ANTIGEN-ANTIBODY REACTIONS BETWEEN LAYERS ADSORBED ON BUILT UP STEARATE FILMS

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(Received for publication, March 2, 1939)

Langmuir and Schaefer (1, 2) have developed a method of conditioning barium stearate films for the adsorption from solution of monolayers of organic substances. This method has been used by them for studying the properties of adsorbed layers of protein (1), enzymes (3), and immunological substances (1).

By adsorbing on barium stearate films of sufficient thickness to give interference colors, a rough estimate of the thickness of the adsorbed layer can be obtained by color comparison with films of known numbers of layers. A more precise method has been described by Blodgett and Langmuir (4) and Blodgett (5) in which the increase in thickness is calculated from the change of angle of incidence of the ordinary Rs[1] ray of sodium light for minimum reflectance.

Using the less accurate method of color comparison, Shaffer and Dingle (6) have investigated the antigen-antibody reactions between adsorbed monolayers of egg albumin and anti-egg albumin and between pneumococcus polysaccharide and antipolysaccharide specific antibody. They used whole immune serum for their studies.

Because of Shaffer and Dingle's unexpected results, we have undertaken a reinvestigation of the pneumococcus antigen-antibody reactions on adsorbing surfaces using the more precise method (4, 5) and antibody solutions purified by dissociation according to the method of Heidelberger and Kendall (7). In addition we have made measurements on the diphtheria toxin-antitoxin system. The results we have obtained are not in agreement with those of Shaffer and Dingle or those

[1] The plane of polarization of the Rs ray is perpendicular to the plane of incidence.

of Langmuir and Schaefer (1) for toxin and antitoxin. The nature of these discrepancies and their possible explanation will be discussed later in this paper.

Materials and Methods

The three types of pneumococcus polysaccharides used, hereafter referred to as S I, S II, and S III, were prepared by the method of Heidelberger, Kendall, and Scherp (8).[2]

TABLE I

Analysis of Dissociated Antibody Preparations

Serum No.	Symbol	Species	Type	Nitrogen per cc.	Antibody nitrogen per cc.	Antibody nitrogen
				mg.	*mg.*	*per cent*
715-13	AH I_1	Horse	I	0.298	0.220	74
733-2*	AH I_2	"	I	0.151	0.066	44
701-20	AH II_1	"	II	0.183	0.083	45
701-19*	AH II_2	"	II	0.136	0.116	85
536-11	AH III	"	III	0.351	0.202	58
J-5	AR I	Rabbit	I	0.271	0.102	38
J-6	AR II	"	II	0.142	0.091	64
J-7	AR III	"	III	0.116	0.090	78

Antibodies prepared from serums marked with an asterisk were obtained by dissociation of specific precipitates formed from solutions of Felton precipitates. Other serums were used without preliminary purification.

The solutions of Type I, II, and III horse and rabbit antipneumococcus antibodies were prepared by dissociation of washed specific precipitates, formed in the region of slight antibody excess, with 15 per cent sodium chloride at 40°C. according to the method of Heidelberger and Kendall (7). The per cent of specifically precipitable nitrogen was determined for each sample of dissociated antibody studied. These analyses are summarized in Table I. All protein solutions were finally dialyzed against M/15 phosphate buffer, pH = 6.9, containing 1:10,000 merthiolate. Solutions of S I, S II, and S III containing 0.25 per cent, 0.06 per cent, and 0.05 per cent carbohydrate respectively were made up in the same buffer.

The highly purified diphtheria toxin used was prepared by one of us (9) and has recently been found to show homogeneous sedimentation in the ultracentrifuge

[2] We are greatly indebted to Dr. Heidelberger for presenting us with samples of highly purified S I (123-4), S II (83E), S III (105-7).

as well as homogeneous migration by electrophoresis (10). An electrodialyzed, antitoxic pseudoglobulin preparation was used which was practically homogeneous by ultracentrifugation and 35 per cent precipitable by diphtheria toxin.

Films for conditioning and adsorbing were deposited on polished stainless steel slides by dipping through a stearate monolayer spread on a substrate 3×10^{-5} molar in $BaCl_2$, 10^{-3} molar in Michaelis' Na acetate-Na veronal buffer, pH 7.4 (11). Under these conditions Y layers are deposited. Castor oil was used to keep the monolayer under compression.

