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A Dialysis Study on the Conformation of Lysozyme and Its Binding Properties with *N*-Acetyl-D-Glucosamine[1]

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The technique of thin-film dialysis has been applied to the study of the conformational mobility of lysozyme in solution. An increase of ionic strength by NaCl in the solvent (0.01 *M* acetic acid, pH 3.3) increases slightly the diffusional size of lysozyme. This change is reversible and does not inactivate enzymatic activity. Bovine pancreatic ribonuclease A and chymotrypsinogen A show a similar behavior. *N*-Acetyl-D-glucosamine (NAG) does not add to the effect of NaCl on lysozyme except at near 0.01 *M* where the diffusional size increases. Tritium–hydrogen exchange studies reveal that 10 fewer hydrogens are accessible to solvent in the presence of 1.0 *M* NaCl than in the absence of salt. At the constant ionic strength of 0.06 in phosphate buffer, the variation of pH from 4.5 to 9.0 results in changes in the rates of dialysis with a minimum diffusional size at pH 5.9 which coincides with the presumed pK_a of the catalytic site. This trough disappears in the presence of 10^{-3} *M* NAG.

The thin-film method is also applied to studies of NAG-lysozyme binding with the result that the binding constant is shown to increase with a decrease in the concentration of lysozyme. A Scatchard plot of the data reveals a behavior suggestive of allosteric interaction at a lysozyme concentration lower than 2×10^{-4} *M*. This phenomenon could be explained by the lower binding capacity of lysozyme dimer. The dissociation of the latter to monomer is a function of both lysozyme and NAG concentrations.

INTRODUCTION

In living tissues remarkably specific transformations take place at great speed which depend on the ability of certain molecules to "recognize" others and associate with them. Therefore, experimental methods for detecting such interactions under relevant conditions such as concentration, pH, ionic strength, etc., are of great importance, a statement to which the enormous literature dealing with binding studies attests. With the improvement of spectroscopic methods such as uv, ord, cd, nmr and x-ray diffraction much greater understanding of the phenomenon is possible. Equilibrium dialysis, however, still offers certain overall advantages and the technique is worth trying to improve. The "thin-film dialysis" methods (*1*) offer possibilities for this purpose partly because this approach permits a choice of calibrated membranes with optimum porosity and allows wider concentration ranges to be covered. At the same time very subtle changes in conformation can be detected in solutions at high dilution.

Studies from this laboratory (*2*, *3*) have established that the dialysis behavior of comparatively large solutes, such as globular proteins under 100 000 in molecular weight, can be studied precisely in cellophane dialysis tubing in much the same way that small solutes can be studied. It was shown that the very slow rate of penetration

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; NAG, *N*-acetyl-D-glucosamine; ACTH, adrenocorticotropic hormone; BPA, bovine plasma albumin.

per cm^2 of such large molecules can be compensated for by employing a high membrane-area/retentate-volume ratio in a suitably stirred cell called a "thin-film dialysis cell." It was further found that the shape and state of aggregation of the solute influenced strongly its rate of diffusion through a membrane when the porosity of the membrane was adjusted (*4*) to a critical size, i.e., one which barely permits the solute to diffuse through the membrane. It was suggested at that time that this type of dialysis study should be of value for studying the changes in the shapes of proteins caused by any change in the temperature or solvent environment. Studies of peptides and proteins with respect to size (*5*) association–dissociation phenomenon (*6, 7*) and conformational stability as influenced by the solvent environment (*8, 9*) have been reported subsequently from this laboratory.

A refinement of the membrane-diffusion method for certain purposes derives from the development of a thin-film countercurrent dialysis column (*10*) with a much larger membrane surface which provides rapid interchange of a diffusing solute from one side of the membrane to the other. This permits rapid equilibrium to be reached in equilibrium dialysis studies and offers the possibility of greater precision than the slow dialysis methods currently used, particularly where weak binding is to be studied. It seemed worthwhile to reinvestigate some of the results of earlier work in greater detail in order to see if more informative interpretations can now be made. This paper reports such a study with hen's egg lysozyme (EC 3.2.1.17). This protein was selected because so much is presently known about its conformation, mainly through the x-ray crystallographic studies of Phillips (*11*) dealing with the enzyme and the enzyme-substrate or enzyme-inhibitor complex. Finally, the much studied binding of *N*-acetylglucosamine to lysozyme (*12–19*) seemed ideal for testing some of the possibilities thought to derive from the thin film countercurrent dialyzer.

EXPERIMENTAL

Materials. Lysozyme (salt free, 9100–9300 units/mg) was purchased from Worthington Biochemical Corporation. Defatted bovine plasma albumin was generously given by Dr. T. P. King. NAG and L-tryptophan were A-grade products from Calbiochem. *N*-Acetyl-[^{14}C]-D-glucosamine, ^{3}H-L-tryptophan and tritiated water were purchased from New England Nuclear Corporation. All dialysis tubings were Visking No. 20 cellulose casings from Union Carbide Corporation and were washed with 0.1% disodium EDTA[1] and 1% sodium dodecylsulfate before use.

Static thin-film dialysis experiments. The cell and procedures were the same as described before (*1*). A zinc chloride treated membrane was used for chymotrypsinogen A. The effective dialyzing area of the dialysis membrane was 50 cm^2. The volumes inside and outside the membrane were 0.5 ml and 4.0 ml, respectively. Experiments were carried out at room temperature (24.5 + 0.5°C) unless specified otherwise. The variation of the half escape time ($t/2$) in repeated runs under the same conditions was no more than 5%.

