Technical Papers

Penetration of Trypsin through Formvar Films

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An orthodox explanation has been advanced recently (*4*) for Rothen's effect (*1*), i.e., the interaction between antigen and antibody through a blanket of inert material like Formvar. Phosphate ions from the buffer that was used were shown to be responsible for a migration of antigen (bovine serum albumin) through the Formvar blanket. The antigen was then able to react with antibody molecules at the blanket surface by direct contact. Those experiments, however, did not account for Rothen's (*2*) finding that enzymes (trypsin, pepsin) are also able to act upon substrate layers through an intervening screen. The medium in his experiments for trypsin was veronal buffer, and the substrate was bovine serum albumin. We have shown (*4*) that bovine serum albumin penetrates Formvar blankets in the presence of phosphate buffer but that it does not do so when veronal buffer, physiological saline, or distilled water is used.

To avoid introducing an explanation of the enzyme behavior by means of unknown forces, let us assume in this case that the enzyme (and not the substrate) is able to penetrate the screen under the exact conditions of Rothen's experiment, bearing in mind that his control experiment with a floating screen does not duplicate precisely the conditions of his original experiment. In this control he used a thin Formvar film floating on veronal buffer and showed that no trypsin activity could be found in this buffer after he placed a droplet of trypsin solution on top of the floating screen and allowed it to remain for some time.

On this assumption we have performed experiments using the same basic type of procedure as in Rothen's and our former work. The results indicate that the concept of long-range forces is not needed to explain enzyme-substrate reactions with intervening inert screens. Active trypsin molecules can penetrate Formvar screens; the amount of penetration is a function of the screen thickness (Figs. 1, 2). Under the conditions of these experiments, the original distance between enzyme and substrate is of the order of 10^6 A, which is well beyond the range of even the most imaginative estimates for long-range forces.

It is true that this experiment does not repeat exactly the conditions of Rothen's original one. He used a trypsin solution, and we used trypsin deposited as an S-layer underneath a screen. We find that trypsin molecules are still active under these conditions. The important consideration is the similarity of the physical state of the screen, as our former experiments and also Singer's (*3*) work have shown. Our new work has the advantage over Rothen's control experiment in that we deposit the screen on a solid surface, as in his original experiment. It might well be that important changes take place when a Formvar screen is transferred from a water surface to a slide. Cracks, or the strain caused by spreading the screen over a slightly irregular surface, or even the influence of the adsorptive forces of the underlying surface, may be responsible for the protein permeability.

The fact is, however, that with veronal buffer the trypsin and not the albumin migrates through the screen. The mechanisms involved are complex. Much more work must be done before we can hope for a better understanding of these reactions of molecular layers, which have been brought into the focus of general attention by Rothen's stimulating work.

In one experiment 5 chromium-coated microscopic slides (*A–E*) and 2 cover-glass slips (*S*) were assembled as shown in Fig. 1 (cross section parallel to the shorter edge

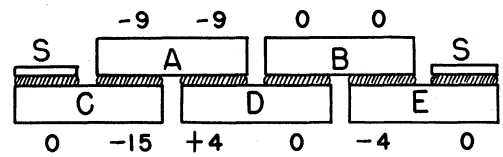

FIG. 1. Action of veronal buffer.

of slides). Plates *A* and *B* lie face down. Plates *C*, *D*, and *E* lie face up. Previous to the experiment proper, all 5 plates were coated simultaneously with an optical base of 39 layers of barium stearate, plus 4 additional steps of 1 double layer each. Then all 5 plates were conditioned in veronal-buffered uranyl acetate solution. Plates *A* and *B* were then dipped for 10 min into a solution of crystallized trypsin (Armour) containing 1 mg trypsin/cc in veronal buffer, 0.067 *M*, pH 7.5. A layer of trypsin molecules about 25 A thick was thus adsorbed from solution. Both plates were then rinsed once with veronal buffer and twice with distilled water. This treatment does not remove the adsorbed layer of trypsin molecules. Plate *A* was then coated with 2 Formvar layers (total thickness, 45 A), and plate *B* with 4 Formvar layers (total thickness, 90 A). Plates *C* and *E* were coated simultaneously with 3 double layers of spread crystallized bovine serum albumin (total thickness, 57 A); and on top of that 1 layer of Formvar (22 A) was placed. Plate *D* was not treated any further and acted as control and pickup. After these preparations and after measurement of each plate on 4 boundary lines, the 5 plates and the 2 cover slips were assembled as shown in

Fig. 1, with 0.05 ml veronal buffer between opposite parts and with enough free horizontal space between adjacent plates to prevent interference. The thickness of the liquid barrier was in all instances about 0.1 mm.

