The "Critical Thickness" of Organic Thin Films[1]

With a Criticism to Long Range Force Hypothesis of Rothen

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Introduction

Several years ago, Rothen[2] postulated specific long range forces to explain the specific interaction of an antibody with the antigen layer through the inert screen film of the thickness extending over 150 Å. Since then, his proposal has aroused considerable interest in biologists, chemists and physists, and has become the focus of much controversy.[3],[4],[5],[6],[7]

But anyone has not been able to formulate any such mechanism that could account for the specificity required to explain Rothen's experimental results. Rothen, himself, has suggested that this specific long range interaction might take place through resonating extended oscillators.

On the other hand, Singer[5] recently has shown the evidence that strongly suggests that holes in the screen films, permitting such macromolecules as antibody to come into contact and react with antigen, are primarily responsible for the effect observed by Rothen. We have also obtained experimental evidences that appear to suggest that the results of Rothen can be explained by a simpler mechanism that does not require the assumption of specific long range forces. This interpretation has been strongly supported by many facts that had already been obtained through the study of

(1) This paper was partly presented before the regular meeting of the Chemical Society of Japan held at Tokyo, Sept. 24, 1949, and the annual meeting of the Chemical Society of Japan held at Kyoto, Apr. 3, 1950.

(2) A. Rothen, *J. Biol. Chem.*, **168**, 75 (1947); *J. Am. Chem. Soc.*, **70**, 2732 (1948).

(3) F. Karush and B. M. Siegel, *Science*, **108**, 107 (1948).

(4) J. Iball, *Science*, **109**, 18 (1949).

(5) S. J. Singer, *J. Biol. Chem.*, **182**, 189 (1950).

(6) H. J. Trurnit, *Science*, **111**, 1 (1950).

(7) B. Carroll, *J. Am. Chem. Soc.*, **72**, 2763 (1950).

thin film. Then, we have found the "critical thickness" of thin film below which the film could not perfectly cover the substrate. There exist similar phenomena on metallic films. It is a well-known fact that metallic films given by evaporation and cathodic sputtering on non-conducting substrate display, below a certain thickness, different properties from those of the metals in bulk. These results have led us to an opinion that Rothen's results did not necessarily prove the existence of specific long range forces. The present paper describes the results of these experiments and some discussion thereupon.

Experimental Procedure and Results

Experiments have been made on three systems as follows: azo-ovalbumin—rabbit antisera against the haptenic group, azo-ovalbumin—ovalbumin, and cholestanol—digitonin.

1. Azo-ovalbumin—Antisera System.—In order to investigate whether specific long range action is demonstrated in artificial antigen—antisera system as well as natural antigen—antisera system used in Rothen's experiments, we have performed an experiment with azo-ovalbumin (ovalbumin coupled with diazotized *p*-amino-phenylarsonic acid) and antibody specifically directed against this haptenic group. After our studies were completed, we found that Singer carried out similar experiments on the same system. His works, however, differ from ours in respect to the condition of the film of antigen; his experiments deal with adsorbed film, while ours with unfolded film.

The techniques used in this experiment will be described elsewhere.(8) Azo-ovalbumin was prepared by coupling ovalbumin with diazotized *p*-amino-phenylarsonic acid in the usual manner. The number of the diazonium groups introduced in one ovalbumin molecule (assuming the molecular weight as 40,000) was found to be 47 analytically. Rabbit antisera was produced against horse globulin coupled with diazotized *p*-amino-phenylarsonic acid.

In order to prepare the antigen film, azo-ovalbumin was spread on distilled water and then, compressing the monolayer at surface pressure of 15 dynes per centimeter, one double layer of azo-ovalbumin was transferred onto the metal slide covered with an optical gauge of multilayers of barium stearate. Under these conditions, the thickness of the transferred antigen layer (one double layer) was found to be 26～30 Å. Subsequently, films of barium stearate, as screen, were deposited on the antigen films by means of the Blodgett—Langmuir technique. These slides were immersed into undiluted antiserum until the adsorption of the antibody was completed. After the slides were taken out of the solution, washed and dried, the increase of thickness was measured optically.

