ON THE UNLIKELIHOOD OF SPECIFIC LONG RANGE FORCES IN IMMUNOLOGIC AND ENZYMATIC REACTIONS*

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PLATES 1 AND 2

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In the past few years, a great many experiments have been performed by Rothen (24, 25) which he has interpreted as suggesting that antigen and antibody, and enzyme and substrate proteins, can react specifically, although separated by distances of hundreds of angstroms. This hypothesis seems to conflict with evidence accumulated from many other investigations indicating that short range interactions, involving distances usually associated with electrostatic and van der Waals' forces, and hydrogen bonds, of the order of 5 A, operate in these reactions. We have therefore engaged in a critical analysis of the experimental techniques used by Rothen to determine whether some interpretation other than that invoking the existence of specific long range forces could be found to explain his experimental results.

Before we proceed with the details of this investigation, it might be worth while to summarize briefly the evidence at hand that short range forces are primarily responsible for the specificity of immunologic and enzymatic reactions. The work of Landsteiner (15), Haurowitz (9), Pauling and his collaborators (21), and others, using as antigens proteins to which known chemical groups (haptens) had been chemically conjugated, has demonstrated the very high order of this specificity in antigen-antibody reactions. Antisera were prepared which contained antibodies directed specifically against a particular haptenic group, and these antisera were allowed to react with a large number of antigens containing somewhat different haptens. By a detailed systematic study of these reactions it was demonstrated that slight structural changes in the hapten (slight, that is, from any long range point of view), such as the substitution of a carboxyl group for an arsonic acid group on a benzene ring or the replacement of an L-tartranilic acid hapten for the D isomer, could have

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profound effects on the extent of the cross-reactions observed. Moreover, Landsteiner discovered that the low molecular weight hapten itself, not associated with a protein, could specifically inhibit the precipitation of the antibody and the hapten-conjugated antigen.

After a more quantitative investigation of these phenomena, Pauling, Pressman, and Campbell (20) proposed a mechanism for the antigen-antibody reaction, more explicit than earlier ones, requiring complementary configurations for the antigen and antibody, so that, over a considerable portion of both molecules, short range van der Waals', electrostatic, hydrogen bond, and similar forces could cooperate to form a bond that was cumulatively sufficiently strong. The phenomenon of hapten inhibition of antigen-antibody precipitation could be ascribed to the competition between hapten and hapten-conjugated antigen for the complementary sites on the antibody. This hypothesis seems to be in accord with most of the experimental results in immunochemistry, as well as with the existing knowledge about the nature of the forces involved in systems of small molecules.

In the case of enzymatic reactions direct evidence has been obtained in certain cases for the existence of short range mechanisms. In recent years it has been possible to demonstrate spectroscopically the existence of certain activated complexes between enzyme and substrate, as suggested long ago by Michaelis and Menten (18). These authors proposed that enzyme and substrate form an unstable compound, the activated complex, which subsequently rearranges to yield the original enzyme plus the reaction products. Keilin and Mann (14) studied the complex formed between peroxidase and hydrogen peroxide, and Chance (5) that between catalase and hydrogen peroxide, by changes in the absorption spectra produced on the formation of the complex. A great amount of material concerning the high order of specificity of various peptidases (1) and their metal ion requirements (27) can also be very well explained by this mechanism, involving the formation of short range chemical bonds between enzyme and substrate molecules.

It would appear, after careful consideration of the facts that have been mentioned only briefly here, that the hypothesis of long range forces requires either of two improbable alternatives, (*a*) either that several or a whole range of mechanisms involving a spectrum of short and long range forces is operative in both immunologic and enzymatic reactions or (*b*) that the principles of structural chemistry acquired through the study of systems of small molecules, which have been used to interpret the evidence cited above, are totally inadequate to explain the reactions of large molecules.

The experiments of Rothen constitute the only evidence that has been

advanced to demonstrate directly that specific long range forces exist. If these experiments can be interpreted satisfactorily by other means, then, in consideration of the improbable alternatives afforded us by the concept of long range forces, we would not be justified in presuming that such interactions exist.

We have obtained experimental evidence that strongly suggests that the results of Rothen can be interpreted by a much simpler mechanism that does not require the existence of long range forces. This evidence and its interpretation make up the major part of this paper. In order to discuss these, we must first describe the experimental techniques and results obtained by Rothen.

Rothen's Technique and Results

The experiments from which the hypothesis of long range forces evolved were concerned with the preparation and accurate measurement of very thin films of antigens, antibodies, and various inert materials. (Henceforth we shall restrict most of our discussion to the immunologic reaction, unless otherwise specified.) On a suitable substrate, such as a highly polished stainless steel slide, an optical gage of barium stearate was deposited by the Blodgett-Langmuir technique (2). This gage consisted of two monolayers of barium stearate on the top half of the slide and four monolayers on the bottom half.[1] The optical gage permitted the use of the half shadow principle with an instrument, developed by Rothen (22), called the "ellipsometer," which is capable of accurate measurements of film thicknesses. The slide with the optical gage was then "conditioned" by contact with a uranyl acetate-veronal buffer solution, which made it possible subsequently to pick up, by the Blodgett-Langmuir technique, more than two monolayers of bovine serum albumin antigen. After the deposition of the antigen layers, the surface was covered with a thin film of inert material (the barrier or screen) such as Formvar, barium stearate, or octadecylamine. A solution of antiserum was then placed on this surface, and allowed to react for several minutes; the slide was then washed in a reproducible manner and dried, and the increment in total film thickness was measured.

By means of this technique, or modifications of it, Rothen obtained the following results. First, let us consider those experiments in which antibody was adsorbed directly on the antigen without intervening barrier films being present. Although in the case of the egg albumin-anti-egg albumin rabbit serum system the amount of antibody specifically adsorbed directly on the antigen was independent of the number of monolayers of antigen on the slide, in the bovine serum albumin-antiserum system the

[1] In our experiments we used three and five monolayers for the optical gage.

amount of antibody adsorbed increased linearly with the number of monolayers of antigen up to eight layers. It was argued that thickness measurements demonstrated that the antigen films consisted of completely unfolded molecules, since the thickness per monolayer was found consistently between 8 and 9 A. Under these circumstances the topmost layer of antigen should have protected underlying layers from the action of antibody if only short range forces were involved, and the amount of antibody adsorbed should have been independent of the number of layers of antigen on the slide. This was not the case in the bovine serum albumin-antiserum system. Other experiments dealt with the pneumococcus type III polysaccharide and rabbit antisera. In certain cases as much as 700 A of antibody were adsorbed on only 5 A of the polysaccharide, and from these experiments it was argued that long range forces were specifically immobilizing antibody which could not have come into direct contact with antigen.

In his experiments with inert barrier films interposed between antigen and antibody, Rothen used Formvar, barium stearate, and octadecylamine screens, the latter two applied by the Blodgett-Langmuir technique. He found that the amount of antibody adsorbed on top of the barrier covering the antigen fell off approximately linearly with barrier thickness, and was independent of the nature of these barriers within the experimental errors involved. In subsequent experiments with enzymatic reactions (25), however, he found that thin films of gold, evaporated from a hot filament in a high vacuum, were effective in inhibiting the reaction.

