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A Force-Area Study of Mixed Films of Trypsin and Ovomucoid

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ABSTRACT

An investigation of the surface chemical behavior of the enzyme-inhibitor complex between ovomucoid and trypsin was undertaken. Monomolecular films of this complex were deposited on ammonium sulfate substrate solutions of pH 4.0 and pH 7.0. Spreading solutions were employed in which the molecular ratios between trypsin and ovomucoid (T:O) were close to 3:1, 1:1, and 1:3.

For films spread on the acid substrates, extrapolation of the linear portion of the force-area (F-A) curves to zero film pressure gave apparent areas per molecule of 3100, 2560, and 3150A^2, respectively, for the three molecular ratios (T:O) of 1:3, 1:1, and 3:1. On the neutral substrates, the corresponding areas were 2660, 1750, and 2470A^2 for the same order of molecular ratios.

No evidence that molecular interaction was accompanied by an area change occurred in films in which the molecular ratio (T:O) of the constituents was 1:3. Some interaction was evidenced for the ratio 3:1. For those films in which the molecular ratio was 1:1, there was evidence to indicate a strong attraction between the trypsin and ovomucoid. This occurred at both substrate pH levels. Further analysis of the data indicates that on the acid substrate, each unit of the complex is probably composed of the two proteins in a 1:1 ratio, while on the neutral substrate each complex unit may contain a higher molecular ratio of trypsin.

PROBLEM STATUS

This is a final report on one phase of the problem; work on other phases is continuing.

AUTHORIZATION

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A FORCE-AREA STUDY OF MIXED FILMS OF TRYPSIN AND OVOMUCOID

INTRODUCTION

This Laboratory has been actively engaged in investigating the chemical and physical characteristics of surface active materials when spread as monomolecular films at air/liquid interfaces. Current effort is directed toward the study of the behavior of selected biologically active proteins as they exist in monomolecular films and the mechanisms by which the active sites on the surface of these proteins are deactivated by inhibitors which are also proteins. Of specific interest are the interacting protein systems catalase-ovomucoid, described by Abrignani and coworkers (1, 2), trypsin-ovomucoid by Lineweaver and Murray (3), and chymotrypsin-ovomucoid by Weil and Timasheff (4).

The investigation of the force-area characteristics of catalase (5) and ovomucoid (6) films and mixed films of these proteins (7) have been presented in earlier reports. This report is limited to an investigation of the force-area characteristics of mixed films of trypsin and ovomucoid.

THE OVOMUCOID-TRYPSIN COMPLEX

Although the inhibition of trypsin by egg white had been known for some time the active component remained unidentified until Lineweaver and Murray (3) demonstrated that the antitryptic activity was quantitatively resident in the ovomucoid fraction. They also showed that the ovomucoid had no effect on proteolytic enzymes other than trypsin. While the activation of ovomucoid by heat is a prerequisite of anticatalase activity, this treatment serves to destroy its antitryptic activity.

Anticatalase activity is brought about by the formation of sulfhydryl groups from the disulfide bonds present in the ovomucoid molecule (2). It is the formation of these sulfhydryl groups that is responsible for this observed loss in antitryptic activity (8). Thus ovomucoid is a rather unique protein in that through a small, reversible alteration in its molecular structure it can inhibit two biologically active proteins. The presence of intact disulfide groups does not, however, constitute the full requirement for antitryptic activity. It appears that carboxyl, phenolic, and other polar groups of the ovomucoid molecule must be free in varying degree. With respect to the trypsin molecule, it appears that amino groups of the enzyme are essential in formation of the enzyme-inhibitor complex, since acetylated trypsin is completely resistant to inhibition by ovomucoid (8).

