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Salted-Out Protein Films

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Proteins can be adsorbed from aqueous solutions onto a plate, covered by barium stearate multilayers, which has been conditioned[1,2] by previously dipping it into a solution of thorium nitrate. When a 1% solution of insulin is applied in this way to a conditioned plate and this is then washed in distilled water, and dried, a film of a thickness of about 45 Å. is obtained. However, if the protein solution is washed off the plate with an 0.8% sodium chloride solution instead of with water and the plate is then washed with distilled water and dried, the film thickness is much greater, ranging from 70 to about 200 Å. The variations in thickness seem to depend upon the rapidity of mixing the salt solution with the underlying protein solution.

These results suggested that the increased thickness due to the application of the salt solution directly to the protein solution on the plate was caused by the concentration gradient in the solution while the salt diffused into the insulin solution. Confirmation of this hypothesis was obtained from experiments in which 0.8% sodium chloride was added to the insulin solution before applying this to the conditioned plate. After washing this off with sodium chloride solution and then with water, the normal thickness of about 45 Å. was found.

The effect seems to be analogous to the Ludwig–Soret effect in which a temperature gradient causes a migration of a solute to the cold part of a solution. The theory of this phenomenon has not been satisfactorily worked out. A simple explanation often given is that the molecules of the solute are bombarded by the solvent molecules with greater intensity on the high temperature side than on the low temperature side. Such an explanation seems particularly useful in accounting for the Soret effect in air commonly manifested by the slow accumulation of dust particles at cold spots on the ceiling of a room.

According to S. Chapman[3] particles having a large diameter compared to the free path of the molecules in a liquid should distribute themselves in such a way that cD is constant throughout the solution, c being the concentration of the particles and D the diffusion coefficient. Thus, since the diffusion coefficient increases with temperature, the concentration should be less in the hotter parts. Bruzs[4] attempts to develop a thermodynamic theory and concludes that the Soret coefficient σ depends upon the entropy and the partial molar specific heats. There are, however, practically no experimental data for checking this theory.

Hartley,[5] after discussing the preceding theories, advances reasons for believing that the relative solubilities of the solute in the solvent, at different places in the tube, are important in determining the Soret coefficient.

In a second paper,[6] Hartley considers the distribution of solute molecules in a solvent of graded composition. Apparently in all cases if there is one component A originally uniformly distributed throughout a solvent, the effect of a concentration gradient in a second component B is to drive the molecules of A toward the region where B has a low concentration. We shall designate this as the carrying effect.

Hartley believes that there are three factors that determine the magnitude of this forced migration. First there is the Chapman effect, according to which the particles should migrate toward the region where the diffusion coefficient is lowest. Thus, if the presence of a substance B increases the diffusion coefficient of substance A, A should tend to move into the region where B has a low concentration. It seems improbable, however, that the general effect of dissolved substances should be to raise the diffusion coefficient of other substances, so that Chapman's theory seems inadequate as a general explanation of the carrying effect.

As a second factor, Hartley considers the effect of the added substance B on the solubility of A.

The third factor he describes as a "push effect." The larger molecules are subjected to greater bombardment by diffusing molecules than small ones.

(1) I. Langmuir and V. J. Schaefer, THIS JOURNAL, **59**, 1406 (1937).
(2) I. Langmuir and V. J. Schaefer, *ibid.*, **59**, 1762 (1937).
(3) S. Chapman, *Proc. Roy. Soc.* (London), **A119**, 34 (1928).
(4) B. Bruzs, *Z. physik. Chem.*, **A162**, 31 (1932).
(5) G. S. Hartley, *Trans. Faraday Soc.*, **27**, 1 (1931).
(6) G. S. Hartley, *ibid.*, **27**, 10 (1931).

Hartley assumes that the pressure exerted is approximately proportional to the mean cross-sectional area of the large molecules. Thus an enormous push effect is to be expected in the case of colloidal particles. This simple explanation of the "push effect" cannot be generally valid, for it would indicate that a sphere the size of a baseball placed at the boundary between a molar salt solution and overlying pure water should be forced upward with a force of about 1000 lb. It is obvious, however, that any pressure difference greater than that due to gravity will be equalized by a flow of liquid. It is only when the particles are of a size comparable to the free path of the molecules of the liquid that an appreciable push effect can exist.

