Surface Films of Azo-proteins

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Introduction

Numerous attempts[2] have been made to interprete the observed properties of the protein surface films in terms of the structure and the orientation of the film molecules. Though various hypotheses have been advanced, no definite conclusion has yet been reached. In order to throw some light on this problem, it seemed desirable to study the films of artificially modified proteins and to compare their behavior with that of the original native proteins. For this reason we made a study of the surface films of azo-protein, *i. e.* the protein to the side chains of which known chemical groups have been chemically conjugated through diazonium groups. As a sample of azo-protein, *p*-azo-phenylarsonic acid-conjugated ovalbumin (henceforth referred to as azo-ovalbumin) was chosen. With azo-ovalbumin, experiments were carried out to determine the force-area curves of the films spread on water.

Further, films were transferred, in various ways, onto metal slides and their antigenic activity to the antibody directed specifically against the diazonium groups was tested. In connection with the activity tests, the structure of protein monolayer was discussed. These are described in the present paper.[3]

Experimental

Material.[4]—The samples of azo-ovalbumin with various amounts of diazonium groups were prepared according to the method of Haurowitz[5] from purified hen ovalbumin by treating with different amounts of diazotized *p*-phenylarsonic

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(2) H. B. Bull, "Advances in Protein Chemistry," Vol. 3, 1947, p. 95—121.

(3) Following the present study, investigations were made on the relationship between the number of haptenic groups in azo-proteins and the number of adsorbed antibody molecules. These results will be reported elsewhere.

(4) Details on the material will be given by one of the present authors, S. Yamaoka, elsewhere.

(5) F. Haurowitz, *Hoppe-Seylers Z. physiol. Chem.*, **245**, 23 (1936~37).

acid. Each preparation was purified thoroughly until no further color was observed in the supernatant fluid after precipitation by adding hydrochloric acid followed by dialysis. The nitrogen analysis of the compounds was made by the Micro-Kjeldahl method modified by Elek and Sobotka[6] and the arsenic determination by the method of Sandell.[7] Each sample thus obtained was found to be electrophoretically uniform by the Tiselius method.

The number of diazonium groups per one molecule of azo-ovalbumin, x, was estimated by referring to Boyd and Hooker's[8] method of calculation. Then we may write the following equation

$$F_c = F(1-0.373\,R)+3.05\,R \qquad (1)$$

where F_c represents the ratio of the weight of azo-ovalbumin to total nitrogen in it, F, that of ovalbumin to nitrogen which was estimated as 6.62 according to Calvery,[9] and R, that of arsenic to total nitrogen in azo-ovalbumin.

When F_c is obtained from the value of F and R with an azo-ovalbumin, the percentage of arsenic content in azo-ovalbumin, As %, is calculated from the following equation,

$$\text{As}\,\% = 100\times\frac{R}{F_c} \qquad (2)$$

Thus, the value of x is obtained from the following equation

$$\text{As}\,\% = \frac{75\,x}{40000+228\,x}\times 100 \qquad (3)$$

where the molecular weight of ovalbumin is approximately taken as 40,000.

Procedure.—Preliminary experiments showed that although azo-ovalbumins had a tendency to spread on pure water, they appeared to dissolve partly into substrate. Therefore, solution of 0.01 N HCl or, more satisfactorily, a half saturated solution of ammonium sulfate was used as a substrate in a trough of chromium plated brass (60.0×14.6×2.0 cm.) In order to spread azo-ovalbumin monolayer, 0.05% aqueous solution of azo-ovalbumin was given on the surface of substrate solution from a capillary pipette. Force-area curves were obtained by using the Wilhelmy's method based on the principle of tensiometry.[10] The sensitivity of the instrument was of the order ±0.1 dyne per cm. For the measurement in low pressure regions, the sensitivity was raised to 0.01 dyne per cm., using thinner torsion wire (diameter 60 μ). All the experiments were made at a room temperature (about 20°C.). Next, the transfer of azo-ovalbumin monolayers onto metal slides was attempted. However, when acidic substrate water was used, it was frequently unsuccessful to obtain the built-up films of azo-ovalbumin using piston oil of castor oil (pressure, 15 dynes per cm.), because the detachment of the films occurred during the deposition. Thus, azo-ovalbumin was spread on distilled water at pH 5.5 and transferred onto chromium slides covered with the optical gauge of barium stearate multilayers, using castor oil as piston oil. One monolayer of azo-protein film can be transferred either by immersing the slide into the trough before the protein has been spread and withdrawing it through the protein monolayer (up-trip) or by immersing the slide through the protein monolayer previously spread (down-trip) and removing the monolayer from the surface before the slide is withdrawn.

