THE ADSORPTION OF PROTEINS AT OIL-WATER INTERFACES AND ARTIFICIAL PROTEIN-LIPOID MEMBRANES

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Emulsions of oil in water can theoretically be thermodynamically stable only if the interfacial surface tension γ between the two phases is zero. This follows from the fact that the pressure in a droplet of radius r exceeds that in the surrounding liquid by an amount

$$\Delta p = 2\gamma/r. \tag{1}$$

When two drops coalesce the pressure within the drops decreases and there is a decrease in free energy.

If a liquid is heated to its critical temperature γ vanishes when its separate phases disappear. Similarly if phenol and water are mixed and heated to the critical solution temperature the two liquids become miscible when $\gamma = 0$. These considerations would seem to indicate that all emulsions must be inherently unstable or metastable since on the one hand stability requires $\gamma = 0$ and on the other hand $\gamma = 0$ requires that there shall be only one phase.

Closer examination, however, shows that even when $\gamma = 0$, droplets may be stable and there may be no tendency for the separate phases to disappear.

If an insoluble non-volatile substance is spread as a monolayer at an air-water interface of surface area S, the lowering of surface tension may be looked upon as being due to a spreading force F exerted by the adsorbed molecules as the two dimensional analog of a pressure. Here F is defined by

$$F = \gamma_0 - \gamma \tag{2}$$

where γ_0 is the surface tension of the pure solvent and γ is that of the surface covered by the monolayer.

In general F depends upon σ, the number of molecules/cm.^{2}, in the monolayer and upon the temperature. The relation connecting these three quantities may be called an equation of state, and is the two dimensional analog to the equation which relates the pressure with the density and temperature of a gas.

If the free surface S is altered, as by moving a barrier, the total number of adsorbed molecules $S\sigma$ remains constant. Thus for insoluble adsorbed substances F depends on S in a manner that can be calculated from the equation of state: In general F increases rapidly as S decreases.

A substance which lowers the surface tension of water but is appreciably soluble in water also forms an adsorbed film on the surface, and F is still related to σ and T by an equation of state. But now if the free area S is decreased $S\sigma$ does not remain constant, for some of the adsorbed molecules can go into solution. The relation between the properties of the adsorbed film and the concentration c of the dissolved substance in the solution is given by Gibbs' equation

$$dF/d \ln c = \sigma kT \tag{3}$$

By combining this with the equation of state so as to eliminate F it is possible by integration to obtain an "adsorption isotherm" which gives σ in terms of c and T. On the other hand, if we eliminate σ we obtain an equation which we may call the "force-isotherm" that expresses F as a function of c and T.

With a solubility as great as one part per million, the amount of solute in the water phase is usually far greater than the amount adsorbed and therefore if S is changed c does not change appreciably and F remains constant. Thus even a slight solubility may have a profound effect on the relation between F and S.

Consider a droplet of oil in water containing a soluble substance which tends to be adsorbed at the oil-water interface. Thus σ in equation (3) is positive and F increases (γ decreases) as c increases. In some cases it may be possible to increase c to a point where $\gamma = 0$. Then no work is required to break a droplet into small ones. The total interfacial area is increased by subdivision of the drops but γ remains zero since new adsorbed substance at the interface can be taken up from solution.

Let us now take the case in which the substance adsorbed at the surface of the oil drops is insoluble in both the water and the oil. Consider a single droplet that has N molecules adsorbed on its surface S, so that

$$\sigma = N/S \tag{4}$$

With a sufficiently large value of N it is possible that F may be so great as to make $\gamma = 0$, which will occur if $F = \gamma_0$.

It is now no longer necessary that the two phases should disappear. Consider, for example, a droplet of spherical form for which $\gamma = 0$. If the drop becomes distorted its area increases, and therefore σ and F decrease and γ becomes positive. Any distortion of the drop from the spherical form brings into existence a force which causes the drop to return to spherical form.

If the amount of adsorbed substance is increased beyond that needed to make $F = \gamma_0$, or $\gamma = 0$, for the spherical drop, the drop will automatically increase its surface by assuming some non-spherical form, but γ remains zero.

According to this theory it seems possible to explain the existence of stable oil-water emulsions having an insoluble emulsifying agent adsorbed on the surfaces of the drops which have zero surface tension.

Protein Monolayers on Water

Henri Devaux in 1903 found that egg albumin forms insoluble monolayers at an air-water interface. In a recent paper[1] he described experiments to measure the degree of insolubility. Egg albumin monolayers formed either by spreading from a solid fragment of the substance or from dilute solutions can be reversibly compressed to one-half area.

