The Inhibitory Interaction of Cationic Detergents with the Active Center of Lysozyme. I. Site of Interaction*

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ABSTRACT: When the stability of the lysozyme–substrate complex was studied by difference spectrophotometry under various circumstances, it was found that some cationic detergents inhibit the formation of the lysozyme–substrate complex, and therefore the activity of lysozyme. When a detergent solution was added to the lysozyme solution a difference spectrum, with its main peak at 297 mμ, originating in the tryptophyl residues of the enzyme was observed. The addition of substrate prevented the interaction of lysozyme with the detergent. The presence of excess substrate resulted in the complete disappearance of the difference spectrum caused by the interaction of the detergent. The magnitude of the difference spectrum was only two-thirds as great when a modified lysozyme, in which the tryptophyl residue at position 62 was selectively oxidized by *N*-bromosuccinimide, was used. This suggests that the difference spectrum of the lysozyme–detergent system is attributable to three of the six tryptophyl residues of the enzyme. Phillips and his collaborators have studied the binding site of the lysozyme molecule using complexes formed by substrate analogs. This site is located in a cleft in the molecule, and involves three tryptophyl residues (62, 63, and 108). From these facts, it is concluded that the detergent interacts with the active center.

The way in which substrates are bound to lysozyme has been analyzed recently by Blake *et al.* (1967a,b) by X-ray diffraction studies of the crystal of various complexes formed between the enzyme and substrate analogs. Several hydrogen bonds between the enzyme molecule and the analogs have been shown to exist and several others postulated for the natural substrate. It is not likely of course that the only binding forces are hydrogen bonds (parts of the active center cleft of the molecule are nonpolar and likely take part in hydrophobic interaction with the substrate).

One way of investigating the importance of hydrophobic contributions to binding is to study the interaction of hydrocarbons or molecules containing large hydrocarbon moieties with the enzyme (Miles *et al.*, 1963; Hymes *et al.*, 1965). In the present study we have investigated the effect of several cationic detergents on lysozyme, and have found that some, but not all of them, interact with the active center of the molecule. Cationic detergents have been preferred over anionic ones in this study because some acids or polyanions inhibit the activity of lysozyme by neutralizing the basicity of lysozyme, *i.e.*, by electrostatic interaction, in which we are not at present interested (Nihoul *et al.*, 1952; Skarnes and Watson, 1955).

Lysozyme has three tryptophyl residues in the active center (Blake *et al.*, 1967a,b). We have found that detergent binding, like substrate binding (Hayashi *et al.*, 1963), can be detected by its effect on the environment of some or all of these residues. The binding of detergent is thus conveniently followed by difference spectrophotometry, which is the method we have used throughout the present study. Glazer and Simmons (1965, 1966) have previously studied the effect of the anionic detergent sodium dodecyl sulfate on the optical rotatory dispersion and circular dichroism of lysozyme. This detergent eliminates the circular dichroism band due to the aromatic residues.

Materials and Methods

Reagents. Lysozyme and the substrate (glycol chitin) were prepared by the method described previously (Hayashi *et al.*, 1963). Acetyltryptophan was synthesized according to the method described by du Vigneaud and Sealock (1932). This compound did not give a positive ninhydrin test and it showed only a single spot in paper chromatography. DBMA[1] and other detergents supplied by the Kao-Soap Co., Ltd., were used without further purification. All reagents used were of analytical grade.

Preparation of NBS-Oxidized Lysozyme. The oxidation of lysozyme with NBS was carried out according to the method described in a previous paper (Hayashi *et al.*, 1963). Samples oxidized to various extents were prepared. All samples were dialyzed against deionized water for several days, and lyophilized.

Assay of Activity by Viscosimetry. The viscometric assay of the enzymatic activity of lysozyme was carried out as described in a previous paper (Hayashi *et al.*,

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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.

TABLE I: Systems for Measurement of Difference Spectra.

System	Sample Compartment 1	Sample Compartment 2	Ref Compartment 1	Ref Compartment 2	Expression[a]
1	E + A + B	B	E + B	A + B	[E + A]/[E][A]
2	(E + S) + A + B	B	E + S + B	A + B	[(E + S) + A]/[E + S][A]
3	(E + A) + S + B	B	E + A + B	S + B	[(E + A) + S]/[E + A][S]
4	E + S	B	E + B	S + B	[E + S]/[E][S]

[a] Parentheses indicate that two components were mixed previously in the cell; brackets indicate the spectrally significant components in a cell. The term to the left of the oblique stroke identifies components in the experimental cell; to the right, the reference cell. Abbreviations are: A, additive such as detergents; E, lysozyme or aromatic amino acids; S, substrate; B, buffer solution.