Considering the possibility that holes in the barrier films were responsible for the effects observed, Rothen attempted to show that they did not exist, or in any event were not important. We shall defer presenting these arguments until the section under "Discussion," and proceed to the experimental part of our investigation.

Experimental Results and Interpretation

We have obtained evidence that strongly suggests that holes in the barrier films are indeed responsible for most of the effects observed. Three different approaches have been utilized: (*a*) an investigation of certain barrier films other than those used by Rothen, (*b*) an electron microscope study of the various surfaces involved in these experiments, and (*c*) experiments with hapten-conjugated protein antigens and antibodies specifically directed against the hapten group.

Barrier Films—In order to carry on this investigation as effectively as possible we attempted to duplicate Rothen's technique in complete detail. We built an ellipsometer[2] of comparable sensitivity for film thickness

[2]Made to our specifications by the Instrument Development and Manufacturing Company, 3018 East Foothill Boulevard, Pasadena, California.

measurements and the necessary troughs and auxiliary equipment. In only two minor regards did our technique differ from Rothen's: First, instead of using highly polished stainless steel slides as the film substrates, we used carefully cleaned microscope glass slides coated with a film of aluminum approximately 1000 A thick. The aluminum was evaporated on both sides of the slides in a high vacuum mirror-coating assembly. We found these slides considerably easier to prepare than the stainless steel slides, and moreover we were able to discard them after one use. In some of his later experiments Rothen had reported (25) that chromium-coated or gold-coated glass slides gave the same results as those obtained with stainless steel slides. Secondly, in the deposition of the protein monolayers on to the conditioned barium stearate surface, Rothen used a movable bar and a Wilhelmy balance on the trough to maintain the film pressure at 8 dynes cm.$^{-1}$. Instead, we used a paraffined mercerized cotton thread as the barrier to contain the protein film, and purified triorthocresyl phosphate[3] as the piston oil, in the usual manner of depositing monolayer films (2). Proper care was taken to eliminate the possibility of contaminating the protein film with the oil. Langmuir and Schaefer (17) have recommended the use of triorthocresyl phosphate as a piston oil, and report its spreading pressure to be 9.5 dynes cm.$^{-1}$. The difference between 8 and 9.5 dynes cm.$^{-1}$ is not significant for our purposes. However, we found that, while we were able to pick up a double layer on the first down and up trips of the slide through the compressed protein monolayer, further layers were picked up only after allowing the slide to dry and then only on the up trips. This is somewhat different from Rothen's findings, since he was able to pick up a layer on each down trip and up trip. This slight discrepancy, however, presumably has little effect on the results obtained.

Our experiments with different barrier films are recorded in Table I and Text-fig. 1.[4] To avoid too much detail in Text-fig. 1 we have included experimental points only in the cases of Curves A and G. These experiments were all performed with four layers of bovine serum albumin (Armour) as the underlying antigen film. The pooled rabbit antiserum, provided through the kindness of Professor Dan H. Campbell, contained about 5 mg. of antibody per ml., as determined by quantitative precipitin

[3] Technical triorthocresyl phosphate was purified by extensive washing with dilute NaOH, and then with water, and was then dried over $CaCl_2$. The oil distilled at about 200° at 350 μ pressure.

[4] Formvar is a polyvinyl alcohol partially converted to the acetal by treatment with formaldehyde, and is sold by the Shawinigan Products Corporation, New York. VYN-W is a vinyl chloride-vinyl acetate copolymer manufactured by the Bakelite Corporation, New York. The ethyl cellulose and the cellulose acetate, designated N-22 and LL-1 respectively, were obtained from the Hercules Powder Company, Wilmington, Delaware. Parlodion is a partially esterified nitrocellulose supplied by the Mallinckrodt Chemical Works, St. Louis.

TABLE I

Experiments with Different Barrier Films

Nature of barrier	Barrier* thickness, A	Antibody† adsorbed, A	Nature of barrier	Barrier thickness, A	Antibody adsorbed, A
No barrier	0	81 ± 4‡	Ethyl cellulose in	28	55
Formvar in ethylene	17	67	acetone (Curve	33	40
chloride (Curve A)	39	36	D)	35	40
	43	61		53	35
	54	56		54	49
	84	32		56	18
	92	40		58	35
	94	24		61	56
	94	48		64	44
	102	18		69	41
	104	24		80	37
	125	26		88	13
	133	34		90	30
Barium stearate	48 ± 4§	56 ± 10§		101	34
(Curve B)	92 ± 4§	33 ± 7§		111	25
VYN-W in methyl-	8	65		111	4
ethyl ketone	46	65	Cellulose acetate	10	65
(Curve C)	46	49	in acetone	17	65
	69	53	(Curve E)	25	43
	71	65		45	50
	78	32		48	17
	83	48		48	54
				49	30
				58	26
				62	38
				75	32
				77	23
				78	0
				81	0
				85	20
Parlodion in methyl-	13	20	Ethyl cellulose in	13	39
ethyl ketone	23	66	ethylene chloride	14	24
(Curve F)	28	59	(Curve G)	15	48
	29	36		15	27
	30	30		20	26
	31	32		22	49
	32	30		22	31
	41	40		30	5
	42	19		41	32
	49	30		44	9
	55	29		50	0
	57	13		58	0
	64	13		72	0
	69	10		73	8

TABLE I—*Concluded*

Nature of barrier	Barrier* thickness, A	Antibody† adsorbed, A	Nature of barrier	Barrier thickness, A	Antibody adsorbed, A
Parlodion in methyl-	71	0	Ethyl cellulose in	76	3
ethyl ketone	80	10	ethylene chloride	79	0
(Curve F)—con-	88	32	(Curve G)—con-	111	0
tinued	122	9	tinued	115	0
	136	0		120	0

* Acetone, methylethyl ketone, and ethylene chloride, when applied to the slides on which the antigen was deposited on the conditioned optical gage, caused an apparent decrease of about 10 A in the measured film thickness, presumably due to the solubilization of some of the stearic acid in the optical gage by the organic solvents. Higher boiling solvents, like amyl acetate, completely obliterated the line of demarcation between the top and the bottom half of the optical gage. The measured barrier thicknesses given, except in the case of barium stearate, are therefore somewhat smaller than the true values.

† Control experiments in which normal rabbit serum was used in place of antiserum, under otherwise identical conditions, resulted in a small decrease, about 5 to 10 A, in the total film thickness. No correction was made for this effect, however.

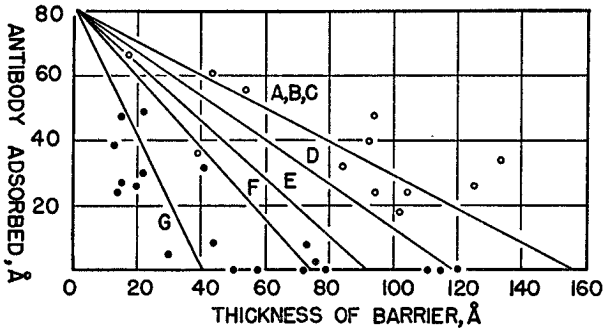
‡ Average of twenty experiments.