The stearate was deposited, by varying the depth of dip of the slide, to give films graduated in steps of two from 41 layers at the upper to 51 layers at the lower end. The purpose in building a stepped film for adsorption is to provide a series of bands of varying reflecting power for polarized monochromatic light. By properly choosing the angle at which the film is viewed a pair of adjacent steps can be found of equal brightness. For an unconditioned film the 47 and 49 layer bands match in brightness for the Rs ray under sodium light at an angle of incidence of about 71°. On increasing the total thickness this angle increases. If the thickness increase is large a match may more easily be obtained between a pair of steps of fewer stearate layers. In calculating the total thickness increase this shift of the matched bands as well as the angle of incidence must be taken into account. Thicknesses were calculated from the following equation:

$$T = \frac{\lambda}{4\sqrt{n^2 - \sin^2 i}}$$

in which T is total thickness, i the angle of incidence, $\lambda = 5893$ Å the average wave length of sodium light, and $n = 1.495$ the refractive index of barium stearate given by Blodgett (5).

Conditioning of the surface for adsorption was accomplished by the improved Langmuir-Schaefer method (2) which consists of successive immersions for 30 seconds in 10^{-3} molar thorium nitrate and dilute potassium silicate solutions followed by rinsing in distilled water and drying.

The adsorbate was applied according to the following technique: The slide was flooded with M/15 Sørensen phosphate buffer solution pH 6.9; a few drops of the adsorbate solution were added; then the mixture stirred for 30 seconds by moving a glass rod, in contact only with the upper surface of the solution, back and forth across the slide. The preparation was rinsed first with M/15 phosphate buffer, then with distilled water and dried. Application in this manner was repeated until the thickness became constant within the accuracy of the method, that is to ±3 Å, the equivalent of 0.5 degrees change in the angle of incidence at minimum reflectance of the monochromatic light. The next adsorbate solution was then applied by the same procedure. We have found it important to keep the composition of the solution in contact with adsorbing surface constant during the adsorption process in order to obtain consistency and reproducibility of thickness measurements. A possible explanation of this necessity is to be found in a recent paper by Langmuir and Schaefer (12).

The Pneumococcus Capsular Carbohydrate-Antibody System

Pneumococcus antibodies occur in the euglobulin fraction of horse serum. When adsorbed on properly prepared slides an average

TABLE II

Reactions between Pneumococcus Specific Carbohydrates and Horse Antibodies

For order of application, read down

	Adsorbate	Thickness increase Å ± 3						
Series I	S III						−2	
	S I			−2				
	AH I_1	42	50	47	56	60	48	40
	S I	−1	1	0				0
	S II				−3			
	S III					−2	−1	
	AH I_1	36	45	62	6	8	11	
	AH III							3
	S I	−7		−28				
	AH I_1	64		105				48
Series III	S II	−1						
	AH II_1	46	36	40	31	29		
	S I				2			
	S II	3	1	0				
	S III					3		
	AH II_1	11	4	7	4	5		
	AH I_1			4				
Series V	S III	2						
	AH III	53	49	48	50			
	S I			−2		−1		
	S II				1		1	
	S III	−1	−1					
	AH III	45	48	6	11	51	46	
	S III		−3					
	AH III		62					

	Adsorbate	Thickness increase Å ± 3		
Series II	AH I_2	47	45	
	S III	0		
	AH III	−1		
	AH I_2	1		
	S I	1	−1	
	AH I_2	17	20	
	S I		0	
	AH II_2		−1	
	AH I_2		18	
Series IV	AH II_2	43	40	37
	S II	−2	−1	−2
	AH II_2	23	25	30
	S II	−1	−2	−2
	AH I_2	1		
	AH II_2	46	57	46

Each column in each series represents a complete experiment. Blank spaces indicate no treatment with the corresponding adsorbate.

thickness of 45 Å was obtained with an average divergence regardless of sign from this value of 6 Å. No systematic difference between the three types of horse antibodies was observed. None of the three

carbohydrates gave measurable adsorbed films on the conditioned stearate. When applied to an underlying layer of type specific antibody, however, they produced changes in the surface, without significantly increasing the total thickness, which caused the adsorption of a second layer of the homologous antibody of approximately the same thickness as the first layer provided the antibody solution contained a sufficiently large percentage of specifically precipitable nitrogen.

TABLE III

Reactions between Pneumococcus Specific Carbohydrates and Rabbit Antibodies

For order of application, read down

	Adsorbate	Thickness increase Å ± 3					Adsorbate	Thickness increase Å ± 3				
Series VI	AR I	15	16	33	32	Series VII	AR II	15	32	20	15	12
	S I	−2	1	1			S II	0	2	1	1	1
	S II				2		AR II	6	7	6	10	10
	AR II	1	0				S II				2	0
	AR I		12	29	3		AR II				28	36
	S I		−2		0							
	AR I		17		21							
Series VIII	AR III	15	16	20	19							
	S III	0	0	0	0							
	AR II			1	2							
	AR III	27	29	19	20							
	S II			2								
	AR II			2								
	S III	−2	0	−2								
	AR III	45	48	28								

Each column in each series represents a complete experiment. Blank spaces indicate no treatment with the corresponding adsorbate.