Recently Seastone[7] found it impossible to spread monolayers of tobacco mosaic virus by Gorter's method on the surface of water adjusted to a *p*H corresponding to the isoelectric point of the protein. At *p*H 1 there was a small amount of spreading corresponding to about 0.1 sq. m. per mg. Since the protein solution when applied sank to the bottom of the tray and so could diffuse only slowly to the surface, Seastone attempted to spread the protein on a solution of higher density, using for this purpose a 25–90% saturated ammonium sulfate solution. The protein solutions, and even distilled water, spread with almost explosive rapidity on such strong salt solutions, for these have surface tensions that exceed that of pure water by several dynes per cm. With 90% saturated ammonium sulfate at *p*H 7.1, a film of virus covering an area of 0.06 sq. m. per mg. was obtained. Measurements of the virus activity proved that little or none of the tobacco virus remained in the liquid under the film. If this film has a density 1.3 and if it contains all the protein applied to the solution, its thickness should be 126 Å. In another case Seastone reports that a film of a thickness of 1000 Å. was obtained by applying tobacco mosaic virus to a 50% saturated ammonium sulfate solution. He suggests that these thick films may correspond to monolayers in which the rod-shaped molecules of diameter 150 Å. lie flat in the surface in some cases and lie with their long axes perpendicular to the surface in other cases.

The work of Gorter and Philippi shows that many proteins spread to give films of thicknesses from 7 to 20 Å. under compressions to 0 to 10 dynes per cm. Some samples of tobacco virus sent to us over a year ago by Dr. Stanley gave films of approximately this thickness. About six months ago, Dr. Stanley sent us some new samples of 1.9% tobacco virus purified by ultracentrifuge sedimentation. With a spreading technique which we had used previously, which was a modification of Gorter's, we were unable to obtain monolayers. When this solution of tobacco virus was spread upon ammonium sulfate solution we obtained very thick films, ranging from 100 to 2000 Å.

Our experiments showed clearly that the film thickness was not determined by the molecular diameter but depended on the concentration of the salt solution and on the amount of protein applied in relation to the area available for spreading.

When a drop of tobacco virus solution is placed upon a concentrated ammonium sulfate solution, the drop spreads over the surface because the surface tension of the water is less than that of the salt solution. We have observed that one drop of distilled water (0.05 ml.) spreads on a saturated ammonium sulfate solution to form a film 5×10^{-4} cm. thick and of an area of about 100 sq. cm., as shown by the pushing back of a film of indicator oil on the surface of the solution. A pressure of only one dyne per cm. causes the immediate collapse of the circular area covered by the water film.

The rate of diffusion of salt from a solution into an overlying layer of initially pure water can be calculated by methods used in problems of heat conduction.[8]

Consider a salt solution of concentration c_0 on which is placed at time $t = 0$, a layer of water of thickness b. Then, if x is the depth below the surface, the concentration c is initially 0 between $x = 0$ and $x = b$, and is c_0 for $x > b$. The concentration gradient has a maximum value at $x = b$ which is given (for not too large values of t) by

$$dc/dx = (c_0/2)(\pi Dt)^{-1/2}$$

where D is the diffusion coefficient of the salt.

For values of x small compared to b, the concentration gradient is at first very small but rises to a maximum and then decreases toward zero. The maximum value of dc/dx occurs when $t = b^2/6D$ and is given by

$$(dc/dx)_{max.} = 0.93c_0x/b^2$$

(7) C. V. Seastone, *J. Gen. Physiol.*, **21**, 621 (1938).

(8) Ingersoll and Zobel, "Mathematical Theory of Heat Conduction," Ginn and Co., New York, N. Y., 1913; see especially Eq. (25) on p. 70.

At this time the concentration at the surface $x = 0$ is only $0.084c_0$; at $x = b/2$ it is $0.198c_0$ and at $x = b$ it is $0.5003c_0$. The concentration gradient at $x = b/2$ is then $0.45c_0/b$.

The diffusion coefficient D for salts in water is of the order of magnitude of 10^{-5} cm.^{2}/sec. Thus the maximum concentration gradients close to the surface will occur within less than a second, even if the water film has a thickness as great as 0.08 mm., which corresponds to 0.8 ml. per 100 sq. cm.

Although the concentration gradient reaches its maximum within such a short time, the actual concentration of salt in the surface (at $x = 0$) approaches its limiting value very slowly. Thus to reach $0.9c_0$ will require a time $32b^2/D$ and to reach $0.99c_0$ takes $t = 3200b^2/D$. With $b = 5 \times 10^{-3}$ cm., $D = 10^{-5}$ cm.^{2}/sec. One hundred and sixty seconds would be required for $c = 0.9c_0$ and four and one-half hours for $c = 0.99c_0$.

