[CONTRIBUTION FROM THE RESEARCH LABORATORY, GENERAL ELECTRIC COMPANY]

Activities of Urease and Pepsin Monolayers

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Many proteins, including urease and pepsin, can be spread on an air–water interface to form monolayers covering areas of the order of 1 m.²/mg. under a force F of about 2 dynes/cm. The amount of protein per sq. cm. in such a film is sufficient to form a layer 8 Å. thick of dry protein of a density 1.3. These protein films are very compressible, so that the area decreases to about one-half at $F = 25$ and to one-quarter at $F = 34$.

Monolayers of egg albumin, under a compression of $F = 16$ dynes/cm., have the characteristics of a 2-dimensional plastic solid capable of withstanding shearing stresses of 0.7 dyne/cm. with only elastic deformation, the elastic modulus being of the order of 10 dynes/cm. Larger shearing stresses cause flow at a rate proportional to the increment of shearing stress, the coefficient of viscosity being of the order of 400 g. sec.$^{-1}$.

Urease and pepsin form monolayers which have mechanical properties very similar to those of egg albumin. These monolayers appear to be wholly insoluble in water, even when subjected to pressures of $F = 30$ or more which gradually crumple the film.

If the protein films were in thermodynamic equilibrium with an underlying protein solution, the solubility c should increase with F in accord with Gibbs' equation

$$dF/d \ln c = \sigma kT \qquad (1)$$

where σ is a measure of the amount of protein per unit area in the film in terms of the molecular units which exist *in solution*.

Assuming a molecular weight of 35,000 for the protein in the solution, a film having a specific area of 1 m.²/mg. corresponds to $\sigma = 1.7 \times 10^{12}$ molecules/sq. cm. (regardless of any assumption regarding the molecular weight of the protein in the monolayer). Thus at 20° there should be a 3×10^{14}-fold increase in solubility for each increment of 1 dyne/cm. in the value of F.

Since even at the highest compression these protein monolayers have no measurable solubility, we must conclude that the spreading of a monolayer of protein from solution is a process involving irreversibility to an extraordinary degree.

It is apparently generally believed by those familiar with the physics and chemistry of proteins that the spreading of a protein to form a monolayer causes its denaturation. The insolubility is in some cases taken as the criterion of denaturation. When proteins such as egg albumin are shaken with water forming a froth, the protein is gradually converted to an insoluble form in which it has lost its antigenic properties. It should be noted, however, that continued shaking not only produces monolayers on the surfaces of the bubbles but crumples and destroys the monolayers when the bubbles break. It thus seems possible that the observed loss of chemical and biological activity may be due to the *destruction* rather than to the *formation* of the monolayer.

Gorter[1] has found that pepsin and trypsin monolayers lifted off the surface of water on a wet silk fabric, transferred to a casein solution and shaken, possess nearly the full peptizing activity. This may indicate either that the monolayer is active or that the monolayer has been reconverted to a soluble form possessing the full activity, *i. e.*, the spreading process has been reversed.

Wrinch's theory,[2] according to which proteins have a characteristic 2-dimensional network structure, suggests that the synthesis of proteins involves the adsorption of amino acids or other constituents upon a protein template, very much as the outer layer of atoms in a growing crystal has its structure determined by the surface lattice of the underlying layer. The fact that the tobacco mosaic virus is produced, under proper conditions, in the tobacco plant by an autocatalytic reaction lends further support to this hypothesis. Northrop's observation[3] that the formations of pepsin and trypsin from their precursors are autocatalytic reactions is additional evidence of the same kind.

Unless we assume some mysterious and improbable action at a distance it seems inconceivable that a molecule of pepsin of molecular weight 39,000 and diameter of 46 Å. can act as a nucleus to determine the growth of another molecule like itself unless at some stage in the process it is spread out into a thin sheet so that all of its parts are made accessible.

(1) E. Gorter, *Trans. Faraday Soc.*, **33**, 1125 (1937).
(2) D. M. Wrinch, *Proc. Roy. Soc.* (London), **A160**, 59 (1937).
(3) J. H. Northrop, *Physiol. Rev.*, **17**, 144 (1937).

This mechanism requires, however, that the molecule in the extended form should retain its specificity. Such considerations have led us to make the hypothesis that monolayers of proteins in general are highly reactive.

To test this hypothesis we have made experiments with urease and pepsin, two enzymes whose activity can be tested easily by simple chemical means.

The techniques which we have described recently for depositing protein monolayers or multilayers on properly prepared barium stearate surfaces[4] and for adsorbing protein monolayers from solution on conditioned plates[5,6] seem to be particularly adapted to a study of the reactivities of protein films made in various ways.

I. Urease Monolayers

A solution of crystalline urease was kindly given to us in July, 1937, by Dr. James B. Sumner and was kept in a refrigerator. Unfortuntely the experiments described below were made in September and November. At the latter time there was a precipitate in the solution and the urease had lost much of its activity, so further experiments were discontinued. We hope to make quantitative measurements of the activities of fresh samples of urease. The data given here are qualitative and of a preliminary nature only.

Films of urease were produced in various ways on an area of about 1 sq. inch (6.3 sq. cm.) of a metal or glass plate. The reactivity of the film was determined qualitatively by dipping the plate into 25 ml. of a 0.1% urea solution containing 0.1 mg. of phenol red, the solution having been adjusted to *p*H 7 by the addition of a trace of potassium bicarbonate.

