The Inhibitory Interaction of Cationic Detergents with the Active Center of Lysozyme. II. The pH Dependence of the Interaction*

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ABSTRACT: Some cationic detergents were previously shown to interact with lysozyme resulting in a competitive inhibition. The detergent was shown to bind at the active center of the enzyme, probably by a combination of hydrophobic bonding and electrostatic bonding. Consequently, it was concluded that the active center of lysozyme consisted partly of a hydrophobic region. In the present experiments, we have attempted to characterize in more detail the nature of the interaction between the enzyme and detergents, and to use the results to determine features of the active center of the enzyme molecule. The pH dependence of interaction was just the reverse of that of the activity, *i.e.*, at pH 4.5, where the activity reached its maximum value, the interaction disappeared and no inhibition was observed, whereas at higher and lower pH values when the activity was lower, the interaction was greater. However, when fully active acetyl lysozyme in which five out of six amino groups were modified by acetic anhydride, was used, the interaction did not show significant pH dependence; even at pH 4.5, the detergent associated quite strongly with the protein, and caused significant inhibition.

These facts apparently mean: (1) that certain carboxyl groups are involved in both the interaction and the activity; (2) that one or more of these carboxyl groups must be in the ionized form for the enzyme to be active; (3) that these latter groups, when in the charged form, may help bind the cationic detergent electrostatically to the protein; (4) that these latter groups, when titrated, allow the enzyme to become active and also can no longer contribute to detergent binding so that this binding is lowered; and (5) that the acetylation of ε-amino groups makes the binding of detergent easier, so easy that it is unaffected by pH.

It was reported in the previous paper that some sort of interaction, probably due to hydrophobic bonding, was found between lysozyme and a cationic detergent, dimethylbenzylmyristylammonium chloride (DBMA),[1] by difference spectrophotometry (Hayashi *et al.*, 1968). From the observation that the substrate, glycol chitin, interfered with the interaction and that the interaction was reduced by the selective oxidation of the tryptophyl residue at position 62 by *N*-bromosuccinimide (NBS), it was concluded that some tryptophyl residues (probably three residues) included in the active center of lysozyme were involved in the interaction. This interaction resulted in the reversible, competitive, inhibition of the enzymatic activity of lysozyme. Thus, the detergent seems to be a suitable tool with which to study the characteristics of the active center of lysozyme and one which may lead to an increased understanding of the mechanism of the enzymatic action. The present paper deals with the important question of the pH dependence of the interaction, a question whose answer does indeed throw some light on the mechanism of action of the enzyme.

Materials and Methods

Preparation of Acetyl Lysozyme. The acetylation of lysozyme was carried out according to the method of Fraenkel-Conrat (1950) with minor modifications. Acetic anhydride (7.2 g) was added dropwise, with vigorous stirring, to a mixture of 40 ml of 5% aqueous lysozyme solution and 40 ml of saturated sodium acetate solution. This addition was carried out over a period of 2.5 hr while the reaction vessel was kept at 0°. The reaction mixture was dialyzed against running water for 1 day and against deionized water for 2 days using $^{18}/_{32}$ Visking tubing. The solution was centrifuged and the supernatant was lyophilized. The yield of dried acetyl lysozyme was 1.7 g.

The number of free amino groups in acetyl lysozyme was determined spectrophotometrically using a specific reagent for the amino group, trinitrobenzenesulfonate, according to the method of Okuyama and Satake (1960). It was found that 1.7 amino groups were trinitrophenylated while 6.7 were trinitrophenylated in native lysozyme. The acetyl lysozyme prepared as described here was fully active toward glycol chitin.

Microanalysis of Cationic Detergent. The concentration of the cationic detergent was determined by a

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[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: DBMA, dimethylbenzylmyristylammonium chloride; NBS, *N*-bromosuccinimide.