§ Average of four experiments.

tests. It was kept sterile, and aliquots were diluted 1:6 with a phosphate-saline buffer just before use. This buffer is the same as that described by Rothen. The precision of the data obtained in the experiments with polymer barrier films is not high. This lack of precision appears to be characteristic of the technique; yet it is not due to any irregular behavior of the antigen layers or the antiserum, since the results of the experiments in which antibody was adsorbed directly on four layers of bovine serum albumin were considerably more precise. In twenty experiments performed on independent batches of slides, an average of 81 A of antibody was adsorbed on the antigen with an average deviation from the mean of ± 4 A. In order to ascertain that differences in antibody adsorbed on different barrier films were real and not due to possible batch to batch variations, each set of slides was usually divided into two subsets after the deposition of the antigen layers, and then one subset was coated with barrier films of one polymer, the other subset with films of another, and antibody adsorption studied on both types of barriers at the same time. Often one of the experiments in which antibody was adsorbed directly on the antigen was also performed on one of the slides in a set. In view of the experimental scatter, the straight lines we have drawn to characterize the data are not to be taken too seriously; nevertheless, certain features of these results are significant and worth noting.

The curve we obtained with Formvar screens (Curve A) is roughly parallel to the one obtained by Rothen under similar conditions; the lack of duplication of these curves is accounted for by the use of different antisera in the two studies. Moreover, we find that, within the experimental error, the results obtained with Formvar and barium stearate screens coincide. These data confirm those of Rothen, and indicate that our experimental technique effectively duplicates his.

A family of curves was obtained, depending upon the particular cellulose derivative used as the inert barrier. Despite the experimental scatter, there is no question but that less antibody was adsorbed on ethyl cellulose films cast from ethylene chloride than was adsorbed on parlodion and cellulose acetate films of the same thickness; and in turn less antibody was



TEXT-FIG. 1. Screening action of various materials deposited on four layers of bovine serum albumin. Curve A, Formvar in ethylene chloride; Curve B, barium stearate; Curve C, VYN-W in butanone; Curve D, ethyl cellulose in acetone; Curve E, cellulose acetate in acetone; Curve F, parlodion in butanone; and Curve G, ethyl cellulose in ethylene chloride. ○, the data through which Curve A is drawn; ●, the data for Curve G.

adsorbed on these films than on those of Formvar. On the average, films only 40 A thick of ethyl cellulose cast from ethylene chloride sufficed to inhibit the adsorption of antibody.

It was found that the same polymer, ethyl cellulose, in two different solvents, ethylene chloride (Curve G) and acetone (Curve D), gave two entirely different curves.

Let us consider the meaning of these results. The cellulose derivatives used in this study differ from one another chemically only in the replacement of certain groups for similar ones along the cellulose chain. The chemical fabric of these cellulose derivatives is not very different from that of Formvar; in fact, these derivatives are chemically much more like Formvar than Formvar is like barium stearate, and yet the latter two exhibit the same behavior when used as inert barriers. It would indeed

be astonishing if the postulated long range forces were susceptible to such large fluctuations upon changing the nature of the dielectric medium so slightly. It is more probable to conclude that the *physical states* of these films, which might very well depend on such slight chemical changes in the cellulose chains, are considerably different. Other evidence supporting this view is presented in the section on electron microscopy.

It is also difficult to understand, from the point of view of long range forces, how films of the same dielectric material, ethyl cellulose, can behave in two such radically different ways when cast from two different solvents. The conclusion would seem unavoidable that the physical state of the barrier film is of fundamental importance, and that films cast from ethylene chloride are better films than those prepared from acetone solution. It is known from the osmotic pressure and viscosity studies of Spurlin, Martin, and Tennant (28) that ethyl cellulose is appreciably associated, that is, not molecularly dispersed, in acetone solution, while it is probably not associated in ethylene chloride. The presence of aggregates presumably prevents the film cast from acetone solution from having as uniform a structure as the one cast from ethylene chloride. And yet Curve D is not very different from that obtained with Formvar screens (Curve A); nor do the surfaces of such ethyl cellulose films appear to contain holes or be otherwise unusual when examined in the electron microscope.

Two other explanations might be advanced to account for this family of curves. First, partial denaturation of the antigen might somehow have occurred in these experiments differing in extent from one barrier film to the next. This is not likely, however, since the same solvent, ethylene chloride, was used in the experiments of Curves A and G; nor is there any reason to suspect that the polymers themselves have any specific deleterious effects on the antigen or antibody, particularly since the same polymer, ethyl cellulose, exhibited the behaviors characterized by the two curves, D and G. Secondly, in using the ellipsometer, we assumed all films to have the same refractive index in order to measure their thicknesses, and this assumption might at first sight seem objectionable. However, Rothen has shown (24) that variations in the refractive index such as exist among the various polymers used in this study have only a small effect on the results of the thickness measurements.

We have also examined evaporated films of silica as inert barriers. Electron microscope studies have revealed the relatively uniform structure of such films (10); moreover from the similarity between these amorphous SiO_2 films and glass, which is known to have a surface smoother than that of any polymer film (29), it may be inferred, although it has not yet been conclusively demonstrated, that silica films are more uniform in structure than those of Formvar or parlodion. The silica (b.p. 2200°) was evapo-

rated from a hot tungsten basket filament at a distance of about 15 cm. from the antigen-covered slide, at a pressure between 10^{-3} and 10^{-4} mm. of Hg. Films of 18 A or more of silica,[5] deposited directly on four layers of bovine serum albumin, completely inhibited the subsequent adsorption of antibody. In order to determine whether this was an artifact due to inactivation of the antigen, the following experiments were performed. Under identical conditions, $CaCl_2$ (b.p. $>1600°$) was evaporated directly on the antigen layers, and was subsequently dissolved by the antibody solution placed on top of it. The same amount of antibody, about 80 A, was found to be adsorbed in these experiments as in the usual ones in which antibody solution was placed directly on the antigen. This indicates that neither the high vacuum nor the radiation energy from the tungsten filament played any rôle in the inhibition of antibody adsorption by silica barriers. Experiments were also performed in which the antigen layers were first covered with Formvar films about 40 A thick, and then 18 A or more of silica was deposited on the Formvar. Such combined films also completely inhibited the reaction,[6] although 60 A of Formvar alone permitted 50 A of antibody to be adsorbed (Text-fig. 1). The same results were obtained when six, instead of four, layers of bovine serum albumin antigen were used. These experiments demonstrate that the inhibition of the reaction by silica films is not due to some kind of chemical inactivation of the antigen by the silica.

Attempts to use other films evaporated onto the antigen surfaces ran into difficulties. Intervening films of TiO_2 25 A thick permitted only 10 A of antibody to be adsorbed on four layers of bovine serum albumin. TiO_2, however, appears to liberate oxygen when heated to its evaporation temperature; the pressure inside the evaporation assembly rose sharply upon heating the filament containing the dry TiO_2. No such change of pressure occurred on evaporating SiO_2. MgF_2 was found to be too soluble in water. Barriers of AgCl about 50 A thick permitted 25 A of antibody to be adsorbed on four layers of bovine serum albumin. On examination in the electron microscope, however, the film of AgCl was observed to be crystalline, unlike that of silica, and accordingly it would not be expected to be as effective a screen. On the other hand, it is considerably more effective than Formvar.