Some of the urease films were found to be so active in converting urea into ammonium carbonate that within thirty seconds the surface of the plate was covered by a thin film of solution in which the indicator had changed to a deep red color. This layer gradually increased in thickness and was slowly carried into the bulk of the solution by convection currents, but even after five minutes the color of the solution as a whole had changed very little. No change of color occurred if the urea was left out or if the urease film was not placed on the plate.

(4) I. Langmuir, V. J. Schaefer and D. M. Wrinch, *Science*, **85**, 76 (1937).

(5) I. Langmuir and V. J. Schaefer, THIS JOURNAL, **59**, 1406 (1937).

(6) I. Langmuir and V. J. Schaefer, *ibid.*, **59**, 1762 (1937).

The urease films whose activities we have studied in this way were of two classes:

1. **Deposited Monolayers of A or B Types.**— These are monolayers that are transferred from a water surface to a prepared plate (PR) by dipping (A during down-trip, B during up-trip). At low film compressions ($F < 5$ dynes/cm.) it is difficult to deposit uniform films on PR by dipping. We find, however, that even at $F = 0.4$ good A films can be deposited by holding the plate face down in a nearly horizontal position and lowering it onto the surface of the water in a tray covered with a monolayer of urease. The surface pressure is adjusted to some predetermined value, such as $F = 0.4$, by placing a small drop of a calibrated indicator oil[7] on the surface after having spread the protein and then moving a barrier until the indicator oil changes to the color that corresponds to this particular value of F. Before lifting the plate from the water it is important to remove the protein film from the surface surrounding the plate, by sweeping with barriers and blowing away any residual film, so that no B-layer of protein is deposited when the plate is lifted.

The film produced by this technique is an A-type of film, for it presumably has the same orientation as that deposited on a vertical plate during the down-trip into water. We shall describe a film of this kind as a *lifted film*, and denote it by A_L.

One would expect from the manner of formation of these films that the A- or A_L-layer would be hydrophilic and the B-layer hydrophobic. Measurements of contact angles, however, when a drop of water or other liquid is placed on the surface, show practically no difference in this respect. Protein films deposited at very low pressures are often hydrophobic, whether of the A or the B type, whereas when pressures of 15 dynes or more are used both A- and B-films are hydrophilic. These observations seem to indicate that the protein monolayers can overturn, *i. e.*, the hydrophilic and the hydrophobic groups in the molecule are able to come to or be buried below the surface according to the nature of the substance that is brought in contact with the deposited monolayer.

It is of interest to know whether the chemical activity of the urease film depends on the orientation of the hydrophilic and hydrophobic groups in the monolayer. We find that it is often possible

(7) I. Langmuir and V. J. Schaefer, *ibid.*, **59**, 2400 (1937), see p. 2403.

to anchor monolayers, by properly conditioning the underlying surface, so that the monolayer cannot overturn.

A barium stearate film conditioned by dipping for ten to thirty seconds into a thorium nitrate solution (0.001 to 0.1 M, pH 3) is hydrophilic when removed from the water[6] but becomes hydrophobic after drying, indicating that the thorium atoms and the carboxyl groups attached to them have been drawn down between the hydrocarbon chains. If after the thorium treatment, but before drying, the plate is treated with 1% sodium silicate and washed, the surface remains extremely hydrophilic, even after drying; *i. e.*, it can readily be wetted by water. This suggests that the silicic acid binds the thorium atoms together so they cannot bury themselves in the hydrocarbon part of the film.

By conditioning a barium stearate film in this way with thorium silicate we produce a surface which attaches itself so firmly to the hydrophilic side of a B-protein layer that this layer cannot overturn, and thus the hydrophobic side remains uppermost.

On the other hand, it is possible to so anchor the hydrophobic parts of the urease monolayer that the hydrophilic side is uppermost. This can be done by first conditioning the barium stearate plate with thorium, washing with water, and then dipping into a 0.1% solution of sodium desoxycholate adjusted to a pH of 6.5. This solution wets the plate, but as soon as the plate is washed with distilled water (pH 5.8) the water peels off suddenly and the plate is then very hydrophobic, giving a contact angle with water of 75°.

Desoxycholic acid has a particularly strong tendency to combine with CH_2 groups in hydrocarbon molecules, although it apparently has little affinity for CH_3 groups at the ends of hydrocarbon chains. When an A-layer of a protein is deposited on a surface conditioned with desoxycholate, the hydrophobic groups in the side chains of the protein are firmly anchored and prevented from turning over. Experiments with many proteins have shown that A-layers deposited on desoxycholate-conditioned surfaces are very hydrophilic, whereas protein B-layers deposited on thorium silicate are hydrophobic, except at high values of F.

Since it was desired to determine the thickness of the urease monolayers whose activity was to be measured, these layers were deposited upon one side of chromium-plated slides, 1 × 3 inches (2.5 × 7.6 cm.) on which barium stearate stepped films of critical thickness 45, 47 and 49 layers have been built. The methods of producing these films and measuring their thickness by using monochromatic light have been previously described.[8-10]

Table I gives a summary of the experimental results. The second column describes the type of urease film and its substrate. Here PR represents the prepared plate with the requisite number of barium stearate layers; C denotes the conditioning layer, further described in the parenthesis. The symbols A_L and B describe the method of depositing the urease, A_L being a lift film of A type and B a film deposited on an up-trip (the monolayer having been spread on the water after the down-trip).