This "sandwiching" effect (Table II) can be carried to a third layer of antibody, but generally at this stage the deposition becomes irregular making thickness measurements difficult. It is evident that the sandwich effect is specific from control experiments in which all attempts to obtain cross reactions failed as seen in Table II.

Evidence that the specific carbohydrates are not adsorbed on conditioned stearate surfaces from buffer solutions at pH 6.9 to produce even very thin layers is provided by the failure of any type

carbohydrate to prevent the subsequent adsorption of heterologous antibody.

Similar experiments were performed with antibodies obtained from immune rabbit serum. The results are presented in Table III. The average thickness of rabbit antibody layers adsorbed directly on conditioned barium stearate films is 20 Å with an average divergence from this value of 6 Å. As with horse antibody the capsular carbohydrates failed to produce significant increases of thickness when applied to the specific antibody. Sandwiching effects were obtained also with rabbit antibodies.

The Diphtheria Toxin-Antitoxin System

An average of 13 determinations of diphtheria toxin protein adsorbed on conditioned barium stearate films gave a value of 33 Å for the thickness. The average divergence regardless of sign from this value was 6 Å. A 35 per cent precipitable preparation of antitoxic pseudoglobulin from immune horse serum gave an average thickness for 6 determinations of 23 Å, with a 2 Å average divergence. Toxin did not produce detectable increases in thickness when applied to an underlying adsorbed antitoxin layer, but, conversely, antitoxin applied to an adsorbed toxin layer led to a marked increase, the average of 5 determinations being 49 Å with an average divergence of 2 Å. However, whole normal horse serum, egg albumin, and normal horse pseudoglobulin containing no detectable antitoxin (less than 0.001 unit per cc.) also deposited on adsorbed toxin though giving layers somewhat less thick than antitoxin. For example, in two cases normal pseudoglobulin layers 35 Å and 31 Å thick were adsorbed on toxin.

It is possible that toxin would adsorb to layers of antitoxin if the latter could be obtained in a more nearly pure state.

DISCUSSION

In a recent paper Danielli, Danielli, and Marrack (13) report an investigation of reactions at an air-water interface between pneumococcus carbohydrate and purified antibody. They were unable to demonstrate specific reactions either by change in thickness of the antibody monolayers or change in surface potentials. They give 9 Å for the thickness of the antibody monolayer. This value agrees well

with measurements of all protein monolayers formed on dilute aqueous solutions and has been explained as the result of an unfolding and flattening out of protein molecules at water surfaces.

We have shown, on the other hand, that on barium stearate surfaces, adsorbed layers of pneumococcus antibody can be specifically conditioned with homologous carbohydrate so that a second layer of the antibody will deposit. This sandwich effect which is specific, as shown by the control experiments using heterologous carbohydrate or antibody, can easily be carried to three layers of antibody. The capsular carbohydrates were not themselves adsorbed from solution on conditioned barium stearate surfaces, while the layers which they formed on homologous antibody were too thin to be measured by the method employed. This latter observation is in keeping with the accepted opinion that these polysaccharides are long chains (8, 14) which may be adsorbed parallel to the surface; but is contrary to the findings of Shaffer and Dingle (6) who reported layers 50 Å thick of S III, S V, and S VIII. It should be noted, however, that these authors used 1 per cent solutions of carbohydrate for their experiments and applied the solutions to dry unconditioned stearate surfaces by vigorous mechanical treatment. Under these conditions they may have obtained multilayer deposition.

Our experiments show that we have been able to obtain the most reproducible and significant results by the use of highly purified antibodies. Thus the preparations AH I_1, 74 per cent precipitable, and AH II_2, 85 per cent precipitable, gave more clear cut sandwich effects than the preparations AH I_2, 44 per cent precipitable, and AH II_1, 45 per cent precipitable. A further reason for using purified reactants is to avoid non-specific adsorption of unlike proteins. For example, horse antibody protein will add to rabbit antibody regardless of type or previous conditioning with carbohydrate (Table IV), and normal pseudoglobulin containing no demonstrable antitoxin and even egg albumin will add to a monolayer of diphtheria toxin, thus obscuring specific reactions.

Although we confirm the findings of Shaffer and Dingle (6) that the surface reactions are specific and that rabbit antibody is smaller than horse, we did not duplicate the enormous values they reported (100 Å to 240 Å) for the thickness of antibody layers. Their values may be

due to the fact that they applied whole immune serums to dry slides and to the possibility of adsorption from whole serums of large aggregates the existence of which have been reported by Goodner, Horsfall, and Bauer (15).

Molecular weights of proteins may be calculated, by the use of dissymmetry factors, from the thicknesses of adsorbed layers assuming the layers to be only one molecule thick. However, because of certain features of this calculation the results should be interpreted cautiously. Thus, calculated molecular weights are extremely sensitive to variation in measurements of the thickness of the adsorbed layer because they are proportional to the third power of molecular diameters. Also, axial ratios are very sensitive to variation in dissymmetry factors.