1963). In this assay 0.2 ml of 0.025% lysozyme is added to 10 ml of 0.08% glycol chitin (both solutions are in 0.1 M phosphate buffer at pH 5.6) and the fall in the specific viscosity of the solution is followed. We express the specific viscosity of the solution at t = 3 min as a percentage of specific viscosity of the solution at zero time and use this as a measure of activity. This parameter is equal to $(\eta_S - \eta_B)/(\eta_G - \eta_B) \times 100$, where η_B, η_S, and η_G are the viscosities of the buffer, the solution at t = 3 min, and the original glycol chitin solution, respectively.

We have found it convenient in the text to indicate solutions of enzyme, substrate, and detergent by (E), (S), (D), and so on. In studies of the effect of the mixing order on the enzymatic activity an expression like (E) → (S + D) indicates that a solution of enzyme was added to a solution of substrate plus detergent. Square brackets have been used similarly in describing the difference spectral experiments to indicate spectroscopically significant cuvet contents.

Difference Spectroscopy. Difference spectra in the ultraviolet region were recorded using a Beckman DB or Cary 14 spectrophotometer equipped with a thermostatable cell holder, according to the method described in a previous paper (Hayashi *et al.*, 1963). For convenience, the systems used have been listed in Table I.

Optical Rotatory Dispersion. Optical rotatory dispersion was measured with the Jasco Model ORD-5, an automatic recording spectropolarimeter, using 1-cm quartz cells at room temperature. The Moffitt-Yang equation was used to calculate the parameters a_0 and b_0

$$[M] = \left(\frac{3}{n^2 + 2}\right)\frac{M_0}{100}[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

where $[M]$ is the mean residue rotation at any wavelength corrected for the refractive index, n, of the solvent, λ_0 is the absorption wavelength associated with the rotation (assumed to be 212 mμ), and M_0 is the average molecular weight per residue. The refractive index of the detergent solutions was assumed to be the same as that of buffer solution, 1.33. The dispersion of refractive index was neglected. The lysozyme concentration was kept at 0.904% and the detergent concentration was varied.

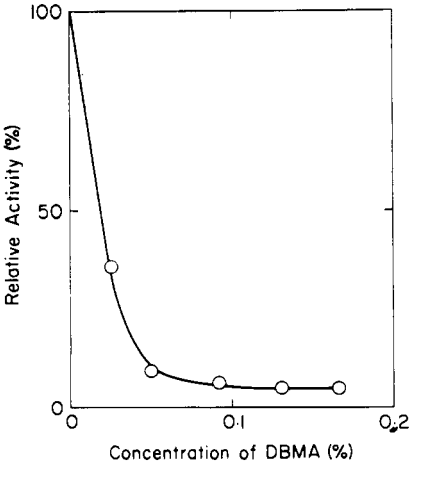

FIGURE 1: The effect of DBMA on lysozyme activity.

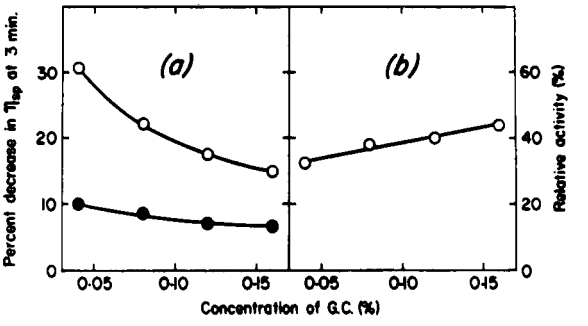

FIGURE 2: The effect of the substrate concentration on the inhibitory effect of DMBA. (a) The activity, $(\eta_s - \eta_b)/(\eta_g - \eta_b) \times 100$ at 3 min, of lysozyme plotted as a function of the concentration of the substrate in the presence and absence of DBMA. (○) No DBMA and (●) 0.05% DBMA. (b) Relative activity of lysozyme in 0.05% detergent solution is represented as a function of substrate concentration. Relative activity was defined as the ratio of activity in 0.05% DBMA to that in the absence of DBMA.

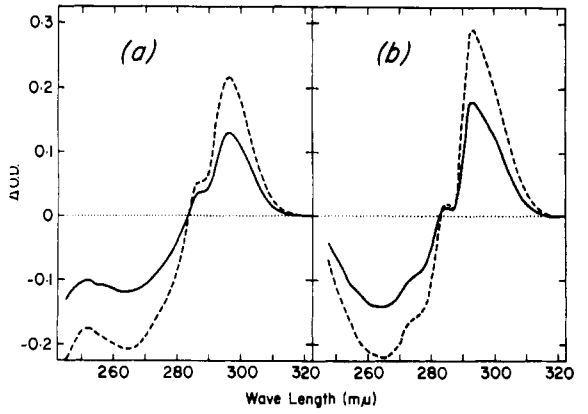

FIGURE 3: Difference spectra. (a) Of lysozyme in the presence of DBMA. Lysozyme concentation, 0.1%; DBMA concentrations, 0.05 (—) and 0.10% (----); 0.1 phosphate buffer (pH 5.6). The enzyme and detergent were mixed together 40 min before measurement. (b) Of acetyltryptophan in the presence of DBMA. AcTrp concentration, 4.09×10^{-4} M; DBMA concentrations, 0.05 (—) and 0.10% (----); 0.1 phosphate buffer (pH 5.6).