TABLE I

ACTIVITIES OF DEPOSITED UREASE MONOLAYERS

Expt.	Substrate and film	F	Hydrophilic? Å.	Before	After	Reactivity
1	PRA_L	0.4	21	No	No	Strong
2	PRC(ThO_2SiO_2)B	.4	18	Yes	No	Strong
3	PRC(ThO_2SiO_2)A_L	.4	11	Yes	Yes	Slight
4	PRC(ThO_2Desoxy)A_L	.4	7	No	Yes	None
5	PRC(ThO_2Desoxy)A_L	16.0	43	No	Yes	Slight

The urease monolayers were spread on water (quartz distilled, pH 5.8) by Gorter's method or by wetting a narrow band along the edge of a glass or nickel plate with the urease solution and then dipping this edge slowly into the water in a tray. The monolayer, even at $F = 0$, is a plastic solid which tears into a jagged star-shaped figure when a small drop of indicator oil is placed on the surface.

The third column of the table shows the surface pressures used in depositing the monolayers. The barium stearate layers upon which the protein film was deposited were built from monolayers of stearic acid spread on distilled water containing $10^{-4}M$ barium chloride, 2×10^{-4} potassium bicarbonate and $10^{-6}M$ potassium cyanide, pH 6.8. In experiments such as those we have made previously with sterols,[9] where it was not necessary to condition the surfaces with thorium dioxide, we have found it desirable to add $2 \times 10^{-6}M$ cupric chloride, since this makes it possible to dip the plates repeatedly into clean water without loss of stearic acid. However,

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

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

(10) K. B. Blodgett, *J. Phys. Chem.*, **41**, 975 (1937).

the presence of copper greatly interferes with the conditioning treatment with thorium nitrate and therefore in the present experiments we omitted copper and purposely added potassium cyanide to eliminate possible traces of copper from the brass tray (although the surface was covered by paraffin and glass).

The thickness given in the fourth column is that of the A_L or B layer alone. The increment of thickness due to the C layer was measured in separate experiments by drying the plate after the conditioning treatment. The $C(ThO_2SiO_2)$ gave about 11 Å. and the $C(ThO_2Desoxy)$ 18 Å.

In carrying out the conditioning treatment it is important to avoid dipping a dry or hydrophobic film into any solution without cleaning the surface of the solution by means of a barrier just before the dipping. Otherwise monolayers of some contamination may be deposited on the plate as an A-layer on the down-trip. In the case of the ThO_2SiO_2 conditioning our procedure is to put the thorium nitrate solution into a carefully cleaned cylindrical glass vessel (5 cm. deep, 10 cm. in diameter) having a ground upper rim. Two cleaned glass strips as barriers are moved several times across the surface and the prepared plate is immersed into the solution near the rim just after the barrier has been moved to produce a fresh surface. The plate is kept thirty seconds in the solution and can be removed without special precaution. It is immediately washed in a running stream of distilled water. As long as it is kept wet there is no danger of its acquiring a deposited monolayer from accidental contamination. The sodium silicate solution (or the sodium desoxycholate) is applied in the form of drops from a dropper directly onto the wet surface. The surface is again washed with a stream of distilled water.

The fifth and sixth columns of the table give data on the hydrophilic or hydrophobic character of the plate before and after depositing the urease monolayer.

The last column describes the reactivity of the film as evidenced by the development of a red color when the plate was immersed in the urea solution containing phenol red. Check runs were made for Expts. 1 and 4 with essentially similar results.

It is seen from these data that the activity of the monolayers depends greatly on the type of film deposited. The strongest action was obtained in Expts. 1 and 2 which gave hydrophobic films. When the hydrophobic groups in the monolayer were anchored by desoxycholate in Expt. 4, leaving only hydrophilic groups on the surface, there was no detectable activity. In Expt. 5, where the film was highly compressed, $F = 16$, before being deposited, the thickness had risen to 43 Å. In such a thick film the number of hydrophobic groups per unit area is presumably greater than can be anchored by the desoxycholate and the unanchored ones account for the observed slight activity.

The low activity of the A_L film in Expt. 3 as compared with that of Expt. 1 may be due to an anchoring action of the $C(ThO_2SiO_2)$ on some of the hydrophobic groups. Barium stearate surfaces, PR, are oleophobic and hydrophobic but $PRC(ThO_2SiO_2)$ surfaces when dry are highly oleophilic as well as being hydrophilic. Thus a PRC surface should anchor hydrophobic groups more effectively than does a PR surface.

These experiments suggest that the activity of the urease in decomposing urea is associated with the hydrophobic groups among the side chains, and that when these groups are not on the surface the activity is lost.