According to Langmuir,[11] the monolayer deposited by down-trip is called an A-layer and that deposited by up-trip, a B-layer. In order to deposit a B-layer directly onto the optical gauge, it was necessary to condition the surface of barium stearate film by thorium nitrate solution (0.001 mol). One double-layer (AB-layer) of azo-ovalbumin can be transferred on stearate layers as easily as native proteins. It was found that a transfer of more than one double-layer on the barium stearate films was not successful, while successive transfer of B-layer was performed without difficulty. However, the increasing number of B-layer made further successive transfer gradually difficult. Possible mode of deposition will be described later.

To measure the deposition ratio, a floating barrier of a circular waxed paper disk was used. The displacement of the disk multiplied by the width of the trough gave the area of monolayer consumed.

The thickness of the built-up films of azo-ovalbumin was measured by using the Blodgett and Langmuir[12] method. The optical thickness (T) of the films was calculated from the following formula:

$$T = 24.4\left(N_1\frac{\cos r_1}{\cos r_2}-N_2\right), \qquad (4)$$

where T is the thickness in Å., N, the number of stearate layers which gives a minimum intensity in the reflected light for the angle of the refraction r_1 before the transfer of the film, N_2 the corresponding number of stearate layers after the film has been transferred, and r_2, the corresponding angle of refraction. The refractive index of protein film was assumed to be 1.495, being the same as that of barium stearate films. It was possible by using polarized sodium light to

(6) A. Elek and H. Sobotka, *J. Am. Chem. Soc.*, **48**, 501 (1926).

(7) E. B. Sandell, *Ind. Eng. Chem., Anal. Ed.*, **14**, 82 (1942).

(8) Boyd and Hooker, *J. Biol. Chem.*, **104**, 329 (1934). In Boyd and Hooker's paper the second term of the righthand side of formula (1) is represented as $3.20\,R$, but the coefficient should be corrected to 3.05.

(9) Calvery, *J. Biol. Chem.*, **94**, 613 (1931~32).

(10) A. Dognon and M. Abribat, *Compt. rend.*, **208**, 1881 (1939). T. Sasaki, *J. Chem. Soc. Japan.*, **62**, 796 (1941).

(11) I. Langmuir, *Cold Spring Harbor Symp.*, **56**, 171 (1938).

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

measure the thickness of built-up films within the error of approximately ±2Å. The increment of thickness by the adsorption of a antibodies onto azo-ovalbumin films was also measured by the above method.

Results and Discussion

Force-Area Relationships.—(i) High pressure region. Fig. 1 gives the force-area curves for the monolayer of native ovalbumin and azo-ovalbumin on a half-saturated solution of ammonium sulfate. These curves, A, B, C and D, show the relationships between pressure and specific area (m.²/mg.) for a series of azo-ovalbumins of increasing number of diazonium groups. As the number of diazonium groups in azo-ovalbumin molecule increases, the limiting specific area decreases. However, with the area per molecule, instead of specific area of azo-ovalbumin, practically identical force-area relationships were obtained for each sample of azo-ovalbumins irrespective of the number of diazonium groups. In other words, the limiting area (area extrapolated to zero pressure) per one azo-ovalbumin molecule is independent of the number of diazonium groups and is around 7000 Å.², although it tends to increase slightly with the coupling of diazonium groups. This means that diazonium groups introduced contribute not to the area but mainly to the thickness of azo-ovalbumin monolayer. The thickness of monolayer at 15 dynes per cm. can be evaluated from the correspondent specific area and the film density (assumed to be 1.3, the same as density in bulk). The results obtained with the samples of azo-ovalbumin are given in Column VIII of Table 1.

