as a function of the elapsed time between spreading and the start of the compression.

The results in Fig. 1 show that the surface area is independent of time up to an elapsed time of twenty minutes. Extreme care was not exercised in these measurements and, accordingly, there is more variation of the film area than more carefully performed determinations would have shown. The results are, however, sufficiently exact to establish the independency of the area of the film of the time which had been allowed to elapse between spreading and the start of compression.

Figure 2 shows a force–area curve for egg albumin. The measurements upon which this curve are based were done as carefully as possible. The film was compressed slowly and in small decrements. Ample time was allowed after each decrease in area for the attainment of equilibrium surface tension. The completion of compression required about two hours. The elapsed time between spreading and the start of compression was five minutes. While no detailed study has been made of the reversibility of the force–area curves, within the gaseous region, the curves are certainly perfectly reversible. In fact, the curves are reversible up to a film pressure of at least 5 dynes per centimeter.

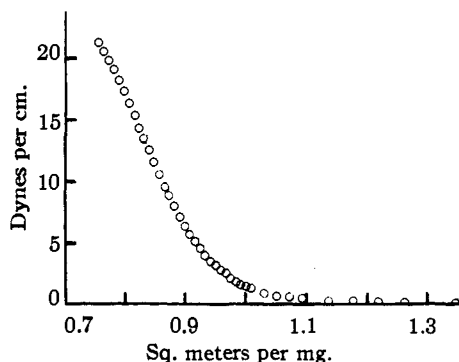

Fig. 2.—Force–area curve for egg albumin on 35% ammonium sulfate solution at 25°.

Discussion

To facilitate the consideration of the results we will divide our discussion into two parts. First, we will consider the force–area curve for egg albumin at film pressures below one dyne per centimeter and second at high pressures.

When the area in square meters per milligram is multiplied by the surface tension lowering in dynes per centimeter at pressures below about one dyne per centimeter pressure, a straight line is obtained. This is shown in Fig. 3.

The results in Fig. 3 illustrate the typical behavior of a so called "gaseous" film. We believe it more realistic to speak of such a film as a dissolved film. The measurement is exactly analogous to an osmotic pressure measurement in 3 dimensions and the gas laws in 2 dimensions should apply.[10] At 25° and for one mole of film FA should equal 24.6 $\times$ 10^5 ergs when F is expressed in dynes per centimeter and A in square meters per milligram.

(9) Blodgett, THIS JOURNAL, 59, 2189 (1937).

(10) Adam, "Physics and Chemistry of Surfaces," 3rd ed., Oxford University Press, London, 1941.

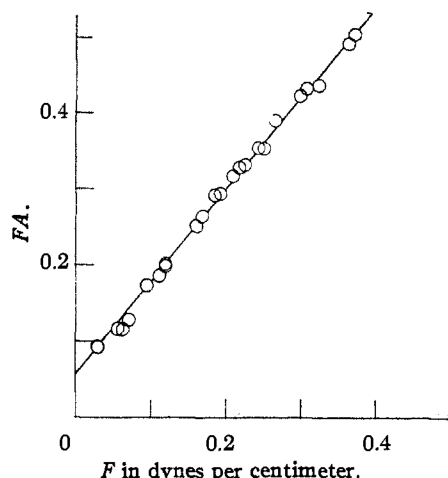

Fig. 3.—Film pressure multiplied by the film area plotted against the surface tension lowering in dynes per centimeter.

The best straight line through the points shown in Fig. 3 was calculated by the method of least squares. The resulting equation is

$$FA = 0.970F + 0.0554 \quad (1)$$

The extrapolated value of FA at zero film pressure is, accordingly, 0.0554 ergs. At zero film pressure the ideal gas laws in two dimensions should be valid and the molecular weight of the egg albumin in the surface film is therefore $24.6 \times 10^5/0.0554$ or 4.44×10^7 milligrams or the molecular weight is 44,400. The molecular weight of egg albumin in volume solution as determined from osmotic pressure measurements is 45,160 ± 180.[11] We conclude, therefore, the egg albumin molecule undergoes no dissociation or association when spread on the surface of the ammonium sulfate solution.

