[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Long Range Enzymatic Action on Films of Antigen

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The work reported here deals with the mode of action of two proteolytic enzymes, trypsin and pepsin, as well as that of the specific enzyme capable of depolymerizing the polysaccharide from Type III pneumococcus. This is a logical continuation of work previously published[1] on reactions between films of antigen and antibody molecules, where it was shown that specific forces between antigen and antibody seemed to extend considerably in space. In these experiments antigenic protein films were spread on water from which they were transferred onto polished metal slides. The slides were then coated with blankets of various inert materials and finally a drop of dilute solution of the corresponding immune serum was spread over each blanket. After the slide was washed, specific adsorption of antibody could be detected, the amount of which decreased regularly with the increase in thickness of the blanket. The probability that the antibody molecules reached the antigenic layers by diffusing through the blanket was for several reasons considered to be remote. The conclusion was therefore reached that the antibody molecules might actually be held on top of the slide by specific forces extending through the thickness of the blanket.

The analogy between immunological and enzymatic reactions naturally led to the question whether enzymes might not also exert their action through a blanket. In this case the interaction would be of such a nature as to bring about the breakdown of substrate molecules. Therefore, the film technique was extended to investigate possible long range enzymatic action.

When a slide covered with one or many monolayers of protein antigen is treated with a homologous immune serum, a specific adsorption of antibody occurs. However, no such adsorption takes place if the layers have been first treated for a few seconds with a pepsin or trypsin solution at the proper *p*H. Similarly, a monomolecular layer of the polysaccharide from Type III pneumococcus will adsorb a considerable thickness of homologous antibody but is no longer able to do so after being treated briefly with a solution of the enzyme which depolymerizes the polysaccharide. Thus the immunological reaction occurring between films of antigen and antibody molecules may be utilized as a very convenient and highly sensitive detector of enzymatic action.

The experiments devised to detect a long range enzymatic action were very simple. The antigenic layers—bovine albumin or polysaccharide from Type III pneumococcus—were deposited on a metal slide and coated with a screen of inert material, such as barium stearate or formvar (a formaldehyde polyvinyl polymer). A drop of the appropriate enzyme solution was then spread on the screen and allowed to remain for five to ten minutes. After the enzyme solution was washed off, the slides were treated directly with homologous antibody and the increase in thickness which followed was compared to that obtained on a screen of similar thickness when no enzyme treatment had been applied. (Advantage is thus taken of the long range interaction between antigenic films and antibody molecules to disclose a possible long range enzymatic action on the antigen films.) A far more satisfactory method, however, was to dissolve away the blanket after the enzyme treatment, and then to apply the antiserum directly on to the antigenic layers. It was found possible to remove formvar films with ethylene dichloride without impairing the immunological reactivity of either the protein antigen films or the adsorbed polysaccharide molecules underneath. The removal of the barium stearate screens was more difficult to carry out and was always accompanied by a partial loss of the immunological reactivity of the layers.

The experiments showed indeed, as reported in two preliminary notes,[2] that enzymatic action takes place in spite of an intervening blanket and that this action does not appear to result from actual diffusion of the enzyme molecules through the blanket. If our conclusions are correct it means that no intimate contact between substrate and enzyme molecules is necessary for the enzymatic action to proceed. This is of fundamental importance from a physical as well as from a biological point of view.

Experimental

The technique used in this work is essentially the same as that described in the preceding paper[1] dealing with immunological reactions. The thickness of transferred or adsorbed films was determined optically by measuring the change that occurs in the ellipticity of polarized light after reflection from a film coated slide. The instrument developed for this purpose is the ellipsometer, which is characterized by a half shadow end-point made possible by reference films deposited on the slides to serve as optical gage.[3] Previous papers[1-3] should be consulted for the description of this instrument as well as for the experimental details concerning the preparation of the optical gages and their conditioning with uranyl acetate, the spreading of films and their transfer onto metal slides, the deposition of the blankets or screens, the treatment with antisera, the washing of the slides, and the optical measurement of the film thickness.

Enzymes.—Crystalline pepsin and crystalline trypsin in concentrations of 0.04% with respect to protein were used, unless otherwise stated. The samples of crystalline trypsin contained 60% magnesium sulfate. Pepsin was

(1) A. Rothen, *J. Biol. Chem.*, **168**, 75 (1947).

(2) A. Rothen, *ibid.*, **163**, 345 (1946); **167**, 299 (1947).

(3) A. Rothen, *Rev. Sci. Instruments*, **16**, 26 (1945).

dissolved in 0.02 *M* hydrochloric acid and trypsin in a veronal buffer *p*H 7.5 (0.05 *M*). The solution containing the depolymerase for the polysaccharide was of unknown concentration but a range of dilutions from one to five were used.[4] The medium was a phosphate buffer *p*H 7.2.

Antisera.—Antisera were diluted 0.1 with a phosphate buffer *p*H 7.2 containing 1% sodium chloride as described previously. Rabbit antibovine albumin sera and rabbit and horse antipneumococcus Type III sera were used.

Bovine Albumin Films—Antibovine Albumin Rabbit Sera. Pepsin Action.—Two to six unfolded monolayers of bovine albumin were transferred onto slides covered with an uranyl conditioned optical gage of one and three (or two and four) monolayers of barium stearate. A drop of pepsin solution deposited on the layers for one minute brought about a decrease in thickness from 8 to 30 Å. depending on the number of monolayers of bovine albumin. No increment in thickness occurred after subsequent treatment with homologous antiserum, which demonstrated that the remaining unfolded antigen films had been completely inactivated by the enzyme. A solution of 0.02 *M* hydrochloric acid alone could detach in one minute all layers transferred onto a slide except the first two. In this case, however, these remaining layers were not inactivated, as was indicated by an increase in thickness of 60 Å. after homologous antiserum treatment. When a blanket of one double layer of barium stearate about 50 Å. thick was deposited on two transferred double layers of bovine albumin, no change in thickness resulted from pepsin treatment and no adsorption of antibody occurred on subsequent antiserum treatment. If a drop of 0.002 *M* hydrochloric acid was substituted for the pepsin–hydrochloric acid solution, the antiserum treatment produced an increment of 40 Å. Similarly, slides with three double layers of bovine albumin coated with two double layers of barium stearate exhibited significant differences between those treated with hydrochloric acid alone and those treated with pepsin. A layer of antibody about 40 Å. thick could be adsorbed on the hydrochloric acid treated slide, but no change or even a considerable decrease in thickness occurred on the pepsin treated slides.

