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ENZYMIC ACTIVITY AT INTERFACES

II. ENZYMIC ACTIVITY OF MICROSOMAL NUCLEASE AND BOVINE PANCREATIC RIBONUCLEASE AT THE AIR/WATER INTERFACE

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Summary

The adsorption isotherms and the spreading tendency of microsomal nuclease (nucleate 3′-oligonucleotidohydrolase, EC 3.1.4.7) and bovine pancreatic ribonuclease (ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) in the presence of isopropyl alcohol as spreading agent have been determined using enzymes radioactively labelled by acetylation. In parallel, the concentration-surface pressure relations have been established.

The enzymic activity of microsomal nuclease spread from isopropyl alcohol containing aqueous solutions was only a few percent of its activity in bulk, while the activity of the adsorbed enzyme was only slightly reduced.

Adsorbed monolayers of RNAase were almost inactivated, while the spread monolayers in the presence of isopropyl alcohol became reactivated after exposure to the substrate for several hours. The exposure time for the reactivation decreases with increasing surface concentration.

In a previous paper we described the relation between the surface activity of trypsin and its enzymic activity when spread or adsorbed at the air/water interface [1]. Since the degree of helicity is very little affected by surface forces [2], it was assumed that the surface forces influence the biological activity of the enzymes by modifying their tertiary structure. It was therefore of interest to compare two enzymes having a similar function but differing in the stability of their tertiary structure. We have chosen micorcoccal nuclease, which has no cystines or any other internal bonds and bovine pancreatic ribonuclease, whose structure is stabilized by four S-S bonds.

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As stated in our previous paper [1], it is impossible completely to eliminate dissolution of the enzyme during spreading. By successive exchange of the solution in the subphase before adding the substrate, the enzyme concentration in the bulk can be reduced until it is negligible with respect to the enzyme in the monolayer, and thus its contribution to the total activity can be accounted for. The low residual concentrations in the bulk, as well as the surface concentrations were determined with the aid of tritium-labelled enzymes. Determinations of minute concentrations of enzymes in the subphase permits investigation of specific activities of enzymes dissolved from monolayers into the subphase.

Use of ^{3}H-labelled enzymes also made possible investigation of enzymic activity of their adsorbed monolayers, albeit only from very dilute solutions.

Experimental

Materials

Micrococcal nuclease (nucleate 3'-oligonucleotidohydrolase, EC 3.1.4.7) was kindly supplied to us by Dr. Sara Fuchs of the Deparment of Chemical Immunology. Among its 149 amino acids there is no cysteine. Its molecular weight is 16 800 [3,4] and its optimal enzymic activity is at about pH 9.2 depending somewhat on the concentration of Ca^{2+} which activates the enzyme. Its concentration was determined by absorbance at λ = 280 nm, where the extinction coefficient is $E^{1\%}_{1cm}$ = 11.6 [5]. The enzyme hydrolyses denatured DNA > native DNA > RNA at the indicated relative rates [6] producing 3 nucleoside phosphates.

As substrates for the nuclease, we used calf thymus DNA (purchased from Worthington Biochemical Corporation) in its native and denatured forms (denaturation by heating native DNA in boiling water for 20 min and then quenching in ice-water). The melting hypochromicity at λ = 258 nm was 40%. Bovine pancreatic ribonuclease (ribonucleate 3'-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) (RNAase) was purchased from Worthington. The enzyme is specific to cytosine and uracyl nucleosides. We used as a substrate the sodium salt of cyclic cytidine 2',3'-monophosphate. Analytical grade Tris buffer and salts, and double-distilled water were used in all the experiments. Isopropyl alcohol was redistilled before use.

Radioactive materials. Tritium-labelled oleic acid and acetic anhydride were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Tritiated *Escherichia coli* DNA of specific radioactivity of 31 000 cpm/μg was obtained from Professor C. Paoletty, Institute Gustave Roussy, Villejuif, France.

The labelling of proteins by acetylation is described elsewhere [7]. The method is based on adding a benzene solution of tritiated acetic anhydride to the freeze-dried protein and storing the moistened protein for days or weeks at 0°C, the freezing point of benzene.

Methods

The methods are described in the first paper of this series [1] and only a short account will be given here.