The nature of the inhibition of trypsin by ovomucoid has been the subject of rather extensive investigation by many workers. Earlier kinetic studies by Fraenkel-Conrat, Bean, and Lineweaver (8) and by Green and Work (9) indicated the inhibition to be noncompetitive with the substrate. In later work, however, the inhibitory action of ovomucoid was shown to be of a competitive nature (10). This competitive inhibition was found by Green (11) to differ from the usual type of competitive inhibition in two respects. First, the association constants of the enzyme-inhibitor complex were found to be so high that the concentration of free inhibitor could not be assumed to be that of the total inhibitor added. Thus, according to Green, the ideal relation of Lineweaver and Burk (12)

between the reciprocal velocity and reciprocal concentration does not apply. Second, trypsin combines with the inhibitor much more slowly than with the substrate so that the inhibitor-trypsin-substrate system does not normally attain equilibrium (11). Based on this latter investigation by Green, it has been concluded (13) that the inhibitor combines with the enzyme at or near the enzymatically active site.

It has been considered that trypsin and ovomucoid (T:O) combine in a 1:1 ration (14); however, evidence is presented by Ram and coworkers (10) that other complexes between the two constituents may be formed. These authors concluded from ultracentrifuge and electrophoresis data that while trypsin was capable of binding only one ovomucoid molecule, the latter could bind more than one trypsin molecule. Using the molecular ratios (T:O) of 3:1, 1:1, and 1:3 in their ultracentrifuge studies, they found that with an excess of ovomucoid there appeared two sedimentation boundaries having sedimentation rates which were characteristic of those rates previously determined for the 1:1 trypsin-ovomucoid complex and ovomucoid alone. In the presence of an excess of trypsin, however, only one boundary was observed, sedimentation occurring at a rate at first characteristic of the 1:1 complex, but decreasing as the amount of excess trypsin was increased. This behavior was construed by the authors as being caused by the association of one or more of the excess trypsin molecules with the complex. Similar results were obtained from the electrophoresis experiments.

EXPERIMENTAL PROCEDURE

Apparatus

The force-area measurements were made on a modified Wilhelmy film balance. A detailed description of this apparatus as it was constructed and used in this Laboratory appears in an earlier report (6). Briefly, films are spread on an aqueous substrate, the surface area of which may be adjusted by a movable barrier. Film pressures are measured as surface tension reductions of the film-covered substrate as the film area is decreased. These reductions appear as changes in apparent buoyancy of a quartz slide of known perimeter suspended from the beam of an analytical balance and partially immersed in the substrate (15,16). Modifications to the instrument include the electromagnetic device of Augenstine (17) for measuring the restoring force applied to the slide, and the Teflon tray and barriers of Fox and Zisman (18). A simple optical lever 5.5 meters in length was used to indicate slide displacement and the electromagnetic force necessary to restore the slide to its null position was measured as an IR drop across a standard one-ohm resistor by a Rubicon potentiometer. From calibration data, a suitable factor was obtained for translating the IR drop in millivolts to the corresponding film pressures in dynes/cm.

Materials and Methods

Crystalline trypsin was obtained from Worthington Biochemicals Corp. and crystalline ovomucoid was obtained from Mann Research Laboratories. These proteins were of the highest grade of purity available and no further purification was attempted. The ammonium sulfate used in preparing the substrates was a special enzyme grade obtained from Mann Research Laboratories. All other chemicals used were of an analytical grade. Distilled water was obtained from a tin-lined still.

The films were formed from spreading solutions of 0.05M phosphate buffer (pH 7.5) in which the total protein concentration (ovomucoid plus trypsin) never exceeded 0.05 percent by weight (19). In preparing the spreading solutions the relative proportion of trypsin to ovomucoid was varied in accordance with the work of Ram et al. (10) so that

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the final solutions contained the two constituents in the molecular ratio (T:O) of 3:1, 1:1, and 1:3.

The films were prepared on 4.85% ammonium sulfate substrates of pH 4.0 and 7.0, the former being the isoelectric pH of ovomucoid. This concentration of salt was chosen so that the substrate would be convenient to handle and the "salting-out" effect on the protein molecules (20), which tends to oppose underfilm formation, would minimize loss of material into the substrate on spreading. In addition, the relatively high concentration of ammonium sulfate tends to minimize the effect of extraneous ions. Preparation and handling of the substrate and the mechanics of film compression are described in earlier reports (5,6).