The experimental results are given in the curve A of Fig. 1. Each of the points represents an

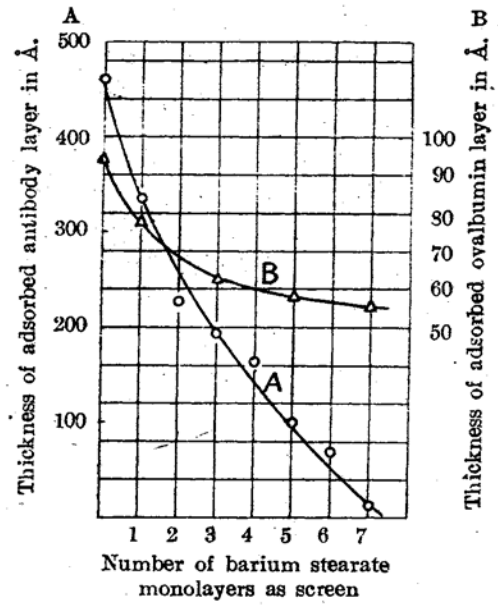

Fig. 1.—Screening action of barium stearate films on one double layer of azo-ovalbumin. Curve A, azo-ovalbumin—antiserum system; curve B, azo-ovalbumin—ovalbumin system.

average of about four independent determinations. Control experiments demonstrated that none of the antibody in the antiserum used in these experiments was adsorbed on the film of original ovalbumin when it was treated in the same manner, and that no measurable amounts of normal serum proteins were adsorbed on the stearate-coated or non-coated films of azo-ovalbumin. These results demonstrate that the antibody specifically directed against the haptenic group was adsorbed on the stearate-covered antigen through the screen in the range of thickness indicated.

Pauling, Pressman and Campbell,(9) on the other hand, based on the investigations of the hapten-conjugated antigen and the antibody, proposed a mechanism for the antigen-antibody reaction that, over a considerable portion of both molecules having complementary configuration, the short range forces, as van der Waals', electrostatic, hydrogen bond, and other similar forces could cooperate cumulatively to form a sufficiently strong bond.

In the case of azo-ovalbumin and antisera

(8) T. Tachibana, K. Fukuda, T. Ogata, K. Suzuki and S. Yamaoka, unpublished.

(9) L. Pauling, D. Pressman and D. H. Campbell, *Physiol. Rev.*, **23**, 203 (1943).

directed against the diazonium groups, antibody activity is directed specifically against them. If Rothen's interpretation is applied to our experiments, long range forces should operate between antibody and haptenic group of which chemical structure is well known. However, long range forces hypothesis was advanced on the basis of the assumption that holes in the screen films are not responsible for the effect observed. If thin screen films were actually preventing the diazonium groups of azo-ovalbumin and antibody molecules from coming into direct contact, this facts is inconsistent with the consideration put forth by Pauling and his co-workers.

On the other hand, similar phenomena observed by Rothen in immunologic and enzymatic reactions, might occur in the case of the adsorption of large molecule on films of any adsorbent as well. In order to investigate this problem, the following two experiments were performed.

2. Azo-ovalbumin—Ovalbumin System.—Adsorption of ovalbumin on the film of azo-ovalbumin. It was at first reported by Haurowitz[10] that the mixing of azoprotein and ovalbumin in solution of some *p*H range brought about precipitation reaction. In this case, the interaction would be of electrostatic nature as pointed out by Haurowitz. We found, by monolayer technique, that the ovalbumin molecule was adsorbed on the spread film of azo-ovalbumin when the slide covered with azo-ovalbumin film was brought into contact with the solution of ovalbumin. Then, experiments were extended to detect whether both molecules might not also exert their action throughout a screen film of barium stearate.

As described previously, ovalbumin coupled with diazotized *p*-amino-phenyl arsonic acid was spread on the distilled water and transferred, as one double layer, onto the metal slide covered with an optical gauge of multilayers of barium stearate. The thickness of one double layer was found about 28 Å. The number of the diazonium groups per molecule of the sample was found 58 analytically. Then azo-ovalbumin film was coated with the screen films of barium stearate in the same manner as in the previous experiments. Hereafter the slides to be tested (with or without screen) were immersed into the solution of *p*H 4 containing one percent of ovalbumin for 45 minutes at room temperature of about 10°C. The thickness increment was measured optically. The results (the thickness increment caused by the adsorption of ovalbumin) are given in curve B of Fig. 1. Ovalbumin molecules were adsorbed on azo-ovalbumin film, despite of the screen films of barium stearate over 100 Å. thick in a manner similar to that found for the antigen-antibody system. Control experiments demonstrated that ovalbumin layer of about 20 Å. thick was adsorbed directly on the stearate films alone, when they were treated in the same manner.