Assay of lysis activity. The standard lysozyme assay (*20*) was used in which the decrease of the absorbancy of 600 nm, during a 3-min period, was measured in a Cary Model 14PM recording spectrophotometer. In all cases the final lysozyme concentration was adequately adjusted to 1–10 μg/ml in order to yield a linear response curve.

Equilibrium dialysis with the countercurrent dialyzer. The detailed description and operational procedures of the thin-film dialyzer, as shown in Fig. 1, have been published (*10*). Lysozyme was dissolved in a solution of 0.05 *M* sodium phosphate buffer, pH

5.9, ionic strength 0.06 containing NAG and [^{14}C]NAG (10–20 μCi/liter). Lysozyme concentrations were 0.1, 0.2, 0.5, 1.0, and 2.5 m*M*. These solutions were injected consecutively into the dialyzer through the retentate stream after the whole system had been fully equilibrated with the identical buffer solution containing NAG, which was used to dissolve the lysozyme. The flow rates of the retentate and the diffusate were adjusted to 0.4 ml/min and 1.2 ml/min, respectively, which gave a residence time of

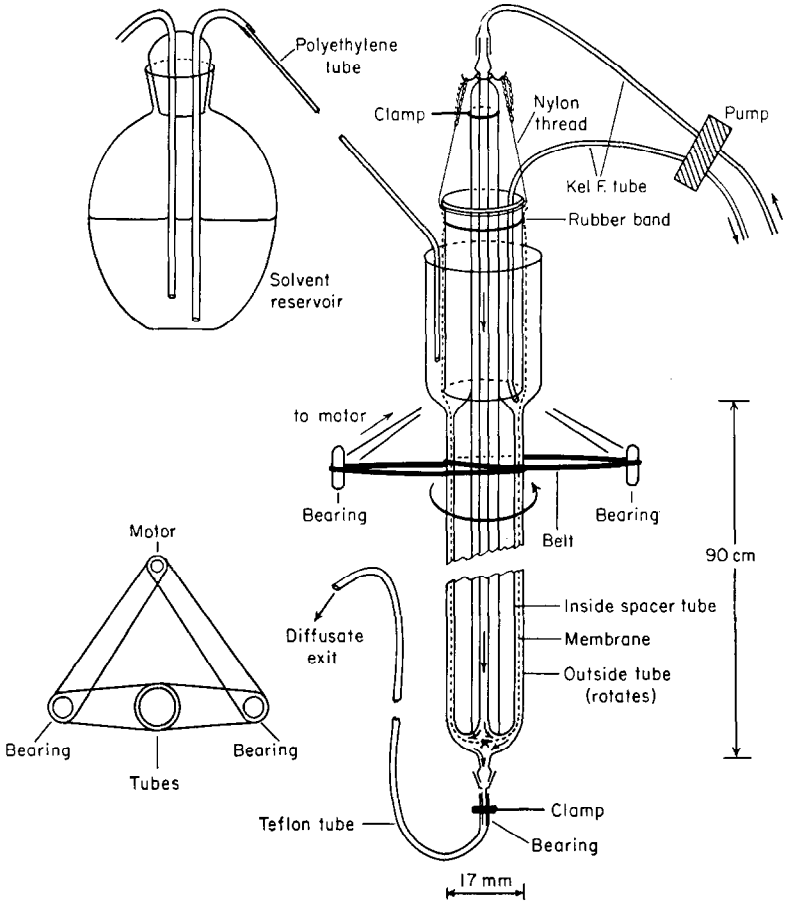

FIG. 1. Schematic drawing of the thin-film countercurrent dialyzer.

10 min. Notwithstanding the shortness of the time it was shown that equilibrium is reached under these conditions by a test in which a ligand concentration of the retentate stream increased by virtue of the countercurrent effect from zero to that of the diffusate inlet. The exit retentate was collected every 2 min by a fraction collector. For the determination of lysozyme concentration, 0.1 ml of the solution was diluted to 1.0 or 5.0 ml. The molar extinction coefficient, $\epsilon_{280}^{1.0} = 3.5 \times 10^5$ was taken for the calculation of the lysozyme concentration throughout these experiments. An aliquot (0.4 ml) was pipetted into a scintillation vial which contained 10 ml of Bray's solution (*21*) and counted for [^{14}C] in a Packard scintillation spectrometer model 3320 or 3380 with automatic external standardization.

Calculation of NAG binding. The average number of moles of NAG bound per mole of lysozyme, ν, was calculated by the following formula:

$$\nu = \frac{C_t - C_0}{C_0} \times \frac{\epsilon}{A} \times (NAG)_0,$$

where C_0 is the radioactivity in cpm of [^{14}C]NAG at the initial molar concentration of NAG, $(NAG)_0$, and C_t is the radioactivity from the retentate after equilibration. The optical absorbancy of lysozyme was determined on the equilibration mixture and after 10 or 50 times dilution with buffer.