The thick films of tobacco virus observed by Seastone and confirmed by our experiments thus appear to be essentially salted-out films in which the protein is forced to the surface by the concentration gradient of the salt until the protein concentration becomes so high as to cause a crystallization of the protein into a thin sheet.

Recent work[9,10] has shown that highly purified tobacco virus solutions, if stronger than 2%, separate into two phases on standing. The lower layer, usually water clear, is liquid crystalline; the upper layer, which is slightly turbid, shows, on gentle agitation, anisotropy of flow.

X-Ray examination has shown that in the denser phase the rod-shaped molecules lie with their axes parallel, but in a plane perpendicular to this direction the molecules are arranged in a hexagonal close packed lattice with a spacing which varies continuously from 150 to 600 Å., depending upon the concentration of the solution. The lower limit of 150 Å., obtained when the water content is very low apparently corresponds to the diameter of the molecular rods.

The forces that hold the molecules apart in the hexagonal lattice are recognized as being electrostatic forces associated with the negative charges on the molecules. The intervening water must therefore carry an excess of ions of the opposite polarity. The spindle-shaped crystals of tobacco virus observed by Stanley and others have been shown to have a similar structure and are thus tactoids in which the distance between molecules depends on the water content.

We believe that in our experiments the concentration gradient of ammonium sulfate forces the tobacco virus to the surface in such concentration that this denser phase appears. Probably the molecules are arranged with their axes parallel to the surface and in transverse directions are packed in a hexagonal lattice.

Films Salted-Out onto Plates.—The salted-out films of insulin produced on a plate by applying a few drops of insulin solution and then washing this off by pouring over it a salt solution were very non-uniform in thickness. We attempted in several ways to develop a technique of producing uniform films.

Using one method, we saturated filter paper, blotting paper or silk fabrics with an insulin solution, applied this in one or more layers onto a wet, conditioned barium stearate multilayer and placed on top of this other layers of porous paper or fabric wetted with salt solution. After two minutes the layers of paper were lifted off, the film was washed with water and dried, and the thickness was determined by matching the color of the specularly reflected light with that given by a barium stearate stepped color gage.[11,12] With 0.05 ml. of 0.1% insulin on 3 sq. cm. filter paper covered with paper saturated with 90% saturated ammonium sulfate solution, a film 220 Å. thick was produced.

This represents nearly 20% of the protein applied to the paper. With more concentrated insulin solutions (up to 10%) film thicknesses, in some cases as great as 650 Å. were obtained, but the fraction recovered was much less. The films produced by this method were usually of unsatisfactory uniformity.

The best method we have found for producing salted-out films directly on a plate is as follows. A prepared plate covered with barium stearate multilayers is conditioned by thorium nitrate so as to render it hydrophilic and it is then dipped into a solution of the protein. It is important that the plate first should be rendered hydrophilic for otherwise when it is dipped into the protein solution a film is deposited on the down trip.

The plate is lifted from the protein solution at a rapid uniform rate, the adhering liquid is allowed to drain for a few seconds and the surplus removed from the lower corner of the plate by filter paper. The plate is then quickly immersed into a strong salt solution in a beaker and allowed to stand for a few minutes. It is then taken out of the salt solution and dipped successively for twenty seconds into each of several beakers containing 1% tannic acid solution. The plate is then washed thoroughly in distilled water, dried and the thickness of the protein film is measured optically.

The tannic acid renders the protein film insoluble so

(9) F. C. Bawden, N. W. Pirie, J. D. Bernal and I. Fankuchen, *Nature*, **138**, 1051 (1936).

(10) J. D. Bernal and I. Fankuchen, *ibid.*, **139**, 923 (1937).

(11) K. B. Blodgett and I. Langmuir, *Phys. Rev.*, **51**, 964 (1937).

(12) I. Langmuir, V. J. Schaefer and H. Sobotka, THIS JOURNAL, **59**, 1751 (1937).

that it can be washed with water without loss. Salted-out films of insulin, however, suffer no loss, even without the tannic acid treatment. When tannic acid is used with insulin the film is 12 Å. thicker. We assume that with other proteins the use of tannic acid gives a similar increment of thickness.