This pile was left for 10 min in a wet chamber. The plates were taken apart rapidly and carefully washed with distilled water and measured after drying. The figures inserted in the diagram indicate the respective loss or gain in A on each treated part. The experiment shows clearly that plate *A* lost material equally on both locations, that plate *C* lost material only on the part opposite the trypsin plate *A*, and that plate *D* adsorbed some material on the part opposite plate *A*. On the other hand, plate *B*, which had the thicker Formvar screen, did not lose a measurable amount of material, and the part of plate *D* opposite *B* is also unchanged. On that part of plate *E*, however, which was opposite plate *B* there is a slight loss of material. There was no change on the two parts of plates *C* and *E* over which cover glasses had been placed. The only plausible explanation for these results is this: Trypsin molecules were able to pass through the thinner Formvar screen on plate *A* and diffused through the buffer solution toward plates *C* and *D*. On plate *C* they penetrated the single Formvar screen and split part of the substrate underneath, which in turn diffused through the screen back into the solution and was removed by washing. On plate *D*, however, the trypsin has been adsorbed. On the other hand, the thicker Formvar screen on plate *B* prevented diffusion of trypsin almost completely, so that no adsorption could be detected on the control plate *D*; but the slight loss on plate *E* indicates that the 4 layers of Formvar still permitted some trypsin molecules to reach the substrate layers on this plate. The cover-slip controls on plates *C* and *E* show that the veronal buffer itself does not remove substrate from underneath the screen. This experiment has been performed several times with essentially the same results.

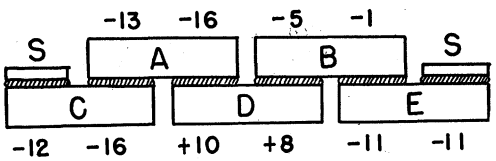

FIG. 2. Action of phosphate buffer.

If the same experiment is done with distilled water instead of veronal buffer, we do not find changes on any of the plates exceeding 2 A–3 A. The standard deviation for different boundary lines was between ± 0.5 A and ± 1.5 A in this set of experiments.

In another experiment, an identical set of plates, as described in Fig. 1, was used. The thickness of adsorbed trypsin on plates *A* and *B* was 28 A. The Formvar film on plate *A* was 46 A thick (2 layers) and on plate *B*, 75 A thick (4 layers). The 3 bovine serum albumin double layers on plates *C* and *E* were 48 A, and the single Formvar layers on top, 16 A in thickness, respectively.

Phosphate buffer was used instead of veronal buffer. After a 10-min period in the wet chamber the plates were rinsed once with veronal buffer and twice with distilled water.

As can be seen from Fig. 2, there is a combined effect of the phosphate buffer on the substrate, as well as on the enzyme. The leaching action on the bovine serum albumin of phosphate buffer through Formvar films, which has been reported previously (*4*), is demonstrated here again. But, furthermore, we find that trypsin is removed through a Formvar film by phosphate buffer. The phosphate action is even stronger than the veronal action, as a comparison of Fig. 1 and Fig. 2 shows.

The difference in loss on plates *C* and *E* can be explained by a double action of trypsin activity and phosphate leaching on plate *C*. The interesting fact that the 2 trypsin plate parts that are opposite plate *D* show a larger loss than the 2 parts opposite the substrate plates (*C* and *E*) can be explained by assuming that the substrate is removed relatively rapidly through the thin Formvar film on plates *C* and *E* and is adsorbed to a certain extent on the opposite parts of plates *A* and *B*. Either this adsorbate interferes somewhat with the diffusion of the trypsin, or it is still present after washing the plate at the end of the experiment. Also, a combination of these two effects seems to be possible and would account for the observed differences.

A detailed discussion of these experiments will be published elsewhere.

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On the Interaction of Protein Films

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In view of two recent notes by H. J. Trurnit (*4*, *5*) on the interaction of protein films, it seems appropriate to state clearly the point of view held in this laboratory.

In previous publications (*1*, *2*), we have shown that thin blankets of barium stearate or of plastic material, coating antigenic layers deposited on metallic slides, do not prevent a specific adsorption of homologous antibodies and, further, that certain enzymes, which would inactivate the adsorbed substrate layers, are capable of inactivating them also in spite of intervening blankets.

In the first of the above-mentioned notes, Trurnit concluded that the observed interaction between antigen and antibody through a blanket can be explained by a simple diffusion process of the antigenic layers. This explanation was based on the fact that the ions of a phosphate buffer are capable of leaching out, through a relatively thin Formvar blanket, a fair amount of bovine albumin