Tritium–Hydrogen Exchange

a. Exchange-in study. Lysozyme (30 mg) was dissolved in 3 ml of either 0.01 *M* acetic acid or 0.01 *M* acetic acid plus 1 *M* NaCl. To each, 20 μl of tritiated water (1.0 Ci/ml) was added and mixed vigorously. This constitutes zero time for the experiment. At suitable intervals 0.4-ml samples were passed through the 90-cm countercurrent dialyzer (Fig. 1) against the respective solvent systems, and the emerged retentate solutions were assayed for both lysozyme concentration and radioactivity. The flow rates for the retentate and the diffusate were 0.5 ml/min and 3.0 ml/min, respectively, which yielded the residence time for the protein of 8 min in the dialyzer. It had previously been shown (*10*) that a single pass under these conditions would reduce a tritium count of 10^8 per min to background.

b. Exchange-out study. Lysozyme (60 mg) was first dissolved in 3 ml of weak buffer (10^{-3} *M* Na–cacodylate, pH 7.0) and then incubated with 40 μl of tritiated water (1.0 Ci/ml) at 60 to 62.5°C for 14 hr to insure complete exchange-in. After incubation, this solution was divided into two portions. To one of them, 0.1 *M* acetic acid was added until the solution reached a pH of 3.45. To another portion sufficient 0.1 *M* acetic acid and 4 *M* NaCl were added to make the final concentration of NaCl to be 1.0 *M* and pH 3.45. In order to strip the excess tritium, 2.5 ml of the tritiated lysozyme solution was passed through the 90-cm dialyzer at the flow rates mentioned above. The emerging fractions of the retentate were collected in a fraction collector. Peak tubes were pooled. At intervals 0.4 ml of this pooled sample was taken and passed through the dialyzer against the respective solvent systems and treated in the same way as described in *Exchange-in study.*

RESULTS AND DISCUSSION

Effect of salt on the diffusional size of three proteins. In 0.01 *M* acetic acid (pH 3.3) egg white lysozyme passed through the unmodified membrane at the half escape time of 3.2 hr at 25°. When NaCl was added to increase the ionic strength, the rate of dialysis was found to decrease with the increase of NaCl concentration. When three membranes with porosities adjusted for three globular proteins of different size were used for the experiments, a point of "transition" at near 0.005 ionic strength was observed as shown in Fig. 2. An identical result had been reported previously for ribonuclease in the presence of NaCl and $MgSO_4$ (*3*).

Although the increase of salt concentration would increase slightly the viscosity of the solution and thereby slightly retard the rate of dialysis, it could scarcely account for the observed striking decrease in the rate of dialysis. The possibility that the salt might decrease the porosity of the membrane is ruled out by previous studies (1). It was checked again with [^{14}C]-labelled inulin. In fact our studies with ACTH analogues

and with mono- and dinucleotides and tRNAs from *E. coli* show that an increase of salt concentration often strongly increases the rate of dialysis. These are polyelectrolyte solutes with like charges distributed along the chain. Salt masks the mutual repulsion of the like charges. Another point in regard to the association of protein molecules at high ionic strength should also be made. In the case of lysozyme (mol wt 14 500) the dialysis was performed in a selective membrane which completely excluded chymotrypsinogen A (mol wt 25 000) in solutions of both low and high salt concentrations.

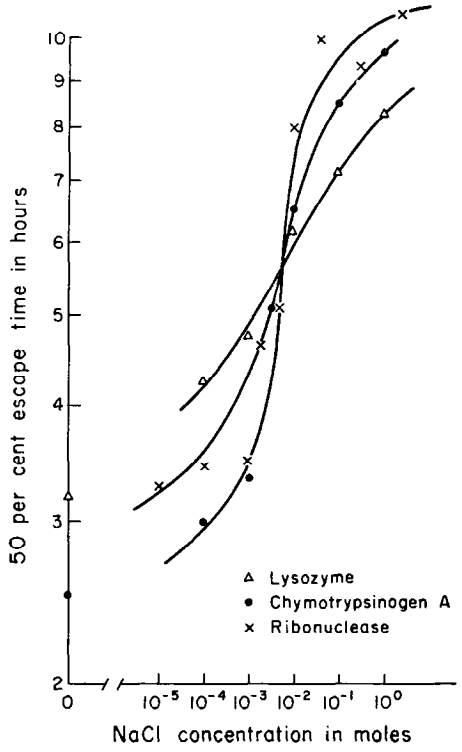

FIG. 2. Effect of NaCl concentration on the dialysis rates of lysozyme, ribonuclease A, and chymotrypsinogen A. The initial amounts of proteins inside the membrane were 5 mg in 0.5 ml for lysozyme, and 10 mg in 0.5 ml for ribonuclease A and chymotrypsinogen A. The results were obtained from three different membranes for three proteins.

Therefore, the passage of aggregates of lysozyme was excluded and if salt promoted aggregation this could contribute to the reduced rate of dialysis. In all cases, the escape curves were straight lines. Moreover sedimentation equilibrium studies reported by Sophianopolous and Van Holde (*22*) have indicated that lysozyme is mostly monomeric at a pH below 4 and 0.1 ionic strength. Thus the slower rate of dialysis in salt suggests a significant conformational change and an increase in the diffusional size of the molecule.