The thicknesses of films of insulin, salted-out from a 0.5% insulin solution by applying sodium chloride solutions, increase from 90 Å. with 0.3% salt to a maximum of 340 Å. with 1% salt, decreasing to 190 Å. at 3% and to 140 Å. with salt solutions between 10 to 35%. Variation of the *p*H of the salt solution between 4 and 7 gave little or no change in thickness.

Salted-out films of pepsin (Eli Lilly's, 8000 units per gram), formed by using saturated ammonium sulfate solutions at *p*H from 2 to 5 gave 100 Å. from a 0.1% protein solution, and up to 600 Å. with a 5% protein solution. With a 0.5% pepsin solution, thicknesses of only 50 Å. were obtained with ammonium sulfate up to 25% saturated, and the thickness increased from 150 to 225 Å. as the concentration of the sulfate solution was raised from 50 to 100% saturation.

The salted-out films produced in this way were very uniform, but scattered a good deal of light (more than the films produced by the use of impregnated filter paper).

Films Salted-Out on the Surfaces of Salt Solutions.—A tray is filled with a strong solution of some salt such as ammonium sulfate, or magnesium sulfate and its surface is cleaned in the usual way. Barriers are placed across the tray to delimit a definite area.

A graduated 1-ml. hypodermic syringe is filled with the protein solution and by a micrometer screw the plunger is forced down a definite distance so as to deliver a given volume of solution. By Gorter's method the protein solution is introduced from the needle just under the surface of the salt solution. Because of the relatively high density of the salt solution, it is equally satisfactory to let drops of the protein solution fall upon the surface. Another method, which we call the "band method," consists in distributing the protein solution along a line near the edge of a thin nickel or platinum band which has a length equal to the width of the tray and has been cleaned by heating in a flame. Before the protein solution has dried, the band is lowered slowly edgewise into the tray close to one end of the delimited area.

The protein solution applied by either of these methods spreads rapidly over the salt solution, and the protein is driven to the surface by the concentration gradient. If the bottom of the tray has been made black (by black Bakelite varnish, baked on, or by a sheet of black glass) and the thickness of the protein film on the solution exceeds about 200 Å., it is visible because of the increased reflection from the surface. There is usually a part of the surface farthest from the point of application that is covered by an invisible monolayer, 10–20 Å. thick, which was formed from the first portion of the applied protein solution and which was pushed against the barriers and so retarded the further spread of the protein solution. A surface pressure of about 15 dynes per cm. can be observed against a movable barrier under these conditions.

The salted-out film on the surface should be allowed to age for two minutes so that it may reach its maximum thickness and stability.

Optical Measurement of Thickness.—Sometimes the salted-out protein films on the salt solutions are so thick, 1000–7000 Å., that interference colors are seen. There are two convenient methods of producing films of known thickness on water which serve as comparison standards. A few drops of 10% sulfuric acid applied to the edge of a barium stearate multilayer (49 to 120 layers) on glass creeps under the film and detaches it so that it may be floated off onto water in a tray. Since water has a lower refractive index ($n = 1.33$) than the film ($n = 1.50$), the interference colors are complementary to those observed when the same film is on chromium or on glass of refractive index higher than 1.55.

Table I gives the number of layers and the thickness of barium stearate multilayers on water which give various colors when examined at an angle of incidence of 45°.

The second method consists in spreading a weighed amount of indicator oil (partially oxidized lubricating oil) on water and compressing the film between barriers to confine it to known areas so that the thickness can be calculated. The relation between the thickness and the color of the oil films was approximately the same as that of the stearate films given in Table I.

TABLE I

COLORS OF THIN OIL OR STEARATE FILMS ON WATER OBSERVED WITH UNPOLARIZED LIGHT AT $i = 45°$

Color	No. of layers	Thickness, Å.
Gray	33	800
Faint yellow, first order	49	1200
Yellow	57	1400
Dark yellow	65	1600
Yellow-red	73	1800
Bluish-red	81	2000
Purple-blue	89	2200
Blue	97	2400
Bluish-green	105	2600
Yellow-green	115	2800
Yellow, second order	122	3000

A salted-out film produced by applying 0.3 ml. of a 1% solution of commercial egg albumin in successive drops to the central portion of a 100-cm. area of a 90% saturated ammonium sulfate solution gave at the point of application a disk of about 5-cm. diameter showing a light yellow color, while on the solution. The film around this disk showed only a gray color. Some of the film from the colored disk, transferred to a chromium plate by the "lift method" (to be described later), fixed by dipping into 1% tannic acid, then washed and dried, gave a purple-blue, about the same as that given by 45 layers of barium stearate on chromium observed at $i = 50°$. A portion of the same film transferred to a fused quartz plate, $n = 1.46$, showed a yellow color. On a microscope slide ($n = 1.51$), the color was light blue, while on lead glass ($n = 1.6$) the film gave an intense blue color. These data show that the film had a refractive index between 1.46 and 1.51, probably close to 1.48, and that the thickness was about 1100 Å. The refractive index of protein calculated from that of solutions is usually found to be approximately