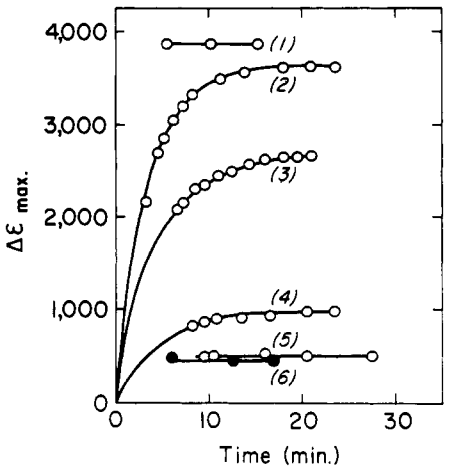

FIGURE 1: The time dependence of interaction of DBMA with lysozyme measured at various pH values. The concentrations of lysozyme and DBMA were both 0.1%. Difference spectra were measured at 35° in 0.2 M phosphate–0.1 M citrate buffer system (McIlvaine buffer). (1) pH 7.0, (2) pH 6.0, (3) pH 5.6, (4) pH 5.0, (5) pH 4.0, and (6) pH 3.0. The wavelengths of maximum difference were 297 mμ for 1–3, 295 mμ for 4, and 292 mμ for 5 and 6.

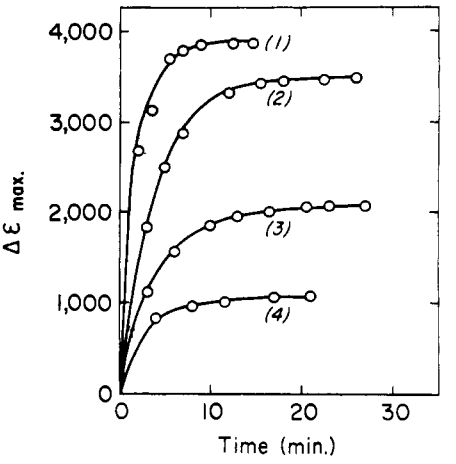

FIGURE 2: The time dependence of interaction of DBMA with lysozyme measured at various pH values. Conditions for measurements were same as that in Figure 1 except that 0.1 M Sørensen buffers were used. (1) pH 1.5, (2) pH 2.0, (3) pH 2.5, and (4) pH 3.0. The wavelengths of maximum difference were 297 mμ for 1 and 2, 296 mμ for 3, and 295 mμ for 4.

technique which combined the methods of Auerbach (1943) and Few and Ottewill (1956). Detergent solution (1 ml), 10% sodium carbonate solution (1 ml), 0.12% bromophenol blue solution (1 ml), and chloroform (5 ml) were pipetted into the glass-stoppered test tube. The tube was then shaken for more than 1 min, allowed to stand in the dark until the chloroform layer became clear, and the optical density of the chloroform layer at 500 mμ was measured. The concentration of detergent was calculated from a previously prepared standard curve.

Equilibrium Dialysis. The method reported by Klotz *et al.* (1946) was applied to the dialysis equilibrium with minor modifications. The tubing was filled with 10 ml of 0.1% lysozyme solution containing a known concentration of the detergent. A sealed, glass tube with diameter slightly less than that of the tubing was inserted in the solution. The dialysis bag was immersed in 20 ml of a detergent solution of the same concentration as the inside solution and it was allowed to dialyze at approximately 10° for 7–15 days. The Visking tube was then removed and the amount of detergent in the external solution was analyzed. For each detergent concentration, a control experiment was also performed by dialyzing a detergent solution alone against the same solution to estimate the amount of the detergent bound to the dialysis tubing. The amount of detergent bound to the lysozyme was determined by subtracting the detergent concentration in the external solution of the sample tube from that of the control tube.

Critical Micelle Concentration. The method developed by Corrin and Harkins (1947) was used to determine the critical micelle concentration of DBMA. Sodium 2,6-dichlorophenolindophenol solution (2 ml of 1×10^{-4} M) containing 6×10^{-4} M hydrochloric acid was mixed with 2 ml of varied concentration of the detergent. The change in the absorbancy of dye at 660 mμ was measured as the detergent concentration was increased. The optical density at 660 mμ was plotted as a function of the detergent concentration. The critical micelle concentration was represented as a concentration corresponding to the inflection point on the curve.