This phenomenon is obviously not due to an electrostatic effect alone. If this were true, one would expect contraction of the molecule instead of expansion since these proteins are predominantly positively charged under the experimental conditions, and since proteins tend to undergo expansion at a pH far from their isoelectric points. In the latter case the expansion can be eliminated by the addition of an electrolyte, a salt

concentration as low as 0.1 *M*, to reduce charge–charge repulsion. It has been proposed by von Hippel and associates (*23*, *24*) that proteins undergo progressive change in conformation with a change in concentration of neutral salts. The change noted by dialysis may be related but detectable at lower salt concentrations because of the sensitivity of the method. In any case in our study the initial change was significant even at 10^{-4} *M* of NaCl at room temperature. When lysozyme in the expanded form in the presence of NaCl was dialyzed against salt free 0.01 *M* acetic acid the rate of dialysis was identical to that when no NaCl was added from the beginning. Diffusates containing lysozyme in the different NaCl concentrations showed the identical specific activity toward the lysis assay under the conditions described by Prasad and Litwack (20). Therefore, it is obvious that the conformational change due to the NaCl is rapidly

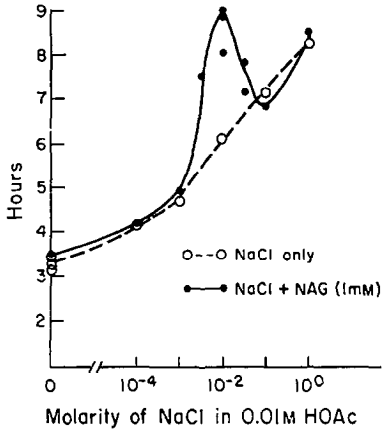

FIG. 3. Effect of NaCl concentration in the presence of 1 m*M* NAG on the dialysis rate of lysozyme. The curve without NAG was taken from Fig. 2. Data for two curves were obtained from the same membrane.

reversible. It should be mentioned, however, that the lysis activity was low under the 0.01 *M* acetic acid solvent conditions at which these dialysis studies were undertaken, approximately 10–12% of the optimum.

ORD studies failed to show the change caused by salt at this low ionic strength. This result can be taken to indicate that these proteins in solution have a degree of conformational flexibility at low pH under the conditions used here which does not perturb the chromophor essential to their measurement by ord. At a higher ionic strength sufficient perturbation for detection by ord has been reported (*23*, *24*).

When 10^{-3} *M* of NAG was added to the dialysis solution, the rates of dialysis were similar to those with no NAG except at near 0.01 ionic strength (Fig. 3). This correlates with the possibility that a particular interaction of NAG with lysozyme may require an optimum ionic strength of 0.01 and with the known observation that salt is required to achieve optimum activity in the lysis of the cell wall (*25*).

Salt effect on the exchangeable protons in lysozyme. In order to provide independent evidence that NaCl at these concentrations does indeed cause a conformational shift in lysozyme and also to investigate the possible involvement of hydrogen bonding in the salt-induced conformational change, hydrogen-exchange studies were carried out at two conditions, with and without 1.0 *M* NaCl (Fig. 4). A definite difference was noted which seems to derive from the numbers of exchangeable protons observed by

this technique. There were 10 fewer hydrogens exchanged-in in the presence of 1.0*M* NaCl within 28 hr. A similar difference in the exchange-out is noted. Thus in the presence of salt there seems to be a decrease in the availability of protons in the protein for exchange with water. This suggests that NaCl strengthens directly (or indirectly) conformations or hydrogen bonds at regions where solvent is easily accessible. The strengthening of hydrophobic interaction by increase in the dielectric constant may play a role. These experiments were performed in 0.01 *M* acetic acid at pH 3.45, a pH range where most proteins show the slowest rate of proton exchange.

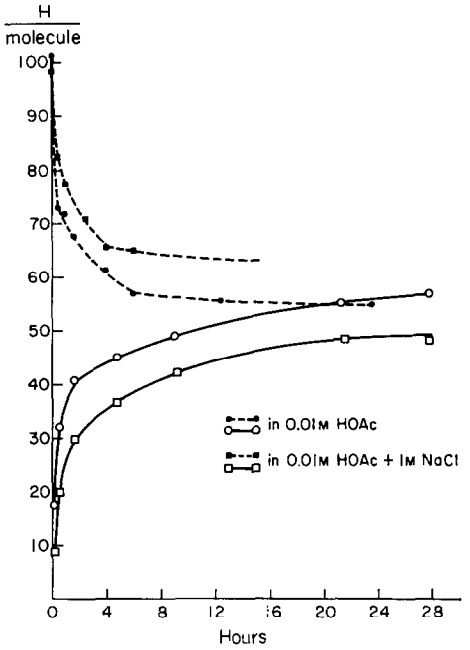

FIG. 4. Tritium–hydrogen exchange-in and -out curves. The ordinate shows numbers of proton exchanged per mole of lysozyme. Since 2 min were allowed for mixing and sampling in addition to 8-min residence time in the dialysis column, the first point was plotted at 10 min. Other points were plotted at the actual time of sample injection plus 8 min of residence time. Experiments were carried out at room temperature (24.5 ± 0.5°C). Flow rates are indicated in Experimental section.

Praissman and Rupley (*26*) reported results which indicated that in solution at pH 4.7, increased salt concentration decreased "zero time" exchange. However, they found that after a 24-hr exchange period, more hydrogens had exchanged in salt solutions than in salt-free media, a result in contrast to our data. However, their conditions were different (0°C and at pH 4.7) and a different experimental method was used. The high precision of the dialysis method has been well documented by two separate studies from this laboratory (*27*, *28*).