1.58. It has been shown previously[13] that built-up films of egg albumin gave a refractive index for Na light of 1.50. Thus it is probable that the salted-out films and the built-up films contain water or air which lowers the refractive index. Experiments show that paper wet with a drop of octane held above a thick salted-out protein film on water or over the same film deposited on a chromium plate produces striking changes of color just like those observed by bringing these vapors in contact with skeletonized barium stearate films. These facts seem to indicate that these thick protein films are skeleton films which contain a considerable proportion of water or air.

Similar films of 1200 Å. thickness were produced by applying 0.5 ml. of a 1% solution of crystalline pepsin (Northrop) to 90% saturated ammonium sulfate.

Dr. W. M. Stanley gave us a 1% solution of some centrifuged monodispersed tobacco mosaic virus. When 0.1 ml. was applied to 100 sq. cm. of 90% saturated ammonium sulfate solution, a grayish colored film of considerable rigidity was formed covering an area of about 55 sq. cm. This was deposited upon a chromium plate and fixed in 1% tannic acid. After washing and drying, the film, when examined with the R_s ray at large angles of incidence, showed a second-order yellow corresponding to a thickness of 120 barium stearate layers or 2900 Å. At an angle of incidence $i = 45°$, this gives a purple color. Thus we should expect the film on water before deposition on the plate to have shown the complementary color, *viz.*, a second-order yellow (see also Table I). During the growth of the film, the colors should have changed from first-order yellow through purple, blue-green, to second-order yellow. Actually, however, the films on water show only a strong grayish reflection, which forms a strong contrast with a clean water surface.

The probable explanation of the absence of interference colors of the tobacco virus films on water is a graded lower boundary which does not provide the definite reflection needed for interference. When the film is deposited on a plate, fixed, washed and dried, the upper surface which was previously the lower surface now has a definite boundary and gives good interference colors.

With several proteins, egg albumin, pepsin, urease, edestin and horse globulin, we have observed that the salted-out films formed are of a composite type. Thus, if a drop of a 1% solution of one of these proteins is applied to the center of the clean surface of a 90% saturated ammonium sulfate solution about 100 sq. cm. in area, a faintly visible gray film instantly covers the surface followed within a few seconds by the formation in the central part of a circular area 2 to 5 cm. in diameter which first appears black (similar to a clean surface). This area is soon filled in with a grayish film, starting at the outer edge and gradually filling the interior with a uniform film which gradually increases in reflecting power. A second drop applied to the same place sinks into the salt solution and then rises and dissolves this film, producing a black region as previously described. This is followed by the appearance of a film similar to the previous one in formation but much thicker, often showing intense interference colors extending even into second- or third-order colors, depending largely on the purity and concentration of the protein solution used.

(13) K. B. Blodgett, *J. Phys. Chem.*, **41**, 975 (1937); see p. 980.

A large area of the film on the surface, including the colored spot, is now deposited on a chromium plate and is fixed by gently flooding over the surface a 1% tannic acid dissolved in 90% ammonium sulfate saturated solution. After washing and drying, it is usually found that interference colors are seen not only on the central disk but also far out into the surrounding area. Within the disk the thickness of the film as determined by the colors observed after deposition agrees with that determined from the color seen on the salt solution. The portions surrounding the disk, however, now give colors which correspond to very great thicknesses, often as large as 7000 Å.

These phenomena seem to show that these salted-out protein films are essentially composite and consist of two layers, a *diffuse layer* which has an indefinite lower boundary and thus produces no interference effects while on the solution, and a second or *compact layer* which overlies the diffused layer on the solution and which has a definite lower boundary giving good interference colors. The compact layer is of uniform thickness within the central disk and gradually decreases with increasing radii over the surrounding surface. The diffuse film is far thicker than the compact film and decreases steadily in thickness as the radius increases. Originally, while the film is on the solution, the diffuse film has a maximum thickness under the central disk; but while depositing this on the plate and subsequently fixing it with tannic acid, the thickest parts of this diffuse film usually break away, within a circular area, leaving within this only the compact film on the plate. By agitating the salt solution under the film or by more vigorous treatment with tannic acid fixing solution, the area from which the diffuse film is removed may be increased greatly without altering the thickness of the compact film.