These experiments demonstrate that pepsin solutions are capable of destroying, through at least two double layers of barium stearate, the specific capacity of bovine albumin layers to react with antibody molecules. The fact, however, that the acid medium used for dissolving the pepsin is by itself capable of detaching some of the antigenic layers from the slides, made this enzyme unsuitable for a systematic research on account of the difficulty in differentiating the true enzymatic action from that of the medium.

Trypsin Action.—Trypsin proved an ideal enzyme to investigate, since a veronal buffer at neutral *p*H, in which the enzyme is most active, does not remove multilayers of protein from the metal slides except in one case which will be discussed later.

Conditioned slides covered with one, two or three double layers of bovine albumin were treated for three minutes with trypsin. A decrease in thickness of about 9 Å. occurred when there was one and a decrease of about 18 Å. when there was more than one double layer of bovine albumin. No significant increase in thickness followed antiserum treatment. No change in thickness and no inactivation resulted from treating the slides with the buffer alone.

When one to three double layers of bovine albumin were coated with one double layer of barium stearate, no change in thickness followed trypsin treatment. There was an increase of 9 to 17 Å. when the blanket consisted of two or more double layers of barium stearate. Subsequent treatment with a homologous antiserum caused the removal of most of the stearate layers except when there was only one underlying double layer of bovine albumin, in which case no change or a very slight decrease of a few Å. units was observed.

The results showed that in spite of a blanket as thick as five double layers of barium stearate, the trypsin molecules were capable of undermining the architecture of two or three double layers of bovine albumin underneath. The anchorage of the blanket was weakened to such an extent that the barium stearate molecules were washed away by the antiserum treatment. It appears as if the foundations upon which the structure of the layers of barium stearate were built had disintegrated under trypsin action. No such removal of the blanket took place after serum treatment if the slides were not submitted to trypsin action.

It was shown in a preceding article[1] that conditioning deposited layers of bovine albumin with uranyl acetate reduced the amount of antibody which could subsequently be adsorbed. In an analogous way, trypsin action on antigenic layers of bovine albumin is considerably reduced by conditioning. For example, inactivation of conditioned multilayers of bovine albumin does not occur following a two-minute trypsin treatment. The results obtained on inactivation by trypsin of three conditioned double layers of bovine albumin through intermediate blankets of barium stearate are summarized in Table I.

TABLE I

TEN MINUTE TRYPSIN ACTION THROUGH BLANKETS OF BARIUM STEARATE MULTILAYERS ON THREE CONDITIONED DOUBLE LAYERS OF BOVINE ALBUMIN (U BOV ⇵)$_3$ DEPOSITED ON A GAGE OF BARIUM STEARATE

The figures in the table stand for the increase in Å. units observed after treatment with antiserum

	Blanket, number of monolayers of barium stearate					
	0	1	2	4	6	10
Trypsin treatment	40[a]	70	30	15	18	10
No trypsin treatment	135	110	100	85	60	25

[a] 10 Å. in 20′.

There was one conditioning treatment after each "round trip" (⇵) deposition but none after the last one. The system of layers can be represented by the symbol (U bov ⇵)$_3$ where U stands for one uranyl conditioning. The trypsin solution was left for ten minutes on the slides. If we compare the figures of the two horizontal rows it appears that a significant difference in the thickness of the adsorbed layers of antibody, between the slides treated with trypsin and the slides not so treated, is still noticeable with blankets up to three double layers of barium stearate (≃150 Å.). These experiments indicate that the range of action of trypsin extends at least as far as the distances at which interaction between antigenic layers and antibody molecules can be demonstrated. By the very nature of the test, it cannot be said whether this action extends even farther. Uranyl conditioning between the deposited antigenic layers produced such a stabilizing influence that, in spite of the enzyme treatment, the blanket of barium stearate was never removed by the antiserum treatment. This stabilizing action of the uranyl ions may be twofold, first, in restricting the disintegrating effects set up in the bovine albumin layers by the enzyme, second, in holding together the fragments of the broken down molecules of the layers in such a way as to offer still a firm anchorage for the molecules of the blanket.

Obviously, in order to evaluate the maximum thickness of blankets through which trypsin molecules can act, the blanket should be removed after the enzyme treatment and prior to the deposition of the antiserum. It will be seen later than this can be accomplished easily with blankets of formvar. The removal of the blankets of barium stearate without impairing the optical gage of barium stearate underlying the antigenic layers proved a more

(4) I am very much indebted to Dr. M. Kunitz for the samples of crystalline trypsin, and to Dr. O. T. Avery for the depolymerase solution.

delicate task. The problem was solved by using an optical gage of one and three, or three and five monolayers of octadecylamine instead of barium stearate. The slides were conditioned by uranyl acetate as usual. To remove the barium stearate blankets the slides were first treated with a citrate buffer *p*H 3.6 (0.057 *M*) for a minute or so to liberate the free stearic acid which was then leached off with ethylene dichloride or benzene. The optical gage of octadecylamine was unaffected by virtue of the insolubility of the salt of the amine in organic solvents.

Some experiments were made with blankets of octadecylamine which could be removed without affecting an optical gage of barium stearate layers. The blankets of amine were removed by treating the slides with a dilute solution of ammonia (0.01%) and then with benzene. Thus by taking advantage of the large difference in solubility in organic solvents between the salt of the acid and the free base (or between the free acid and the salt of the amine) it was possible to remove a blanket made of barium stearate without affecting an amine optical gage and vice versa.

The influence of blankets of octadecylamine on trypsin action have been summarized in Table II.

TABLE II

SIX MINUTE TRYPSIN ACTION THROUGH BLANKETS OF OCTADECYLAMINE COVERING MULTILAYERS OF BOVINE ALBUMIN DEPOSITED ON CONDITIONED BARIUM STEARATE GAGES

The figures represent the increment in Å. units observed after treatment with the immune serum, following removal of the blanket. The subtitles "buf." and "try." indicate whether the slides were submitted to "buffer" or "trypsin" before the dissolution of the blanket. The duration of the "trypsin" or "buffer" treatment was six minutes. The letter R indicates that the blanket was detached by the trypsin treatment.