Electron Microscope Studies—The barrier films used in this investigation were too thin to be examined by direct transmission in the electron micro-

[5] Silica has a similar refractive index, around 1.46, to the protein and polymer films.

[6] Control experiments with normal sera indicated that, while either Formvar or silica films on the antigen did not adsorb measurable amounts of protein, silica films on Formvar films on the antigen adsorbed about 12 A of protein nonspecifically under similar conditions.

scope. Apart from their fragility in thicknesses of the order of 100 A, the degree of contrast between the films and any small holes that might exist within them would be too low to be observed with any certainty. We therefore resorted to examining the various films involved in this study by making replicas of their surfaces by well known shadow-casting techniques (29), and observing these replicas in the microscope.

We have reproduced several of these micrographs in Figs. 1 and 2. Except for the first specimen, all of the surfaces studied were directly replicated with chromium at a shadowing angle whose tangent was about one-fifth and were subsequently backed up with a polymer film to lend the requisite strength to the replica, and the metal and polymer films were then stripped from the underlying surfaces with Scotch tape (29). In examining the surface of the aluminum film on glass, the mirror substrate used in all our experiments, a parlodion replica of the surface was cast from amyl acetate solution and was stripped with Scotch tape, and this replica was then shadowed with chromium.

In the course of these studies we found it impossible to strip parlodion-backed chromium replicas from a considerable number of surfaces. Upon substituting ethyl cellulose films cast from ethylene chloride solution for the parlodion, we were able to strip the metal from nearly all the substrates we tried. For example, one to six monolayers of bovine serum albumin on a conditioned optical gage of barium stearate on the aluminum-glass substrates completely retained parlodion-backed chromium films, but easily permitted ethyl cellulose-backed replicas to be stripped. Certain surfaces, such as those of Formvar on top of such antigen layers, permitted both types of polymer-backed replicas to be stripped, but in no case was it possible to remove parlodion-backed chromium replicas from surfaces that retained those backed with ethyl cellulose films cast from ethylene chloride. This improvement in the replica technique has been mentioned elsewhere (26). It is interesting that ethyl cellulose films are more effective in this stripping procedure than are parlodion films, which are in turn more effective than Formvar films. This adhesiveness to the chromium films is in the same order as the capacity of these films to inhibit the adsorption of antibody on underlying antigen layers. It would seem likely that this correlation is due to the physical structure of these films; the more closely knit the structure, the stronger the short range van der Waals' attractions between the polymer and the metal, and the greater the adhesiveness; and the more closely knit the structure, the fewer holes or loci capable of forming holes to permit the antigen and antibody to come into direct contact.

A direct chromium replica of the surface of four monolayers of bovine serum albumin on five layers of barium stearate on the aluminum-glass

substrate is reproduced in Fig. 1, *b*. Separate micrographs have shown the barium stearate layers to have little structure compared to that of the bovine serum albumin layers. The structure of the protein film is characteristic, and different from that of the underlying substrate. A micrograph of a similar bovine serum albumin surface was recently published by Karush and Siegel (13). However, the deposition of bovine serum albumin layers in their experiments was not accompanied by measurement of the optical thickness of the layers. They were therefore unable to prove that their films were the same as those prepared by Rothen. In our case, however, the films were measured with the ellipsometer, and, despite the fact that the surface was quite rough, each layer produced an increment in the ellipsometer reading corresponding to 9 A.

We have therefore demonstrated that measuring 9 A per layer of protein deposited is no proof that the layers on the slide are uniformly spread out. On the contrary, we have shown in these experiments that the ellipsometer is unable to distinguish between a smooth film and an irregular one of about the same average thickness, when the irregularities are much smaller than the wave-length of light. This situation can be understood theoretically as well, as has been demonstrated by Weigle.[7] Let us suppose that the film on the metal surface consists of patches of larger thickness than that of the hypothetical uniform film, but that the patch dimensions and the average distance of separation from each other are smaller than the wavelength of light. From consideration of the Lorentz-Lorenz equation, it can be shown that the effective refractive index, n', of the patchy film is reduced and is given by $(n'^2 - 1)/(n'^2 + 2) = f((n_1^2 - 1)/(n_1^2 + 2))$, where f is the fraction of the metal surface covered by the film and n_1 the refractive index of the film material. Rothen has stated (24) that, under the conditions of operation of the ellipsometer, the ellipse of polarization is mainly affected by changes in the phase difference between the two components vibrating in and perpendicular to the plane of incidence (22). This phase difference is given by $\Delta = -A(1 - (1/n^2))\ l$, where A is a constant, n the refractive index, and l the thickness of the uniform film. Upon substituting a patchy film for a uniform one, while the thickness of the patches is now greater than that of the uniform film, the effective refractive index is smaller. The net result may be, therefore, as Weigle has shown, that very little change is produced in the ellipse of polarization because of a balancing of these two effects, and hence it would be difficult with this technique to distinguish between smooth and patchy films.

The significance of this proof that the antigen layers are not smooth and uniform structures is that it explains the experiments in which more antibody was adsorbed, the greater the number of layers of bovine serum

[7] Weigle, J. J., personal communication.

albumin. For, since the layers did not completely cover underlying ones, considerably more antigen than just the topmost layer was exposed to the action of antibody. This explanation was suggested by Karush and Siegel (13), and earlier by Kabat (12) and Pauling (19).

In Fig. 1, *c* and *d*, we have reproduced replicas of the surfaces of antibody adsorbed directly on five layers of bovine serum albumin, and of a Formvar film on the antigen, respectively, while in Fig. 2 there is shown a replica of the surface of antibody adsorbed on Formvar films on top of the antigen. It is to be noted that, while the first two are relatively free of any large irregularities, the last one shows that antibody is aggregated when adsorbed on bovine serum albumin films that have been covered with Formvar. These aggregates are not present in the original antisera, nor are they artifacts produced by the Formvar films. (The specimen of Fig. 1, *d* was washed with phosphate-saline buffer and water, and dried in the same manner as those of Figs. 1, *c* and 2.) The particular areas of the replicas that have been pictured are representative of the entire specimens, and all of these pictures have been reproduced several times under a variety of conditions.

If long range forces were operating between antigen and antibody, one would expect the antibody to be spread uniformly over the Formvar film above the antigen layers. Instead, the presence of aggregates strongly suggests the formation of antigen-antibody precipitates about local sites, presumably holes, in the Formvar films. With barium stearate screens (Fig. 1, *e*) it is not so easy to discern discrete antigen-antibody aggregates, possibly because the holes present or produced in these films are smaller and closer together (see "Discussion" below). Electron micrographs of antibody deposited on parlodion and cellulose acetate barriers on top of the antigen showed even smaller irregularities.