Rothen has performed, on the other hand, similar experiments with protamin and insulin to demonstrate that barium stearate and Formvar were effective screens for this system. A double layer of barium stearate covering the film of protamin clupein sulfate (10 Å. thick) cut down the adsorption of insulin completely, whereas 220 Å. of insulin could be adsorbed directly on the protamin. This fact was ascribed to the non-existence of holes in screens through which even smaller molecule, such as insulin, than antibody globulin molecule could permit to pass. However, as stated by Singer, the barium stearate screens may not be inert barrier in these experiments, but may rather react with the protamin film.

Such complications are excluded in our experiment with azo-ovalbumin-ovalbumin reaction. Therefore, the present results may show that apparently long range forces also might exert their effect in the reaction system mentioned above. The adsorption of ovalbumin on azo-ovalbumin film is commonly thought to be brought about due to the electrostatic forces between the oppositely charged molecules, since azo-ovalbumin molecule is charged negatively while ovalbumin molecule is positive at *p*H 4. Consequently, if long range force operates in this case, it may be of electrostatic nature. However, two possibilities may also be considered: 1) that there may be holes to permit the diffusion of ovalbumin molecules in the screens: 2) the electrostatic interaction between azo-ovalbumin film and ovalbumin molecule may take place through induced polarization through the screen. Which possibility is true, would be difficult to decide only from this experiment. At any events, it is important that there could be found a phenomenon that appeared to be caused by long range force, in non specific reaction also.

3. Cholestanol-Digitonin System.—The deposition of digitonin on cholestanol films was studied by Langumir, Schaefer and Sobotka.[11]

We have performed experiments to see whether the adsoption of digitonin does not take place through the screen of barium stearate. The techniques used are essentially the same as that described in their paper, except for the covering of cholestanol films with the screen of the built-up films of barium stearate.

The results are summarized in Table 1. It is noticeable that, regardless of the thickness of screens, the thickness increments after treatment with digitonin were found to be about 15 Å. Control experiment demonstrated that the optical gauge of stearate multilayers alone decreased 6 Å. in thickness after being treated with digitonin solution. The amount of digitonin adsorbed on cholestanol films was independent of the number of the monolayers of cholestanol on the slide. Although the amount of digitonin adsorbed on cholestanol film was independent of the thickness of screen films, the surfaces after adsorption of digitonin were, depending upon the thickness of

(10) F. Haurowitz, *Kolloid-Z.*, **74**, 208 (1935).

(11) I. Langumir, V. J. Schaefer and H. Sobotka, *J. Am. Chem. Soc.*, **59**, 1751 (1937).

screen film, markedly different in their adhesion toward water. This is given in column marked "hydro-affinity" in Table 1. When screen films

Table 1

Screening Action of Barium Stearate Film against the Adsorption of Digitonin on Cholestanol Film

Cholestanol film		Screen of barium stearate		After treatment with digitonin		
Mode of deposition**	Thickness observed, Å.	Number of layers	Thickness observed, Å.	Thickness increment, Å.	Hydro-affinity of the surface	θ
AB	42	0	0	11	++	
$(AB)_2$	72	0	0	12.5	++	
$(AB)_5$	187	0	0	7	++	64°
AB	34	1	20.5	18.5	++	
AB	32	2	48.5	14	++	76°
AB	37	5	116	15	++	79°
AB	36	8	195	10	+	
AB	41	10	244	7	+	92°
				14.5*	+	

The time of adsorption was 10 minuutes in each experiment, but the value marked * was obtained by adsorption of one hour. Symbol ++ indicates to be more hydrophilic than symbol +. θ denotes the contact angle towards water. ** Symbols AB, $(AB)_2$ and $(AB)_5$ stand for one, two and five double layers, respectively.

of stearate are less than 5 monolayers, the slide has relatively high hydro-affinity. However, when they are thicker, the slide become less hydrophilic. Cholestanol films and barium stearate films themselves are both originally hydrophobic. Therefore, the effect observed might probably suggest that digitonin molecule, penetrating the screen film of stearate, came into contact directly with the underlying cholestanol film.

If this is the case, there may probably be holes to permit molecule as large as digitonin to diffuse in screen of stearate film.

Discussion

From the present studies, it was found that some kind of chemical reactions involving large molecule, such as the adsorption of ovalbumin on azo-ovalbumin film and the adsorption of digitonin on cholestanol film, as well as the adsorption of antibody on antigen film could occur although the adsorbent films were separated by the screen films of barium stearate. Consequently the phenomena that long range forces appear to operate are not always specific in biochemical reactions alone. Therefore, it seems to be unreasonable for us to require specific long range forces in immunologic and enzymatic reactions alone. It may be rather plausible to admit the possibility that holes in screen films are responsible for the effect observed, although Rothen attempted to show that the possibility did not exist or in any events was not important. At least, our experiment with the cholestanol-digitonin system might prove that there are holes to permit digitonin molecules to diffuse in screen film of barium stearate, since this system probably reacts by a short range mechanism.