The thickest compact films are obtained by using protein solutions of very high purity. For example, we find that solutions of pure crystalline egg albumin (given by Dr. P. A. Levene) give far thicker compact films and form them in far less time than when an impure egg albumin is used.

The compact film, which is usually very uniform in thickness over the central disk, acts as though it were a single crystal sheet; the diffuse film frequently may be seen upon stirring the underlying solution as a veil which scatters an appreciable amount of light and can be moved about under the compact film. It perhaps consists of a suspension of very fine crystals.

With insulin we have not been able to produce these composite films. When this protein is applied to a 90% ammonium sulfate solution, a white cloudy precipitate is formed but no coherent film. On 1% ammonium sulfate the insulin spreads to form a monolayer. When a 1% insulin solution is applied to an unlimited area of a 1% sodium chloride solution, an insulin monolayer is formed, but with a restricted area the pressure builds up to $F = 18$ dynes per cm. and a circular grayish area appears, which after deposition on a plate gives a thickness of 115 Å. It is not necessary to fix this deposited film with tannic acid, for it can be washed gently with water without loss. However, the salted-out film formed on the salt solution gradually decreases in thickness to 50 Å. if left for five minutes before deposition.

In many experiments we have made estimates of the total amount of protein which can be recovered in the salted-out films by transferring them to metallic surfaces, or we have determined the protein content per sq. cm. by a method which we shall describe later involving the production of monolayers on another water surface. In general, with pure crystalline egg albumin, tobacco virus and pepsin, there is a very high recovery in the film.

With impure proteins such as commercial egg albumin or pepsin, the fraction of applied protein which is recovered in the salted-out film is much lower. Apparently there is a purification of the crystallizable protein involved in the formation of the salted-out film, so that the yield serves to measure the crystallizable protein content.

Properties of Salted-Out Films.—The thick salted-out films of protein, particularly the compact ones, possess considerable mechanical strength and are relatively incompressible. When subjected to moderate pressure, they can be lifted off the solution on a loop of platinum wire.

A film produced from crystalline egg albumin, on an ammonium sulfate solution, having a thickness of 1000 Å. and showing a light yellow interference color, was found to be under a pressure of $F = 23$ dynes per cm. at the time it was formed. On raising the pressure to 30 dynes per cm. the area decreased only 2%, corresponding to a compressibility of 0.0030 for each dyne per cm. On raising the compression to $F = 50$, the area decreased 17%, corresponding to a compressibility of 0.0080. At this higher pressure groups of parallel wrinkles began to appear, especially when slight shearing stresses were applied. At $F = 54$, the whole surface became wrinkled and collapse set in. For comparison we note that a monolayer of egg albumin on water decreases to half its area when F is raised from 1 to 25 and to one-quarter the original area at $F = 34$. In the F range from 23 to 30 the compressibility was 0.07 for the monolayer as compared to 0.003 for the salted-out film.

Transference of Salted-Out Films to a Water Surface.—If a piece of nickel or platinum foil cleaned and made hydrophilic by heating in a Bunsen flame, is lowered into a salt solution covered by a salted-out protein film under compression of $F = 30$ dynes per cm. produced by a drop of oleic acid, and is then raised out of the solution, the film is deposited on the foil as a hydrous B-film. If, now, before the water film under the protein film has dried, the foil is lowered into clean water in another tray, the protein film escapes onto the water surface.

Salted-out films of many proteins, such as pepsin, insulin and egg albumin, when transferred in this way to water, which preferably has its pH adjusted to the isoelectric point of the protein, spread out apparently without appreciable loss to form typical monolayers. By measuring A_2, the area of the monolayer at some value of F, such as 16 dynes per cm., and determining the thickness T_2 of the monolayer at the same compression, we can calculate the weight, W_2, of the protein transferred to the water by the relation

$$W_2 = 1.3 \times 10^{-5} A_2 T_2 \qquad (1)$$

where W_2 is expressed in mg. and T_2 in Å.

The thickness T_2 can be measured optically after depositing the monolayer on a barium stearate multilayer film of critical thickness (45–47–49 layers), preferably by the "lift method" in which the plate is lowered horizontally onto the monolayer (H-layer) and then after the surface has been cleaned, raised from the water. By this method a single monolayer of A_L-type is deposited.