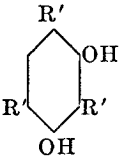
Conjugated Protein Antigen-Antisera Systems—At the beginning of this paper we outlined the immunochemical evidence, largely based on studies of hapten-conjugated proteins and antisera directed against the hapten groups, that indicates that short range forces operate in antigen-antibody reactions. The hapten groups are well known chemical structures, and there is no doubt concerning the fact that antibody activity is directed specifically against them. In the case of bovine serum albumin and similar molecules, however, no one has as yet been able to locate or define any antigenic foci on the molecules. This is not surprising, since so little is known about the detailed structure of proteins in general. Lacking this direct evidence for the existence of short range forces in these systems, however, we must at least recognize the possibility that Rothen's results with these systems and the great body of work involving conjugated proteins could be reconciled by postulating that a whole set of mechanisms

might be involved, ranging from a long range one in systems of the type of bovine serum albumin and its antibodies to a short range interaction in conjugated protein systems. If this were true, thin Formvar films should prevent antibody directed against the hapten from being adsorbed on films of hapten-conjugated protein antigens, if the Formvar films were actually preventing the antigen and antibody molecules from coming into direct contact.

In order to investigate this problem we performed some experiments with *p*-azophenylarsonic acid-conjugated proteins (henceforth referred to as R-azo proteins) and antibodies specifically directed against this hapten. Rabbit antisera were produced against R-azo bovine globulin, and R-azo gelatin and R-azo human albumin were used as test antigens.[8] The conjugated antigens were prepared in the usual manner by slowly adding cold diazotized arsanilic acid to a cold dilute solution of the protein, keeping the pH about 9 by adding the necessary amount of dilute NaOH. The resultant mixture was placed in the refrigerator overnight, and was then exhaustively dialyzed against cold distilled water until no further color was observed in the solution outside the dialysis bags. On analysis the R-azo gelatin and R-azo human albumin yielded about 0.8 per cent As.

These conjugated proteins would not spread on distilled water, apparently because the introduction of arsonic acid groups had affected the solubility properties of the original proteins. They spread on buffers adjusted to pH ~4, but it was felt that there was no particular advantage in picking up films spread on buffered solutions over adsorbing these proteins directly on the slides from solution. A drop of a 2.4 per cent solution of R-azo gelatin, or a 0.9 per cent solution of R-azo human albumin in water, was accordingly smeared on a uranyl acetate-condi-

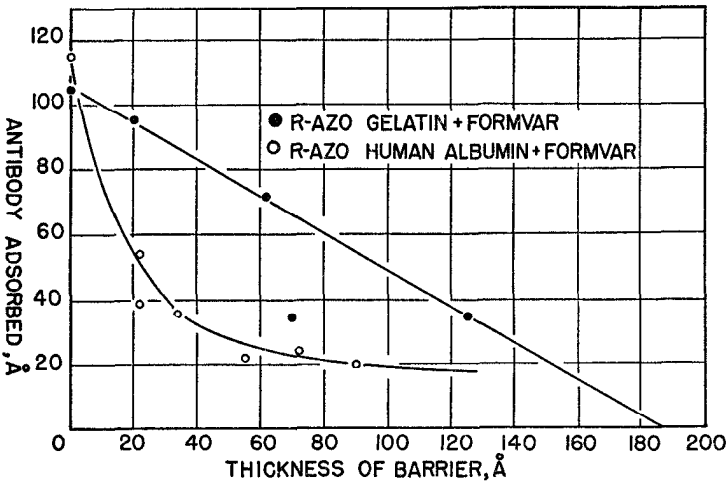
[8] The proteins and the anti-R-azo bovine globulin serum were obtained from Professor Campbell. The antiserum on analysis yielded about 1.5 mg. of antibody per ml. in precipitin tests with the dye antigen,



where R′ = —N=N⟨ ⟩—N=N⟨ ⟩AsO_3H_2 (4). The bovine globulin, obtained from Armour, was designated as Fractions II and III, the human albumin as Fraction V; and the gelatin was a specially prepared sample obtained from the Knox Gelatin Company, designated C-1, and characterized in blood substitute studies (3).

tioned optical gage of barium stearate and allowed to remain for a few minutes in a moist atmosphere. The unadsorbed residue was then washed off with distilled water. Under these conditions about 18 A of the R-azo gelatin and 45 A of the R-azo human albumin were fairly reproducibly adsorbed. Undiluted anti-R-azo bovine globulin serum was employed in the adsorption experiments, and the slides were washed and dried in the manner described previously.

The results of antibody adsorption experiments are given in Text-fig. 2. Each of the points represents an average of about four independent determinations. Antibody specifically directed against the *p*-azophenylarsonic acid group was adsorbed on the hapten-conjugated protein anti-



TEXT-Fig. 2. Anti-R-body adsorption on films of R-azo proteins coated with Formvar barrier films.

gens, despite intervening films of Formvar, in a manner similar to that found for the bovine serum albumin-antiserum system. Control experiments demonstrated that none of the antibody in the antiserum used in these experiments was adsorbed on the original human albumin and gelatin proteins when they were treated in the same manner, and no measurable amounts of normal serum proteins were adsorbed on the Formvar-coated films of the R-azo proteins. The shape of the R-azo human albumin curve is unusual, but there is no doubt that antibody specifically directed against the R group was adsorbed on the Formvar-covered antigen throughout the range of barrier thickness indicated.

These experiments provide us with the following alternatives. If holes in the barrier films are *not* responsible for the effects observed, then the long range force hypothesis must now be able to explain *all* the results

concerning the nature of the specificity of the conjugated protein-antibody system, as well as the phenomenon of hapten inhibition of antigen-antibody precipitation. On the other hand, if one is convinced by the available evidence that short range forces control the specificity of the reactions of these conjugated protein systems, then these experiments prove that antigen and antibody must be coming into direct contact through holes in the Formvar films. We have eliminated the possibility of a range of mechanisms in the immunochemical reaction such as we discussed at the beginning of this section.

DISCUSSION

Before discussing the interpretation of our experimental results in greater detail, let us consider the arguments presented by Rothen to show that holes in the barrier films are not responsible for the effects observed.

The results of Germer (8) on the study of films of barium stearate and stearic acid by electron diffraction are quoted to imply that these films are very uniform. However, the fact that these diffraction diagrams are usually characterized by diffuse bands, and by the absence of sharp spots, definitely indicates the lack of uniform, crystalline order in these films. The presence or absence of a small proportion of holes in these layers could not be ascertained by this method. More recent investigations by Williams and Epstein (7)[9] with electron microscope and electron diffraction techniques indicate that monolayers of barium stearate consist of closely packed, pyramidal micelles about 100 A in diameter, rather than of the more or less continuous structure ascribed to them by Germer. This would make for a film of considerably less cohesive strength, and with an increased probability for hole formation, than would be expected for a uniform film.

The suggestion made by Iball (11) might be introduced here. He proposed in effect that, even if a film of barium stearate monolayers deposited on antigen were free of many holes initially, then, under the stresses produced by the growth of antigen-antibody precipitates in relatively few places, considerably more holes or cracks could develop in the fragile film to permit more antigen and antibody to come into contact. This suggestion might be extended to apply to barrier films of Formvar and other polymers as well.