Blanket, number of double layers of octadecylamine	Number of bovine albumin double layers (⇃↾) 1		2		3	
	Buf.	Try.	Buf.	Try.	Buf.	Try.
0	60	0	120	0	185	0
1	57	25		17		
2	38	38		17		
3	34	36		18		
4		37		48		R0
5			59	58		R0
6				54		
7			58			18
8				58	73	46
10					67	67
						or less

In all these experiments the blankets were dissolved before the application of the antiserum. It is shown in the table that the removal of the screen sufficiently disrupted the system of multilayers of bovine albumin so that even without enzyme treatment the thickness of the antibody layer which could subsequently be adsorbed was considerably reduced. The thickness of the antibody layer dropped from 185 to 73 Å. in the case of three double layers of antigen. The difference, however, between "buffer" and "trypsin" treated slides is sufficiently large to permit definite conclusions to be drawn. A screen of two double layers of octadecylamine completely protects one underlying double layer of bovine albumin. Five double layers of octadecylamine are necessary when there are two double layers of bovine albumin underneath. Finally, a blanket as thick as ten double layers was needed to protect three double layers of bovine albumin. It also appears that with two double layers of bovine albumin, whether the blanket had been one, two, or three double layers thick, the thickness of antibody adsorbed was about 17 Å. This probably results from the fact that the first antigenic layer directly attached to the gage is more resistant toward trypsin than the others. Indeed, without a blanket, multilayers were nearly completely inactivated in a few seconds; there remained, however, a small but definite power for adsorbing specific antibody, corresponding roughly to that of one single layer. Even after a three-minute trypsin treatment a specific increment of antibody of 10 Å. could still be observed. Complete inactivation occurred, however, in a few seconds if the slides coated with a conditioned gage were first covered with one single or one double layer of egg albumin before the deposition of the bovine albumin layers. This procedure thus ensured a more uniform sensitivity of the bovine albumin multilayers toward trypsin and it is for this reason that it has been used in nearly all the following experiments.

Some of the results obtained with screens of barium stearate have been summarized in Table III.

TABLE III

SIX MINUTE TRYPSIN ACTION THROUGH BLANKETS OF BARIUM STEARATE COVERING MULTILAYERS OF BOVINE ALBUMIN DEPOSITED ON CONDITIONED GAGES OF ONE AND THREE LAYERS OF OCTADECYLAMINE

All slides after deposition of the blanket were treated for six minutes with a trypsin solution (active) or a solution of trypsin which had been brought to a boil for a few minutes (inactivated). After dissolving the blankets, the slides were treated with the antiserum. The figures stand for the increments observed in A units. The letter R indicates when the blanket was removed by trypsin treatment. "ov" and "bov" stand for ovalbumin and bovine albumin, respectively.

System of antigenic layers	Enzyme treatment	Blanket, number of double layers of barium stearate 1	2	3	4	5
ov ↑ bov ↑	Active	25				
	Inactivated		45			
ov ↑ bov(↑)$_2$	Active	15	26			
	Inactivated		46			
ov ↑ bov(↑)$_3$	Active	0	2			
	Inactivated		57			
ov ↑ bov(↑)$_4$	Active	0	0	0	0	0
ov ↑ bov(↑)$_5$	Active	0	0	R	R	R
ov ⇃↾ bov ↑	Active	10				
ov ⇃↾ bov(↑)$_2$	Active	0	0	8		
ov ⇃↾ bov(↑)$_3$	Active	0	0	0		
ov ⇃↾ bov(⇃↾)	Active	0	18	36	42	45
	Inactivated		48			
ov ⇃↾ bov(⇃↾)$_2$	Active	0	0	0		10
	Inactivated			86		
ov ⇃↾ bov(⇃↾)$_3$	Active	0	0	0	0	0

As in the experiments of Table II, the removal of the blanket produced partial inactivation (see Fig. 4 of ref. 1). The table shows that the greater the number of layers of bovine albumin the thicker the blanket needed for protection. It also appears that the mode of deposition of the antigenic layers is of importance. Inactivation could occur through blankets of greater thickness when the bovine albumin layers were deposited on the way up (↑↑)$_n$ by successive emersions than when they were deposited by successive immersions and emersions (⇃↾)$_n$. The depositions by emersion were accomplished by immersing the slides into the tray before the protein had been spread. In the deposition by successive immersion and emersion the protein film was spread before immersing the slides. With the antigenic system ov ⇃↾ bov (↑)$_6$, complete inactivation occurred through twenty double layers of barium stearate and twenty-five double layers were needed to ensure complete protection. This very