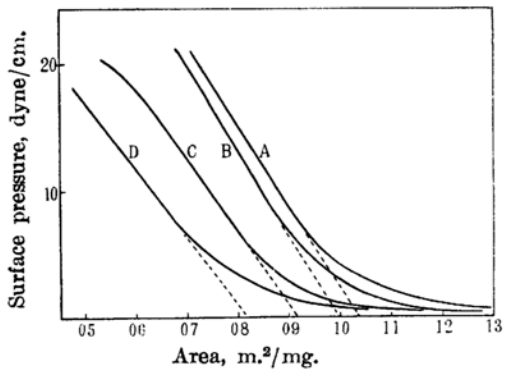

Fig. 1.—Force-area curves for azo-ovalbumin samples: curve A, Native ovalbumin; curves B, C and D, Azo-ovalbumin with 14, 30 and 58 diazonium groups per molecule.

On the other hand, the thickness is also found by the following consideration. Since the area per molecule of each sample is nearly the same at the pressure of 15 dynes per cm. *i. e.*, about 5500 Å.², the thickness (h_A) of their monolayers may be calculated from the following equation.

$$h_A = h_0 \frac{M_A}{M_0}, \qquad (5)$$

h_0 being the thickness of ovalbumin monolayer at the pressure of 15 dynes per cm., calculated from the specific area and density, M_A and M_0, molecular weight of azo-ovalbumin and ovalbumin, respectively, where the latter is taken as 40,000. The thickness values calculated are given in Column IX of Table 1, being in good agreement with that obtained from the specific area and density.

Thus, Eq. (5) is applicable for the estimation of molecular weight of azo-ovalbumins, on the basis of data available from force-area curves. The thickness of a single layer of azo-ovalbumin molecule, when measured directly using the built-up films, gave somewhat higher value than that obtained from force-area relationships (Column X, Table 1). As the deposition ratio of any of the samples of azo-ovalbumin was 0.83, the same as that of native ovalbumin, much higher thickness value should be expected with the film spread on distilled water. This may probably be due to incomplete spreading of azo-ovalbumin which was spread on distilled water so as to form the

Table 1

I	II	III	IV	V	VI	VII	VIII	IX	X
Sample	Number of azo-groups per molecule	Molecular weight	Limiting area per milligram, m.²	Area/mg. at 15 dynes/cm., m.²	Limiting area per molecule, Å.²×10³	Area per molecule at 15 dynes/cm., Å.²×10³	Thickness of monolayer calculated from specific area and density, Å.	Thickness of monolayer from Eq. (5), Å.	Thickness of monolayer observed at 15 dynes/cm., Å.
A	0	40000	1.04	0.81	6.9	5.3	9.5	9.5	9
B	14	43300	1.00	0.78	7.1	5.6	9.9	10.2	11
C	30	47100	0.92	0.69	7.4	5.4	11.1	11.1	12.5
D	58	53800	0.82	0.62	7.3	5.5	13.1	12.7	14

Temperature of measurements: 20~21°C.

built-up films.

It has now been established that the coupled diazonium groups do not contribute to the area of the compressed protein film molecule, but tend to increase the thickness. According to Pauly,[13] and Kapellar-Adler and Boxer[14] the diazonium groups are introduced into the side-chains of polypeptides, such as tyrosine and histidine residues. Accordingly, the present results seem to support the view[15] that under higher pressures the side-chains will be forced out of the surface until they are oriented perpendicular to the surface.

(ii) Low pressure region (below one dyne per cm.). The force-area curves of ovalbumin and azo-ovalbumin with 58 diazonium groups per molecule are shown in Fig. 2. It was found for both samples that a break occurs in force-area curves, denoting a higher order transformation point. A detailed description will be given in future.