Furthermore, we might show some evidences that the physical state of screen film is of fundamental importance for the explanation of the effect observed. Let us consider the barium stearate alone as screen film for convenience' sake.

If we examine the screening action of barium stearate films from the results of Rothen, Singer and the present authors, we see that there is an almost constant "critical thickness" of barium stearate films to inhibit the combination of antibody on antigen film.

This critical thickness is found around 150 Å. in most systems investigated, as summarized in Table 2. Particularly it would be difficult to explain from the long range forces hypothesis that the same value of critical thickness was obtained independently of whatever the antigens used may be natural or artificial, or whatever the amount of antibody adsorbed directly on antigen film may be (See Table 2).

The critical thickness of stearate film was also observed in other phenomena. The clean glass surface is hydrophilic and gives a zero

Table 2

The Critical Thickness of Barium Stearate Film to Inhibit the Specific Adsorption of Antibody on Antigen Layers

Varieties of the system	I One double layers of ovalbumin and anti-ovalbumin system	II One double layers of bovine albumin and anti-bovine albumin system	III Adsorbed layer of Pneumococcus polysaccharide III and homologous antibody system	IV Adsorbed human albumin and homologous antibody system	V One double layers of azo-ovalbumin and homologous antibody system
Thickness of antibody layer adsorbed directly on antigen film, Å.	26	65	270 125	80	465
Critical thickness of barium stearate film, Å.	70~100	150	150~160	150~160	150~170

Critical thickness was observed directly or calculated from the number of layers multiplied by the thickness per monolayer, 24.4 Å. I, II, III, were taken from the results of Rothen; IV from Singer; V from the present authors.

contact angle with water. However, after the stearic acid monolayers have been deposited on glass, the surfaces becomes hydrophobic and the contact angles increase with the number of the stearate monolayers until they attain a critical value, about 115°, where the thickness of stearate films is 70～120Å. (3～5 monolayers) as shown in Table 3 (column 3).

Table 3

Screening Action of Stearic Acid Multilayers on Solid Surface

1 Number of layers	2 Thickness, Å.	3 Contact angle towards water	4 Surface potential, mV.	5 Static friction coefficient
0	0	0°		1.0
1	24.4	66°11′ 101°46′	191	0.12
3	73	115°23′	355	0.09
5	122	115°56′	467	0.06
7	171	112°10′	457	0.06
9	220	117°36′		0.06

column 2 ; calculated from the thickness per monolayer, 24.4 Å.: column 3 ; S. Mitsui and T. Sasaki, private communication (1948): column 4 ; T. Isemura, Private communication (1950): column 5 ; T. Isemura, this Bulletin, **15**, 467 (1940).

The surface potential of stearate monolayers on metal slides shows also a similar behavior. In this case, the critical thickness of stearate monolayers is 120～170 Å. (5～7 monolayers) as indicated in column 4 of Table 3. It might also be significant to quote the results of Isemura who studied on the effect of monolayers on the static friction of glass surface. We see from his results that the static friction decreases with the increase of the number of stearate monolayers, until it becomes nearly constant in the region of the thickness exceeding five monolayers, *i.e.* about 120 A., as shown in column 5 of Table 3. These results appear to be as if the character of underlying substance was transmitted across the screen film of stearate. Furthermore, as far as stearate films are used as screen, the critical thickness to interrupt the effect of underlying substance is always about 120 Å., irrespective of independent phenomena observed. This value of the critical thickness is approximately the same as that found in immunologic and enzymatic reaction. This coincidence is regarded as more than accidental. This seems to indicate that multilayers of stearate, when they are less than 150 Å. thick, consist of fragile structure. Namely such a thin film may be such as the network of structure loose enough to permit the diffusion or penetration of a large molecule. The detailed mechanism may be complicated. The present suggestion might be extended to apply to screen films of Formvar and other polymers as well.

Moreover, Rothen showed that in the bovine serum albumin—antiserum system, the amount of antibody adsorbed increased linearly with the number of monolayers of antigen up to eight layers where the thickness of adsorbed layer of antibody is 149 Å. We also obtained a similar relation from the experiments with azo-ovalbumin—rabbit antiserum. These results are represented in Fig. 2, the details of which will be described elsewhere.