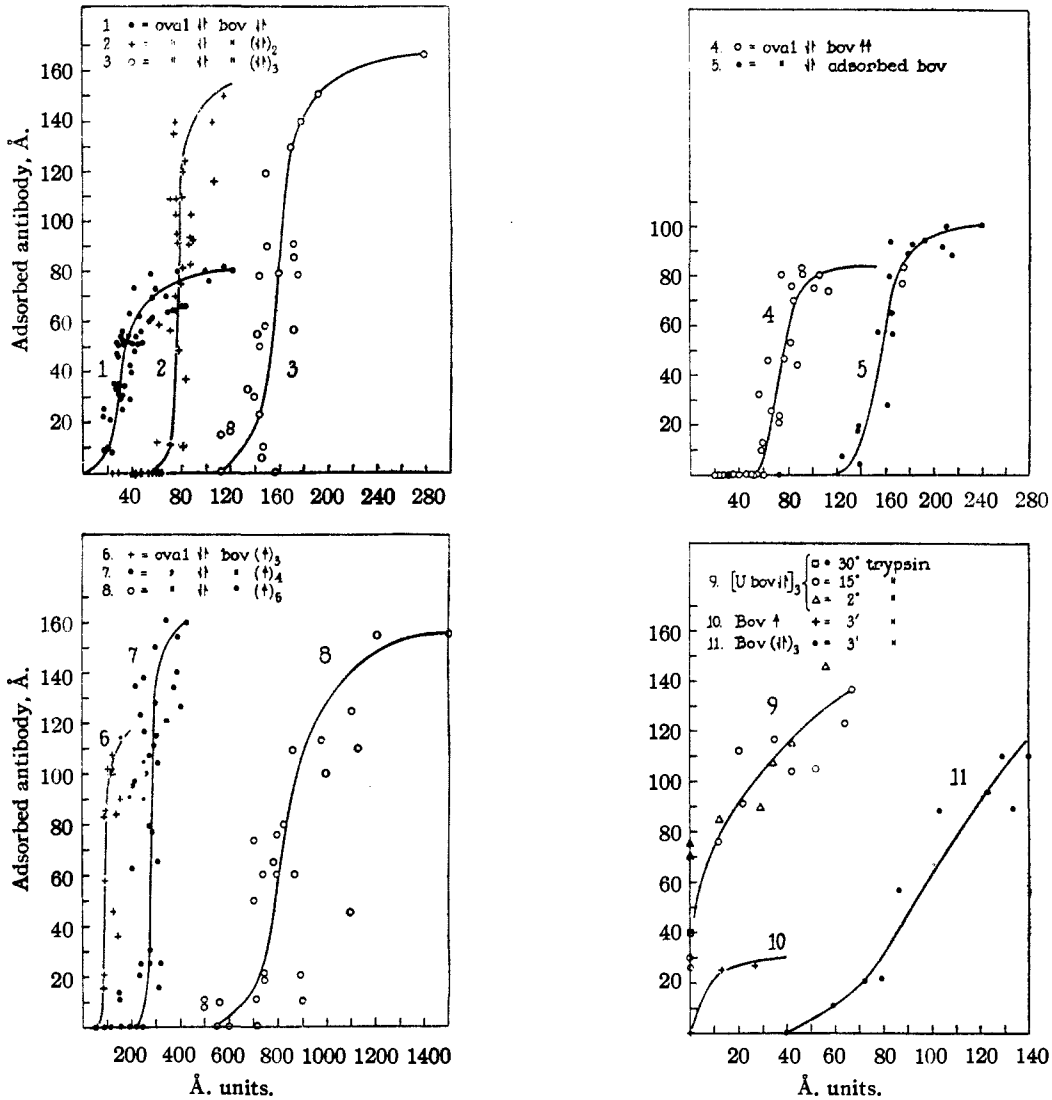

Fig. 1.—Inactivation of films of bovine albumin by trypsin through intervening blankets of formvar.

fundamental difference in the behavior of "up" and "down-up" layers will appear even more strikingly in the results obtained with blankets of formvar described in the next section.

Formvar Blankets.—Blankets of barium stearate or octadecylamine were not entirely satisfactory for two reasons, first the removal of the blankets produced a certain amount of inactivation, second the blankets were often removed by the enzyme treatment when there were three double layers of antigen underneath. Such disadvantages were not encountered with formvar blankets.

All the results obtained with screens of formvar have been summarized in Fig. 1. The abscissas give the thickness in ångström units of the formvar blankets present during the trypsin treatments and the ordinates, the amount of antibody adsorbed after removal of the blanket.

The conditions required for reproducibility of the results are very rigorous and traces of impurity appear to play an important role. Variations in results were more apt to occur between experiments carried out on different days than between results obtained with slides treated simultaneously. Experiments with one or two monolayers were more consistent than similar experiments made with multilayers. In other words, the phenomenon of coöperation between successive layers is very sensitive to small variations in experimental conditions.

Figure 1 summarizes experiments made with one, two and three double layers of bovine albumin deposited by the "down-up" process or the "up" process on slides covered with a conditioned gage of three and five layers of barium stearate plus one double layer of egg albumin. The abscissas show the thickness of the blanket of formvar upon which the trypsin solution was deposited, and the ordinates, the thickness of antibody specifically adsorbed after removal of the screen with ethylene dichloride. It is evident from the curves that the thickness of a formvar blanket needed for protection against trypsin action increases with the number of bovine albumin layers, in harmony with what was found with blankets of barium stearate and octadecylamine. A blanket of formvar about 180 Å. thick is necessary to protect three double layers of bovine albumin whereas for one double layer of bovine albumin 70 Å only will suffice. It is to be noted that all curves rise very sharply ($\partial y/\partial x \rightarrow \infty$) at a critical thickness of blanket.

Time Factor.—In all these experiments, the trypsin solution was left on the blankets for ten minutes. The time factor was investigated wtih the system ov ⇵ bov

$(\Updownarrow)_2$. It was found that essentially the same curve was obtained whether the enzyme solution stayed for five minutes or fifteen minutes on the slides. The amount of specifically adsorbed antibody, however, was only 35 Å. instead of 80 to 85 Å. if the trypsin solution was left for four hours on a blanket 160 Å. thick deposited on one double layer of egg albumin plus one double layer of bovine albumin.

When the layers of bovine albumin were deposited on the way "up" curves 4, 6, 7, 8 and 10 of Fig. 1 were obtained. It is evident from comparing the results presented in Fig. 1, that the mode of deposition of the antigen layers has a tremendous effect on the thickness of the blanket needed for protection. A blanket of 100 Å. is sufficient to protect the antigenic pile ov ⇂ bov $(\Updownarrow)_2$, but the protective thickness has to be increased to about 260 Å. when the antigenic pile has the structure ov ⇂ bov $(\uparrow\uparrow)_2$. The total thickness of the protein layers was the same in both cases.

A formvar blanket 600 Å. thick offered practically no protection at all for six layers of bovine albumin deposited on the way up and the impressive thickness of 1,000 Å. was necessary to keep the protein layers completely active. It was, therefore, of importance to determine whether or not a system of "up" layers without blankets was much more labile toward trypsin action than a system of "down-up" layers. If this were so, it could be argued that the difference in behavior of the two types of systems toward trypsin in the presence of a blanket was due to a small amount of trypsin which might have diffused through the blanket and would be capable of inactivating the "up" layers and not the "down-up" layers on account of difference in stability. The inactivation of the two types of multilayers without blanket, was therefore investigated as a function of the concentration of the trypsin solutions. The experiments were carried out at 4° for the following reasons. It was observed that at room temperature (20°) the veronal buffer was by itself capable of removing the equivalent of three to four layers of an "up" system of multilayers, but none at all if the layers had been built up by the "down-up" process. At 4°, however, the veronal buffer did not remove any of the layers of either system. It is of importance to note that a system of six "up" layers once coated with a formvar blanket does not lose any thickness following a veronal buffer treatment of ten minutes at room temperature. In other words, unfolded bovine albumin molecules cannot diffuse through a formvar blanket. The results which have been summarized in Table IV show that a system of "up" layers is inactivated slightly faster than a system of "down-up" layers, but this small difference cannot account for the large difference in the thickness of formvar blankets needed for protection against trypsin action.