RESULTS AND DISCUSSION

The force-area curves obtained from the mixed films were found to be similar in shape to those obtained from trypsin films over the corresponding film-pressure range. Representative curves for the three molecular ratios (acid substrate) are shown in Fig. 1.

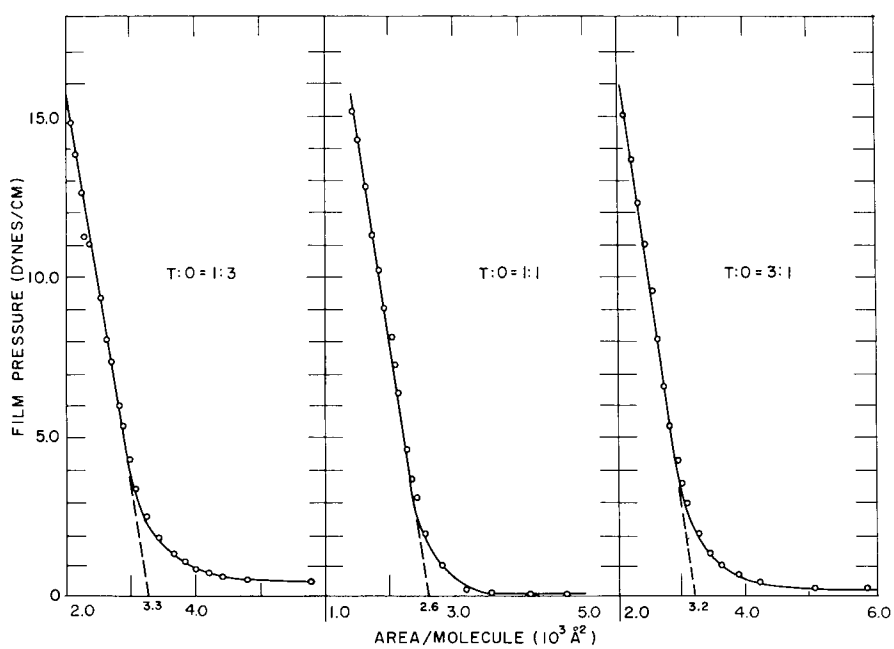

Fig. 1 - Typical force-area curves of trypsin-ovomucoid mixed films for the three molecular ratios (T:O) on 4.85 percent ammonium sulfate, pH 4.0

Curves obtained from films prepared on neutral substrates were similar in shape. Although all ovomucoid F-A curves contained a double inflection (6), only a few of the mixed film curves exhibited this property. There was no correlation between the relative proportion of ovomucoid and the appearance of the second inflection, which one might expect with the increasing population of ovomucoid molecules in the films. Table 1 lists the averages and standard deviations for limiting areas, linearity points, and slopes obtained from all the mixed films of trypsin and ovomucoid. The corresponding values obtained from the separate ovomucoid and trypsin films are included for comparison.

Table 1
Properties of Mixed Films of Trypsin and Ovomucoid at Various Molecular Ratios as a Function of Substrate pH

pH	Ovomucoid*	Trypsin*	T:O Ratio of Mixed Films		
			1:3	1:1	3:1
Limiting Area/Molecule ($10^3 A^2$)					
4.0	2.86 ± 0.07	3.4 ± 0.2	3.1 ± 0.1	2.56 ± 0.07	3.15 ± 0.04
			(3.00)†	(3.16)	(3.30)
7.0	2.51 ± 0.06	2.0 ± 0.2	2.66 ± 0.07	1.8 ± 0.2	2.5 ± 0.7
			(2.41)	(2.26)	(2.13)
Linearity Point (dynes/cm)					
4.0	4.3 ± 0.9	7.2 ± 0.4	4.2 ± 0.3	4.2 ± 0.7	4.8 ± 0.6
7.0	3.2 ± 0.4	5.7 ± 0.2	3.0 ± 0.1	3.6 ± 0.6	3.9 ± 0.2
Slope (dynes/cm × molecules/A^2)					
4.0	13.8 ± 0.8	16.2 ± 0.8	12.5 ± 0.6	15.8 ± 0.8	14.2 ± 0.7
7.0	11.1 ± 1.2	26.5 ± 1.3	8.2 ± 0.6	14.5 ± 1.1	12.8 ± 1.0

*Data on ovomucoid (6) and trypsin included for comparison.
†Parenthesized figures refer to theoretical area values calculated according to Payens (21).

