The studies of Gorter (1), Hughes and Rideal (2), Neurath (3), and Langmuir (4) have shown that proteins are capable of forming surface films of a thickness and area independent of the diameter of the molecule. Depending on the pH and salt concentration of the solution upon which the protein is spread, the surface area may be more than 5 times greater than that to be expected from a monolayer of contiguous spherical molecules. The thickness of such surface films is correspondingly less than the assumed spherical molecular diameter. The proteins so far examined by Gorter develop their maximum spreading at pH 1, and again at the isoelectric point, 1 mg. covering about 1 sq. meter. The same maximum spread may be induced at any pH by the addition of salts (5). This remarkably constant area has been demonstrated for pepsin, trypsin, ovalbumin, and insulin, all having a molecular weight of about 35,000, as well as for zein (6) having a molecular weight 6 times greater.

Recently, Philippi (7) has reported film measurements on some of the high molecular weight respiratory proteins. He has found that one of these, derived from Palinurus vulgaris, spread to the same extent as egg albumin at pH 1, but did not spread on solutions of a higher pH. He points out the fact that at pH 1 the molecule of this protein is broken down into smaller components to which he attributes the spreading.

It should be noted that proteins which form films of 1 sq. meter per mg. at pH 1 and at their isoelectric point develop smaller films of the same thickness at other hydrogen ion concentrations. The area of the film is conditioned by the amount which remains on the surface,
MEASUREMENT OF SURFACE FILMS

since the thickness of the film appears to be invariable. If a film of egg albumin occupying 0.1 sq. meter per mg. at pH 3 is brought to pH 1, it will not expand to 1.0 sq. meter per mg., but will retain its original area.

An indirect confirmation of this fact was obtained by determining the amount of pepsin entering a subsurface solution at pH 4.6. According to Gorter, pepsin forms a film of only 0.1 sq. meter per mg. at this pH. One would then expect to recover 90 per cent of the pepsin from the solution under the film. Actually, from 70 to 90 per cent was demonstrated under a film of almost exactly 0.1 sq. meter per mg. (0.2 mg. = 210 sq. cm.). However, the accuracy of activity measurements with such necessarily dilute solutions (4 × 10⁻⁷ gm. protein per cc.) is not great.

The results to be presented deal with film area measurements applied to Limulus hemocyanin and tobacco mosaic virus. In addition, the surface behavior of protein-containing particles, namely vaccinia virus and a Gram-negative non-motile bacillus, was examined.

Materials and Methods

The Langmuir tray (8) was used to measure surface areas. Certain minor modifications were introduced. Instead of air jets to prevent leakage of the film around the ends of the movable barrier, paraffined threads were attached to the edges of the tray and to the movable barrier. Solid sticks of high melting point paraffin were used to push the film toward the movable barrier. A strip of thin celluloid for the movable barrier was found to be more durable than paraffined paper and equally satisfactory. Each film area was measured not longer than 30 seconds after the protein was applied to the surface, at pressures ranging from 0 to 60 dynes per cm. The areas given in the accompanying curves represent the areas of the films at 0 dynes per cm. The pH of the tray solutions was determined electrometrically by means of the quinhydrone electrode. The pH from 1 to 3.1 was obtained with HCl. From pH 3.3 to 5.2, 0.02 M acetate buffers were used, and from pH 6.0 to 7.9, 0.02 M phosphate buffers were used. The protein solutions were applied to the surface by means of a platinum loop delivering 0.006 cc. Entirely reproducible results were obtained by this method, which was simpler than the micro-pipette employed by Gorter.

The proteins to be tested were obtained in as pure a state as possible. A sample of 3 times crystallized, dialyzed swine pepsin was made available by Dr. R. M. Herriott. The ovalbumin was also crystallized 3 times and dialyzed free of ammonium sulfate. Hemocyanin (molecular weight, 3 million) (9) was obtained from the blood of Limulius polyphemus and purified in the quantity ultracentrifuge by a method previously described (10). A sample of ultracentrifuge-purified
tobacco mosaic virus protein (molecular weight, 17 million) (11) was kindly supplied by Dr. H. S. Loring. Vaccinia elementary bodies were prepared by the method described by Craigie (12), which yielded a final product showing practically nothing but Paschen bodies when examined by the Morosow stain. The isoelectric point was found by cataphoresis measurements to be pH 5.0. The suspension of B. gallinarum (fowl typhoid) was made from a 5 hour culture in digest broth which had been passed through a Berkefeld N filter to remove larger particulate matter before inoculation. The organisms were sedimented and resuspended 4 times in pH 7.6 buffered saline. The isoelectric point was determined by cataphoresis to be pH 4.1.