It is of interest to form some estimate of the relative activities of urease monolayers and urease in solution. According to Sumner, 7.4×10^{-6} g. of crystalline urease is equivalent to 1 unit of urease which by definition produces 0.2 mg. of ammonia per minute from a urea–phosphate solution at 20° and *p*H 7. If we assume that the 1 sq. inch (6.25 sq. cm.) of urease monolayer in Expt. 1 consisted of pure urease, it would correspond to 0.24 urease unit and if distributed uniformly throughout the solution should produce 4.8×10^{-5} g. of ammonia per minute which would be equivalent to 2.8 ml. of 0.001 *N* hydrochloric acid solution per minute. We have recently placed 0.1 ml. of 0.001 *N* ammonium hydroxide solution on a 1 inch square (6.25 sq. cm.) of filter paper, and immersed this in a 0.1% urea solution with phenol red. The coloration during the first minute was approximately like that observed in Expt. 1. The activity of the monolayers is thus about 0.02 of that which we might expect from a similar amount of pure urease in solution. The lower activity of the monolayers may, however, be due to a loss of activity of the urease solution during the three and one-half months it was kept before use.

The activity of urease, being that of a catalytic enzyme, should continue indefinitely unless acted on by some poison.

In our experiments, however, we found that the activity of the monolayers on the plates gradually decreased. The plate was usually left in the solution for five minutes during which the film of red liquid slowly reformed each time after it was removed by agitation. At the end of five minutes the plate was removed, dried and the film thickness determined. There was usually no change in thickness, but in a few cases a slight increase was observed. When the plate was put into a fresh urea–phenol red solution, the activity was found to be greatly reduced. In some cases, five minutes later, a third solution was tried, but in no case was there any activity.

These experiments were made in September, 1937. Two months later when the urease was of low activity, experiments were undertaken to see if phenol red acted as a poison toward the urease.

We tested the activity of the urease solution by taking 10 mg. of it, adding 10 ml. of 0.1% urea containing 20 drops of 0.02% phenol red. After five minutes this was titrated with 0.001 *N* hydrochloric acid. We then repeated the test except that the phenol red was not added until the end of the five minutes, just before titrating. The first test showed 3.5 units of urease per ml. of original solution, while the second gave 8.0. Other tests showed that the amount of ammonia formed within five minutes was directly proportional to the amount of urease used, indicating that the change in the *p*H of the solution during the reaction did not appreciably alter the velocity of the reaction. These results show that the phenol red acts as a catalytic poison on the urease causing the activity to decrease to $^1/_7$ value every five minutes (assuming a monomolecular reaction rate). This observation affords a sufficient explanation of the decreasing rates observed with the monolayers. With a fresh supply of pure urease and by avoiding the use of phenol red until the urease monolayer has been removed from the solution we hope to make quantitative measurements of the activities of urease monolayers of various types and to study the dependence on the film thickness.

2. **Urease S-Layers.**—A barium stearate film conditioned by thorium dioxide or ThO_2SiO_2 can adsorb proteins from *solution*.[5,6] We shall call such films S-layers. These layers, when saturated, frequently have thicknesses equal to or even greater than the diameters of the normal protein molecules, while deposited monolayers are usually far thinner. There is thus no apparent reason for believing that S-layers may not retain the specificity and reactivity of the native protein.

We have measured the activities of PRC-(ThO_2)S urease films of thickness 35 Å. and find that the activity is considerably stronger than that of PRA_1 films. The procedure for preparing the S-layers is to produce PRC(ThO_2), wash it, and while the plate is still wet place a few drops of the urease solution upon it. This is agitated or stirred for one minute with a 60-cycle solenoid vibrator and then washed in a stream of distilled water. Its activity may then be tested while wet or it may first be dried so that the thickness can be measured.

To determine the thickness of the S-layer it is necessary to know that of the underlying PRC film. The thickness of PR is readily determined, but that of the C-layer (ThO_2), which is about 4 Å., must be determined by separate experiments with other plates since the surface of PRC is made partly hydrophobic by drying.

With surfaces conditioned with ThO_2SiO_2 it is possible to dry them and determine the thickness, but it is important to wet the surface with pure water before adding drops of protein; otherwise the monolayer on each protein drop builds an A-layer on the plate when it first touches it.

Urease S-layers were formed on glass microscope slides by cleaning these in a sulfuric acid–bichromate mixture, washing well with distilled water and applying a few drops of urease solution. After one minute this was washed off with a vigorous stream of distilled water and dried. On placing this in the urea solution with phenol red, a strong reaction was observed, of about the same intensity as that found with the PRCS films. In another experiment the S-layer of urease on glass, after drying, was heated for sixteen hours to 100°. This also gave a strong reaction when immersed in the urea solution.

II. Pepsin Monolayers

Pepsin from two sources was used in these experiments; Dr. J. H. Northrop kindly supplied us with a sample of dry crystalline pepsin and we also used a commercial grade of pepsin made by Eli Lilly Company.

The activities of these samples of pepsin and of

the monolayers produced from them were measured in terms of arbitrary "units" by determining the time needed for the clotting of a standard skim milk solution into which the pepsin had been introduced. The milk solution contained 16.7% of dry powdered skim milk (Breadlac made by the Borden Company) in 0.1 M acetate buffer adjusted to pH 5 by acetic acid. The pepsin unit is ordinarily defined as the amount which causes the clotting of 6 cc. of this solution at 37° in one minute. Since the clotting time is inversely proportional to the pepsin concentration, we have calculated U, the number of pepsin units, by the equation

$$U = V/6t \qquad (2)$$

where V is the volume of the solution in ml. and t is the clotting time at 37° in minutes. We have used this equation to measure the apparent activities of monolayers even when the pepsin is not uniformly distributed throughout the solution. The activity of the sample of Northrop pepsin was found to be 50 units/mg. while the Lilly pepsin gave 8.1 units/mg.