TABLE IV

INACTIVATION OF MULTILAYERS OF BOVINE ALBUMIN BY TRYPSIN

Figures in columns 2 and 3 represent the thickness in Å. units of adsorbed antibody after treatment of the multilayers by trypsin solutions at 4° for ten minutes.

Concentration trypsin mg. per cc.	ov⇂ + bov$(\uparrow)_3$	ov ⇂ + bov$(\Updownarrow)_3$
0.4	0	0
.008	48	66
.004	80	95
.0008	160	160

The case represented by curve 5 of Fig. 1 is particularly interesting. All the bovine albumin films described so far were formed on a water surface and then transferred onto the slides. Bovine albumin molecules, however, can be adsorbed directly by depositing a drop of solution on the metal slides. In this case whatever unfolding of the molecules takes place is not complete, since the average thickness of an adsorbed layer was consistently found to be between 17 and 18 Å. This thickness is equal to that of two monolayers of unfolded molecules. The thickness of the blanket needed for protection, however, was about 180 Å., twice the thickness necessary to protect two "up" layers.

Conditioning of the antigen layers with uranyl acetate greatly diminished the thickness of the blankets in formvar needed for protection, as is plainly shown in curves 9 and 11 of Fig. 1. Fifty ångströms of formvar offer no protection for three double layers of bovine albumin, but ensure nearly total protection for the system of layers (U bov ⇂)$_3$.

Curve 10 shows that a blanket of formvar as thin as 20 Å. completely protected against inactivation one monolayer of bovine albumin deposited on the way "up."

Since the thickness of a blanket needed for protection increases with the number of layers of bovine albumin underneath, it was of interest to determine whether a thicker blanket was also needed if layers of egg albumin were substituted for some of the bovine albumin layers. As it will be seen later trypsin acted upon egg albumin layers just as easily as upon bovine layers in destroying their property of reacting with immune sera. The following systems of layers were deposited on conditioned gages of barium stearate: (ov $(\Updownarrow)_2$ bov ⇂), (ov $(\Updownarrow)_4$ bov ⇂) and (ov ⇂ bov ⇂ ov ⇂). The curve of inactivation as a function of the thickness of the screen was very much the same for all three systems and intermediate between curves 1 and 3 of Fig. 1.

Polyvinyl Chloride Blankets.—A few experiments carried out with blankets of polyvinyl chloride indicated that the screening action was analogous to that offered by blankets of formvar.

Metallic Blankets.—Metallic blankets of gold were deposited by evaporation in high vacuum directly onto transferred films of bovine albumin. It was found, however, that a thin blanket of gold 20 Å. thick evaporated onto three double layers of bovine albumin prevented any specific adsorption of homologous antibody. (In the case of the polysaccharide from pneumococcus Type III, a specific adsorption of antibody still occurred through 50 Å. of gold.) It was impossible to remove the gold once it was evaporated on the protein layers. Thus slides coated with bovine albumin layers and a blanket of gold obviously could not be used to detect any trypsin action through the blanket because of the lack of means to determine whether or not inactivation had occurred. Of all the methods tested to detect trypsin action through metallic blankets the one most satisfactory was as follows. Gold films were deposited by evaporation *in vacuo* on clean microscope slides. Thin films of formvar were then deposited on top of the gold by dipping the slide into a solution of formvar in ethylene dichloride. When the formvar film was detached from the slide and floated on a water surface, the gold film adhered to the formvar, with the result that the gilded face of the formvar film was in contact with the water surface. Such gilded blankets were transferred from the water surface onto the antigenic films coating a metallic slide in such a manner that the gilded surface was on top. Trypsin solution was deposited on the gilded blanket for ten minutes. After washing off the enzyme solution the slides were treated with a strong jet of ethylene dichloride which dissolved the formvar and removed the gold at the same time. If the blanket was transferred with the gilded face in contact with the antigenic layers, the gold could not be removed by ethylene dichloride treatment. It was observed that a blanket consisting of a formvar film 130 to 200 Å. thick, plus a gold film 40 to 60 Å. thick, offered complete protection against trypsin to six "up" layers of bovine albumin. Partial protection was ensured if the gold film was ≃30 Å. thick, and with no gold, as it has been shown above, complete inactivation still occurred with films of formvar as thick as 600 Å.

Ovalbumin and Antiovalbumin Rabbit Sera

The inactivation of ovalbumin films by trypsin was not studied extensively, because the amount of rabbit anti-

body which can be specifically adsorbed is independent of the number of deposited ovalbumin layers (1) and, therefore, does not afford a sensitive indication of inactivation. Some of the results are presented in Table V, which shows that a blanket of 70 Å. of formvar offers ample protection for two as well as for four double layers of ovalbumin.

TABLE V

TRYPSIN ACTION THROUGH BLANKETS OF FORMVAR COATING MULTILAYERS OF OVALBUMIN

The protein layers were deposited on conditioned gages of two and four layers of barium stearate. The blankets of formvar were dissolved in ethylene dichloride before treatment with the antiserum. The figures in the last column represent the thickness of the adsorbed antibody layer in Å. units.

Protein films	Thickness formvar blanket, Å.	Trypsin treatment, min.	Antiovalbumin rabbit serum treatment
ov(⇵)₂	96	0	23
ov(⇵)₂	71	5	18
ov(⇵)₄	50	5	11
ov(⇵)₄	74	5	18
bov ⇵ ov(⇵)₂	64	5	0
bov ⇵ ov(⇵)₄	60	5	0
bov ⇵ ov(⇵)₄	51	0	20

It should be noted, however, that if the ovalbumin layers were deposited on one double layer of bovine albumin complete inactivation occurred in spite of a screen of 64 Å. This would seem to indicate that a layer of ovalbumin anchored directly on the barium stearate gage is much more resistant to trypsin action than if it is anchored on a double layer of bovine albumin. As mentioned earlier, an analogous situation was encountered with films of bovine albumin.