The methods for the measurement of difference spectra, lysozyme activity, and optical rotatory dispersion have been described in previous papers (Hayashi *et al.*, 1963–1965, 1968). Unless otherwise stated, the assays were performed in 0.1 M phosphate buffer at pH 5.6 and 0.1 M acetate buffer at pH 4.5.

Results

The pH Dependence of the Interaction of Lysozyme and Acetyltryptophan with DBMA. The pH dependence of the interaction of lysozyme with DBMA was examined by following the time-dependent change of the intensity of the main peak of the difference spectrum ($\Delta\epsilon_{max}$) in different buffers at various pH values. In Figures 1 and 2 are shown the time-dependent changes of $\Delta\epsilon_{max}$ at various pH values in the McIlvaine and Sørenson buffers. Similar data at pH 5.6 were presented in the preceding paper (Hayashi *et al.*, 1968) and other similar data have been collected in other buffers. Acetyltryptophan was also studied in the same way.

In Figure 3 are plotted the equilibrium values of $\Delta\epsilon_{max}$ as a function of pH. It is seen that there is a very marked pH of minimum interaction, from about 3 to 5. The interaction of acetyltryptophan, in contrast to that of lysozyme, is nearly independent of the pH from 1.5 to 7.0, as can also be seen in Figure 3. The equilibrium values of the optical density differences reported here for lysozyme and acetyltryptophan were, within experimental error, independent of the

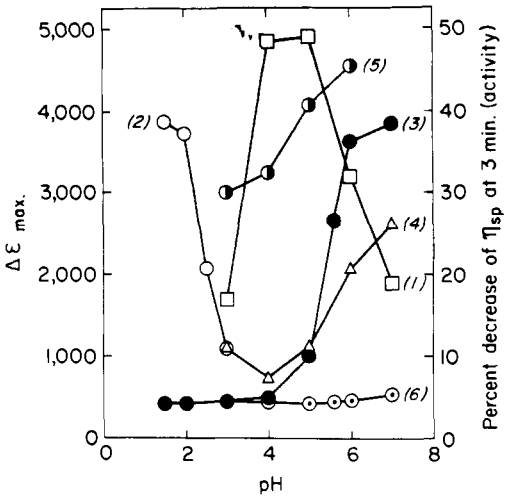

FIGURE 3: The pH dependence of the activity and interaction. (□) Activity; (○) interaction of lysozyme in Sørensen buffer; (●) interaction of lysozyme in McIlvaine buffer; (△) interaction of lysozyme in buffer with constant ionic strength (M = 0.1); (◑) interaction of acetyl lysozyme; (⊙) interaction of acetyltryptophan (3–5 × 10^{-4} M).

temperature between 25 and 40°. Also shown in Figure 3, for comparison, is the pH dependence of the enzymatic activity (in the absence of DBMA), which clearly exhibits a maximum around pH 4 or 5.

The Influence of DBMA on Lysozyme Activity at pH 4.5. The influence of DBMA on the activity of lysozyme at pH 4.5 was investigated as it was previously investigated at pH 5.6 (Hayashi *et al.*, 1968) by mixing an enzyme solution containing DBMA with a substrate solution containing the same concentration of DBMA. (The only significant difference between the two procedures is that because of the relatively slow combination of the detergent with the enzyme at pH 5.6 it was necessary to preincubate the enzyme and detergent for 30 min at that pH. This is not required at pH 4.5 because the combination is complete by the time 10 min has passed; see Figures 1 and 2.) The relative activity at pH 4.5 is plotted in Figure 4, as a function of the DBMA concentration along with the earlier data for pH 5.6. The figure shows that DBMA, which is a powerful inhibitor at pH 5.6, has only a very small inhibitory effect at pH 4.5.