The pepsin monolayers to be deposited on the prepared plates were spread from dry fragments or powder (Cary and Rideal's method) placed on the distilled water of the tray, which was adjusted to the isoelectric point pH 2.6 by hydrochloric acid. The monolayer was then compressed to a definite value, such as $F = 9$, as measured by a surface balance. The thickness of the deposited films was usually measured optically (within ± 1.5 Å.). During the course of the experiments it was found that slight differences in the technique of spreading films caused variations in the A_L-films, deposited at $F = 9$, from 11 to 23 Å., although the films made in any one day were uniform within 2 or 3 Å. These variations, which also occur with egg albumin and other plastic solid protein films, appear to be due to internal strains in the films and are increased by too rapid spreading. They can be avoided largely by placing a small drop of indicator oil on the monolayer near the part that is to be used for deposition and controlling the rate of application of pepsin so that no pressure is built up (a pressure of 0.2 dyne/cm. changes the color of the indicator oil).

Our first studies of the activity of pepsin monolayers were made by the following method.

Drop-on-Plate Method.—A drop of the standard milk solution is placed on a plate covered by a protein monolayer. It is important that this plate be wet with water before the drop of milk is added, for otherwise the protein monolayer on the surface of the drop forms an A-layer on top of the pepsin layer on the plate. A microscope cover glass 0.5 inch (1.26 cm.) in diameter is placed on the drop of milk, and the milk is observed with a low-power microscope, with dark field illumination. The Brownian movement of the particles of milk is very striking. With sunlight this movement can be seen even with the naked eye. At any given point the Brownian movement ceases suddenly, and shortly afterwards it is seen that the milk has clotted by a chain-like arrangement of its particles.

It is interesting to watch this clotting of a drop of milk on a plate having only part of its surface covered by a pepsin monolayer. The clotting begins close to the surface of the monolayer and soon extends throughout the thickness of the layer of milk under the cover glass. Along the boundary of the monolayer (*i. e.*, between the PRA and PR films) there is then a striking contrast in appearance over the two surfaces. Gradually, however, the clotting extends further into the milk over the PR film. It can be seen that the visible particles do not move into contact with the monolayer, but clot because of the presence of a diffusible substance, presumably pepsin liberated from the monolayer.

Pepsin monolayers were deposited on surfaces conditioned in various ways, and the clotting times t of a drop of milk (volume 0.025 ml.) were observed. The pepsin activity U was then calculated by Eq. (2). Column 4 of Table II gives the activity of the monolayers found in this way expressed in pepsin units/sq. cm. of surface. The fifth column gives the monolayer thickness determined optically. From this we can calculate the weight of pepsin per sq. cm., assuming the density to be 1.3. Dividing the activity per

TABLE II

ACTIVITY OF PEPSIN MONOLAYERS BY DROP METHOD

Plate at 37°, Lilly pepsin, $F = 9$

	Film	t, min.	10^3 U/sq. cm.	Thickness T, Å.	U/g.
1	PRA_L	3.3	1.0	14	5500
2	GB[a]	2.5	0.5	..	..
3	PRAB	2.0	1.6	35	3500
4	$PRC(ThO_2Tan)A_L$	6	0.6	18	2600
5	$PRC(ThO_2Tan)B$	4	0.8	14	4500
6	$PRC(ThO_2Desoxy)A_L$	3.3	1.0	..	..

[a] GB denotes a B-layer on a cleaned glass plate.

unit area (U/sq. cm.) by the weight per unit area ($1.3 \times T$) we get the specific activity $U/g.$ given in column 6. These data show that the specific activity of the pepsin after being spread as a monolayer is only a little less than the observed specific activity of the original substance (8000).

The activities were not greatly dependent upon the type of film or the conditioning treatment given the plate. The lowest activity was found in Expt. 4 for the pepsin on a surface conditioned with thorium nitrate followed by tannic acid (ThO_2Tan). This conditioning treatment gives a surface which remains very hydrophilic after drying.

Plate-in-Tube Method.—Monolayers of pepsin were deposited by the lift method, on one side of slides 0.5 × 2.0 inches (1.26 × 5.04 cm.) which previously had been built to critical thickness by barium stearate layers. Usually two of these PRA_L plates having a total monolayer surface of 10 sq. cm. were wetted by water after the film thickness had been measured and were then immersed, back to back, into 6 ml. of standard milk in a test-tube 1.4 cm. inside diameter. This tube was maintained at 37° and tested from time to time for clotting by gently tilting the tube. In some experiments, after a time t_0, the two plates were removed from the milk and transferred to another tube containing 6 ml. of milk. In Table III t_1 and t_2 represent the clotting times in minutes in the first and second tubes, respectively, each measured from the moment when the plates were put into that tube.

Examination of the plates removed from the milk before clotting occurred showed that the clotting began almost immediately on the surface of the plate and gradually extended to form an increasingly thick clotted layer, which adhered firmly to the plate. A PR plate (without pepsin), when immersed in milk to which pepsin had been added, did not adhere to the curd which finally formed.