The above discussion should be qualified by pointing out that some surface films show a minimum in the FA *vs.* F curve at low values of F.[10] If this were true of the egg albumin film, the intercept on the FA-axis at zero value of F would be larger than 0.0554 and, accordingly, the molecular weight of the protein in the film would be smaller than 44,400. While there is no evidence for such a minimum in the present case, the possibility cannot be excluded. If the minimum is absent, it means that the forces of attraction between the egg albumin molecules on the surface are negligible. If the film pressure were made infinitely large, it is evident from equation 1 that the area occupied by the gaseous protein molecules would be 0.97 square meter per milligram of protein and accordingly it is considered that 0.97 square meter per milligram of egg albumin is an approximate measure of the area occupied by the gaseous molecules at any pressure. If the area occupied by the gaseous molecules is not constant, a straight line relation could not exist between FA and F as is shown in Fig. 2.

(11) Bull, *J. Biol. Chem.*, **137**, 143 (1941).

Let us now turn to a consideration of the egg albumin film at high pressures. The compressibility coefficient of a surface film is $-dA/AdF$ where A is the area in square meters per milligram of protein and F is the surface tension lowering in dynes per centimeter. In Fig. 4 is shown the compressibility coefficient plotted against the area of the spread film.

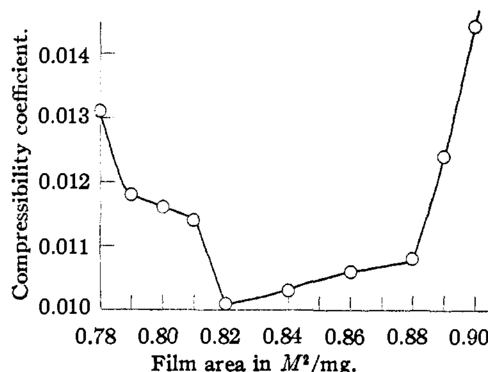

Fig. 4.—The compressibility coefficient of spread film of egg albumin plotted against the area of the film.

The compressibility coefficient shows a rather sharp minimum at 0.82 square meter per milligram. This area corresponds to a film pressure of 14.6 dynes per centimeter. It is believed that this pressure represents the maximum compression to which the egg albumin film can be subject and remain uncollapsed. On this basis, the area per egg albumin molecule when spread into a film and assuming a molecular weight of 45,160 is 6,140 square Å. According to Chibnall[12] the average residue weight of egg albumin is 111.4. It is not clear from his paper whether this is the average residue weight based upon the total egg albumin or whether it is the residue weight after the carbohydrate prosthetic group has been subtracted. Presumably it is the former. The point is not very important, as Neuberger[13] has found that the molecular weight of the carbohydrate fraction is only 1250 and accordingly constituted less than 3% of the weight of egg albumin. Also unknown is the position of the carbohydrate residue when the egg albumin is spread into a surface film. If it is in the film it will contribute to the area, while if it is held beneath the film and directed toward the water phase, it will not contribute to the surface area. Striking a compromise between these various factors we can set certain probable limits for the average area per residue and these fall between 15.6 and 15.1 square Å. per residue. Palmer and Galvin[14] report from X-ray diffraction studies that the average area of the amino acid residues in egg albumin is 15.4 square Å. The agreement between the X-ray and the film area results is good. If the egg albumin film is anhydrous at its maximum compression and has a

(12) Chibnall, *Proc. Roy. Soc.* (London), **B131**, 136 (1942).
(13) Neuberger, *Biochem. J.*, **32**, 1435 (1938).
(14) Palmer and Galvin, THIS JOURNAL, **65**, 2187 (1943).

density equal to that in bulk, the film is 9.12 Å. thick and if the carbohydrate prosthetic group is not in the surface, the thickness will be about 3% greater. If the side chains of the amino acid residues are oriented vertically to the surface, this thickness should correspond to the side chain spacing. Palmer and Galvin[14] report the side chain spacing of egg albumin from X-ray diffraction studies to be about 10.2 Å. The discrepancy between these two values is probably due to the fact that the X-ray side chain spacing refers to the closest approach of the peptide chains in the direction of the side chains. The longest side chain would therefore determine the distance which would be recorded. The thickness of the compressed film, however, corresponds to the average length of the side chains.