The surface measurements on the bacteria and elementary bodies were made the same day they were prepared or within 12 hours. The hemocyanin was stored 3 days in a concentrated solution and diluted just before use. On the basis of Kjeldahl nitrogen estimations all of the preparations were adjusted to a concentration of 2.5 mg. of protein per cc.

RESULTS

The relation of pH to spreading on 0.02 M solutions is shown in Figs. 1 and 2. Tray solutions adjusted to the isoelectric point of each of the materials to be tested were included in the series. Pepsin and ovalbumin developed surface areas in agreement with Gorter’s data. Maximum spreading took place at pH 1 and at the isoelectric point. Hemocyanin and vaccinia elementary bodies showed no spreading at their isoelectric points, but below pH 3.3 both exhibited definite spreading. It may be noted that there is a correlation here with the pH stability. The vaccinia elementary bodies moved out slowly on the surface of the tray solution at any pH; there was no evidence of settling. However, with the suspension of organisms, and the tobacco mosaic virus protein, even at pH 1, an undeterminable amount failed to remain on the surface, since an easily visible ring could be seen settling to the bottom of the tray when either of these materials was applied to the surface. For this reason, it is possible that the failure to demonstrate surface films depends on the technical difficulty of bringing these proteins onto the surface without loss by settling.

In an attempt to eliminate this source of error, tray solutions of greater density, 25 to 90 per cent saturated ammonium sulfate, were used. Preliminary observations using the loop of paraffined thread described by Langmuir showed that bacterial suspensions would

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1 Reagent grade, adjusted to about pH 7 with ammonia.
spread out with almost explosive rapidity on the surface of such solutions and displace the floating thread. However, after a few seconds the thread would of itself return to its former position. The same occurred with tobacco mosaic virus protein. When it was found that distilled water would bring about the same phenomenon it was realized that although the suspended material remained on the surface, the apparent spreading was due to a transitory water effect.

![Graph showing pH vs. square meters per mg. for Pepsin IE PT. pH 2.7 and Ovalbumin IE PT. pH 4.6.](image)

**Fig. 1**

This is borne out in the data presented in Fig. 3. Under conditions which cause pepsin and ovalbumin to spread to 1 sq. meter per mg., the area occupied by the bacteria, and by tobacco mosaic virus, is very small. Hemocyanin occupies about one-fourth the area of the lower molecular weight proteins, while the elementary bodies of vaccinia spread to about one-seventh of 1 sq. meter per mg. There can be no doubt that the materials in question were on the surface
when these measurements were made. In addition to the gross observation of the bacterial suspension moving out from the loop, plate counts of the solution under the surface showed that about 99 per cent of the bacteria had remained on the surface. Compression of the surface area caused the organisms to gather into a visible sheet which could be removed on slides and examined microscopically. It consisted of closely packed organisms which had apparently under

![Graph](image_url)  
**Fig. 2**

gone two-dimensional agglutination. Such aggregation of non-spreading particles would constitute a serious source of error in any activity measurements on surface films.

The tobacco mosaic virus protein was also shown to remain on the surface of ammonium sulfate solutions. If the extremely small films developed by this material resulted from the fact that 99 per cent of the protein entered the subsurface or substrate solution, one should
be able to demonstrate the virus there. Accordingly, virus activity measurements on the liquid under the film were carried out.

0.2 cc. containing 1 mg. of virus protein was allowed to flow slowly onto the surface of the tray containing half-saturated ammonium sulfate solution. After measuring the surface area, a 10 cc. pipette was introduced into the tray behind the barrier in as nearly horizontal position as possible. The pipette was filled and vigorously discharged 15 times causing a definite swirling of the tray contents. 10 cc. were withdrawn, dialyzed 3-4 hours in a rocking dialyzer at about 6°C. and inoculated onto leaves. The final ammonium sulfate concentration was less than 0.5 per cent. Controls consisted of the same

\[
\text{Fig. 3}
\]

\[
\text{PER CENT SATURATED AMMONIUM SULFATE}
\]

\[
\text{pH 7.0 6.7 6.9 6.9 7.1}
\]

\[
\text{SQUARE METERS PER MG.}
\]

\[
\text{0 0.2 0.4 0.6 0.8 1.0 1.2}
\]

\[
\text{1 PEP SIN} \quad \text{2 OVALBUMIN} \quad \text{3 HEMOCYANIN} \quad \text{4 VACCINIA} \quad \text{5 TOBACCO MOSAIC VIRUS} \quad \text{6 S. GALLINARUM}
\]

\[
\text{2 The author wishes to express his gratitude to Dr. H. S. Loring for assistance in carrying out these activity measurements.}
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amount of virus discharged under the surface of fresh tray solutions, stirred, dialyzed, and inoculated in exactly the same manner.