Equilibrium Dialysis. Equilibrium dialysis experiments were carried out at pH 4.5 and 5.6. The moles of DBMA bound per mole of lysozyme are plotted as a function of the concentration of DBMA in Figure 5. The figure shows that only small amounts of DBMA were bound to lysozyme at pH 4.5 while six molecules of DBMA were bound at pH 5.6.

Interaction of Acetyl Lysozyme with DBMA. The interaction of acetyl lysozyme with DBMA was studied as a function of pH by difference spectrophotometry and activity measurements. The difference spectrum was of the same shape as that of native lysozyme. However, no time dependence in $\Delta\epsilon_{max}$ after mixing was observed. The equilibrium value of $\Delta\epsilon_{max}$ at pH 5.6 is plotted as a function of DBMA in Figure 6. A similar result was obtained at pH 4.5 where the equilibrium value was found to be only slightly lower than that at pH 5.6. (As shown above, no significant interaction of native lysozyme and DBMA was observed at pH 4.5.) The difference in the effect of pH on the interaction of acetyl lysozyme with DBMA was also evidenced by the fact that DBMA exerts a strong inhibitory effect on acetyl lysozyme at both pH values as shown in Figure 7.

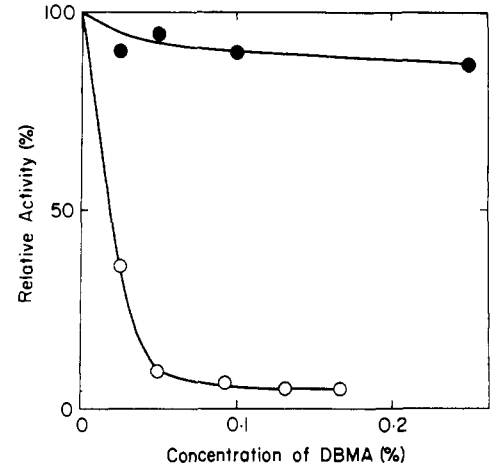

FIGURE 4: Inhibition of activity of lysozyme by DBMA. (○) pH 5.6 and (●) pH 4.5.

The measurement of the optical rotatory dispersion of acetyl lysozyme in the presence of the detergent was carried out to calculate the parameters a_0 and b_0. The values of the parameters are listed in Table I, where the concentration of acetyl lysozyme was kept constant (0.925%) and that of the detergent was changed. The table shows that the parameters did not change on increasing the detergent concentration. This implies that the secondary and tertiary structures of acetyl lysozyme were not destroyed by the interaction with the detergent. Similar data have already been presented for the unmodified enzyme (Hayashi *et al.*, 1968).

Length of Hydrocarbon Chain of Detergents. The interactions of lysozyme, acetyl lysozyme, and acetyltryptophan with several detergents in which the length of hydrocarbon chain was varied were studied by dif-

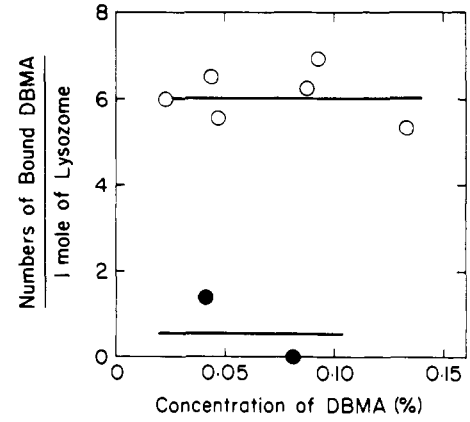

FIGURE 5: The number of molecules of DBMA bound to lysozyme. (○) 0.1 M phosphate (pH 5.6) and (●) 0.1 M acetate (pH 4.5).