The first four experiments of Table III prove that the reaction which causes clotting continues after the plates are removed from the milk. If we use the clotting time t_1 to calculate U by Eq. (2) as in Col. 4, we see that the U/sq. cm. in Expts. 1 to 4 varies roughly in proportion to the square root of t_0, so that

$$U/\text{sq. cm.} = 6.4 \times 10^{-5}(t_0)^{1/2} \qquad (3)$$

These results indicate that the rate of clotting is determined by the amount of pepsin that diffuses out through an increasingly thick layer of clotted milk. As in the case of the growth of ice on a pond the thickness of such a layer increases in proportion to $t^{1/2}$, the rate of diffusion varies as $t^{-1/2}$, and the total amount that escapes during the time t_0 varies with $t_0^{1/2}$.

TABLE III

ACTIVITY OF PRA_L PEPSIN FILMS BY PLATE-IN-TUBE METHOD

Lilly pepsin, $F = 9$, thickness = 21 Å.

Expt.	t_0	t_1	10^3 U/sq. cm.	t_2	10^3 U/sq. cm.	Total
1	>132	132	0.76			
2	100	150	.67			
3	80	185	.54			
4	30	270	.37			
5	15	600	.16	196	0.51	0.67
6	4	92	1.09	198	.51	1.60
7	1	67	1.50	239	.42	1.90
8	0.17	249	0.40	74	1.35	1.75

An examination of the data of Expts. 5 to 8 indicates that the agitation of the liquid close to the surface of the plate, incident to the removal of the plate, distributes the activity throughout the milk if this agitation occurs *before* the milk clots on the plate.

The total activity of 0.0019 unit/sq. cm. shown in Expt. 7, with a film 23 Å. thick, corresponds to 7000 units/g. which is only 13% less than the directly observed activity of the Lilly pepsin in solution.

The importance of agitation when the plates are first placed in the milk, as shown by the data of Table III, suggested the following modification of technique.

Displacement Method.—In this method the plate bearing the pepsin monolayer is immersed for two minutes in 6 ml. of standard milk in a test-tube, during which time the milk is agitated continuously by gently shaking and rotating the tube. The plate is then removed and put into a second tube of milk which is shaken for two minutes.

TABLE IV

ACTIVITY OF A_L-LAYERS OF PEPSIN. DISPLACEMENT METHOD

Lilly pepsin, shaken 2 min. and removed

Expt.	Film	F	T, Å.	t, min.	10^3 U/sq. cm.	$U/g.$
1	$2(PRA_L)$	9	12	33	3.8	24,000
2	PRA_L	9	12	69	3.4	22,000
3	PRA_L(2′ age)	45	106	14	18	13,000
4	PRA_L(16′ age)	45	106	32	8	5,800
5	$PRC(ThO_2Desoxy)A_L$	9	14	40	6.2	34,000
6	$PRC(ThO_2Tannic)A_L$	9	14	115	2.2	12,000
7	$PRC(ThO_2SiO_2)A_L$	9	14	59	4.2	23,000
8	$PRC(ThO_2)A_L$	9	14	68	3.7	20,000

It was found in most cases (all the experiments of Table IV) that no clotting occurred within ten hours in the second tube, showing that practically all the pepsin had been displaced from the monolayer and stirred into the milk of the first tube by the two-minute shaking.

In Expt. 1 of Table IV two plates were placed back to back but in the other experiments only one plate (4 cm.) was used. Experiment 2 shows that the pepsin liberated by the two plates was twice that from one.

In Expts. 3 and 4 the monolayer on the water was compressed to one-tenth of the area which it occupied at $F = 1$. This required an initial compression of about 45 dynes/cm. A film compressed to such a degree is crushed so that it will not expand to its original area if the pressure is removed. The deposited monolayers, measured optically, had about nine times the thickness of the monolayer formed at 9 dynes/cm. The film in Expt. 3 was deposited within two minutes after compressing, while that of Expt. 4 was held (at constant area) for sixteen minutes. The specific activities of these crumpled monolayers are from one-half to one-quarter that of the monolayers formed at $F = 9$, but they have by no means been "denatured."

To test the prevalent opinion that vigorous shaking causes loss of activity we placed 100 ml. of 0.1 M acetate buffer (pH 5) in a 0.5 liter bottle, added 100 mg. of Lilly pepsin and shook by a machine giving a 5-cm. vertical stroke 400 times per minute. At intervals of a few minutes the machine was stopped and 1-ml. samples were removed to test the activity. During the first ten minutes a froth filled half the bottle which subsided very slowly on standing, but after ten minutes there was relatively little frothing. The tests showed that the specific activity of the protein decreased from 8000 units/g. at the start to 4000 after one minute, to 180 after five minutes, and less than 10 after ten minutes. This corresponds to a decrease to half activity for each time interval of 0.90 minute or at the rate of 1.3% per second. Small samples taken at intervals were spread on the water in the tray by Gorter's method so as to produce monolayers. A monolayer obtained at the end of one minute was still a 2-dimensional plastic solid which gave a star-shaped figure with indicator oil. After five minutes, however, the protein monolayer formed a 2-dimensional liquid which gave a circular figure with indicator oil. Also it took a good deal more of the solution to cover the trays with the monolayer than it did with the original pepsin solution, but even after forty minutes there was no difficulty in obtaining a liquid monolayer. At that time, however, the solution was turbid from protein precipitated in a flocculent form.