It is evident from a consideration of the chemical analysis of egg albumin that 15.4 square Å. cannot represent the true cross sectional areas of the side chain residues; many of these residues are massive and would occupy much more space. The film thickness of 9.12 Å. is also much too thick to be the average length of the residues. The conclusion is drawn that the residues alternate above and below the peptide chain so that the true average area per residue would be twice 15.4 or 30.8 square Å. This interpretation is in keeping with the conclusions drawn from X-ray diffraction studies on wool keratin and on other proteins. Clearly, the above dimensions are not unique for any given surface configuration of the peptide chain or chains of egg albumin and they could be satisfied by any configuration of the chains which would allow alternate packing of the side chain residues above and below the level of the peptide chain. There is only one additional restriction imposed on this structure. Since the egg albumin film is stable on the surface, we can conclude that the underside of the film is predominantly hydrophilic while the upper side is predominantly hydrophobic.

It has been suggested that in an uncompressed protein monolayer the protein exists on the surface as stretched β-keratin chains with the side chain residues lying flat on the surface.[15] If this were true, the area per residue in an uncompressed condition would be for egg albumin about 30.6 square Å. and the film molecules should occupy 1.61 square meters per milligram. It has been shown that the area of the dissolved (gaseous) film is actually about 0.97 square meter per milligram. The conclusion is therefore drawn that even at very low pressures extensive orientation of the side chain residues is present and that the major effect of the compression of the film is one of dehydration which attends the fitting together of the protein molecules as they are compressed. It is believed that whatever configurations are present in the uncompressed molecules are likewise present in the compressed molecules and that compression is attended with little rearrangement of the peptide chains in the surface. There exists the possibility, however, that the behavior of a spread protein film on a dilute buffer solution is quite different in this respect than it is on concentrated ammonium sulfate solutions. The compressibility coefficient of the egg albumin film on concentrated ammonium sulfate solution is much less than it is for a film of this protein on a sodium acetate buffer. This may indicate a spreading apart of the peptide chains on a dilute buffer solution which does not occur, at least not over a short time, on an ammonium sulfate solution.

A characteristic feature of the formation of surface films of protein is the speed with which they form. Bull[16] has been able to show that the formation of a layer of spread protein on the surface of egg albumin solutions is complete in less than 0.58 second. The present communication also emphasizes the rapidity of the formation of surface films. These considerations lead one to the belief that the native protein contains layers of peptide chains which resemble closely the structure of that of the spread film and that spreading simply involves the "unleafing" of the native molecule.

Very pertinent to these considerations are the findings of Rothen and Landsteiner[17] who report that spread films of egg albumin when deposited on glass slides retain their power of combining with specific antibody. It is generally believed that the ability of a protein to combine with a specific antibody resides in the surface configuration of the protein molecule The results of Rothen and Landsteiner would seem to indicate that the spread film of egg albumin retains its configuration which it had in the native molecule. These conclusions would appear to support the contention that native protein molecules of the egg albumin type are layer structures as Astbury,[18] Pauling,[19] Dervichian,[20] Boyes-Watson and Perutz,[21] and Palmer[22] have suggested.

Acknowledgment.—It is a pleasure to acknowledge the financial assistance of the Abbott Research Fund of Northwestern University which permitted the employment of technical help, Miss Lorraine Schmidt.

Summary

1. The force–area curve of egg albumin has been investigated for films of the protein spread upon 35% ammonium sulfate solution. A Wilhelmy balance has been used to record the film pressures.

2. It is concluded that the area of the spread film for any given film pressure is independent of

(15) Neurath and Bull, *Chem. Revs.*, **23**, 391 (1938).

(16) Bull, *J. Biol. Chem.*, **123**, 17 (1938).

(17) Rothen and Landsteiner, *J. Exptl. Med.*, **76**, 437 (1942).

(18) Astbury, *Nature*, **137**, 803 (1936).

(19) Pauling, This Journal, **62**, 2643 (1940).

(20) Dervichian, *J. Chem. Phys.*, **11**, 236 (1943).

(21) Boyes-Watson and Perutz, *Nature*, **151**, 714 (1943).

(22) Palmer, *J. Phys. Chem.*, **48**, 12 (1944).

the time which elapses between the spreading and the start of the compression up to an elapsed time of twenty minutes.

3. The egg albumin film is "gaseous" at low film pressures. The molecular weight of the egg albumin in the spread film has been estimated to be about 44,000. It is concluded that the egg albumin molecule probably does not dissociate on the surface.