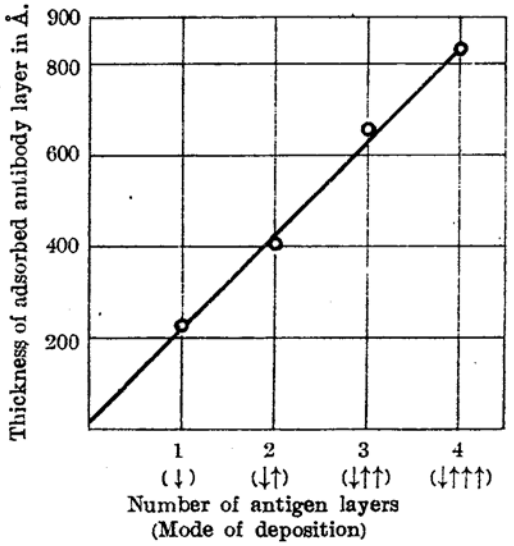

Fig. 2.—Relation between the number of azo-ovalbumin and thickness of antibody layer adsorbed on it.

It is noteworthy that a layer of 800 Å. of antibody, which is larger than the major axis of antibody molecule, was adsorbed directly on the four monolayers of antigen (ABBB). At first sight, this result might appear to support long range force hypothesis. However it may be unfavorable to Rothen's hypothesis that the thickness of antibody layer increases linearly with the number of underlying monolayers of antigen, *i.e.* the number of haptenic groups of antigen per unit area. The total haptenic groups of antigen layers should be available to the adsorption of antibody molecules. Let us notice that the thickness of antigen layer, in this experiment, is lower than the "critical thickness."

In this case, the structure of antigen layer may be fragile. Especially, by bringing into contact with antiserum, the structure of protein

layers may become more loose by swelling. Therefore, the antigen layer might conceivably disrupt, exposing all the haptenic groups of antigen molecules in original monolayers to antiserum. Thus, the underlying antigen layer, also, may become available to the reaction by direct contact with antibody molecules. After drying, the layers may become such structure as that in which antigens and antibodies are mixed together or alternated more regularly. Such conditions are probable in consideration of more strong affinity between antigen and antibody than between each antigen monolayers.

Rothen, also, reported that, on optical gauges of octadecylamine, a layer of 700 Å. of antibody was adsorbed on 5 Å. of pneumococcus type III polysaccharide. He explained this facts by long range forces. But, in this case also, as polysaccharide is a chain polymer, it may extend into bulk of antiserum, like seaweeds on the bed of sea, exposing all determinant groups to antibody. Therefore, adsorbed layer of 700 Å. is not a uniform layer of antibody, but instead may be quite complicatedly composed of antigen and antibody. Thus, the large thickness of 700 ~ 800 Å. of adsorbed antibody layer observed by Rothen and us may be explained without long range forces hypothesis.

In conclusion, in view of the considerations and the evidences presented above, we seem to be facing the problem of a "critical thickness" or structure of thin organic films rather than the long range forces postulated by Rothen.

Summary

By studying the reactions of protein with the aid of monolayer technique, Rothen found that antigen and antibody, and also enzyme and substrate molecules, can react in despite of being apparently separated by some inert screen.

He assumed from these results, that the specific long range forces operate in immunologic and enzymatic reactions. We have found that similar phenomena occurred in the following systems: monolayer of hapten-conjugated protein as antigen and antibody directed specifically against the hapten, azo-ovalbumin and ovalbumin, and even cholestanol and digitonin. Therefore, it leaves some room for consideration to assume specific long range forces in immunologic and enzymatic reaction alone.

Besides, we suggested that there was required an almost constant thickness (about 150 Å.) of barium stearate to inhibit completely the combination of antibody with antigen in transferred film, independent of the variety of the systems.

We have also noticed, from the data of the contact angle towards water, those of surface potential and of static friction coefficient of stearic acid multilayers on glass or metal surface, that the multilayers of stearic acid must have a thickness over about 150 Å. (same as the result of antigen-antibody reaction) to interrupt the effect of underlying substance, regardless of the phenomena concerned.

These facts appear to suggest the fragile nature of screen film which is thinner than the certain thickness which we call the "critical thickness." Such a thin film may conceivably be such as the net-work structure loose enough to permit the diffusion and the penetration of large molecules. Such a physical state of screen film may primarily be responsible for the results of the experiments of Rothen, Singer and the present authors.

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