The surface pressure of the adsorbed and of the spread enzyme monolayers

was determined by an automatic Wilhelmy type surface balance built locally and described elsewhere [8]. The surface concentration of the spread and adsorbed enzymes was determined in a 5 × 5-cm trough compartment with a gas flow counter fitted with an ultra thin formvar end-window [1]. The trough was made of stainless steel on which the adsorption of the nuclease and the RNAase was insignificant. Spread monolayers were prepared by spreading from aqueous solutions containing 20% isopropyl alcohol. The dissolved protein was removed from the subphase by consecutive exchange with buffer solution until the total amount of protein remaining was less than 3% of that in the surfaces layer. At this point half of the subphase was transferred to an equal volume of substrate solution in another trough compartment of equal size to serve as a blank for the enzymic activity of the subphase. The same volume of substrate solution was injected into the subphase underneath the surface layer.

The enzymic activity of adsorbed monolayers of the RNAase and the nuclease were measured in the same trough compartments. After the surface radioactivity count showed that the surface concentration had leveled off (approx. 2 h) half of the subphase was transferred to the equal volume of substrate solution in the other trough compartment (II), and the same volume of substrate solution was injected into the subphase. At the same time an enzyme solution (III) having the same concentration as the solution used in the adsorption experiment, was mixed with an equal volume of substrate. The relative enzymic activity (e.a.) of the surface layer ξ (the e.a. of the surface layer divided by the e.a. of equal amount of enzyme in the solution) was then calculated by the following relation:

$$\xi = \frac{1(\text{e.a.})_{\text{I}} - (\text{e.a.})_{\text{II}}}{2(\text{e.a.})_{\text{III}} - (\text{e.a.})_{\text{II}}} \tag{1}$$

Alternatively, if surface and bulk concentrations are known, no reference bulk $(\text{e.a.})_{\text{III}}$ is needed and

$$\xi = \frac{[(\text{e.a.})_{\text{I}} - (\text{e.a.})_{\text{II}}]}{\text{En}^{\sigma}(\text{e.a.})_{\text{II}}} \cdot En^{\beta} \tag{2}$$

where En^{σ} and En^{β} are the amounts of enzyme in the surface and that remaining in the bulk. All the measurements of surface pressure and of the enzymic activities were carried out in an air-conditioned room at between 21 and 22°C.

Results

Formation and surface pressure of the enzyme monolayers

The calibration procedure of the gas flow counters for surface concentration determination has been fully described in the first paper of this series. We wish only to mention here that the efficiency of the counting and its change with the distance of the end-window from the monolayer was determined with spread monolayers of [^{3}H]oleic acid on an acidic solution. In order to increase the counting efficiency and to diminish the dependence of the surface count on the distance, the gap between the counter and the surface was flushed with He

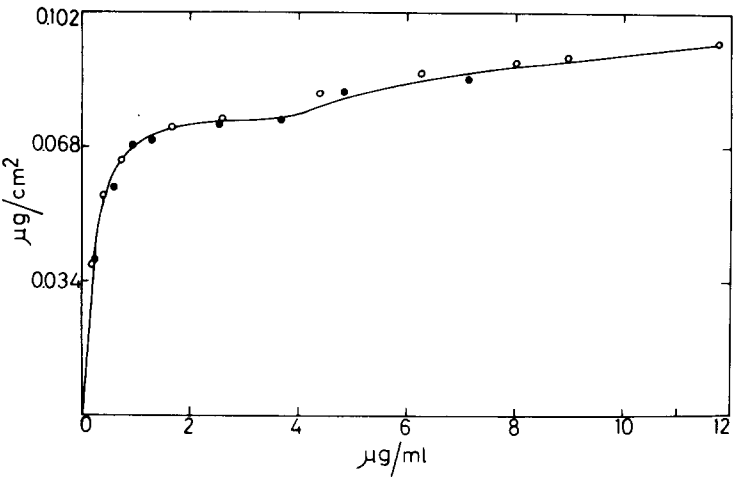

Fig. 1. Adsorption isotherm of nuclease at pH 8.8 (Tris buffer). ○, concentration subsequently increased in solution. ●, subsequent quantities applied to surface from an aqueous solution containing 20% isopropyl alcohol and equilibrated while stirring the subphase. Subphase solution 10^{-2} M Tris buffer pH 8.8.

saturated with H_2O (to eliminate surface evaporation).

Since protein monolayers can be obtained either by adsorption or by spreading, it is of interest to establish the difference or similarity of the monolayers obtained in these two ways. As can be seen from Fig. 1, there is no difference in the relation between the surface concentration and the total concentration of the nuclease whether it be adsorbed from the solution or "spread" from an aqueous solution containing 20% isopropyl alcohol. In these experiments subsequent portions of nuclease were either added to the bulk of the solution or applied to its surface under constant but gentle stirring of the subphase.