The thickness T_1 of the original salted-out film can now be calculated from the equation

$$T_1 = A_2 T_2 / A_L \qquad (2)$$

where A_L is the area of the "lifted film" taken from the salted-out film. Our experience has been that this is usually a more accurate method of determining T_1 than a direct optical measurement of the salted-out film on the water or on a plate upon which it may be deposited. The reason for this is that the optical measurements are often difficult because of a relatively large amount of scattered light from the thick protein films. The method, however, is not applicable to tobacco virus and other proteins which do not spread to form monolayers.

Table II gives a summary of data obtained in studies of salted-out films of various proteins on 90% saturated ammonium sulfate solutions. The insulin (L) was a pure crystalline product obtained from Eli Lilly and Company, while the pepsin (L) was a commercial grade from the same source having an activity (skim milk test) of 8000 units per gram. A similar test of the activity of Northrop's crystalline pepsin (N) gave 50,000 units per gram. The egg albumin (L) was also an impure commercial product.

The third column gives the concentration of the protein solution in percentage by weight. A measured volume of this solution (for example, 0.18 ml. in Experiment 1, and 0.033 ml. in Experiment 2) which contained the weight W_1 of protein as given in mg. in Col. 4 was applied in the middle of the delimited area (Col. 5) by Gorter's technique. The sixth column gives the approximate area of the salted-out film that was produced; Col. 7 gives A_L, the area of the salted-out film that was deposited onto a plate and subsequently transferred to the surface of water (at the isoelectric point of the protein) in another tray where it gave the area A_2 (Col. 8) when subject to a

TABLE II

SALTED-OUT PROTEIN FILMS ON 90% SATURATED $(NH_4)_2SO_4$

T_2 = 16 Å. at F = 16 dynes/cm.

1	2	3	4	5	6	7	8	9	10
Expt.	Protein	% by weight	W_1, mg.	Delimited area, sq. cm.	A_1, sq. cm.	A_L, sq. cm.	A_2, sq. cm. F = 16	T_1, Å.	β
1	Insulin (L)	0.1	0.18	300	170	4	18	72	0.88
2		1.0	.33	300	95	4	63	252	.94
3		10	2.50	300	110	4	340	1340	.77
4	Pepsin (L)	0.1	0.148	300	80	4	6	24	.17
5		1.0	.96	300	100	4	15	60	.081
6		10	9.6	300	50	4	127	510	.035
7	Pepsin (L)	10	2.7	300	30	4	42	170	.025
8		10	4.8		30	4	50	200	.016
9	Insulin (L)	1	1	50	40	5	217	700	.37
10		1	1	100	90	5	185	600	.70
11		1	1	200	140	5	110	350	.64
12	Pepsin (L)	1	1	50	30	5	53	170	.066
13		1	1	100	50	5	30	96	.062
14		1	1	200	100	5	73	116	.150
15	Egg albumin (L)	1	1	100	90	12.5	180	230	.27

compression of F = 16 dynes per cm. The thickness T_2 was found in the various experiments to range from 15 to 18 Å., so we have taken the average value T_2 = 16 Å. The thickness T_1 of the salted-out film, as given in Col. 9, was calculated by Eq. (2) from A_2, T_2, and A_L.

The last column gives β, the fraction of the protein applied to the salt solution which was recovered in the salted-out film. This was calculated by the equation

$$\beta = 1.3 \times 10^{-5} A_1 T_1 / W_1 \qquad (3)$$

Examination of the data in the table shows that with a large delimited area, Experiments 1 to 3, from 77 to 94% of the insulin was found in the salted-out film, but with the L-pepsin only from 8 to 17% was recovered, the highest fraction being obtained with the most dilute protein solution. The fraction recovered was not greatly dependent on the amount of protein solution applied (Experiments 6, 7 and 8). The thickest films were obtained with the most concentrated solutions (Experiments 3 and 6) and when the largest amounts of the protein solution were applied (Experiments 6, 7 and 8). In Experiments 9 to 14 the delimited areas were purposely varied. The fraction recovered is somewhat less when the available spreading area is restricted, but the thickness T_1 is made greater in this way (Experiments 9 and 12).

The high percentage of recovery from the insulin compared with pepsin suggested that this might be due to the difference in purity of the two proteins. We therefore made some experiments with a crystallized egg albumin obtained from P. A. Levene and a crystallized pepsin from J. H. Northrop. With these very high percentages of recovery were obtained. Films of various thicknesses, ranging from 100 up to 7000 Å., could be obtained by regulating the amount of protein in the delimited area.