The area figures represent the apparent limiting area/molecule extrapolated to zero film pressure; the figures in parentheses refer to theoretical areas calculated according to the method of Payens (21) described below.

The data show that at all three molecular ratios the limiting area was smaller on the neutral substrate. This is probably due to a decrease in the repulsive forces within the molecules as the pH of the substrate was shifted toward the isoelectric point of trypsin. A minimum in limiting area was also observed for the films in which the two constituents were present in a molecular ratio of 1:1. On the assumption that only physical packing took place between the molecules on the substrate, such a minimum would not have been predicted, as reference to the parenthesized area values will disclose. The occurrence of this minimum limiting area, therefore, suggests that an intermolecular association has occurred between these two species, and that the 1:1 molecular ratio is the most nearly optimum of those studied for demonstrating this effect.

To determine the extent of this interaction between the trypsin and ovomucoid molecules more graphically, the method of Payens (21) described in an earlier report (7) has been adopted. By this method of analyzing force-area data, ideal force-area curves may be constructed for the mixed monolayers on the assumption that no molecular interaction has occurred and the specific areas of the mixed-film components would be equal to the specific areas of the pure-component monolayers for a given film pressure. The

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apparent limiting area/molecule (at F = 0) in such an ideal mixed monolayer, or the apparent limiting area/molecule at any other film pressure (F ≠ 0) is given by:

$$(Am)_F = (1 - w) \ (A_1)_F + w \ (A_2)_F \tag{1}$$

where $(Am)_F$ is the apparent molecular area in square angstroms at film pressure F, and w and (1 - w) represent the relative proportion of each species present. A_1 and A_2 are the respective specific areas of the component molecules at film pressure F. The resulting curve is actually an enclosed area, and the extent of interaction between the film molecules can then be estimated by comparing the positions of the experimentally determined force-area curves with those of the corresponding ideal curves on the same x-y coordinates.

The procedure for constructing the ideal curves from separate force-area data obtained from trypsin and ovomucoid films is the same as that used in constructing the ideal catalase-ovomucoid curves described earlier (7). The average apparent molecular area at each one dyne/cm pressure increment up to 15 dynes/cm was determined from the force-area curves of the mixed films, for each of the three molecular ratios. The data so obtained for each molecular ratio were then plotted on the same coordinates as were the data for the corresponding ideal curve at that molecular ratio. The resulting ideal and corresponding experimental curves are compared in Figs. 2-4.

Inspection of these curves discloses that within the experimental error no apparent interaction has occurred between the film constituents for the molecular ratio of T:O = 1:3 (Fig. 2). For those curves representing the ratio T:O = 3:1 (Fig. 3), there is evidence of some association of the constituent molecules on the acid substrate. This