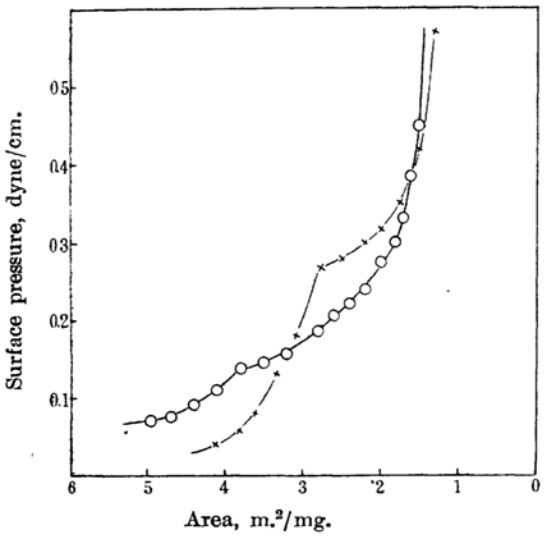

Fig. 2.—Force–area curves of azo-ovalbumin samples: Cross shows native ovalbumin and circle azo-ovalbumin with 41 diazonium groups per molecule. Underlying solution is 0.1 *N* HCl, and temperature, 23°C.

Various Types of Built-up Films of Azo-ovalbumin.—With ovalbumin various types of built-up films have been obtained by Langmuir[11] and coworkers. Langmuir indicated these films with the following symbols: PRA, PRAA, PRAB, PRBBB···, PRBAB, etc., where P stands for a metal plate, R for barium stearate multilayers, A for protein monolayers which is deposited by a down-trip and B for that deposited by an up-trip.

We examined the mode of deposition with azo-ovalbumins and found that it was possible to prepare the following types: PRA, PRABBB ···, PRTB, PRTBB, PRTBAB etc., where RT represents the barium stearate multilayers conditioned by thorium nitrate, the surface of which is hydorphilic. Contrary to ovalbumin, it was difficult to prepare the types of PRB and PRAA. According to our experience, it may be said that the more hydrophobic is the surface of underlying substrate, the easier is the deposition of A-layer, and the more hydrophilic the underlying surface, the easier the deposition of B-layer.

The order of wettability for various type of built-up films gave the following series:

$$\text{PRA} > \text{PRAB} > \text{PRTB}$$

i. e., PRA film is more hydrophilic than PRTB film. Langmuir[11] found, with ovalbumin, that PRA and PRB films differ very little in wettability. This fact has led him to the view that the hydrophobic and hydrophilic portions of protein molecule have no fixed orientations within the film, but can readily overturn by contact with water. In the present experiment, however, B-layer of azo-ovalbumin film was found to have adhered to the PRT film whose surface is highly polar. Then anchoring of hydrophilic groups to the surface of PRT film would occur, leaving the hydrophobic groups on the side exposed to air. This may be the reason why PRA film is more wettable toward water than PRTB film.

Antigenic Activity of Azo-ovalbumin Films.—The reactivity of protein films is evidently associated with the structure and orientation of film molecules. For instance, Langmuir and Schaefer[16] demonstrated that the activity of urease monolayers depends greatly on the type of films deposited. To obtain some insight into this problem, it was desirable to utilize azo–protein–antibody system. For this purpose, various types of the built-up film of azo–ovalbumin containing 29 diazonium groups in one molecule were treated with undiluted antiserum,[17] which contains an

(13) H. Pauly *Z. Physiol. Chem.*, **94**, 284 (1915).
(14) R. Kapellar-Adler and G. Boxer, *Biochem. Z.*, **285**, 55 (1936).
(15) *Cf.* H. Neurath and H. B. Bull, *Chem. Rev.*, **23**, 391 (1938).
(16) I. Langmuir and V. J. Schaefer, *J. Am. Chem. Soc.*, **60**, 1351 (1938).
(17) This serum was obtained from rabbits injected with *p*-phenylarsonic acid-conjugated horse serum and contained appreciable quantity of antibody specifically directed against the diazonium groups.

antibody specifically directed against the *p*-azo-phenylarsonic acid group, according to the following technique. The prepared plates were placed in antiserum during 15~20 hours. The plates were then removed and washed. After drying, the increment of thickness was measured. These results are summarized in Table 2.

Table 2

Type of film	Thickness increment after treatment with antiserum, Å.
PR	—
PRA	225
PRAB	400
$PR(AB)_E$	<20
$PR(AB)_EB$	250
$PR(AB)_EBB$	405
PRTB	90
PRTBA	180
PRTBB	180

The treatments with antiserum were performed at 0°C. The symbol $(AB)_E$ denotes one double-layer of ovalbumin which is ineffective to the adsorption of antibody used in these experiments. All the data were obtained with a pool of antisera in order to eliminate individual variation from serum to serum.