Experiments 5 to 8 show that the various conditioning treatments given to the plate alter the activity in a ratio of about 3:1. As in the data of Table II the lowest activity is found with the A_L-layer on the surface conditioned with tannic acid.

Table V contains some comparative data with Northrop's crystalline pepsin. The rather surprising result was obtained that the activities U_1 calculated from t_1 in the first tube were lower than those found for the Lilly pepsin which contained less of the active substance. Furthermore, in Expts. 10 and 11 the activity observed in the second tube, to which the plates were transferred, was greater than that observed in the first tube.

TABLE V

ACTIVITIES OF VARIOUS PEPSIN FILMS BY DISPLACEMENT METHOD

$F = 9$ for all A_L-films; U_1 and U_2 are the activities in 1st and 2nd tubes.

Expt.	Nature of film	Source of pepsin	T_0, Å.	T_1, Å.	U_1 sq. cm. $\times 10^3$	U_2 sq. cm. $\times 10^3$	U g. Total
9	PRC(ThO_2Desoxy)A_L	N	14	No clot	2.95	<0.6	16,000
10	PRC(ThO_2Tannic)A_L	N	14	Thick clot	<0.7	2.3	13,000
11	PRA_L	N	14	Thick clot	<0.7	3.9	21,000
12	PRC(ThO_2)S	L	13	106	1.94	..	11,400
13	PRC(ThO_2)S	N	29	119	8.6	..	23,000
14	GS	L	..	..	2.3	..	..
15	GS dried, 20′ at 80°	L	..	..	<0.2	..	..
16	10 sq. cm. of film on H_2O	L	14	..	1.2	..	6,500

It appears probable that with the higher activity of the Northrop pepsin the milk clotted so rapidly that a thick adherent clot formed over the surface of the plate before the pepsin could escape into the solution. It is probable that a sufficiently vigorous agitation (solenoid vibrator) during the first few seconds would overcome this difficulty. This explanation receives support from the fact that in Expts. 10 and 11 but not in 9 an adherent clot was found on the plate when it was removed from the first tube at the end of two minutes of gentle shaking.

Evidently with a given intensity of agitation there is an optimum *true* activity which will give

the maximum *apparent* activity of the monolayer. If a conditioned surface could be found which holds the pepsin so firmly that little can escape, the apparent activity would be very low. On the other hand, if the surface is one which liberates the pepsin almost instantly upon contact with the milk, the pepsin concentration may become locally so high that a firmly adherent clot is formed, and so the escape of the pepsin is retarded. This is the probable explanation of the behavior of the PRA_L film in Expt. 11.

Experiments 12 and 13 give a comparison of S-layers of the two kinds of pepsin. The thickness of the adsorbed layer of Northrop pepsin is more than twice that of the Lilly pepsin. The specific activity of the L-pepsin film in Expt. 12 is much higher than that of the original substance, while that of the N-pepsin in Expt. 13 is much lower. In Table IV we see also that the specific activities of the L-pepsin monolayers (example in Expt. 1) may be three times as great as that of the original pepsin. These results indicate that in the formation of monolayers at a water surface by the spreading of a solid protein, or during the adsorption of an S-layer from solution on a PRC plate, there may be a separation or fractionation of the constituents of impure proteins. Since the L-pepsin has about $^1/_6$ the activity of the N, $^5/_6$ of L must consist of a substance which is not pepsin; Expt. 12 suggests that this substance consists of molecules of smaller size which are somewhat less strongly adsorbed on PRC than is pure pepsin.

In Expts. 12 and 13 the plate after being shaken in the milk for two minutes in the first tube was removed and washed. The surface seemed to be free from any clotted milk but, after drying, optical measurements showed the presence of a film, on top of the PRC film, of a total thickness given by T_1 in the fifth column of Table V. The pepsin presumably has been replaced by proteins from the milk.

Experiments 14 and 15 give the activities of S-layers of L-protein on glass plates. In Expt. 15 the plate, after washing, was dried and heated for twenty minutes at 80° which completely destroyed the activity. This result is in marked contrast to the behavior of urease S-layers which lost no activity after heating to 100° for sixteen hours.

In Expt. 16 a rectangular wire frame 1 × 5 cm. (the same size as the plates) was dipped into the water in a tray covered by a pepsin monolayer at $F = 9$. On lifting out the frame a film was obtained (like that from a soap solution) which had a total of 10 sq. cm. of pepsin monolayer on it. This was placed in a test-tube, and 6 ml. of the standard milk was poured into the tube and the milk was stirred with the wire frame. This is a repetition of Gorter's experiment in which he supported the film on silk gauze. The specific activity was about 80% of that of the original pepsin, confirming Gorter's results.

The presence of some substance in milk which can displace pepsin adsorbed on surfaces seems necessary to account for our observations. The fact that enormous quantities of milk can be clotted by small amounts of pepsin would seem to be dependent upon this same phenomenon.

We made a few experiments to determine the distribution of the pepsin between the whey and curd of clotted milk. We took two solutions each of 24 g. of skim milk powder in 150 ml. of acetate buffer. To one we added 0.2 mg. of pepsin (L) and to the other 10 mg. After these had clotted, the whey was separated and tested for pepsin activity. The results showed that no large fraction of the pepsin was adsorbed by the curd.