4. It has been found that the area of the gaseous, uncompressed film of egg albumin is 0.97 sq. meter per milligram of protein from which it is concluded that there is extensive orientation of the side chain residues in the uncompressed state.

5. The film pressure and the corresponding film area have been determined at the point of minimum compressibility. At this point the egg albumin film occupies 0.82 square meter per milligram. The area per egg albumin molecule and the area per residue have been calculated.

6. It is concluded that this study is in keeping with the theory that native protein molecules of the egg albumin type are laminated structures.

CHICAGO, ILL. RECEIVED MAY 27, 1944

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

Monolayers of β-Lactoglobulin on Concentrated Salt Solutions

BY HENRY B. BULL

In a previous paper it has been shown that egg albumin spreads in surface films on 35% ammonium sulfate solutions completely and rapidly.[1] It was also shown that a Wilhelmy balance registers the film pressure with a precision which cannot be attained except with the most carefully and expensively made torsion-float type of film balance. It was decided to employ these improvements in technique in a study of the force-area measurements of spread films of β-lactoglobulin.

Experimental.—The β-lactoglobulin was prepared from fresh, raw, whole milk according to a private communication from Dr. A. H. Palmer. It formed large plate-like crystals which were recrystallized several times. The spreading technique employed was the same as that previously described for egg albumin.[1] The concentration of the β-lactoglobulin solution was 0.0269%. The solution upon which the protein was spread was 35% ammonium sulfate. Particular attention was paid to the purity of the ammonium sulfate solutions; previous to use they were treated with activated charcoal to remove surface active impurities.

Results.—Figure 1 shows a force-area curve for a spread film of β-lactoglobulin on 35% ammonium sulfate.

The time between protein spreading and the start of compression was two minutes. The film was compressed slowly and with ample time for the attainment of equilibrium surface pressures.

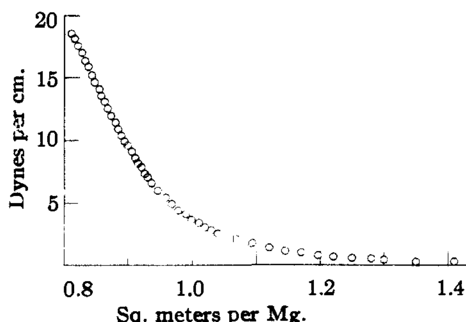

Fig. 1.—Force-area curve of β-lactoglobulin on 35% ammonium sulfate.

Discussion.—When the area in square meters per milligram is multiplied by the surface tension lowering in dynes per centimeter at pressures below about 0.5 dyne per centimeter pressure, a straight line is obtained. This is shown in Fig. 2.

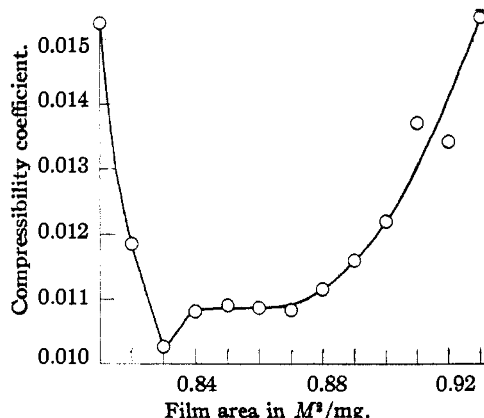

Fig. 2.—Film pressures multiplied by the corresponding film area and plotted against the film pressure.

The results in Fig. 2 illustrate the typical behavior of a so-called "gaseous" film and as we have pointed out in a previous paper[1] the ideal gas laws in 2 dimensions should apply at zero pressure.

The best straight line through the points in Fig. 2, was calculated by the method of least squares. The resulting equation is

$$FA = 1.21F + 0.0555 \quad (1)$$

The extrapolated value of FA at zero film pressure is, accordingly, 0.0555 erg, which corresponds to a molecular weight of about 44,000. The molecular weight of β-lactoglobulin from equilibrium ultracentrifugation is 38,000 while from rate ultracentrifugation it is 41,500.[2]

(1) Bull, THIS JOURNAL, 67, 4 (1945).

(2) Svedberg and Pedersen, "The Ultracentrifuge," Oxford University Press, New York, N. Y., 1940.