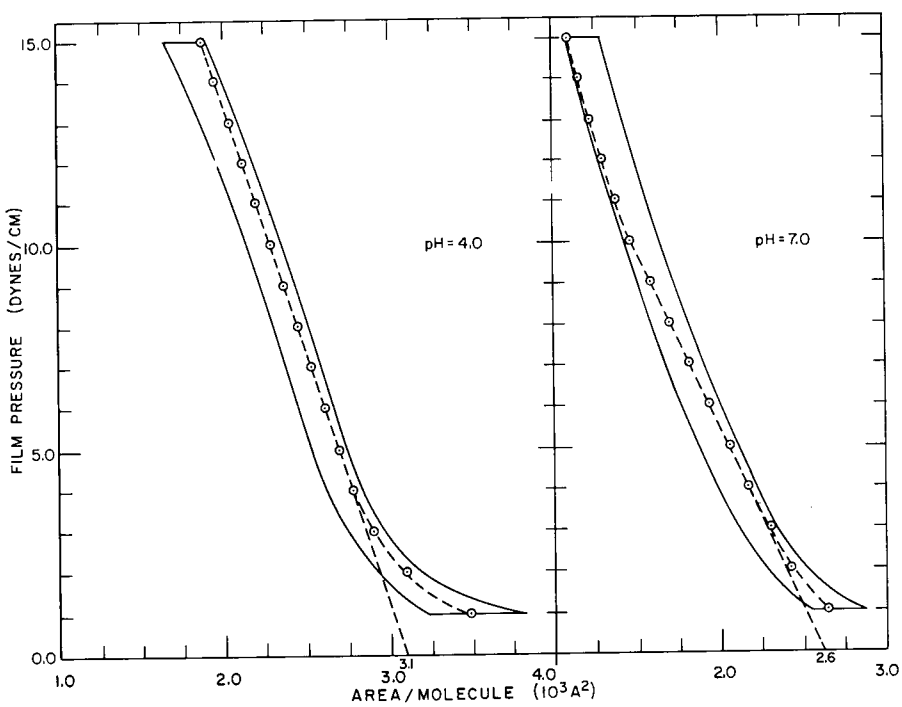

Fig. 2 - Comparison of ideal and experimental curves for trypsin-ovomucoid mixed films (T:O = 1:3) at both levels of substrate pH

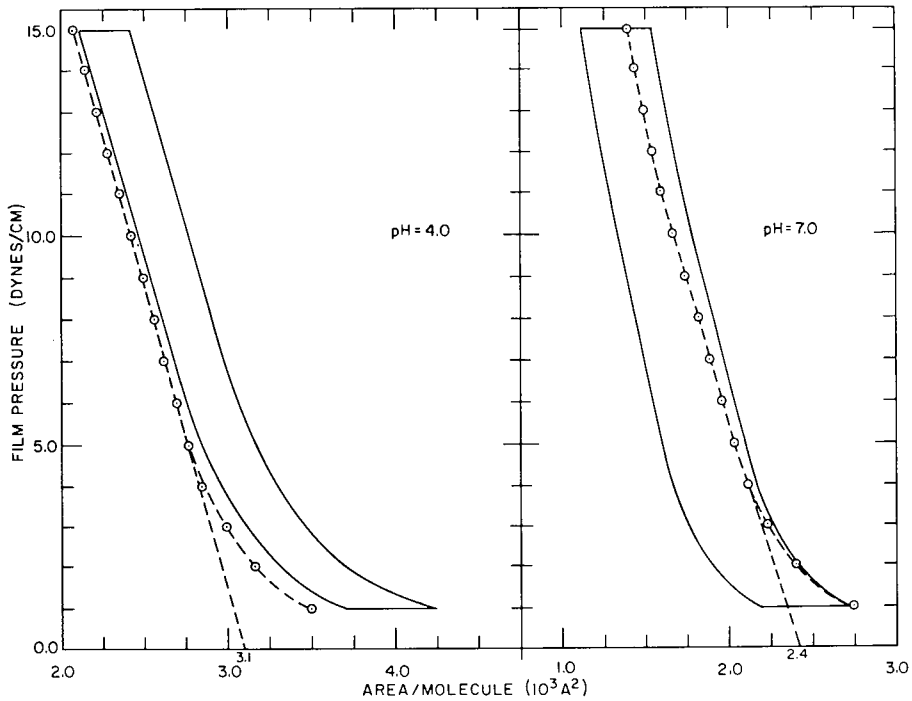

Fig. 3 - Comparison of ideal and experimental curves for trypsin-ovomucoid mixed films (T:O = 3:1) at both levels of substrate pH

association is shown as a displacement of the experimental curve to the left of the ideal curve. At the molecular ratio of T:O = 1:1 (Fig. 4), a strong interaction can be observed where the displacement of the experimental curve from the ideal curve demonstrates a strong attraction between trypsin and ovomucoid, such that the area of each complex unit is much less than the sum of the areas of its constituents. This area decrease may be explained in terms of the formation of intermolecular bonds between the complex constituents which are strong enough to resist the spreading forces and which act to restrict the unfolding of the complex unit on the substrate surface. These results suggest that the molecular ratio of the complex is 1:1 as previously reported (14) and the association does not show up readily in those cases where the calculated molecular ratio is 3:1 in favor of either the ovomucoid or the trypsin because of the diluting effect imposed by the excess of either protein in the film.