The rate of dialysis of lysozyme as a function of pH in the presence of NAG. At a constant ionic strength of 0.06, where the conformation of lysozyme could be affected by the addition of NAG as described above, the variation of pH from 4.5 to 9.0 in phosphate buffer also results in different rates of dialysis as shown in Fig. 5. Without the addition of NAG a trough with a minimum at pH 5.9 is observed. This curve resembles the pH curve for the binding constants of tri-NAG to lysozyme as shown by

Lehrer and Fasman (*14*) from their fluorescence study and by Dahlquist et al. (*12*) from the difference uv spectroscopic study which indicated that the pK_a of the catalytic site is 6.1 which derives from Glu-35 (*29*). When 10^{-3} *M* of NAG is added, the trough in Fig. 5 disappears and a smooth curve results. This indicates that there is a detectable contraction of the lysozyme molecule at the pH optimum for binding which in turn may also suggest that an ionizable group or groups assist in inducing the lysozyme into a conformation favorable for the binding of NAG. The apparent increase in diffusional size above pH 5.9 shown in Fig. 5 which does not occur in the presence of NAG could also arise from dimerization.

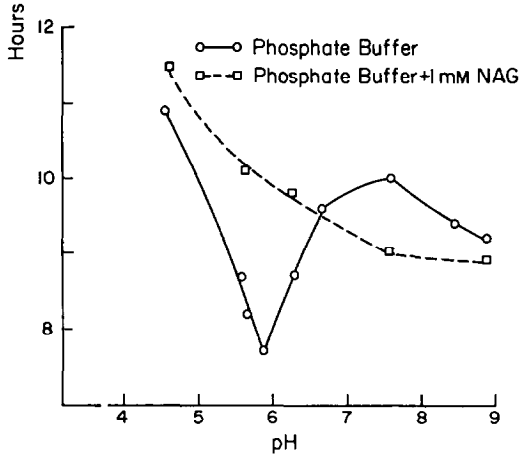

FIG. 5. The dialysis rate of lysozyme as a function of pH at the constant ionic strength of 0.06 with and without 1 m*M* NAG. Sodium-phosphate buffers were used. The initial amount of lysozyme inside the membrane was 5 mg in 0.5 ml of the respective buffers.

Binding studies with the countercurrent dialyzer. The dialyzer shown in Fig. 1 has been used to demonstrate strong binding in the case of 2′-deoxyguanosine-5′-phosphate to the antibiotic actinomycin D (*10*, *30*). A similar procedure was applied to the known weak binding of NAG to lysozyme. In Fig. 6 the clearance of NAG alone is shown to be 99.6%, and the addition of chymotrypsinogen A as an indicator for the nonspecific binding yields an identical clearance. However, the addition of lysozyme to the NAG solution definitely retards the removal of NAG, indicating a detectable degree of binding to lysozyme. Calculation based on the increment of NAG yields a binding constant (K_a) of 5.3 M^{-1}, which is low compared to published data, 20–60 M^{-1}. A reasonable explanation of the result is that since the NAG–lysozyme is a weak complex, NAG is being stripped from lysozyme continuously during the process of dialysis. However, this study demonstrates that the dialysis is rapid enough to reveal a lag in the release of the ligand from the protein even in such weak binding. It is also useful and simple as a preliminary test of interaction.

For gathering precise quantitative data on protein-ligand equilibria in solution equilibrium dialysis is the most useful and revealing technique. The countercurrent dialyzer of the design shown in Fig. 1 is ideal for such a study, since it allows a diffusable ligand to pass rapidly across the membrane and, thus greatly shortens the time of equilibration.

In order to validate the procedure for equilibrium dialysis, the binding of L-tryptophan to bovine plasma albumin was studied as a reference. Figure 7A shows the results when

the binding experiment was carried out at different BPA concentrations as described in the Experimental section for NAG–lysozyme binding studies. It should be noted that ν does not decrease as the BPA concentration increases. In spite of more than a tenfold difference in BPA concentration, ν differs less than 10%. When the binding was studied at different concentrations of tryptophan, a Scatchard plot, as shown in Fig 7B, yields the binding constant of approximately $2.7 \times 10^4\ M^{-1}$ for a single primary site. The secondary binding constant was not determined. These results are in good

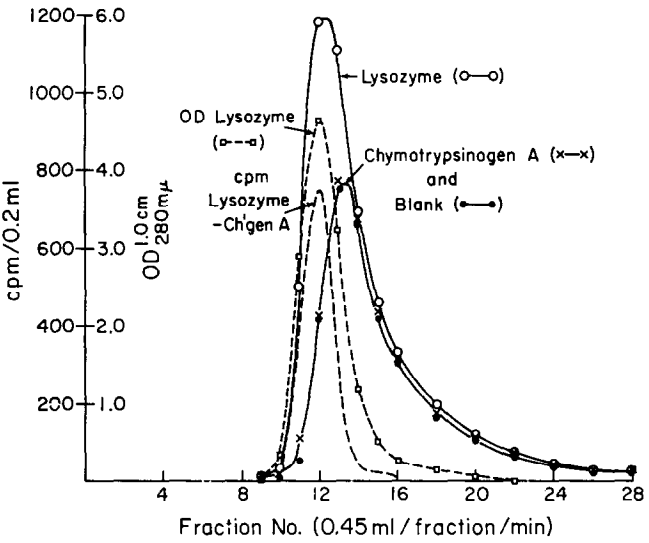

FIG. 6. A pulse experiment with the countercurrent dialyzer to demonstrate the binding of NAG to lysozyme: □—□, optical density of lysozyme in the retentate effluent. NAG (1 μCi in 1.1 ml, 0.1 μCi/μmole) was injected with and without lysozyme (3 mg in 1.1 ml). The NAG (cpm) remaining in the effluent with lysozyme (o—o) was higher than that without lysozyme (blank, ●—●); substituting chymotrypsinogen A (3 mg in 1.1 ml) for lysozyme gave identical cpm (×—×) as blank. The binding of NAG to lysozyme is shown by dashed line (----), which was the cpm with lysozyme minus the cpm with chymotrypsinogen A. In the diffusate stream, 0.05 *M* Na-phosphate pH 5.9 flowed countercurrently at 3.0 ml/min.

agreement with published data where conventional slow equilibrium dialysis was used (31).