In the first case the nuclease was adsorbed directly from the respective solution for several hours and equilibrium surface concentration was considered to be reached when the surface radioactivity remained constant for at least half an hour. In the second case 10 μl quantities of 1 mg/ml aqueous solutions containing 20% isopropyl alcohol were applied by the Trunit method [9] to the surface of a thin layer of aqueous solution over a glass bead. Surface radioactivity was then measured as a function of time until constant values were reached. During this time the isopropyl alcohol did not evaporate appreciably from an aqueous solution at below 1.2% which was the final concentration after spreading.

As can be seen from Fig. 2 there is a difference in the pressure/concentration relations obtained by the two methods. The difference can be at least in part accounted for by the contribution of the isopropyl alcohol to the surface pressure. A better idea of the structure of the surface layers formed by the two methods can be obtained from the pressure/area relation as represented in Fig. 3. In both cases a minimal compressibility at a surface concentration of 0.075 $\mu g/cm^2$ or at an area of 3700 $Å^2$/molecule can be observed. This area can be considered as the surface area of a fully unfolded molecule on the surface which corresponds to 25 $Å^2$ per amino acid residue. The adsorbed monolayer in the absence of isopropyl alcohol seems to reach a collapse pressure of about

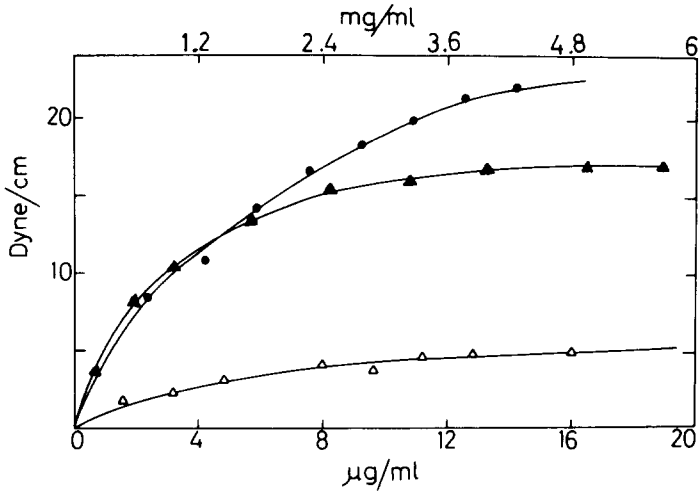

Fig. 2. Surface pressure against the amount of nuclease added per ml of solution. ▲, concentration subsequently increased in solution; ●, subsequent quantities applied from an aqueous spreading solution containing 20% isopropyl alcohol. △, spreading solution only. Bottom abscissa: concentration of nuclease; top: concentration of isopropyl alcohol.

17 dynes/cm at an area of about 2900 Å^2 per molecule or less than 20 Å^2 per amino acid residue. The collapse pressure is higher in the presence of isopropyl alcohol and is reached at somewhat smaller areas.

Fig. 4 shows that unlike in the case of nuclease the adsorption isotherms of RNAase in the presence and absence of isopropyl alcohol are quite different. Considerably hogher surface concentrations are achieved when the RNAase is applied to the surface from a solution containing isopropyl alcohol. One should bear in mind that the adsorption of RNAase as well as that of many other proteins is strongly dependent on the types of ions in the subphase. For example in the presence of phosphate there is a tendency for multilayer adsorp-

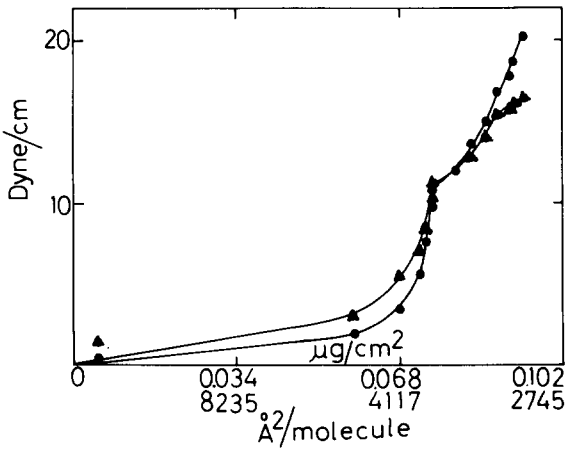

Fig. 3. Surface pressure of nuclease as a function of its surface concentration: ●, in the presence of isopropyl alcohol; ▲, in its absence.