The fact that the same antibody adsorption curves were obtained with screens of barium stearate, octadecylamine, and Formvar is regarded by Rothen as more than coincidental. Our results with cellulose derivatives, however, show that the extent of antibody adsorption is definitely a function of the nature of the screen. Our electron micrographs imply

[9] Williams, R. C., and Epstein, H., personal communication.

that the holes in the Formvar screens are larger but less frequent than those in the barium stearate layers, and that it is merely a coincidence that both screens permit the same amount of antibody to be adsorbed within the large experimental errors involved. It is not at all difficult to appreciate that a polymer film about 100 A thick should, more or less independently of the nature of the surface on which it is cast, exhibit a certain number of holes large enough to permit antigen or antibody molecules to pass through. These films consist of several layers only of randomly coiled molecules with very little order among them. Holes in barium stearate films, considering the structure of these monolayers proposed by Williams and Epstein, might be expected to be more closely spaced and more uniformly distributed.

The argument is advanced that, if there are holes in the barriers, the diffusion of antibody through them should considerably reduce the rate of the reaction, and it was suggested that the fact that the amount of antibody adsorbed on Formvar screens was the same whether the antiserum was in contact with the slide for 15 or 40 minutes is evidence that holes are not significant.

A rough calculation indicates, however, that the time that would be required for the antibody to diffuse through holes in the barrier films is small, and even if the only mechanism by which antibody and antigen could come into contact were through this diffusion process (that is, if possible antigen diffusion or the development of more holes in the barriers upon antigen-antibody precipitate formation is ignored), the reaction might be over in a second or so. Fick's first law of diffusion is given by $dm/dt = -DA\ dc/dx$, where dm/dt is the mass of material of diffusion constant D crossing the boundary of area A in the time dt, and dc/dx is the concentration gradient in the x direction across this boundary. Let us suppose the barrier thickness is 100 A, and the concentration of the antibody solution above the barrier is 10^{-4} gm. per cm.^{3}. The concentration gradient of antibody across the barrier is then 10^2 gm. per cm.^{4}, which we will assume remains constant during the diffusion process, since the antibody in the solution above the barrier film is present in large excess, and antibody that might diffuse through the film would be adsorbed. The diffusion constant of antibody in phosphate buffer is about 4×10^{-7} cm.^{2} per second at room temperature. If we now assume that some small fraction of the barrier film, say 0.01 of its total surface area, has holes large enough to permit antibody to pass, and substitute these numbers into Fick's equation, $dm/dt \backsim 4 \times 10^{-7}\ \sigma$ gm. per sec., where σ is the area of the antigen film covered by the screen. Now the four layers of antigen on the surface are equivalent to about $4 \times 10^{-7}\ \sigma$ gm. In other words, in such a hypothetical case, in one second an amount of

antibody equivalent to the amount of antigen on the slide would diffuse through the holes in the barrier film.

This calculation is no doubt crude, and it is presented only to indicate that the antigen-antibody reaction through holes in the barrier films might very well be completed in a time considerably less than 15 minutes, even if no other factor than antibody diffusion is considered. As a result of this, of course, no difference in antibody adsorption would have been observed at the end of 15 or 40 minutes.

On the other hand, Rothen attempted to prove that diffusion of protein through the screens was not occurring. Slides were prepared on which antibody was adsorbed directly on bovine serum albumin layers, and some of these were then coated with thin Formvar films. These two types of slides were then washed with a 5 to 10 per cent sodium chloride solution for some time. The slides with the Formvar coats did not change in thickness, while the unprotected ones suffered a considerable loss in thickness. These results were interpreted to indicate that antibody molecules could not diffuse through Formvar screens. It is possible, however, that under these conditions the salt dissociation of the antigen-antibody precipitates produced relatively large aggregates of antigen and antibody only partially solubilized, and that these were not able to diffuse through the holes in the Formvar films.

Experiments were performed by Rothen with the protamine insulin reaction to demonstrate that barium stearate and Formvar screens were effective screens for the reaction. A double layer of barium stearate covering 10 A of the protamine clupein sulfate cut down the insulin adsorption completely, whereas 220 A of insulin could be adsorbed directly on the protamine. However, Formvar screens 17 and 30 A thick permitted 120 and 40 A of insulin to be adsorbed, respectively, while for screens thicker than 50 A no specific adsorption took place.

It is our opinion that the barium stearate screens may not be inert barriers in these experiments, but may rather react with the protamine film. Although the details of the reaction mechanism are not completely clear, it seems probable that the insulin and protamine react because the 2 molecules are oppositely charged in the physiological pH range, and attract each other by Coulomb forces. Although the protamine may originally be in the form of a sulfate, the insolubility of the protamine insulinate causes the displacement of a number of the sulfate groups from the protamine in favor of acidic groups on the insulin. Now Langmuir and Schaefer (16) have shown that between pH 7 to 10 from 40 to 5 per cent, respectively, of the carboxyl groups of the barium stearate film are free; that is, are not bound to barium ions. These free carboxyl groups might react with those groups on the protamine molecules that are avail-

able for reaction with insulin. In the process the structure of the barium stearate film might be affected sufficiently, and the reactive groups on the protamine irreversibly bound to an extent large enough so that insulin is no longer adsorbed. On the other hand, the fact that Formvar films permitted some insulin adsorption is significant.

Another set of results, dealing with the absolute amount of antibody adsorbed directly on antigen layers, is cited to indicate that long range forces operate in immunologic reactions. An extreme example of such results follows. On optical gages of octadecylamine, a layer of 700 A of antibody was adsorbed after a long period of time on 5 A of pneumococcus type III polysaccharide, an amount much larger than would be expected if the polysaccharide film were truly immobile and rigid and only short range forces were operating between the antigen and antibody molecules. As Rothen pointed out subsequently (23), however, studies reported by Clowes (6) indicated that up to 450 A of insulin could be adsorbed on layers of protamine only 30 to 50 A thick. Since this system probably reacts by a short range mechanism, such effects are therefore no indication of specific long range forces. Perhaps after the first layers of protein are specifically adsorbed on the substrate molecules, relatively non-specific polarization forces cause the immobilization of subsequent layers. It should also be pointed out that, under the influence of the forces between the reacting molecules, the layers of the substance initially deposited on the surface might not remain intact. In the experiments, for example, in which antibody was adsorbed directly on the pneumococcus polysaccharide, with no intervening barriers present, reaction with antibody might have considerably disrupted the antigen layers, dispersing some of the polysaccharide throughout the antibody. This would have permitted more antibody to be immobilized than might have been anticipated with a rigid film of antigen.

In view of this discussion, we do not consider it proved that holes in the barrier films are either absent or ineffectual. On the other hand, our experimental evidence seems to require that these holes exist. The fact that films of materials of such similar chemical constitution as the cellulose derivatives exhibit quite different screening properties, and that screens of the same polymer, ethyl cellulose, cast from two different solvents, behave very differently, indicates that it is the physical structure of these films that largely determines their screening action. In accord with this conclusion is the very marked correlation between films which exhibit the best film properties and those which inhibit antibody adsorption the most strongly. Silica films, whose surfaces are probably more uniform than those of polymers, and ethyl cellulose films cast from ethylene chloride solution, which are superior to other polymer films in stripping metal

replicas from various surfaces, are both very effective inhibitors of the antibody adsorption. These results imply that holes in the barrier films do exist, and that better films have fewer of them in a given film thickness.