In addition to the advantage of rapid equilibration, the countercurrent dialysis technique offers the advantage that the binding can be studied continuously at varying protein concentrations but at a constant ligand concentration. In the previous sections it has been indicated that the presence of NAG in solution affects the apparent change in the diffusional size of lysozyme at 0.06 ionic strength, pH 5.9. We, therefore, have studied the binding under such conditions.

Figure 8 shows plots of the bound NAG per molecule of lysozyme (ν) against the concentration of lysozyme on a logarithm scale. Since the binding is weak, it is necessary to have a relatively high ratio of radioactive to nonradioactive NAG. The increment of radioactivity due to the binding, however, is always less than 5% over the radioactivity of the base line. Therefore, the data shown in Fig. 8 were obtained from an average of three 20-min countings. The variations nonetheless are still large due to errors in the counting and are exaggerated because they are results of difference above high base-line

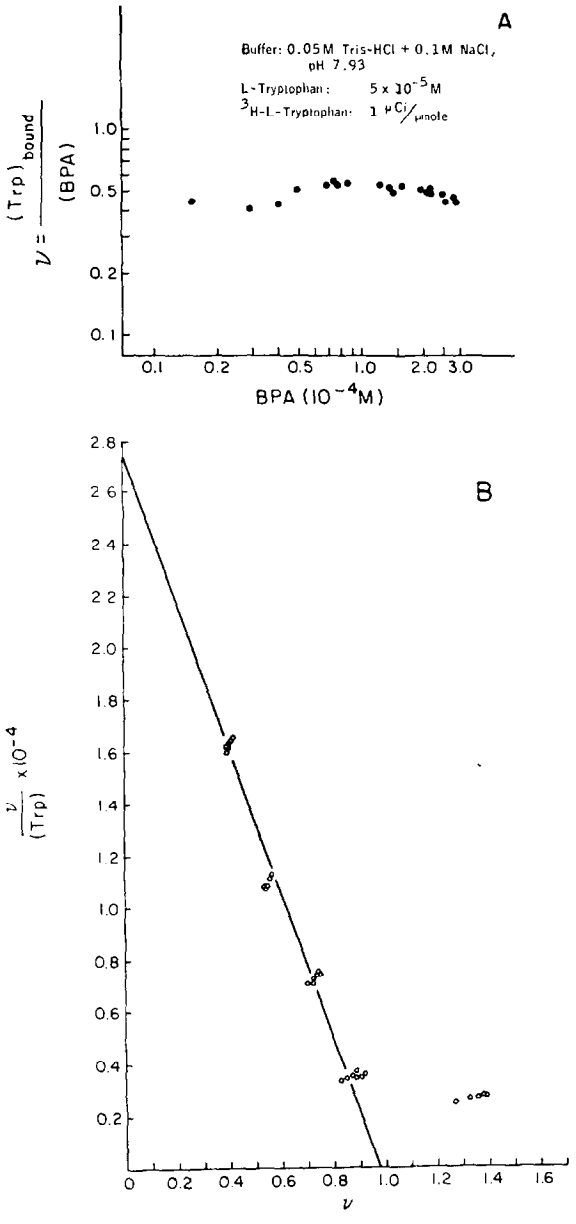

FIG. 7. (A) binding of L-tryptophan to BPA. A constant number of moles of L-tryptophan bound to one mole of BPA at the different BPA concentration. The molar extinction coefficient $\epsilon_{280}^{1.0} = 4.2 \times 10^4$ was taken for BPA. (B) Scatchard plot. The BPA concentration was 0.59×10^{-4} *M*.

counts. Nevertheless, even with the scattering of data a statistically significant straight line can be drawn except when ν is approaching one or higher. In this case the line appears to curve upward. It is reasonable to assume that additional binding sites could come into play due to the high ligand to protein ratio. At present, we do not know if this additional binding is of the specific or nonspecific type. In all cases, the line

clearly shows a slope in which ν increases with a decrease of lysozyme concentration at a given NAG concentration. This phenomenon is not observed in the study of tryptophan–BPA binding (Fig. 7A) and has never been reported in previous studies of NAG–lysozyme binding. The spectroscopic studies of the published results were all conducted at relatively high lysozyme concentrations of 1–2 mM and would reflect primarily the perturbation at a tryptophan site in the cleft region of lysozyme. Although conditions are not identical, the binding constants at the higher lysozyme concentration of the present data based on one binding site per lysozyme molecule agree with the