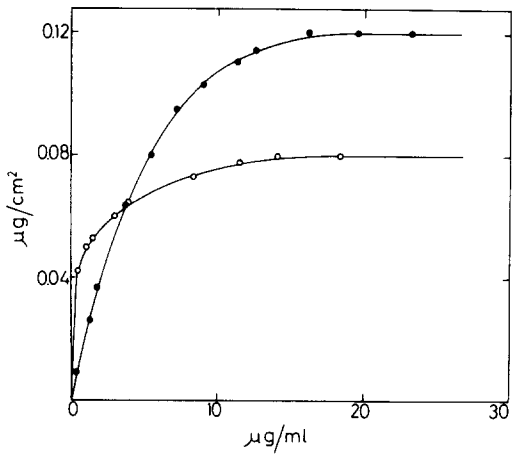

Fig. 4. Adsorption isotherm of RNAase. ○, concentration subsequently increased in solution, ●, applied to the surface in the presence of 20% isopropyl alcohol in the spreading solution. Subphase 10^{-2} M Tris buffer, pH 7.1.

tion [10]. In the present experiments we used Tris buffer.

The surface pressure/concentration relation for the monolayer adsorbed from the bulk and applied on the surface from solutions containing isopropyl alcohol are given in Fig. 5. The corresponding surface pressure/surface concentration or area relations are given in Fig. 6. It is evident from this figure that the limiting area in the presence of isopropyl alcohol (2100 Å²/molecule or 17 Å²/residue) is considerably smaller than in its absence (3000 Å²/molecule or 24 Å²/amino acid residue). It follows from this that at high surface concentrations isopropyl alcohol does not enhance unfolding of the RNAase molecule but, on the contrary, seems to stabilize the tertiary structure in the surface.

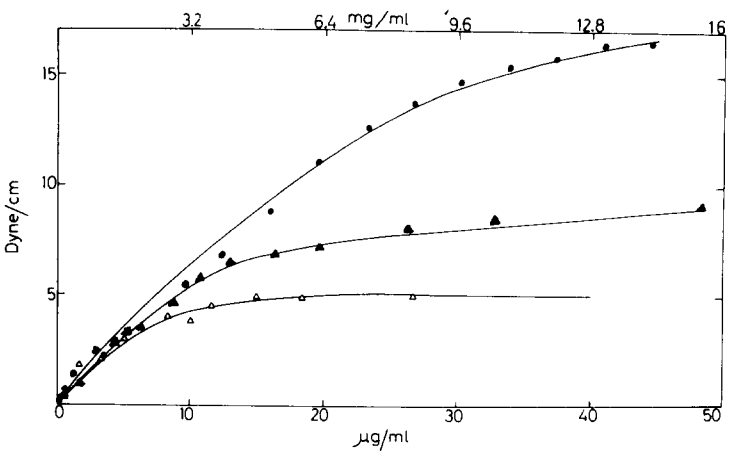

Fig. 5. Surface pressure of RNAase against its total amount added per ml of subphase. ▲, concentration of RNAase subsequently increased in solution; ●, subsequent quantities applied to the surface from an aqueous spreading solution containing 20% isopropyl alcohol. △, Spreading solution only. Bottom abscissa: concentration of RNAase; top: concentration of isopropyl alcohol.

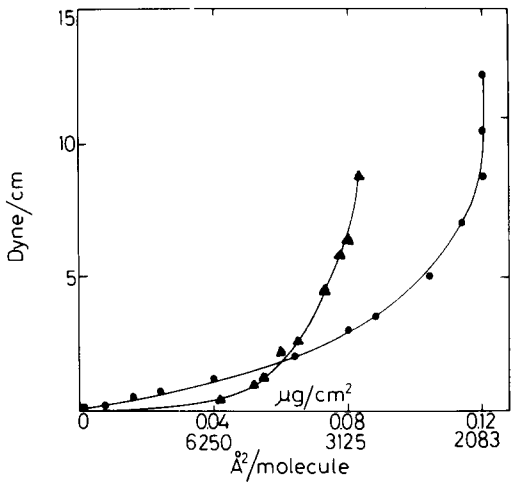

Fig. 6. Surface pressure of RNAase as a function of its surface concentration •, in the presence of isopropyl alcohol; ▲, in its absence.