Action of the Specific Enzyme Hydrolyzing the Polysaccharide from Type III Pneumococcus

The interaction between polysaccharide from Type III pneumococcus and homologous rabbit antibody through blankets of various kinds was described in the previously mentioned article.[1] In the present experiments horse as well as rabbit antisera were used, and interesting differences were brought to light.

The polysaccharide was adsorbed by placing a drop of solution on the slide, as described previously. The layer of polysaccharide, whether adsorbed on a conditioned gage of octadecylamine or on barium stearate, was about 5 Å. thick. When, however, the octadecylamine gage was not conditioned the adsorbed layer was 12 to 15 Å. thick, but the amount of antibody which could be specifically adsorbed was independent of the thickness of the layer of polysaccharide.

Horse Antipneumococcus Sera.—The thickness of the layer of horse antibody specifically adsorbed by the polysaccharide was about 70 Å. Reproducible results were difficult to obtain, values as large as 80 Å. and as low as 30 Å. were occasionally observed. The thickness was not dependent on the nature of the underlying gage, barium stearate or octadecylamine, nor did the coating of the gage with one double layer of protein prior to the adsorption of the polysaccharide influence the results. These findings are in direct contrast to the events observed with rabbit sera where it was found that a layer of antibody about 300 Å. thick could be adsorbed if the polysaccharide was anchored on an amine gage whereas the increment was only 120 Å. if the polysaccharide was on a barium stearate gage. No increment was observed following treatment with an antiserum against Type I pneumococcus or upon treatment of an adsorbed layer of polysaccharide from Type I with a Type III antiserum.

When a drop of a solution of the depolymerase was left for ten minutes on a polysaccharide layer adsorbed on a barium stearate gage or on a barium stearate gage coated with one double layer of protein, complete inactivation of the polysaccharide occurred. The increment observed after treatment with an antiserum was from 0 to 10 Å. If, however, the polysaccharide was adsorbed on an amine gage or an amine gage coated with one double layer of protein, little or no inactivation resulted from the enzyme treatment, the thickness of the subsequently adsorbed layer of antibody being 30 to 60 Å. In other words, the anchorage of the polysaccharide was such that the enzyme was ineffective. It could be argued that the amine by itself acted as an inhibitor for the enzyme. This assumption is disproved by the fact that if a blanket of octadecylamine is deposited on top of a polysaccharide adsorbed on a barium stearate gage, complete inactivation occurred through a blanket of at least one double layer of amine. It was also found that if one double layer of barium stearate was deposited on top of an amine gage prior to the adsorption of the polysaccharide, the enzyme was then capable of inactivating the polysaccharide just as well as if there had not been any amine layer underneath. Thus the direct anchorage of the polysaccharide to the amine is necessary to prevent the enzymatic action.

Rabbit Antipneumococcus Sera.—On account of the thick layers of rabbit antibody which could be adsorbed on a polysaccharide layer from Type III pneumococcus, this system was particularly suited for investigating the screening effect of blankets on the action of the depolymerase. The results have been summarized in Fig. 2, where the abscissa represents the thickness of the formvar or barium stearate blanket which was removed prior to the antiserum treatment. The curve shows that a blanket of about 180 Å. is necessary to protect the polysaccharide whether it was adsorbed on an amine or barium stearate gage. Also, a blanket of barium stearate has the same screening effect as that of an equivalent thickness of formvar. The same curve of inactivation was obtained whether the slides were treated for ten or twenty minutes with the enzyme solution. With no blanket present the enzyme produced complete inactivation when the polysaccharide was adsorbed on a barium stearate gage, but on an octa-

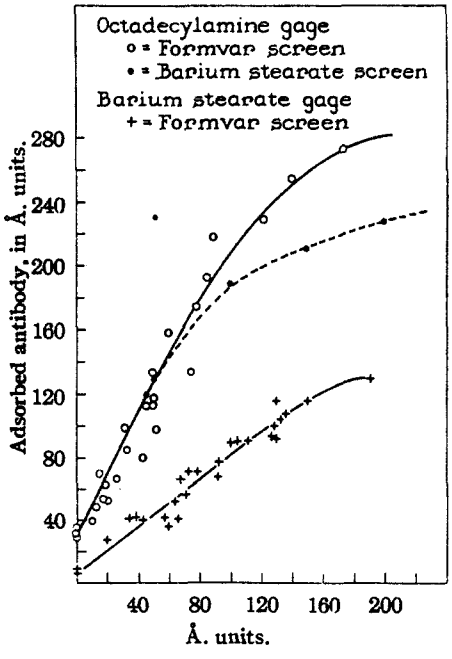

Fig. 2.—Inactivation of polysaccharide from Type III pneumococcus through intervening blankets.

decylamine base enough activity of the polysaccharide remained to produce an increment of 30 to 40 Å. of antibody, the same thickness found when horse serum was used. These experiments show that the enzyme is capable of altering the polysaccharide adsorbed on an amine layer to a much greater extent than the results with the horse serum indicated. The large increment of rabbit antibody adsorbed only a polysaccharide layer anchored to an octadecylamine gage results probably from a delicate adjustment between the structures of the antigen and the antibody. The small increment observed with the horse antiserum under similar conditions indicates that the horse antibody molecules do not respond to the whole antigenic pattern of the polysaccharide permitting the specific fixation of thick layers of antibody, but only to that part of the pattern responsible for short range action.

The enzyme is apparently capable of destroying the "fine" structure of the polysaccharide adsorbed on amine gage but cannot disrupt the whole of the pattern responsible for the specific reaction.

Discussion

All the data presented indicate that pepsin and trypsin, as well as the enzyme depolymerizing the polysaccharide from Type III pneumococcus can act through various blankets made of formvar, polyvinyl chloride, barium stearate and octadecylamine. The question of fundamental importance to answer is: Do the enzyme molecules come in contact with the antigenic layers underneath, or do they act at a distance through the blanket by some mechanism yet unknown? A similar question was raised in the previous study on immunological reaction where it was concluded that the mesh of the fabric of blankets similar to those used in the present experiments did not appear large enough to permit the diffusion of the antibody molecules, and that all available evidence tended to indicate that long range action operated between antigenic layers located on one side of the blanket and antibody molecules adsorbed on the other side.