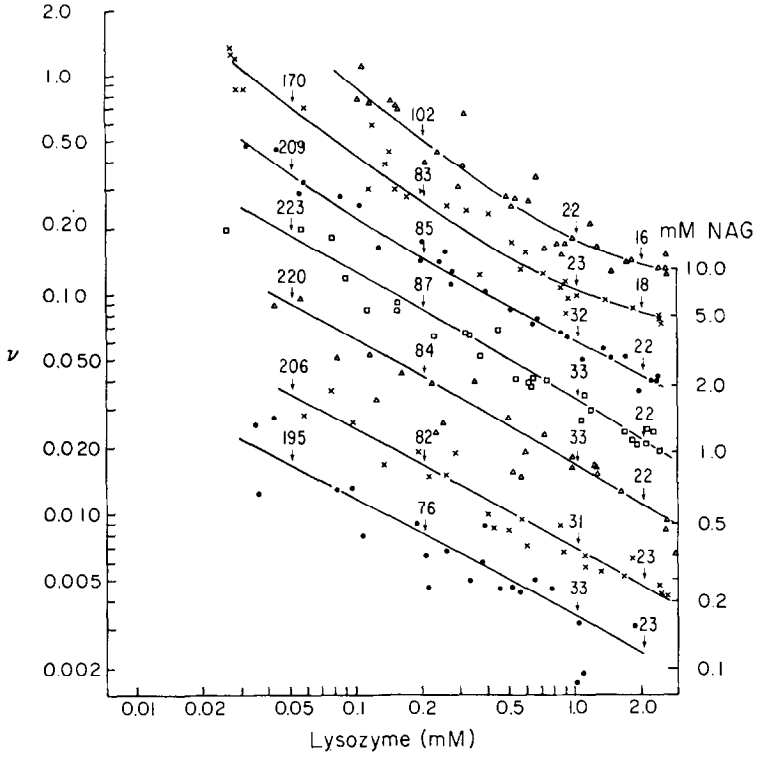

FIG. 8. Nu vs lysozyme concentration at the different NAG concentrations shown on the right. The numbers indicate the binding constant, K_a, at each point shown by an arrow.

published values of $K_a = 15$–$60\ M^{-1}$ where a similar concentration of lysozyme was used.

The possibility that a slightly higher quenching of the counting efficiency in the presence of a high concentration of lysozyme would cause such a slope was investigated. For this purpose, the quenching of samples was examined repeatedly by both the automatic external standardization method and by internal addition of lysozyme to Bray's scintillation solution. Both methods failed to show an increase in quenching as a function of lysozyme concentration. Actually, the white turbid scintillation solution caused by the presence of the higher amount of lysozyme produced the opposite effect; an increase in counting efficiency of the order of a hundredth of one percent. Furthermore, such a consistent slope was not observed at all in the case of tryptophan–BPA binding. When the binding study was carried out at 0.01 M acetic acid, pH 3.4 (Fig. 9),

only a twofold increase in ν was found, instead of the usual eight to tenfold increase over a 20-fold decrease in lysozyme concentration. It seems certain, therefore, that such a slope is not caused by an artifact. Another possible explanation could be the formation of small amounts of di- and tri-NAG through the possible transglucosylation reaction which would appear much more significant at the low enzyme concentration. Since binding constants toward di- and tri-NAG are 10^2 and 10^4 times higher, respectively, a small amount of these oligosaccarides undoubtedly could give a high binding

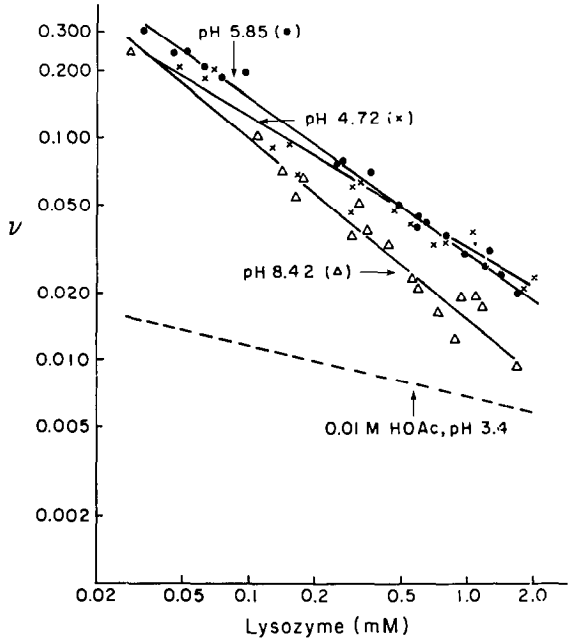

FIG. 9. Nu vs lysozyme concentration at different pH values. (NAG) = 1 mM, pH 4.72, 5.85 and 8.42 were from phosphate buffers, ionic strength = 0.06; pH 3.4 was from 0.01 M acetic acid, no salt was present.

constant if they were included in the mono-NAG binding calculation. Until now we have not been able to detect the presence of these oligosaccarides in the reaction mixture.

Evidence of cooperativity in dimerization of lysozyme and NAG–lysozyme binding. To investigate the characteristics of the binding a Scatchard plot, ν/(NAG) vs ν, was constructed. Straight-line plots were obtained at the higher lysozyme concentration. However, at low lysozyme concentration a complex curve with a maximum was obtained (Fig. 10). This behavior is similar to the binding of GTP to glutamate dehydrogenase in the presence of NADH (*32*) or the binding of succinate to asparate trancarbamylase in the presence of carbamylphosphate (*33*) which may be considered as an example of allosteric interaction according to the two-state model proposed by Monod et al. (*34*). Therefore, the progressive shift in the binding constant with a change in lysozyme concentration, as well as the Scatchard plot showing a maximum, could be attributed to more than one conformational state during the binding process. The failure in the past to detect more than one conformational form could be due to the experimental restriction that the binding studies have been carried out at high lysozyme concentrations in order to be measurable. This may then have restricted

detection of the binding to one site due to the detection method used. Extrapolation of Scatchard plots shows less than one binding site per lysozyme molecule at the high lysozyme concentration, whereas at the low concentration it extrapolates to a value close to one.