The treatment with antiserum produced a considerable adsorption on respective films, whereas the thickness increment on exposure to normal rabbit serum did not exceed a few Å. Obviously the prepared films demonstrated a clear-cut specificity.

Hereupon it would be of interest to compare the activity of A-layer with that of B-layer. For this purpose it seemed desirable to make a comparison between the activities of PRA and PRB films. However, it was impossible to make the film of type PRB with azo-ovalbumin. Therefore, for the sake of convenience the activity of B-layer on $PR(AB)_E$ film (barium stearate multilayer covered with one double-layer of ovalbumin) was compared with that of A-layer on PR film. The thickness increment with non-specific adsorption on one double-layer of ovalbumin alone ($PR(AB)_E$-film) did not exceed 20 Å. If this non-specific increment is taken from the data in Table 2, it would be found that the activity of a B-layer in $PR(AB)_EB$ is almost equal to that of an A-layer in PRA. Antigenic films of type $PR(AB)_EBB$ and PRAB, also, exhibited equal activity, and the activity of PRAB film was twice as much as that of PRA film. These results show that an A-layer and a B-layer of azo-ovalbumin film differ little in antigenic activity.

In the present experiments, however, the substrate films on which an A-layer or a B-layer was deposited were not the same. In this sense, the experiments using PRTBB and PRTBA as antigenic films would enable us to make the comparison between an A-layer and a B-layer, since then the underlying layer PRTB was common to both films. Thereupon, as shown in Table 2, the thickness increment on both films were almost equal and were twice as thick as that on PRTB film. From these data it may also be concluded that an A-layer and a B-layer have almost equal value in respect to antigenic activity. Similar results have been noticed in the behavior of a single layer of ovalbumin, bovine albumin or meta-kentrin by Rothen[18] and coworkers.

The results in Table 2 also show that the antigenic activity of azo-ovalbumin layer on PRT film is markedly less than those on PR or $PR(AB)_E$ film. This means that thorium nitrate conditioning of underlying substrate film has an effect of appreciably decreasing on the antigenic activity of azo-ovalbumin layer. This effect may be due to the anchoring of arsonic acid radicals in haptenic groups to highly polar surface of PRT film. The activity of PRTBB and PRTBA films are just twice as much as that of PRTB film which is less than that of PRA film. In other words, the effect of conditioning extends to the top layer of an antigen double-layer. Protein monolayer is of skeleton-like structure as considered by Blodgett[19] so that haptenic groups of top layer also could anchor to the polar surface of conditioned stearate multilayer.

The fact that the activity of two antigen layers is almost twice as much as that of one antigen layer, would require some explanation. Rothen[20] showed with bovine albumin that the amount of specifically adsorbed antibody is directly proportional to the number of underlying antigen layers up to a certain number. He explained this fact by "Long range forces" hypothesis. We also found a similar phenomenon by our experiments with azo-ovalbumin, some results of which were given in the previous paper.[21] In short, owing to the strong combining forces of antigen and antibody, antibody molecules could bind, penetrating into antigen films of skeleton-like structure, with the haptenic groups in the inside of films. Similarities of behavior in A-layer and

(18) A. Rothen: "Advances in Protein Chemistry," Vol. III, p. 123, (1947).
(19) K. B. Blodgett, *J. phys. Chem.*, **41**, 975 (1937).
(20) A. Rothen, *J. Biol. Chem.*, **168**, 75 (1947).
(21) T. Tachibana and K. Fukuda, This Bulletin, **24**, 4 (1951).

B-layer may also be explained along this line.

Summary

(1) Force-area curves were determined of monolayers of native ovalbumin and azo-ovalbumin with various number of diazonium groups. As a results it was found that the coupled diazonium groups do not contribute to the area of compressed protein film molecule, but tends to increase the thickness.

(2) The mode of deposition, when the monolayers of azo-ovalbumins are deposited on metal slides covered with multilayers of barium stearate, was examined. Various types of deposited azo-ovalbumin films thus obtained were tested as to their antigenic activity. The results were discussed in relation to the structure of protein films.

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