Enzymic activity of the nuclease monolayer

The enzymic activity of adsorbed monolayers of nuclease above a concentration of about 0.02 $\mu g/cm^2$ was, within experimental error, equal to their bulk activity with respect to hydrolysis of native DNA. This is shown in Fig. 7a where the surface concentration and the ratio of the enzymic activity in the surface and the bulk as obtained by Eqn. 2, is plotted against the initial concentration in the bulk phase. Each bar represents the spread of results of three or four experiments. The relatively low accuracy of the determination of the enzymic activity in the surface layer reflects the errors in the three required independent measurements and the difficulty in maintaining constant surface temperature.

In Fig. 7b the enzymic activity ratio ξ of the nuclease in the monolayer spread from a solution containing 20% isopropyl alcohol, to that in the bulk are given. In these experiments different amounts of spreading solution were applied to the surface. With this method of surface layer formation considerably higher surface concentrations were obtained than when the solution was applied in consecutive small portions. In the second method sufficient time was allowed for equilibration after each application and consequently the adsorption isotherm was almost idential to that of the monolayer adsorbed directly from the bulk (Fig. 1).

When relatively large quantities of enzyme-spreading solution were applied at once to the surface, the isopropyl alcohol concentrated in the surface layer presumably causing partial insolubilization of the enzyme. This form of the enzyme, as evident from Fig. 7b) retains, after almost complete removal of the isopropyl alcohol by repetitive washing, only about 1.5% of its bulk activity with respect to native DNA and about 4% with respect to denatured DNA.

These activities are strikingly lower than the enzymic activities of the adsorbed nuclease monolayers which retain, in the surface, their complete enzymic activity from the bulk. One can conclude from this, that while the adsorp-

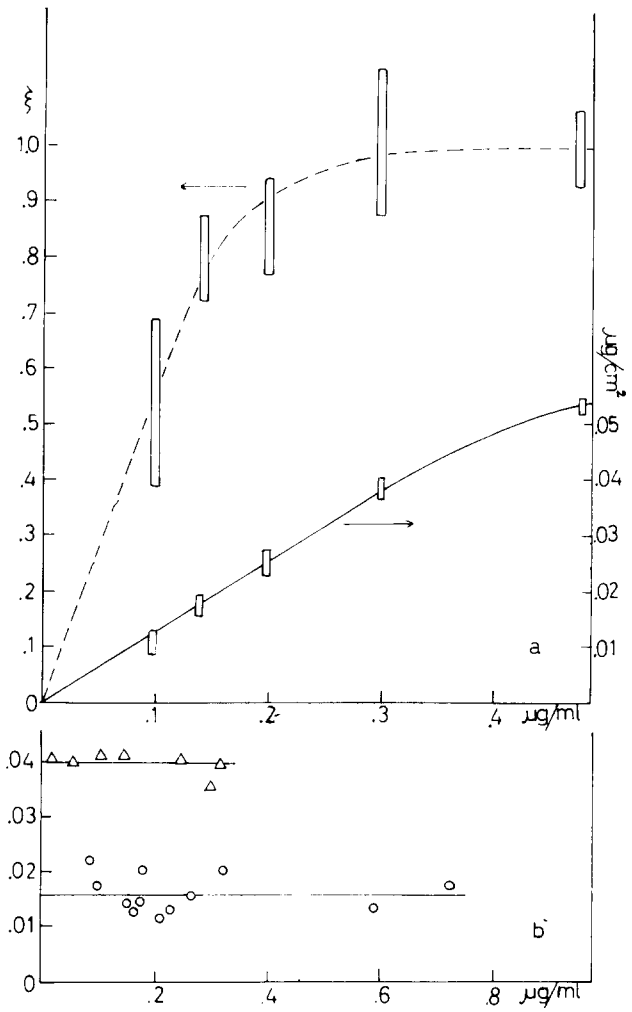

Fig. 7. ξ, the ratio of the specific activity of nuclease in the surface to that in the bulk with respect to the same substrate. (a) Nuclease adsorbed from solutions of different initial concentrations. (b) Different quantities applied to the surface from an aqueous solution containing 20% isopropyl alcohol with subsequent flushing of the subphase with buffer solution. ○, ξ with respect to native DNA, △, with respect to denatured DNA.

tion of nuclease from a dilute aqueous solution is a reversible process, high concentrations of isopropyl alcohol tend to induce an irreversible transformation in the nuclease monolayer resulting in its inactivation. Using the labelled DNA we could show that native DNA does not adsorb onto the spread concentrated surface layer of nuclease, however, up to about 0.05 mg/cm^2 denatured DNA can be adsorbed. The higher activity of the nuclease with respect to denatured DNA is in keeping with the higher binding tendency.