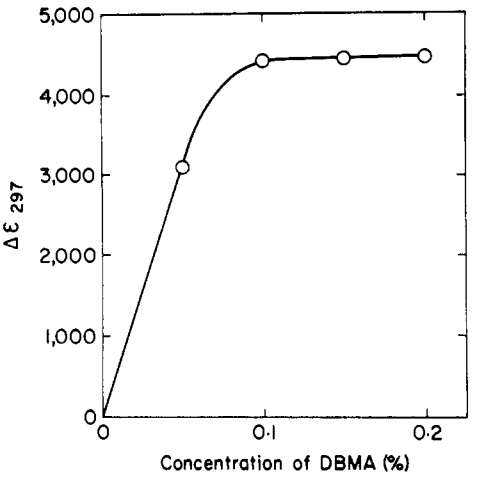

FIGURE 6: Interaction between acetyl lysozyme and DBMA. Difference spectra were measured at 30° in 0.1 M phosphate buffer at pH 5.6. Concentration of acetyl lysozyme was 0.1%.

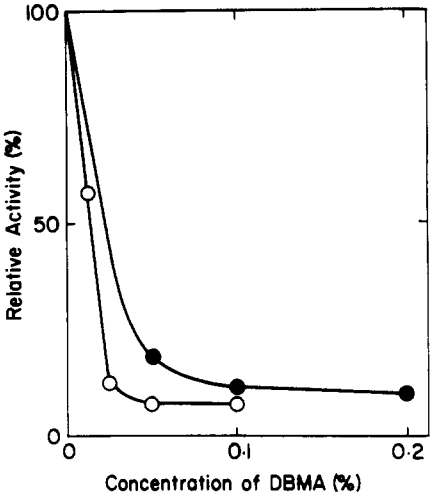

FIGURE 7: Inhibition of acetyl lysozyme by DBMA. (○) pH 5.6 and (●) pH 4.5.

ference spectrophotometry. The results are summarized in Table II. Acetyl lysozyme and acetyltryptophan interact with all detergents used, whereas native lysozyme interacts with only those detergents which contain a hydrocarbon chain of 12 or 14 carbon atoms.

These results suggest that the detergent interacts specifically with the active locus of native lysozyme. When lysozyme was acetylated thereby losing some of its ammonium groups, the limitation on the length of the hydrocarbon chain of the detergent was removed.

Critical Micelle Concentration. As shown in Figure 8, the critical micelle concentration of DBMA in 0.1 M phosphate buffer at pH 5.6 was found to be 0.0125% (3.05×10^{-4} M).

Discussion

It has been shown here that the interaction of DBMA with lysozyme is strongly pH dependent, as followed by difference spectrophotometry. In the pH region between 3 and 5 very little interaction could be detected spectrophotometrically, while on both sides of this pH range the interaction rose steeply as the pH was changed (see Figure 3). The fact that the interaction is strongly dependent on the pH was also shown by a study of the effect of DBMA on the relative activity of lysozyme at pH 5.6 and 4.5. At the former pH the detergent is a good inhibitor of lysozyme (Hayashi *et al.*, 1968) whereas at pH 4.5 it has only a very small inhibitory effect (see Figure 4). Equilibrium dialysis experiments, which measure the binding directly, also lead to the same conclusion (Figure 5). In contrast to the fact that the enzyme interacts minimally with the detergent in the intermediate pH range from 3 to 5 is the fact that the optimal pH for the enzyme's activity (in the absence of DBMA), is around 4 or 5 (Figure 3).

It has also been shown here that neither *N*-acetyltryptophan, a model compound, nor acetyl lysozyme, a fully active derivative of lysozyme with five of its six amino groups acetylated, show any marked pH dependence in the way they interact with DBMA. Acetyltryptophan was studied from pH 1.5 to 7.0

TABLE I: Parameters of Optical Rotatory Dispersion of Acetyl Lysozyme.[a]

Acetyl Lysozyme (%)	DBMA (%)	DBMA/Acetyl Lysozyme (mole/mole)	$-a_0$	$-b_0$
0.925	0	0	286	145
0.925	0.5	19	282	165
0.925	1.0	38	278	160
0.925	2.0	76	286	158

[a] The optical rotatory dispersion was measured after allowing the enzyme-DBMA mixture to stand for 3 hr or more at pH 5.6.