Since this protein is normally soluble in water, one would expect from equation (3) that the solubility would be enormously increased by compressing the film. For example, let us consider a protein monolayer at $F = 0$ having an area of 1.0 sq. meter per mg. and assume a molecular weight of 35,000. This gives $\sigma = 1.7 \times 10^{12}$ molecules/cm.^{2}, and equation (3) at 20°C. gives $d \ln c = 220\, dF$. A compression of the film from $F = 0$ to $F = 15$ should thus increase the solubility by a factor of 10^{95}.

[1] Devaux, H., *Compt. rend. Acad. sc.*, 1935, **201**, 109.

Actually, however, the monolayer at all pressures seems to be wholly insoluble. In fact, the process by which soluble egg albumin spontaneously spreads to form a monolayer on the surface appears to be irreversible. Devaux showed that, after compression of the monolayer to about one-fifth of its area, the area returns nearly to its original value when the force is removed. With a compression to less than one-fifth area, there is a gradual loss of the power to expand completely and one can then observe minute folds in the film which become more pronounced the greater the degree of compression. However, even if the film is compressed to one-twelfth area or less there is no evidence of any solution in the underlying water. By crumpling the film into a very narrow strip it can be lifted off as a thread or fiber by means of a platinum wire. If this is placed, either wet or after drying, upon a fresh surface of water dusted with talc it is seen that there is no tendency whatever for this substance to spread.

Experiments by Mr. Schaefer in this laboratory with egg albumin, pepsin, insulin, and other proteins have confirmed this complete insolubility of protein monolayers.

Devaux,[2] with very dilute aqueous solutions of egg albumin in a tray 2 or 3 mm. deep, found that at concentrations greater than 10^{-7} parts by weight a monolayer is gradually formed which is thicker than that which corresponds to $F = 0$. The diffusion of the protein to the surface at these low concentrations is very slow, so that in each experiment the solution was allowed to stand for 24 hours before the monolayer was tested. A definite portion of the monolayer between two barriers was then allowed to expand by moving these barriers apart, until the force F fell to zero as indicated by the action of talc when blown upon lightly. It was found that the higher the concentration of the solution the greater the expansion ratio. Multiplying this ratio by 11 Å, the thickness of the monolayer at $F = 0$, Devaux obtained the values given in the second column of Table I. With solutions more concentrated than 10^{-6} it was necessary to replace the protein solution under the film by pure water in order to avoid the diffusion of a new supply of protein to the surface during the expansion of the film.

[2] Devaux, H., *Compt. rend. Acad. sc.*, 1935, **200**, 1560.

If we take Philippi's value of 1.15 sq.m./mg. for an egg albumin monolayer at $F = 0$ and use the ratio of expansion as found by Devaux, we obtain the data in the third column.

The data in the last column are the approximate values of F needed to compress egg albumin monolayers to the various thicknesses given in column 2. These are rough determinations by Mr. Schaefer made with a commercial grade of egg albumin.

It is evident that although the reaction between a protein solution and a monolayer is irreversible the pressure F builds up in 24 hours to a value that varies with the concentration of the underlying solution. A kind of pseudoequilibrium results.

TABLE I

The Formation of Monolayers on Egg Albumin Solutions

Protein concentration parts by weight	Film thickness Å	m²/mg.	F
10^{-2}	46	0.28	33
10^{-3}	30	0.42	29
10^{-4}	24	0.53	25
10^{-5}	20	0.63	22
5×10^{-7}	17	0.75	18
4×10^{-8}	13	0.97	4
—	11	1.15	0

The rate of change of F with log c, as given in Table I, corresponds to a value of $\sigma = 4 \times 10^{13}$ molecules/cm.² according to equation (3), in the range of c from 10^{-2} to 10^{-6}. Combining this with the data in the third column, we find that the variation of F is as great as if the molecular weight of the proteins ranged from 2000 to 5000 However, since equation (3) is derived from thermodynamic principles, there is no reason to expect it to be applicable to the irreversible phenomena of Table I; so the foregoing calculation is of doubtful significance.

Protein Monolayers at Oil-Water Interfaces

The property of proteins of forming insoluble monolayers should make these substances particularly suitable for testing our hypothesis that stable emulsions can be formed containing droplets having zero surface tensions.

A third paper by Devaux[3] describes experiments which have an intimate bearing on this problem. He took a solution of 10^{-5} parts by weight of egg albumin in water in a flask, added some pure benzene, and shook it. With this concentration of protein the droplets of benzene rise, join with one another, and form a separate phase floating on the water. The protein is adsorbed at the interface. If the amount of protein is sufficiently small in relation to the area of the interface, the interfacial surface appears clear and brilliant. By increasing the amount of protein or decreasing the area of the interface, the surface becomes suddenly dull and cloudy, and when agitated the cloudiness varies irregularly over the surface. Devaux determined the amount of protein necessary to produce this peculiar appearance, and concluded that the amount needed is just sufficient to cover the interface with a monolayer of from 9 to 22 Å thickness, depending upon the pH of the solution. The cloudy appearance of the surface when the amount of protein exceeded a definite limit is due to the presence of minute folds of the protein membrane in the interface. The presence of folds at the interface should occur when $\gamma = 0$ or when $F = \gamma_0$.