In the case of enzymatic action, one should keep in mind that a single enzyme molecule diffusing through a blanket might damage an extensive area of the antigenic film. It should also be remembered that the size of a trypsin molecule ($M \simeq$ 30,000) is considerably smaller than that of a rabbit antibody molecule ($M \simeq$ 180,000), and that nothing is known about the size of the enzyme depolymerizing the polysaccharide except that it is a non-dialyzable protein molecule.

However, the results obtained with trypsin acting on multilayers of bovine albumin layers through blankets of formvar offer the strongest kind of evidence against diffusion of the enzyme through the blanket. It was shown that the thickness of the blanket needed for protection against trypsin action increased with the number of underlying bovine albumin layers, and that the thickness needed to protect three double layers of bovine albumin was nearly three times that needed for one double layer. It was shown also that the mode of deposition of the layers $(\downarrow\uparrow)_n$ or $(\uparrow\uparrow)_n$ was just as important as their total number. A much thicker screen was needed to protect a system of layers deposited upwards $(\uparrow\uparrow)_n$ than a system of the same total thickness deposited by a round trip process $(\downarrow\uparrow)_n$. It is an amazing fact that a blanket 600 Å. thick offered no appreciable protection to six monolayers deposited upwards, a total thickness of only 48 Å. There is no reason to believe that the permeability of the blanket should depend on the number or mode of deposition of the antigenic layers underneath, especially when the fact is considered that the blanket can be made first on a clean glass slide, floated on water and transferred as one single unit onto the antigenic layers. If the enzyme molecules do actually diffuse through the blanket, they must then diffuse faster or slower depending on the mode of deposition and number of the antigenic layers underneath, a process which in itself would involve a long range action. The fact that the enzymatic action is to a certain extent independent of the time, the same degree of inactivation occurring after ten or twenty minutes in the case of the enzyme depolymerizing the polysaccharide, or after five or ten minutes with trypsin, speaks against a diffusion process. Curve 9 of Fig. 1 indicates that the amount of inactivation of uranyl conditioned layers of bovine albumin through blankets of formvar is the same after two or fifteen minutes. With no screen present, however, the thickness of the adsorbed layer of antibody for slides treated for two minutes with trypsin was more than twice the thickness obtained with slides treated for fifteen minutes. It may be that in the absence of a blanket, the trypsin molecules diffuse slowly through the disintegrating layers and are able to inactivate each layer in turn whereas no diffusion of enzyme molecules could take place through the blanket.

Slides covered with protein multilayers are hydrophilic; they become hydrophobic when the layers are coated with a formvar blanket and are still hydrophobic after trypsin treatment. One must assume, nevertheless, that water and buffer ions must diffuse through the blanket and that the range of action of trypsin molecules should depend on the diffusibility of the buffer ions. It is a fact that trypsin in a phosphate buffer acts at markedly longer distances than in a veronal buffer. There was no appreciable difference in the action of trypsin whether the formvar blankets were formed directly on top of the antigenic layers or whether they were transferred as one unit from a water surface. There was, however, a difference in the shielding action if the blankets were made in two steps, either by forming a blanket directly on the slide and then transferring a second blanket on top of it from a water surface, or by successively transferring two blankets from a water surface. When the antigenic layers consisted of six "up" layers, a screen of formvar 500 Å. thick made in two steps was adequate to protect the system from trypsin action. A considerable amount of inactivation still occurred with a "two step"

blanket 300 Å. thick. This difference in the behavior of the two types of blankets may be due to a diminished permeability to the buffer ions when the blankets have been made in two steps.

It has sometimes been questioned whether, during the treatment of the slides with enzyme solution or antisera, the different layers stay in their original position or whether these are sufficiently mobile to be displaced. Previously mentioned experiments showed that successively deposited layers remain in their order of deposition. It may be added that some of the experiments on the enzymatic action on the polysaccharide from Type III pneumococcus point to the same interpretation. The enzyme can completely inactivate the polysaccharide molecules adsorbed on a stearate layer deposited on an octadecylamine base. The inactivation is incomplete if the polysaccharide is adsorbed directly on an amine base. Consequently the polysaccharide stays on the barium stearate layer and does not diffuse downward toward the amine layer or vice versa the amine base does not migrate upwards. Also the fact that a blanket of amine, deposited on the polysaccharide adsorbed on a barium stearate base, does not prevent inactivation by the enzyme demonstrates that the polysaccharide molecules stay anchored on the stearate and are not displaced when the blanket of amine is deposited on top of them.

It was shown in the study on interaction between films of antigen and antibody molecules that the amount of specific adsorption in the presence of a blanket was practically independent of the nature of the blanket. In the case of the action of the enzyme on the polysaccharide from Type III pneumococcus, a barium stearate blanket has the same protective effect as that of a formvar blanket of equivalent thickness. A different condition prevails when trypsin acts on multilayers of bovine albumin. Inactivation occurs through a larger thickness of barium stearate than of formvar. The interaction between antigenic layers and trypsin is so strong that very often, as we have seen, the blanket of barium stearate is removed following enzyme or serum treatment. When the interaction is diminished by conditioning with uranyl acetate in between the deposition of the antigenic layers, the blankets of barium stearate are never removed and it was shown (Table II) that trypsin acts at least through six, possibly ten, monolayers of barium stearate blanketing three double layers of bovine albumin deposited by the round trip process. In contrast, a blanket of formvar 60 Å. thick entirely cuts off trypsin action on similarly treated layers of bovine albumin.

It has been suggested that the metal slides onto which the layers were deposited have such rough surfaces on a molecular scale, that the results observed might be artifacts due to the valley-mountain profile of the slide. It is known, however, from electron micrographs that an ordinary microscope glass slide has a remarkably smooth surface. Therefore, some glass slides were coated by evaporation *in vacuo* with a film of chromium or gold thick enough to ensure metallic reflection. The same experiments were carried out using these slides and identical results were obtained.