The Activity of Pepsin in Salted-Out Films.—Uniform salted-out films of pepsin were produced by applying pepsin solutions to 90% saturated ammonium sulfate solutions. The thickness of the film was measured optically after depositing it on a chromium plate. Another portion of the film was deposited on a plate of known area and the protein was washed off with 2 ml. of 0.1 M acetate buffer, pH 5, into a test-tube containing 1 g. of powdered skim milk in 4 ml. of the same acetate buffer, and the clotting time at 37° was noted. The weight of the protein on the plate was calculated from the area, and thickness, assuming a density 1.3. In this way the activity per gram was determined. Similar tests of activity were made by introducing known weights of protein directly into the milk solution.

The results showed that Northrop's pepsin (50,000 units per gram) gave salted-out films having at least 90% of the activity of the original protein. On the other hand, with a commercial grade of pepsin (8000 units per gram) salted-out films were obtained which gave (per gram) four times the activity of the impure pepsin applied to the salt solution, although they were still only about 60% as active as the crystalline pepsin.

The production of salted-out films may thus prove to be a rapid and convenient microtechnique for purifying small amounts of various proteins. By the choice of the proper salt and concentration it should be possible to separate proteins from one another and to measure the amounts.

Summary

When a glass or metal plate is dipped into a protein solution, then into a concentrated solution of a salt such as ammonium, sodium or magnesium sulfate, or sodium chloride, a compact film of protein often 200–1000 Å. thick is salted out onto the plate. This can be fixed by 1% tannic acid and can then be washed with water without loss. After it has dried the thickness can be determined optically by interference colors. Insulin films, formed by using 1% sodium chloride solution, need no tannic acid treatment.

Salted-out protein films, in some cases up to 7000 Å. in thickness, are formed on the surface of salt solutions by applying a few drops of the protein solution. These can be deposited as hydrous B-films or as lifted A_L films onto plates or small pieces of metal foil, and they can thus be transferred to clean water surfaces where the amount of the protein can be measured from the area of the monolayer produced, or they can be fixed by tannic acid, washed and dried and the amount of protein per sq. cm. determined optically.

The refractive index of dried salted-out films of crystalline egg albumin was found to be 1.48. This low value and the power of the film to absorb hydrocarbon vapors indicate that the dried film has a skeleton-like structure.

The formation of these films at the surface of the solution or on a plate is aided by the concentration gradient in the salt solution. This carrying effect is analogous to the Ludwig–Soret phenomenon by which a solute originally uniformly distributed through a solution tends to concentrate in a portion of the solution which is cooled.

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NOTES

Esters of Chlorosulfonic Acid[1]

BY W. W. BINKLEY WITH ED. F. DEGERING

While the lower alkyl esters of chlorosulfonic acid have been prepared, they have not been purified by redistillation and there is some disagreement in the values of the physical constants which have been reported.[2] We have therefore again prepared these substances and have purified them by careful rectification using a modified Podbielniak column and have redetermined with care certain of their physical constants.

The esters were prepared by adding dropwise one-fifth mole of the absolute alcohol to one-fifth mole of sulfuryl chloride contained in a test-tube 20 cm. long immersed in an ice-bath, the sulfuryl chloride being agitated by a brisk current of dry air drawn through it. A rough separation of the alkyl chlorosulfonate from the dialkyl sulfate was first accomplished by distillation under diminished pressure at the lowest possible temperature. The distillate was then rectified under diminished pressure in a jacketed electrically heated column which has a continuous nichrome wire gage No. 18 for packing.[3] An average yield of 50% based on the alcohol was obtained. The characteristic physical constants for these esters and other analyses are collected in Table I.

The chlorine content was estimated by a modified Fajans method[4] after the ester was allowed to decompose in a pressure bottle containing calcium carbonate. Sulfur was determined by the method of Carius.

All the lower normal esters of this series are lachrymators. The tear-producing effect decreases as the length of carbon chain is increased.

(1) Abstracted from a portion of a thesis submitted by W. W. Binkley in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry, June, 1939.

(2) Bushong, *Am. Chem. J.*, **30**, 212 (1903).

(3) Podbielniak, *Ind. Eng. Chem., Anal. Ed.*, **5**, 119 (1933).

(4) Mellon, "Methods of Quantitative Chemical Analysis," The Macmillan Co., New York, 1937, p. 297.