The presence of distinct aggregates, which are observed in the electron microscope when the antibody is adsorbed on top of Formvar-coated layers of bovine serum albumin, also strongly suggests that antibody and antigen react through holes in the screens. These aggregates are not artifacts caused by the treatment the surfaces undergo; that is, they are not due to the Formvar itself, to non-specific aggregates in the antiserum, nor to the preparation of the chromium replica of the surface. Nor is it possible to account for them as the products of some kind of surface forces between the antibody and Formvar, since the Formvar surface is completely wet by the solution of antiserum and particularly since the aggregates on barium stearate screens were much smaller. It appears most likely, therefore, that these aggregates are antigen-antibody precipitates which have grown about holes in the Formvar films, perhaps enlarging these holes in the process.

In studying enzymatic reactions Rothen found that a screen of Formvar 500 A thick made in two separate steps could prevent the enzymatic action of trypsin on underlying bovine serum albumin layers, while it took a single Formvar film of about 1000 A to do the same. He ascribed this to a diminished permeability of the buffer ions of the enzyme solution when the blankets were made in two steps. An obvious explanation on the basis of the hole hypothesis is that the holes in the two separately formed films have a limited probability of coinciding to form continuous channels when the films are placed together, thus diminishing the effective number of holes below that found with single films of comparable or greater thickness.

In the light of our experiments and the information available, we suggest that the mechanism of these reactions of proteins in thin films is as follows. The bovine serum albumin layers on the slide are not uniform monolayers, but instead are quite irregular. Up to a certain point, then, the more layers of bovine serum albumin deposited on the slide, the more antigen is exposed to the action of antibody, and the more antibody is adsorbed.

Because of the nature of thin films of the type of barium stearate, and of polymers, a certain proportion of holes may be expected to be formed in them, whatever the nature of the surface on which they are deposited. Karush and Siegel (13) suggested that the roughness of the surface of the antigen layers might be responsible for the production of holes in the screens. Experiments performed by Rothen, however, are not in accord with this suggestion. In these experiments, Formvar screens were first

cast on polished glass surfaces, were floated off onto water, were then deposited on the antigen layers, and subsequently were found to permit the same antibody adsorption as Formvar films of the same thickness cast directly on the antigen layers. Moreover, the fact that enzyme activity occurs through Formvar blankets of 500 to 1000 A thick, dimensions several times those of the largest surface irregularities of the bovine serum albumin films, also requires further explanation. We are of the opinion, therefore, that the presence of holes is an intrinsic property of these films, and that the roughness of the underlying surface is of only secondary importance to their formation. The thicker the screen, the fewer the holes running continuously through it, and the more effective a barrier it becomes. Films of silica presumably have considerably fewer holes than those of Formvar for a given film thickness, and hence inhibit antibody adsorption in much smaller thicknesses. The strength of a film, and its resistance to the strains produced by the growth of antigen-antibody precipitates, probably is of importance as well.

The detailed mechanism by which antigen and antibody come into contact through the holes in the barrier films may be complicated. Whether the antigen diffuses through or protrudes through these holes, whether it is the antibody which diffuses through them to the underlying antigen layers, or whether combinations of these and other factors come into play is at present a matter for conjecture. In the case of the enzymatic reaction of trypsin with bovine serum albumin layers, which is observed to occur through Formvar screens 500 to 1000 A thick, we would presume that the enzyme diffuses through holes in the screens to the substrate below. One might object that Formvar films 500 to 1000 A thick would be expected to have very few holes running continuously through them. As Rothen has noted, however, a single enzyme molecule diffusing through a blanket might damage an extensive area of the underlying substrate film. There need not be many holes, then, to account for the enzymatic activities observed. In the electron microscope we have observed that Formvar films in this range of thickness do have occasional holes running through the films which might account for these effects.

We believe this interpretation of the experimental results obtained by Rothen is self-consistent, suffices to explain the major features of these results in simple terms, and is in accord with our own experimental observations as well. On the other hand, we consider it difficult to interpret some of our results in terms of the hypothesis of long range forces. Our conclusion is, therefore, that it is by no means necessary to invoke the existence of long range forces to account for these experimental results.

In view of these considerations, and of the considerable weight of independent evidence favoring the existence of short range interactions in

immunologic and enzymatic reactions, we feel that it is unlikely that specific long range forces operate in these reactions.

SUMMARY

By studying the reactions of proteins in thin films, Rothen has obtained experimental results which he has suggested indicate that specific long range forces operate in immunologic and enzymatic reactions. He has found that antigen and antibody, and enzyme and substrate molecules, can react despite apparently being separated by thin inert barriers. We have obtained evidence by (*a*) investigating the behavior of certain cellulose derivatives and silica films as inert barriers, (*b*) by an electron microscopic examination of various surfaces involved in these experiments, and (*c*) by a study of the reaction in thin films of hapten-conjugated protein antigens and antibodies directed specifically against the hapten, that strongly suggests that holes in the barrier films, permitting the macromolecules to come into contact and react, are primarily responsible for the effects observed. Rothen's arguments against the existence of these holes are discussed and are considered inconclusive.

In view of this alternative explanation for the results obtained by Rothen, and of the considerable weight of evidence which seems to indicate that short range mechanisms are of primary significance in immunologic and enzymatic reactions, it appears unlikely that specific long range forces operate in these reactions.

The author is grateful to Professor Linus Pauling for suggesting this research and for his continued help and advice. The electron micrographs were taken by Professor R. F. Baker, of the University of Southern California, and Dr. B. Henke, of this Institute, and some of the specimens were prepared by Mr. R. F. Petzold; the author is indebted to them for their assistance.