If the ratio of trypsin to ovomucoid in the complex is 1:1 and a high affinity constant be assumed, the spreading solutions in which the calculated molecular ratio was T:O = 3:1 would be composed of the molecular complex and free trypsin in the ratio T:complex ≅ 2:1. It would be these two species that would be present in the films prepared from these solutions. With this in mind, an ideal curve for trypsin and the complex was constructed over the same pressure range used in the preparation of the other ideal curves. In constructing the ideal curve, area values for the complex were averaged from the experimental curves obtained from those films containing trypsin and ovomucoid in a 1:1 ratio. The assumption was made that the apparent area/molecule observed at each pressure increment on these experimental curves represented the actual film area occupied by one trypsin-ovomucoid unit at that pressure. The corresponding areas, taken from the experimental curves obtained from films having the calculated molecular ratio of 3:1, were then plotted for each pressure increment on the same coordinates as the ideal curve. The resulting comparative curves are shown in Fig. 5.

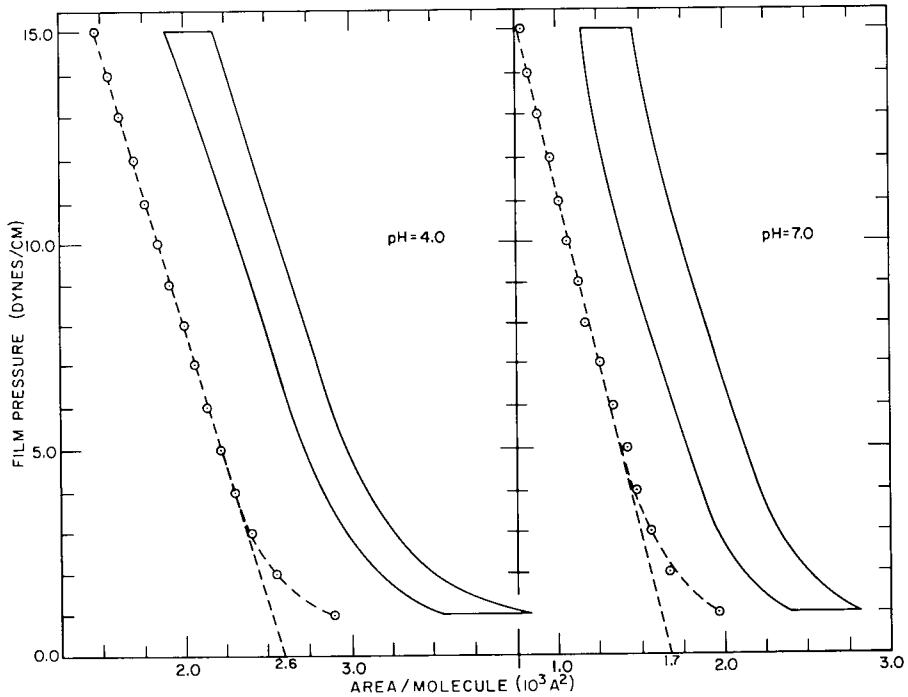

Fig. 4 - Comparison of ideal and experimental curves for trypsin-ovomucoid mixed films (T:O = 1:1) at both levels of substrate pH

For the acid substrate (Fig. 5), the average experimental curve was found to fall within the boundaries of the trypsin-complex ideal curve. This would indicate that the trypsin molecules and those of the complex simply packed together on the substrate surface or associated in such a way that no area change resulted. Additional evidence that no area-changing association took place may be obtained by substituting the limiting area values (Table 1) for trypsin and the 1:1 complex unit into Payens' equations. When this is done the calculated limiting area is $3.14 \times 10^3 A^2$, a value in agreement with the limiting area determined experimentally when the mixed film ratio was T:O = 3:1. These results do not rule out the occurrence of molecular interaction between the film constituents; however, since a decrease in area from that predicted on the basis of simple packing took place when the molecules were present in a 1:1 ratio, it seems likely that the association of more than one molecule of trypsin with one of ovomucoid would also result in an apparent molecular area different from that predicted for simple packing. Thus it is concluded that, at least on the acid substrate, each molecule of ovomucoid binds one molecule of trypsin.