The evidence just presented would indicate that the enzyme does not penetrate the blanket. Nevertheless, the effect of the enzyme does extend through the blanket and this fact should be considered in connection with theories of enzymatic action. The general mechanisms of enzymatic action postulated so far can be classified in two groups. One group assumes an intermediate complex between enzyme and substrate. The existence of such complexes has been demonstrated in the case of peroxidase and catalase by Chance.[5] In the second group, the reaction proceeds from inactivation by collision and by taking a quantum of the energy liberated during the reaction.[6]

It would be very difficult to explain our results on the basis of either type of mechanism if contact between our substrate films and enzyme does not really occur. In the case of proteolytic enzymes, enzymatic activity does not seem to be located in a prosthetic group, but results from steric architecture of the whole molecule. The following experiments, which show that trypsin as well as pepsin requires the native configuration of its molecules to retain enzymatic activity, are in harmony with this view. One or two monolayers of unfolded trypsin deposited on a slide are incapable of inactivating subsequently transferred layers of bovine albumin, even if a drop of a buffer solution at *p*H 7.5 is deposited on the slide for ten minutes. Similar results are obtained if trypsin films are transferred on top of the antigenic films; no inactivation occurs. Unfolding of the enzyme molecule should not abolish its activity altogether if the origin of the enzymatic activity resided in a prosthetic group. Thus this activity must originate from an extensive portion of the active molecule.

One plausible explanation of the long range action at present would appear to be through some resonance phenomenon perhaps involving a characteristic frequency of the substrate and of the enzyme. Appropriate tuning could then result by correspondence in the frequency and polarization of the vibrations, and the vibrations set up in the substrate layers could break down certain bonds characterizing the antigenic pattern. This interpretation has already been proposed by Chaudhury,[7] but is carried one step further. If such hypothetical resonators are extended, and as we have seen the evidence is in favor of an extended "active" part of the enzyme molecule, it is conceivable that resonance may occur at distances of an entirely different order of magnitude from

(5) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943); *Acta Chem. Scandinavica*, **I**, 236 (1947).

(6) G. Medwedew, *Enzymologia*, **2**, 53 (1937).

(7) A. K. R. Chaudhury, *Curr. Sci.*, **14**, 261 (1945).

those involved when small molecules or individual atoms are considered. Resonance might occur in spite of intermediate blankets, no immediate contact being necessary between substrate and enzyme. It might also be conceived that, depending among other things on the frequency involved, certain vibrations would be better transmitted than others by the intermediate blankets. Some types of blankets may have a stronger specific adsorption than others for the particular frequencies involved. The experiments made with blankets of evaporated gold show that such blankets are extremely efficient in preventing enzymatic as well as immunological reactions.

Thus, long range enzymatic action through a resonance phenomenon could be an explanation of the observed facts. If, as the presented data seem to indicate, long range enzymatic action occurs between films of antigen, or adsorbed molecules of antigens, and enzyme molecules, it would seem also likely that the same mechanism could apply to a substrate in solution and should be considered in a discussion of any theory of enzymatic action.[8]

Also it is important to note that in these experiments one is not justified in considering the behavior of single molecules of the substrate independently. The effect of the number and mode of deposition of monolayers of bovine albumin on its range of action is already an indication that considerable interaction takes place between the layers and presumably between the molecules within one single layer. The phenomenon of long range action could be considered as due to the cooperation of a group of molecules. Coöperation phenomena may play a role in biological processes, the degree of coöperation possibly determining the distance at which an enzyme may act. Finally, the possibility of enzymatic action through a thin cell membrane offers a new vista on physiological events.

(8) It is interesting in this connection to mention an article by Vlasow, *J. Physics, U. S. S. R.*, **9**, 25 (1945). The author shows that when considering large polyatomic systems one cannot neglect weak forces of interaction at long distances and that "these interactions reveal new dynamic properties of polyatomic systems, putting the problem of the transition from 'micro' to 'macro' anew." When collective interaction is taken into account, then follows according to the author "the presence and spontaneous origin of eigen frequencies in polyatomic systems." It is worth mentioning that in 1939 Langmuir, *Proc. Phys. Soc.*, **51**, 592 (1939), considered the possible importance of vibrations for the specificity of protein molecules.

Most of the data presented in this work were obtained with the able assistance of Miss Marjorie Hanson. I am indebted to her for her help in the preparation of this article. My thanks go also to Dr. Lyman C. Craig who read with care the manuscript and offered valuable criticism.

Summary

Multilayers of bovine albumin were submitted to the action of trypsin, and films of polysaccharide from Type III pneumococcus to that of a specific depolymerase. In both cases, following enzymatic action, the layers were altered to such a degree that they became incapable of specifically adsorbing homologous antibody. It was observed that blankets of barium stearate, of a plastic polymer (Formvar), and of polyvinyl chloride polymers deposited on the layers did not prevent enzymatic action from occurring when the enzyme solution was deposited on the blanket. The thickness of the blanket necessary to prevent any enzymatic action varied within a wide range depending on the number and mode of deposition of the underlying layers. It seemed unlikely that the enzyme molecules penetrated the blanket and the assumption was made that enzymatic action took place at a distance, the enzyme and substrate molecules being actually separated by an intervening blanket.

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[CONTRIBUTION FROM EASTMAN KODAK COMPANY]

The Determination of Primary Hydroxyl Groups in Cellulose Acetate by Tosylation and Iodination

BY CARL J. MALM, LEO J. TANGHE AND BARBARA C. LAIRD

A study was undertaken of the amounts of primary and secondary hydroxyl groups in various samples of cellulose acetate to determine whether any difference could be detected depending on the history of the sample.

The method of tosylation and iodination for the determination of primary hydroxyl groups in glucose and its derivatives depends on (a) complete tosylation of primary hydroxyl groups and partial or complete tosylation of secondary hydroxyl groups, and (b) subsequent replacement of all primary tosyl groups by iodine and no reaction of secondary tosyl groups. These reactions have been applied to a commercial cellulose acetate by Purves and co-workers,[1,2] who found that slightly more than one third of the hydroxyl groups were primary. Their work indicated that the method should be suitable for comparing the amounts of primary hydroxyl in various samples.

This method, with minor modifications, was used, and the samples chosen for comparison in the

(1) F. B. Cramer and C. B. Purves, THIS JOURNAL, **61**, 3458 (1939).

(2) T. S. Gardner and C. B. Purves, *ibid.*, **64**, 1539 (1942).