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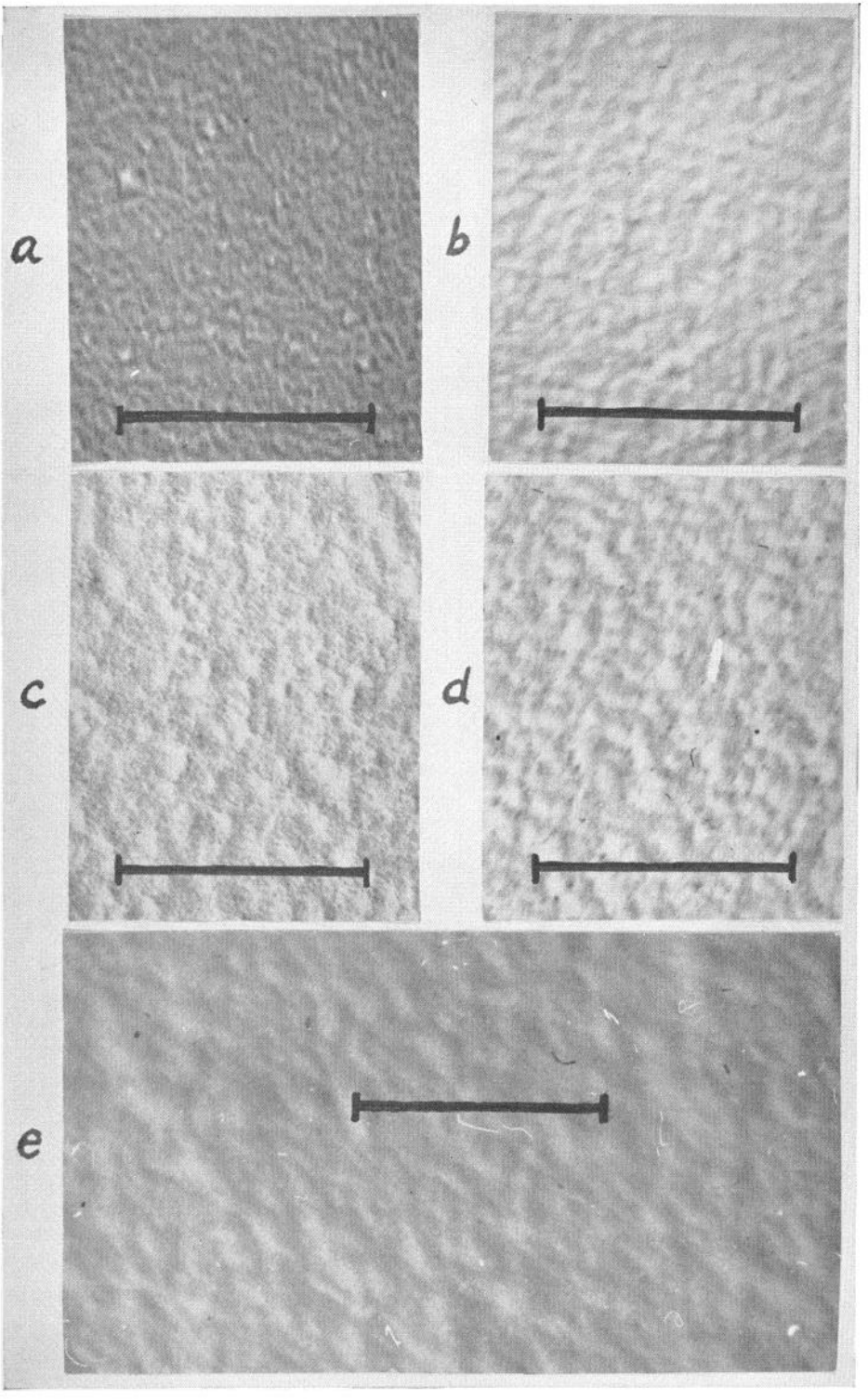
EXPLANATION OF PLATES

Plate 1

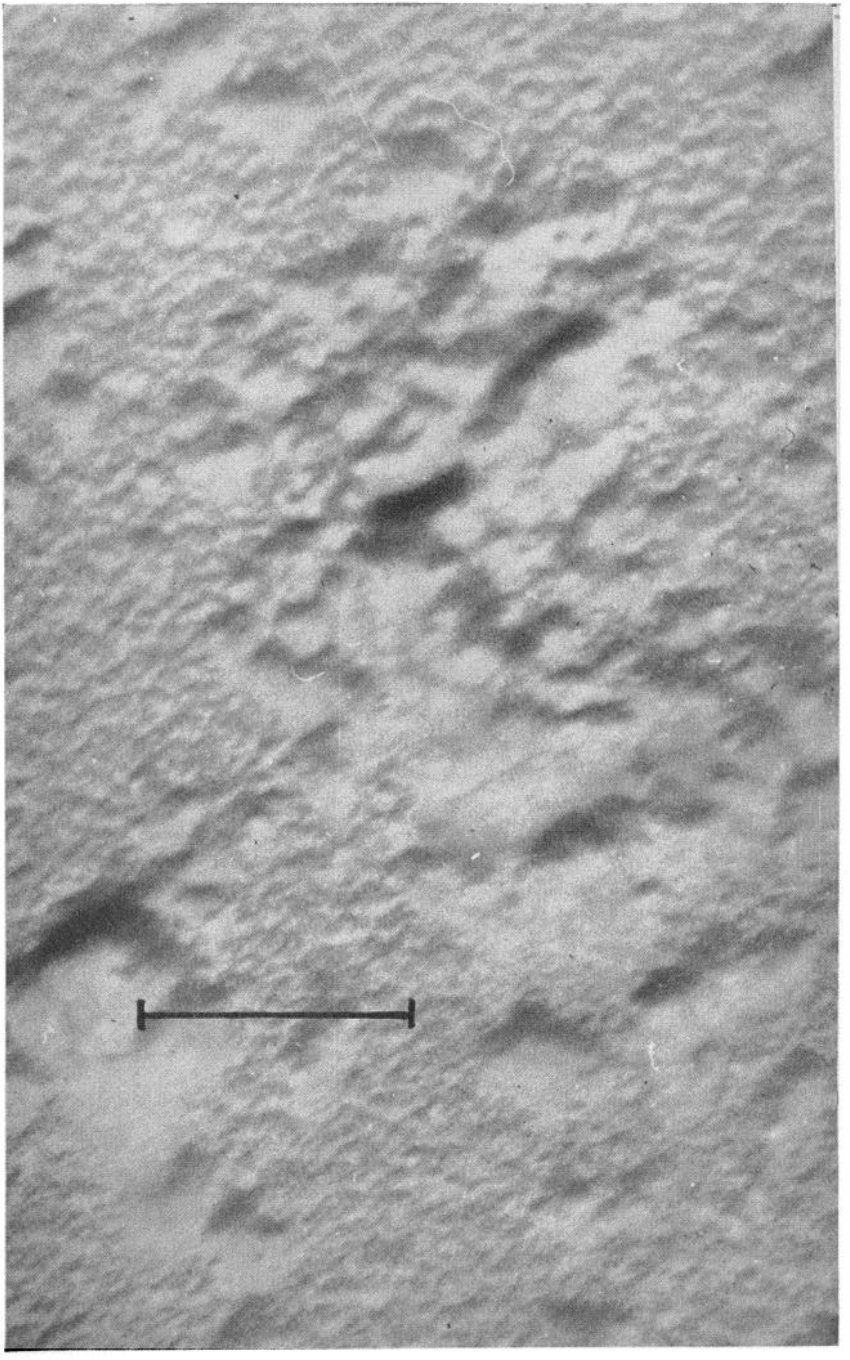
Fig. 1. Electron micrographs of replicas of the surfaces of (*a*) a film, approximately 1000 A thick, of aluminum on glass; (*b*) four layers of bovine serum albumin on a uranyl acetate-conditioned optical gage of five layers of barium stearate on surface (*a*); (*c*) 120 A of antibody on five layers of bovine serum albumin on the conditioned slide; (*d*) a 100 A-thick Formvar film on five layers of bovine serum albumin on the conditioned slide; and (*e*) 90 A of antibody on two layers of barium stearate deposited on five layers of bovine serum albumin on the conditioned slide. The scale marks represent 1 μ, and all the pictures are at the same magnification.

Plate 2

Fig. 2. Electron micrograph of a replica of the surface of 100 A of antibody adsorbed on a 50 A-thick film of Formvar on five layers of bovine serum albumin on a conditioned slide. The scale mark represents 1 μ, and the picture is at the same magnification as those of Fig. 1.



(Singer: Immunologic and enzymatic reactions)



(Singer: Immunologic and enzymatic reactions)