These results may also indicate that lysozyme exists partly in an aggregated form at high concentration where each aggregated molecule could have one or less binding site. The aggregation of lysozyme has already been well characterized (22, 35). Their studies indicate that lysozyme undergoes a reversible dimerization at alkaline pH, and that polymers larger than the dimer appear at higher pH as well as higher ionic

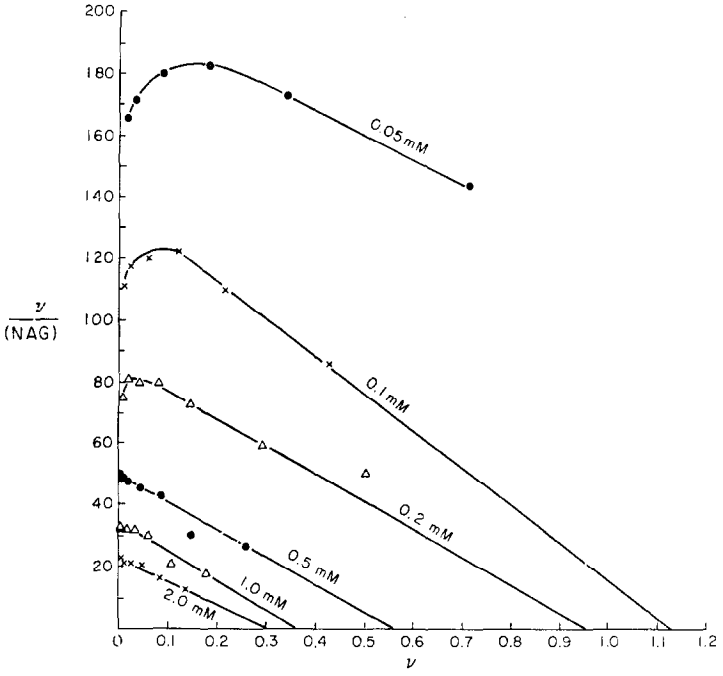

FIG. 10. Scatchard plots of NAG–lysozyme binding at different lysozyme concentrations.

strength, higher protein concentration, and lower temperature. From calculations based on their data, it can be derived that 1 m*M* lysozyme at 0.15 ionic strength contains 30% of dimer at pH 6.0 and 50 to 70% at pH 8.0. In a recent report of Sophianopoulos (*19*), it has been further proposed that the active site of lysozyme is involved in dimerization, possibly in the way of "head-to-tail," suggesting that only one binding site for each dimer would be allowed. If this is true, one should expect 0.85 site per monomeric unit under the assumption that 30% of lysozyme is in the dimeric form. Our results at similar pH and lysozyme concentration cannot be extrapolated close to this number. This apparent difference can be accounted for in part by the fact that the difference in ionic strength of 0.06 and 0.15 changes the conformational form of lysozyme as shown in the study of the dialysis rate as a function of ionic strength (Fig. 3). It seems more likely that a possible "head-to-tail" dimer could have an unfavorable binding capacity in comparison to the monomeric form at the metastable condition of our present study. The poorer binding capacity of the dimer is further illustrated in Fig. 9. At pH 8.4 and where the lysozyme concentration is high the dimer would be predominant. Here

a low ν is found as one would expect. It is interesting to note that at pH 8.4 the slope caused by the change in lysozyme concentration is steeper than that at pH 5.85 and the ν value becomes lower at high lysozyme concentration. At pH 4.72, where lysozyme should be predominantly in the monomer form but is unfavorable for NAG binding, ν is only slightly higher at high lysozyme concentration but lower at low lysozyme concentration. The most distinct decreases in ν value and slope are observed in 0.01 *M* acetic acid in the absence of salt where both dimerization and binding are thought to be highly unfavorable.

The finding that binding becomes more efficient at a higher ratio of NAG to lysozyme in the solution is best explained by the point of view in which the cooperative effect of dimer dissociation of lysozyme to monomer is a function of both lysozyme and NAG concentrations. The protein exists in two states, monomer and dimer. At a low concentration of lysozyme the monomer-dimer equilibrium shifts in favor of the monomer which is the form of higher binding capacity. This shift is influenced by formation of the NAG–lysozyme complex to give a cooperative process. The evidence that the dimerization of lysozyme is reduced when it binds NAG and its oligomer (*19*) support this view.

The literature correlating the details of the structure of lysozyme to its enzymatic activity is now quite large and not all in quantitative agreement (*25*). This confusion may result from the fact that slight changes in the conformation of enzyme and substrate can be very sensitive to small changes in the solvent environment. The experience reported here strengthens this viewpoint particularly in respect to the enzyme at very low concentrations.

As more and more of the details of the interactions between enzymes and substrates become known, evidence for conformational shifts has appeared. It seems important to estimate the overall effect of these shifts on the diffusional volume, e.g., Stokes radius. By careful comparative studies of rigid model solutes it has been shown that the thin film dialysis method is capable of detecting changes in Stokes radius in the order of 2–3% (*1*). From this conclusion it can be estimated that the changes in Stokes radii implied in Figs. 2, 3, and 5 are small, perhaps on the order of 5–10%.

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