With respect to the neutral substrate the limiting area of a trypsin-1:1 complex unit as calculated by Payens' equation is $1.93 \times 10^3 A^2$. This area is considerably smaller than the limiting area determined experimentally when the mixed film ratio was T:O = 3:1 (Table 1). The observed limiting area (T:complex) is larger than that calculated on the basis of simple packing of the film molecules at this ratio. This increase in apparent molecular area is shown in Fig. 5 as a displacement to the right of the experimental curve from the ideal curve.

Whether this increase in area on the neutral substrate is the result of electrostatic repulsion between the film molecules brought about by the change in pH or some other cause, will require further investigation.

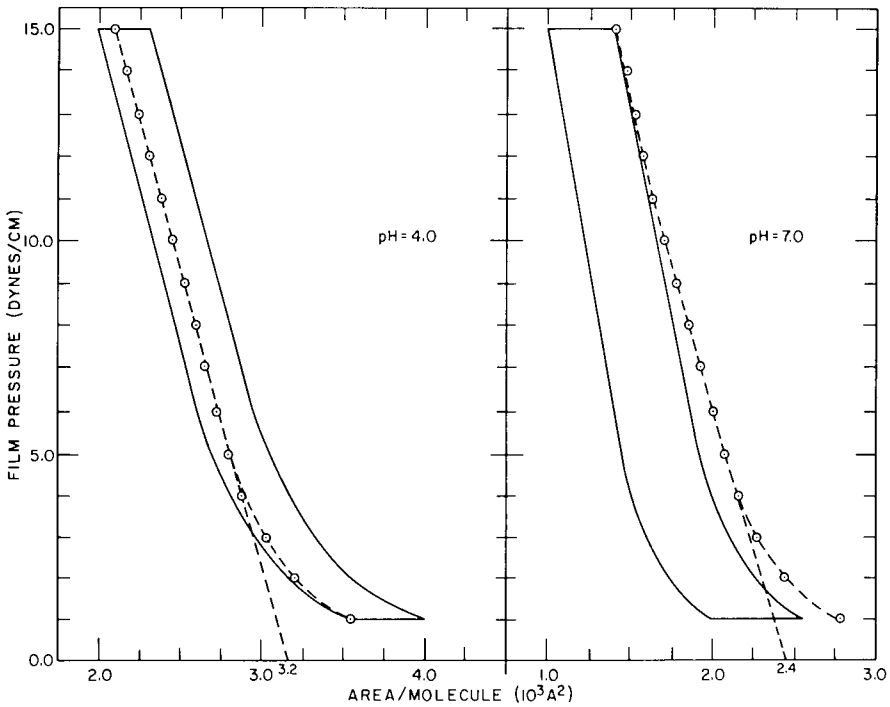

Fig. 5 - Comparison of ideal and experimental curves for trypsin and trypsin-ovomucoid complex mixed films (T:complex = 2:1) at both levels of substrate pH (Based on 1:1 trypsin-ovomucoid complex)

SUMMARY

Force-area measurements have been made on mixed films containing trypsin and ovomucoid in which the molecular ratios (T:O) of the two species were close to 3:1, 1:1, and 1:3. The mixed films were prepared on acid (pH 4.0) and neutral (pH 7.0) substrates. The force-area curves obtained from these mixed films more nearly resembled in shape those curves obtained from trypsin films over the pressure range employed. The numerical values for the film parameters were found to be more dependent on the $[H^+]$ of the substrate than on the relative concentration of the two species present in the films.

Comparisons of experimental curves with constructed curves for each of the three molecular ratios of the film constituents showed evidence of a strong molecular interaction on both the acid and neutral substrates only for those films in which the molecular ratio was T:O = 1:1. The interaction manifests itself as a condensation of the reacting molecules so that the area of each complex unit was less than the sum of the areas of its components.

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