The sodium salt of cyclic 2′,3′-monocytidine phosphate was used as a substrate for RNAase. In contrast to the behavior of nuclease, the adsorbed monolayers of RNAase were completely inactive within the accuracy of determination and calculation by Eqns. 1 and 2.

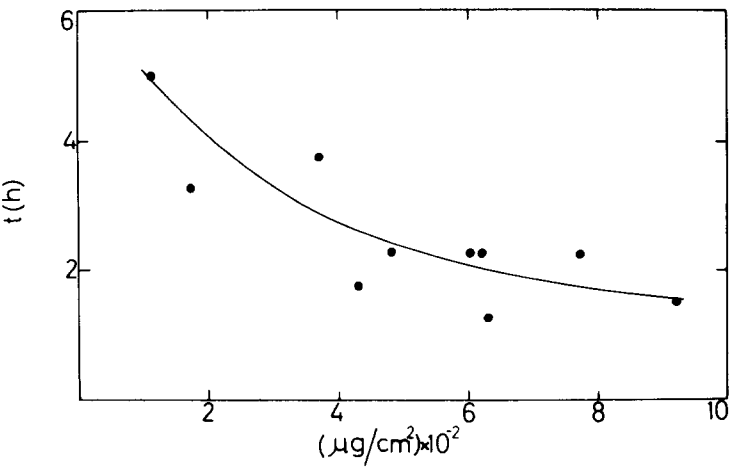

Fig. 8. Induction time, after injection of the substrate, for reactivation of the surface layer of RNAase spread from an aqueous solution containing 20% isopropyl alcohol as a function of surface concentration.

Initially, the spread monolayers, in the presence of isopropyl alcohol, remained inactive after removal of the alcohol. However, several hours after injection of the substrate, the enzyme action started at a rate equal to that in the bulk. As seen from Fig. 8 the time that elapses between the injection of the substrate and commencement of the enzymic action decreases with increasing surface concentration of RNAase. At this stage the concentration of isopropyl alcohol has been reduced to negligible values by the repeated dilution of the subphase with pure buffer solution.

Discussion

RNAase is known to be a very stable molecule. Its three-dimensional conformation is determined by the amino acid sequence along the polypeptide chain and further stabilized by four S-S bonds. Nuclease on the other hand, has no S-S bonds, and changes readily from one conformation to another. This may explain the high enzymic activities of the nuclease in adsorbed monolayers at moderate surface concentrations. Even though the conformation of the adsorbed nuclease is grossly changed it may be converted into the active form upon interaction with the substrate. However, when the surface layers of nuclease are formed in the presence of isopropyl alcohol accumulating in the surface, high surface concentrations (several monolayer equivalents) of enzymatically nearly inactive nuclease are obtained. The isopropyl alcohol seems to induce insolubilization and inactivation of the nuclease in the surface. 20% isopropyl alcohol in the aqueous solution does not reduce the enzymic activity of the nuclease, and the enzyme in the spreading solution was completely active. Thus the surface insolubilization and inactivation is brought about by the influence of the isopropyl alcohol on the surface-oriented and condensed nuclease.

The behaviour of RNAase is quite different in this respect. The adsorbed layer at the air/water interface is completely inactive, while the layer spread

with the aid of isopropyl alcohol becomes active in the presence of the substrate after a certain induction period. Isopropyl alcohol does not really serve as a spreading agent. On the contrary, as evident from Fig. 5 the limiting area of the RNAase in a monolayer spread from a solution containing isopropyl alcohol is smaller than that in an adsorbed monolayer. Presumably the smaller limiting area corresponds to a conformation which can be readily converted into the native form in the presence of the substrate.

On the other hand the adsorbed RNAase if inactivated even by a small conformational change around the active site is not easily reactivated.

In every case the substrate affects the absolute, as well as the relative activity of the enzyme in the surface. The surface-inactivated nuclease in the presence of isopropyl alcohol is more active with denatured DNA than with the native form. The activity differs in absolute value as well as in its ratio to the bulk activity (Fig. 7b). The strength of the interaction between the enzyme and the substrate seems to be an important factor determining not only the enzymic activity but also the rate and the extent of reactivation of an inactivated enzyme.

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