We have repeated these experiments of Devaux, using a long tube about 25 cm. long and 3 cm. in diameter. This tube is filled about one-third with protein solution and about one-quarter with benzene. After shaking, the tube is held in a horizontal position until the phases separate. By tilting the tube gradually toward the vertical position, the interfacial area can be decreased progressively until at a critical angle the cloudy appearance described by Devaux appears. We have also tried the Devaux experiment using light mineral oil and water and obtained substantially the same results.

With higher concentrations, such as 10^{-3}, the droplets of benzene do not readily join together. They obviously have a very low surface tension, and a few drops are often seen which are drawn out into sharp points with a cloudy membrane over their surface, just as would be expected if the interfacial tension were zero. However, even with concentrations of protein considerably above the critical value most of the droplets seem to have definitely a positive surface tension.

[3] Devaux, H., *Compt. rend. Acad. sc.*, 1936, **202**, 1957.

Since γ_0 for benzene-water is about 35 dynes/cm., the adsorbed protein film at the interface must be very highly compressed, and it seemed possible that at such high pressures irreversible changes occur like those observed at air-water interfaces causing F to decrease gradually below γ_0. It would therefore seem better to use a pair of liquids that have a lower interfacial tension than water-benzene.

Protein Membranes

For many biological problems it would be desirable to have one or two monolayers of protein forming a membrane between two aqueous phases. Such a membrane may be expected to have some of the properties of a cell surface. It would be interesting to study its electrical properties and its permeability for various substances.

Mr. Schaefer and one of us, some months ago, made preliminary experiments in the effort to obtain such membranes. We found that a platinum wire loop (8 mm. diameter) or a plate perforated by small holes, when lifted up through water on which there is a monolayer of egg albumin subjected to a pressure of 30 dynes/cm. or more, retained a film like that from a soap solution. These films could frequently be dried in air without rupture. They were very difficult to see, for they reflected practically no light, as their thickness was far less than the wave length of light. Under the microscope with dark field illumination they became visible when punctured, for light was scattered from the edges of the hole in the protein film. Films of this kind in air do not seem to meet the needs of the biologist. We were not able to immerse such films in water without rupturing them.

Attempts to lower a platinum loop or perforated plate into water covered with a protein monolayer seemed in some cases to give membranes, but they were so fragile that the method did not seem promising.

We have recently attempted to build protein membranes by lowering loops or perforated plates through the interface between benzene and water at which there is an adsorbed protein monolayer, at a concentration which gives approximately $\gamma = 0$.

A 0.1 per cent solution of egg albumin in water in a beaker was covered with a layer of benzene. After a short time, on slightly agitating the beaker, the characteristic cloudy appearance of the inter-

face was seen. On lowering a wire loop through the interfacial film a membrane was formed which lasted at best about 20 seconds. It seemed that when the protein monolayers on the opposite sides of the loop came into contact by the gradual up-flow in the intervening benzene film, the membrane ruptured. Observation of the meniscus at the benzene-water interface during the lowering of the loop proved, however, that the interfacial surface tension was not zero. If it had been zero, there should have been no meniscus.

During the lowering of the loop through the interface there must be a radial in-flow of the protein monolayer toward the loop. These highly compressed protein films seem to possess a certain degree of rigidity like those on the surface of water, so that the surface tension may not equalize itself over the whole surface.

If the film is stretched by lowering the loop, the taking up of new protein from the underlying solution seems to be a very slow process especially when the film is highly compressed. For example, Devaux found that fragments of solid egg albumin would build up films of a thickness of 65 Å after 24 hours. Some experiments that we have made showed that within 10 minutes the presence of a solid fragment of egg albumin on the water surface raises F to about 18 dynes, which would correspond to a film of a thickness of only about 17 Å. The rate of arrival of protein molecules into the monolayer from the underlying solution probably slows up very rapidly as the thickness of the monolayer increases.

It seemed to us therefore that it would be desirable to have an interfacial film which had a surplus of folds in it before we attempted to build a membrane. We found that this could be done by lowering a chromium plated slide through the benzene-water interface, holding it under the water for a short time, and lifting it out again. The surface of the plate had been polished and made hydrophobic by covering it with molten ferric stearate and rubbing vigorously with a clean cloth to remove all but a monolayer of stearate.