In the titration of a substrate solution under a film of 0.016 sq. meter per mg., only 1 lesion appeared on 32 inoculated leaves; in the control, with all conditions identical except that the virus protein was introduced beneath the surface, there were 101 lesions on 23 inoculated leaves. In another similar experiment using the same virus preparation, 1 mg. gave a film of 0.012 sq. meter. The sample re-

<table>
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<th>TABLE I</th>
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<td>Diameter of particle</td>
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<td>cm.</td>
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<tr>
<td>Pepsin</td>
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<td>Ovalbumin</td>
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<td>Hemocyanin</td>
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<td>Tobacco mosaic virus</td>
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<tr>
<td>Vaccinia</td>
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<td>B. gallinarum</td>
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* These values were obtained by multiplying the number of particles in 1 mg. by the area of a square the sides of which equalled the diameter of the particle in question. The calculated area represents a maximum value, since circles actually pack into an area less than that occupied by the same number of squares whose sides equal the diameter. The number of particles was obtained by dividing the volume of 1 mg. (assuming the specific gravity to be 1.4) by the volume of a single particle determined from its diameter. Surface area in sq. meters = \( \frac{0.67 \times 10^{-7}}{\text{molecular radius in cm.}} \).

moved from underneath this film and inoculated on 18 leaves showed no lesions; the control showed 27 lesions on 18 leaves. In a third determination the substrate solution gave 1 doubtful lesion on 70 leaves; the control, in which the virus was introduced beneath the surface, showed 78 lesions on 70 leaves. While one would hesitate to conclude that 99 per cent of the virus protein remained on the surface, it seems clear that there is very little loss into the substrate.

In Table I, the observed areas in square meters per milligram on 90
per cent saturated ammonium sulfate solution are presented and compared with the theoretical areas which a monolayer of contiguous spherical molecules would occupy. Pepsin and ovalbumin occupy about 4 times the theoretical area. Hemocyanin occupies twice the expected area; in calculating this area a certain amount of error is introduced since Limulus hemocyanin molecules are not spherical (13). The film of tobacco mosaic virus is smaller than that calculated on the basis of packed spheres. The molecule of this protein is also rod-shaped (14) and may be capable of packing into an area smaller than that calculated for a spherical molecule. Vaccinia and the organism suspension spread out to form a film which, though small, was much larger than the calculated area.

The presence of any low molecular weight protein impurity or breakdown product of a larger particle should be considered as a source of error, especially in the interpretation of a film as small as that produced by B. gallinarum. For example, it was found that a bacterial suspension after standing in the ice box for 10 days gave a surface film of 0.4 sq. meter per mg. However, after sedimenting the bacterial bodies, the almost clear supernatant spread to the same extent.

In spreading at pH 1 it is probable that two factors are operating. One is the effective electrolyte in 0.1 N HCl which would bring about spreading regardless of the H ions. The other is the effect of the H ions in breaking down certain proteins into smaller components. Both Limulus hemocyanin (9) and vaccinia (15) are unstable below pH 3 to 4, which accounts for their spreading below this pH level.

From the measurements presented it may be concluded that not all proteins spread in the same way as the low molecular weight proteins such as ovalbumin and pepsin. This is in agreement with data on respiratory proteins obtained by Philippi (7). A relationship between molecular weight and spreading capacity is suggested. The rapid spreading of low molecular weight proteins at their isoelectric points could not be duplicated with hemocyanin, tobacco mosaic virus, vaccinia, or a suspension of bacteria.

The calculated thickness (volume of protein divided by film area) of films of tobacco mosaic virus protein on half saturated solutions of ammonium sulfate is about 1000 Å. If the molecules are horizontally oriented, it is obvious that such thick films cannot be monomolecular. It is possible that they may be monomolecular if a vertical molecular
orientation occurs, similar to that of a stearic acid monolayer (8). X-ray data indicate a molecular diameter of about 150 Å (14). If the film thickness (corresponding to the molecular length) were 1200 Å, 1 mg. of vertically oriented molecules in a surface film would occupy 76.5 sq. cm. This value is of the same order of magnitude as the observed area of 1 mg., and molecules of these dimensions, having a density of 1.37 would have a molecular weight of about 17 million. Although the existing data on the molecular dimensions and molecular weight of the tobacco mosaic virus protein would permit the assumption of a monolayer of vertically oriented rod-shaped molecules, the rather tentative nature of these data must be borne in mind.

The author wishes to express his gratitude to Dr. J. H. Northrop for his invaluable advice and suggestions.

REFERENCES