Summary

Monolayers of urease or pepsin spread on water and then deposited on plates, or adsorbed from solution by conditioned plates, usually have high chemical activity. The test for the activity of urease was the formation of ammonia from urea at *p*H 7. The urease monolayer remained on the plate with unchanged thickness (about 20 Å.). When the urease monolayer was deposited as an A-layer on a surface such as one conditioned with sodium desoxycholate, which anchors hydrophobic groups of the protein molecule, the activity disappeared. The urease activity thus depends on the presence of hydrophobic groups in the surface.

The pepsin was tested by its power to clot skim milk. There is some substance in milk that rapidly displaces the adsorbed pepsin monolayers on the plate and allows the pepsin to diffuse into the solution in a completely active form. To prevent the imprisonment of the pepsin within a layer of clotted milk, it is necessary to stir the milk when the monolayer is first introduced. The plate can then be removed without delaying the time of clotting. In several cases the specific activity of the pepsin monolayers prepared from a com-

mercial grade of pepsin was several times higher than that of the original pepsin, indicating a selective adsorption of the active pepsin into the monolayer on water or into that adsorbed on the conditioned plate.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Some Reactions of Indene Chloride and the *cis*- and *trans*-Chlorohydrins. Mechanism of Ketone Formation

BY C. M. SUTER AND GARSON A. LUTZ

It was reported by Spilker[1] many years ago that indene in dry ether adds chlorine to give an unstable oil which hydrolyzes readily to a chlorohydrin when boiled with 20% alcohol. Although the chlorination of indene, or of a coal tar fraction containing indene, has been mentioned by other investigators[2] in no case has the indene chloride been described. Courtot and co-workers[2c] were able to isolate a second chlorohydrin from the hydrolysis products and show that it was a stereoisomer of the one obtained by Spilker[1] since both compounds gave 2-chloro-1-indanone when oxidized with chromic acid.

Indene Chloride.[3]—The addition of chlorine to indene in carbon tetrachloride at a low temperature gives an 80% yield of the chloride together with a small amount of 2-chloroindene and higher chlorination products. A careful refractionation of a sample of the chloride indicated that it was homogeneous rather than a mixture of stereoisomers. The formation of two chlorohydrins on hydrolysis does not contradict this since it has been noted[4] that the solvolysis of active α-chloroethylbenzene is accompanied by partial racemization and the α-chlorine of indene chloride would be expected to act similarly. Attempts to obtain a second indene chloride apparently were not successful. The compounds obtained by the action of phosphorus pentachloride upon the higher melting (*trans*) chlorohydrin and of thionyl chloride upon both *cis*- and *trans*-chlorohydrins showed small variations in refractive index but had identical boiling points and upon hydrolysis all gave a mixture of chlorohydrins in which the *trans* isomer predominated. In a recent[5] discussion of reactions where hydroxyl is replaced by chlorine it was noted that thionyl chloride always reacts without inversion when the hydroxyl is attached to a carbon adjacent to phenyl if the reaction is carried out in the absence of a base. If this rule holds for the indene chlorohydrins the isomeric chlorides are indistinguishable in their common physical properties and in their behavior on hydrolysis, an unlikely circumstance. Bodendorf and Böhme[6] have found that an active form of α-chloroethylbenzene is racemized by a variety of chlorides in polar solvents. A preliminary experiment with indene chloride and mercuric chloride in nitromethane gave a product with a refractive index slightly lower than that of the original but the change was too small to be regarded as significant. Apparently the only *cis*- and *trans*-halides of a cyclic olefin that have been isolated are those obtained from cyclohexene and chlorine in the presence of cuprous chloride.[7] Cyclohexene bromide has given every indication of being homogeneous[8] and is probably the *cis* isomer. Cyclopentene likewise gives only one bromide.[9]

Indene chloride decomposes when heated to 225–235° with vigorous evolution of hydrogen chloride and formation of 2-chloroindene. A trace of the olefin is produced when the chloride is distilled at much lower temperatures as shown by refractionation of the distillate. The position of the chlorine is evident since the same compound results from dehydration of the indene chlorohydrins. Von Braun and Ostermayer[10] recently have reported the preparation of 3-chloroindene but

(1) Spilker, *Ber.*, **26**, 1538 (1893).

(2) (a) Heusler and Schieffer, *ibid.*, **32**, 30 (1899); (b) Weissgerber, *ibid.*, **44**, 1442 (1911); (c) Courtot, Fayet and Parant, *Compt. rend.*, **186**, 371 (1928).

(3) Since the halides obtained from olefins necessarily contain two halogen atoms the prefix di- is omitted here as it is in ethylene chloride. Confusion with the possible but unknown higher chlorides of indene seems unlikely.

(4) Steigman and Hammett, THIS JOURNAL, **59**, 2536 (1937).

(5) Cowdrey, Hughes, Ingold, Masterman and Scott, *J. Chem. Soc.*, 1266 (1937).

(6) Bodendorf and Böhme, *Ann.*, **516**, 1 (1935).

(7) Komatsu and Kawamato, *J. Chem. Soc. Japan*, **52**, 685 (1931); *C. A.*, **26**, 5080 (1932).

(8) Rothstein, *Ann. chim.*, **14**, 542 (1930).

(9) Zelinski and Levina, *Ber.*, **66B**, 477 (1933).

(10) Von Braun and Ostermayer, *ibid.*, **70B**, 1006 (1937).