TABLE IV

Non-Specific Reactions

For order of application, read down

Series IX		Series X		Series XI	
Adsorbate	Thickness increase Å ± 3	Adsorbate	Thickness increase Å ± 3	Adsorbate	Thickness increase Å ± 3
AR I	15	AR II	15	AH I_1	42
AH III	24	AH II_1	27	AH III	3

Each column represents a complete experiment.

Nevertheless we present the following comparisons, assuming that the adsorbed molecules are cylindrical and lie with their long axes parallel to the adsorbing surface. Kabat (16) has shown that pneumococcus antibodies are highly asymmetrical molecules having dissymmetry constants of 2.0 for horse antibody and about 1.5 for rabbit antibody. These values give from Perrin's equation (17) axial ratios of approximately 1/20 and 1/10 respectively. Molecular weights calculated from these axial ratios and the average thicknesses of adsorbed layers are for horse antibody, 45 Å thick, approximately 1,300,000 and for rabbit antibody, 20 Å thick, approximately 57,000. The molecular weights given by Kabat from sedimentation and diffusion constants are 990,000 for horse antibody and 157,000 for rabbit antibody. Making the same calculations with Shaffer and Dingle's values, we

get for horse antibody giving adsorbed layers by their technique 240 Å thick a molecular weight of 190,000,000, and for rabbit antibody with layers 100 Å thick 6,700,000. Even if we assume that the molecules in Shaffer and Dingle's experiments are oriented with their long axes perpendicular to the adsorbing surface, an unlikely situation, the calculated molecular weights are not in any better agreement with those of Kabat. Thus for horse antibody we get 23,000 and for rabbit antibody 6,800, values which are much too small. Adsorption of aggregates remains, therefore, as the only explanation of their results.

Assuming the dissymmetry factor for antitoxic pseudoglobulin to be the same as normal horse pseudoglobulin, its molecular weight may be calculated by the same method. Using the factor 1.4 given in a paper by Svedberg (18) and the average thickness of adsorbed layers of 23 Å, a molecular weight of 73,000 is obtained. The value given in Svedberg's paper for normal horse serum globulin calculated from the sedimentation constant is 150,000.

From Lundgren, Pappenheimer, and Williams' data (10) the dissymmetry factor for diphtheria toxin was calculated as 1.25, and from Perrin's equation this value gave approximately 1/5 for the axial ratio. Using this ratio and the average thickness of adsorbed layers of 33 Å, the molecular weight of toxin was determined as about 120,000, which is in fair agreement with 72,000 given by these authors from sedimentation and diffusion constant measurements. Our thicknesses are, therefore, in no way inconsistent with the present knowledge regarding molecular weights.

Our experiments with the pneumococcus system would appear to give support to the lattice theory of antigen-antibody precipitin reactions of Marrack (19) and Heidelberger (20). In the protein systems studied by the quantitative precipitin method and in the ultracentrifuge, no evidence has been obtained that antigen and antibody can unite in a proportion of more than one molecule of antigen per molecule of antibody, although it is clear that many molecules of antibody can react with one of antigen (Heidelberger, 21). The sandwich effect obtained with the pneumococcus system suggests that antibody as well as antigen can be "multivalent" in conformity with the views of Marrack and Heidelberger.

Langmuir and Schaefer with one of us (1) have reported obtaining

with diphtheria toxin and antitoxin alternate adsorbed layers 36 Å and 75 Å thick respectively. They state that the alternation can be continued indefinitely. Our value for adsorbed toxin is in good agreement with this, while our value for purified antitoxin on toxin is somewhat less. As for indefinite alternation of layers we have been completely unable to corroborate this finding. The most we could obtain was a layer of antitoxin on toxin but not the converse. A non-specific deposition might be the explanation of this discrepancy for Langmuir and Schaefer used a pseudoglobulin preparation only 9.6 per cent precipitable by toxin, for their experiments. We have already pointed out the necessity of using purified preparations and have shown that normal pseudoglobulin will deposit on toxin.

SUMMARY

By adsorbing antigens and antibodies on barium stearate multilayers immunological reactions at surfaces have been studied.

Pneumococcus polysaccharide specific antibody systems using purified antibodies from both horse and rabbit sera were investigated. The polysaccharides failed to show visible adsorption, but by alternate treatment with antibody and polysaccharide several layers of antibody could be specifically deposited.

With the diphtheria toxin-antitoxin system antitoxin was found to adsorb to layers of toxin but not conversely. The reaction, however, was not specific.

Molecular weights calculated from the thickness of adsorbed protein layers, using dissymmetry factors, roughly correspond to molecular weights calculated from sedimentation and diffusion constants.

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