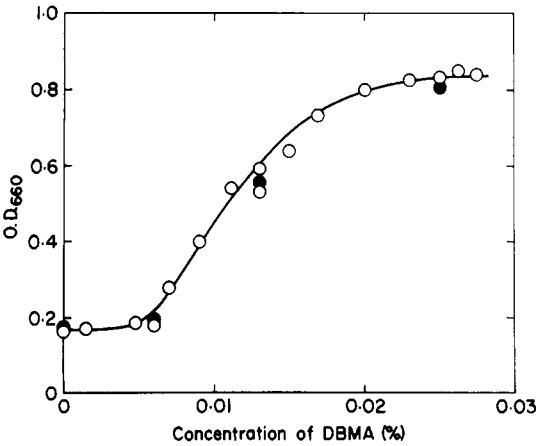

FIGURE 8: Critical micelle concentration. (○) DBMA solution alone; (●) DBMA solution containing equal concentration of lysozyme.

TABLE II: Interaction of Detergents.

Cationic Detergents	Carbon Atoms in Longest Chain	AcTrp[a]	AcLyso	Lyso
Dimethylbenzylstearylammonium chloride	18	+	+	−
Dimethyl-2-hydroxyethylhexadecylammonium chloride	16	+	+	−
Dimethylbenzylmyristylammonium chloride	14	+	+	+
Dimethylbenzyldodecylammonium chloride	12	+	+	+
Trimethyldodecylammonium chloride	12			+

[a] Abbreviations are: AcTrp, acetyltryptophan; AcLyso, acetyl lysozyme; Lyso, lysozyme. The plus signs mean that difference spectra were observed, and the minus signs mean that they were not.

and the interaction, measured as $\Delta\epsilon_{max}$, was constant (Figure 3), showing that the interaction is independent of whether the carboxyl group in the model compound is ionized or not. Acetyl lysozyme was studied between pH 3 and 6, and it was shown that this derivative interacts strongly with DBMA at all pH values studied. The spectral shift observed for acetyl lysozyme at all pH values between 3 and 6 was quantitatively of the same order as observed for native lysozyme at pH values below 2 and above 6. This indicates that the detergent probably interacts with the identical chromophoric groups in the native enzyme and in the derivative. There is, however, a slight pH dependence of the interaction between acetyl lysozyme and DBMA (Figure 3).

The fact that acetyl lysozyme interacts with DBMA at pH values where lysozyme does not is also attested by a study of the effect of DBMA on the relative activity of the acetylated enzyme. As Figure 7 shows inhibition is observed at both pH values, in contrast to the behavior of the unmodified enzyme (Figure 4).

The difference in behavior between lysozyme and the acetylated lysozyme cannot reasonably be attributed to a conformation change which might be supposed to occur in one but not the other. In the first place, we know that both proteins are in the native conformation at pH 5.6, a pH at which they interact about equally with DBMA. Secondly, neither lysozyme (Hayashi *et al.*, 1968) nor acetyl lysozyme (Table I) undergoes a conformation change detectable by optical rotatory dispersion even at high concentrations of the detergent at pH 5.6. (Although the fact that the extinction change is time dependent (Figures 1 and 2) seems to show that a small conformational rearrangement occurs when the detergent is bound.) As the pH is lowered in a lysozyme solution the enzyme reaches an optimal activity at pH 4–5, so that lowering the pH has no conformational effect on the protein, and this is true whether DBMA is present or not. As the pH is lowered in the acetyl lysozyme solution binding of DBMA is not significantly changed as observed both spectrophotometrically, and by the measurement of inhibition, so it would appear that pH has no conformational effect on the derivative either. To put it in other words, we would expect that if a conformation change occurred in one of these proteins as the pH was lowered it would show up by a change in *both* the binding of detergent and the loss of enzymatic activity in the presence of DBMA. However the native enzyme changes its binding properties but remains fully active. The derivative, on the other hand, is inactive in the presence of DBMA, but the binding of the detergent is largely unchanged. It is quite possible, we believe, that a conformation change is responsible for the increase in binding observed with lysozyme at pH values lower than 3. Here the activity is decreased while the binding is much increased. This possibility will be discussed further below.