The effect of lowering the plate through the interface was to carry down a film of benzene and to stretch the interfacial film, thus decreasing its thickness so that new protein could rapidly be taken up by the film. On raising the plate, the protein monolayer which had been carried down by the plate returned to the interface between the bulk

phases of the water and the benzene, producing a circular area in the middle of this interface which was obviously greatly folded and far more cloudy than the surrounding surface. This *prestretching* of the surface gives an interfacial area much larger than the apparent one. After 10 to 20 seconds, however, the folds that were produced in this way seemed gradually to disappear, leaving the interface in its original condition. If the platinum wire loop was lowered through the interface, within a few seconds after prestretching, the membrane formed on the loop persisted for about 1 minute. While the wire frame was being lowered the folds produced by the prestretching were used up during the formation of the first part of the membrane. During this time there was practically no meniscus. The remaining portion of the membrane was formed under slight tension, as indicated by the meniscus; and this was probably responsible for the subsequent rupturing. When the membrane did break it disappeared completely.

In cell surfaces, besides proteins, there are present such substances as sterols and lecithin, which may be responsible for the stability. We tried some experiments therefore in which we added cholesterol to the benzene, but without appreciably improving the results.

The addition of 0.5 mg. of lecithin per cc. of benzene greatly improved the results. A layer of this solution over a 0.1 per cent solution of egg albumin in water gave membranes which lasted several minutes without rupturing and when they finally broke it was seen that there was only a small hole near the center of the film. The hole, however, grew rapidly in size until the Devaux crinkling effect was seen around the edges, indicating that the interfacial surface tension had then become zero. A partly ruptured membrane having a hole covering one-fifth of its area remained unchanged for several hours.

The rupturing of these membranes was overcome by prestretching the interfacial film between the dilute lecithin solution and the aqueous protein solution. The hydrophobic chromium plate is lowered into the interfacial film for about one-half minute. On withdrawing it there is a large circular area on the interface having a great number of folds which show little tendency to disappear, indicating that the lecithin greatly stabilizes the interfacial film and prevents it from contracting or collapsing. If a wire loop is dipped into the vicinity of these folds, a membrane is formed which, although covering the

total area of the wire loop, shows the Devaux cloudiness. These membranes if undisturbed show no tendency to rupture. We made several of these membranes on separate wire loops suspended in the water phase from the sides of the beaker. By perfusing the vessel with a very slow stream of distilled water, the benzene, the interfacial film, and the protein solution were slowly washed away, leaving the intact membranes in pure water. The benzene which was originally between the two protein monolayers was presumably dissolved by the water, since it is appreciably soluble in water. The membrane probably therefore consists of two layers of protein separated by one or two monolayers of lipoid.

The failure to obtain good protein membranes without the lecithin and the disappearance of the folds in the interface produced by prestretching are probably caused by a gradual crumpling of the protein monolayer at the interface due to formation of cross linkages between the molecules, resulting in a kind of denaturation of the film. The difficulty thus seems to be due to the fact that $F = 35$ is too large a spreading force for a pure protein to stand indefinitely. A pair of liquids showing lower interfacial energy might solve the problem. The lecithin, however, seems to enable the film to withstand this pressure.

It seems possible that by the addition of a substance that would lower the interfacial tension a similar result might be obtained. We tried the effect of the addition of ethyl alcohol, but we did not find any improvement in the protein films by its use. This may well be due to a displacement of the adsorbed alcohol by the protein.

5 ml. of saturated cetyl sulfate solution injected into 100 ml. of the protein solution under the benzene made it much easier to get the Devaux effect and it persisted longer. Traces of such substances may facilitate the building of membranes. Using an egg albumin solution without added substances the Devaux effect produced by gently tapping the beaker was very much more pronounced after the protein solution had been allowed to stand for 24 hours in contact with the benzene. This suggests that after long periods of time the value of F can finally rise to 35 in spite of the initial tendency to collapse.

Stabilization of Oil-Water Emulsions by Proteins

Observations of the surface tensions of globules of benzene in dilute protein solutions seemed to indicate that in most cases the surface

tension was not quite zero. The difference in density between the two liquids, however, somewhat interfered with the interpretation of these results. We therefore added enough carbon tetrachloride to the benzene to make the density nearly that of water, and this was then shaken in about twice its volume of water. Upon addition of a few drops of 1 per cent egg albumin solution the tube was gently shaken and allowed to stand. The hydrocarbon phase remained suspended almost motionless in the form of large drops throughout the aqueous phase. Some of these drops were not even approximately spherical, but were of very irregular shape, showing that the surface tension was actually zero. In some cases it was found that a large drop of the hydrocarbon phase contained a large drop of water inside it. It would seem that by perfusing such a double drop with water it would be possible to dissolve out the benzene and the carbon tetrachloride, leaving a double shell of protein which would have considerable resemblance to a cell wall.

We believe that the technique we have described for building protein membranes across holes in plates may be useful to the biologist in the study of the properties of these membranes. Such a plate having a hole covered by a membrane could form the partition between two separate aqueous solutions, so that permeabilities and conductivities, etc., could be studied.