The facts reported here, in conjunction with conclusions reached in the accompanying paper (Hayashi *et al.*, 1968), show that DBMA interacts with the active center of the lysozyme molecule at pH 5.6 but not at pH 4.5. The interaction therefore is very sensitive to the pH, that is, to the effect of pH on the protein. Since the protein is known to be conformationally independent of the pH (Hamaguchi and Sakai, 1965) in this range, one is led to believe that titratable groups in or near the active center can exert a control over the interaction depending on whether they are ionized or not. Since these groups are titrated in the pH range between 2 and 6 (Figure 3) they are undoubtedly carboxyl groups. The experiments with acetyl lysozyme show that the acetylation of five ϵ-amino groups (out of six present in the native molecule) effectively destroys this control.

The pH dependences of the catalytic activity and of the DBMA interaction are inversely related as shown in Figure 3. It would therefore seem that the same carboxyl groups that influence the activity of the enzyme also influence the binding of detergent. Blake *et al.* (1967) have suggested that the carboxylic groups in the side chains of Glu_{35} and Asp_{52} are of great importance in the mechanism of the enzyme, and further that Asp_{52} should be ionized and Glu_{35} unionized at the optimum pH. Our evidence, while it cannot of course identify the individual residues involved, is consistent with the crystallographic results of Blake *et al.*

If we assume that the suggestions concerning residues

35 and 52 made by Blake *et al.* (1967) are correct, it is possible to reach some tentative conclusions concerning the binding of DBMA to lysozyme and acetyl lysozyme. In lysozyme, first, it is found that DBMA binds in the active center of the enzyme at pH 5.6, interacting hydrophobically with three typtophyl residues (Hayashi *et al.*, 1968) which are likely Trp_{62}, Trp_{63}, and Trp_{108}. As Glu_{35} is titrated, binding is reduced, perhaps because there is an electrostatic interaction between Glu_{35} and the cationic charge of the detergent. As the pH is further decreased, Asp_{52} is titrated too, the activity falls off, and binding of detergent increases again. Asp_{52} is involved in a complicated network of hydrogen bonds (Blake *et al.*, 1967) and titrating it will undoubtedly have a severe effect on this network. It is not unlikely that a small local conformation change occurs which facilitates detergent binding, even though no independent evidence for such a conformation change has been presented hitherto. Second, several possibilities might explain the increased detergent binding observed with the acetylated lysozyme. The acetyl groups are inserted on the ϵ-amino groups of the lysyl residues, but it appears that none of these are near the active center, with the possible exception of Lys_{97}. It would therefore seem that the increased binding is most easily attributed to the lowered positive charge, the slightly increased hydrophobicity, or a combination of both. It is also possible that acetylation breaks a hydrogen bond between an ϵ-amino group and a side-chain carboxyl group, and the liberated carboxyl then facilitates detergent binding electrostatically.

This description of the effect of pH on the binding of DBMA to lysozyme must, of course, be regarded as a speculative one. Other interpretations could be reached, especially because other carboxyl groups (Asp_{101} and Asp_{103}) have been implicated in the active center by Blake *et al.* (1967). Nevertheless the behavior of Glu_{35} and Asp_{52}, hypothesized by Blake *et al.*, on other grounds does seem to provide a convenient framework for our own results.

Finally we can say something about the form in which the detergent interacts with the lysozyme molecule. The critical micelle concentration of DBMA is 0.0125%, and it is independent of whether lysozyme is present or not (Figure 8). At this concentration DBMA reduces the activity of lysozyme (at pH 5.6, Figure 4) and acetyl lysozyme (at pH 4.5 and 5.6, Figure 7) by about 50% whereas considerably less than 50% of the spectral shift has occurred at this concentration (Hayashi *et al.* (1968) for lysozyme; Figure 6 for acetyl lysozyme). It would thus seem that individual detergent molecules may effect most of the inhibitory action by binding in the active center, but that the binding of a micelle is required to give the maximum spectral shift.

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