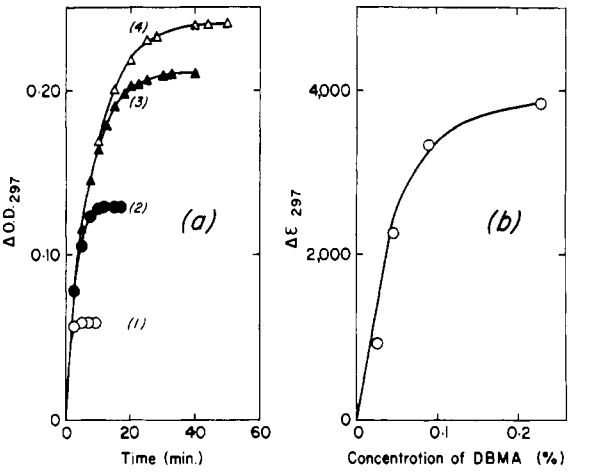

FIGURE 4: The intensity of the difference spectrum at the peak wavelength as a function of time and concentration of DBMA. Measured by system 1 at 25° in 0.1 M phosphate buffer at pH 5.6. The lysozyme concentration was 0.1% (6.26×10^{-5} M). The concentrations of DBMA were (1) 0.024, (2) 0.047, (3) 0.09, and (4) 0.23.

Influence of DBMA on Lysozyme Activity. The inhibitory effect of DBMA on the activity of lysozyme at pH 5.6 was studied by incubating the enzyme (0.025%) with the desired concentration of detergent for 30 min, until time-dependent changes had stopped (see below). After this time, 0.2 ml of this solution was mixed with 10.0 ml of a solution of substrate (0.08%) and detergent in the same concentration as the enzyme solution. The inhibitory effect is shown in Figure 1 where it is seen that 0.05% detergent nearly completely inhibits the action of lysozyme. If this experiment was carried out with no detergent in the enzyme solution almost no inhibition was observed even though the final detergent concentration was nearly the same as in the first experiment. The reason for this is simply that the assay procedure requires a measurement 3 min after mixing enzyme and substrate; but as will be shown below the enzyme and detergent require 30 min or longer to equilibrate.

In order to determine the nature of the inhibition of lysozyme activity by DBMA, the activity was measured for various concentrations of the substrate in the presence of a fixed concentration of DBMA. The DBMA was preincubated with the enzyme as in the first mixing procedure described above. The activity parameter, $(\eta_S - \eta_B)/(\eta_G - \eta_B) \times 100$ at 3 min, and the relative activity are plotted against the substrate concentration in Figure 2a,b, respectively. Figure 2b shows that the relative activity increases when the substrate concentration is increased. This suggests that lysozyme activity was inhibited competitively by DBMA. However, this could not be confirmed by kinetic experiments, apparently because the substrate is a heterogeneous high polymeric substance.

Optical Rotatory Dispersion of Lysozyme in the Presence of DBMA. The values of the optical rotatory parameters, a_0 and b_0, of the lysozyme in the presence of DBMA are shown in Table II. The table shows that the parameters do not change with an increase of the detergent concentration or the preincubation time. These results imply that the secondary and tertiary structures of the lysozyme molecule are not altered by the detergent. A similar conclusion was reached by Glazer and Simmons (1965) in their study of the effect of dodecyl sulfate on the optical rotatory dispersion of lysozyme.

Interaction of Lysozyme with DBMA. When lysozyme and DBMA are mixed, a specific difference spectrum, with a main peak at 296 or 297 mμ, can be observed (Figure 3a). A similar difference spectrum is observed when acetyltryptophan is mixed with the detergent (Figure 3b). When the enzyme is used, the difference spectrum is time dependent for about 30 min, but the difference spectra in Figure 3a were measured after equilibrium was reached. The time dependence of ΔOD_{max} is shown in Figure 4a. In Figure 4b the equilibrium values of $\Delta\epsilon_{max}$ are plotted as a function of the DBMA concentration. The equilibrium values of $\Delta\epsilon_{max}$ increased on increasing the detergent concentration

TABLE II: Parameters of Optical Rotatory Dispersion.[a]

Lysozyme Concn (%)	DBMA Concn (%)	DBMA/Lysozyme (mole/mole)	$-a_0$	$-b_0$
0.904	0	0	272 (283)	155 (150)
0.904	0.5	19	288 (270)	142 (167)
0.904	1.0	39	260 (274)	170 (161)
0.904	2.0	77	270 (283)	167 (152)

[a] The optical rotatory dispersion was measured after allowing the enzyme–DBMA mixture to stand for 3 hr or more, and for 24 hr at room temperature. The latter values are given in parentheses.

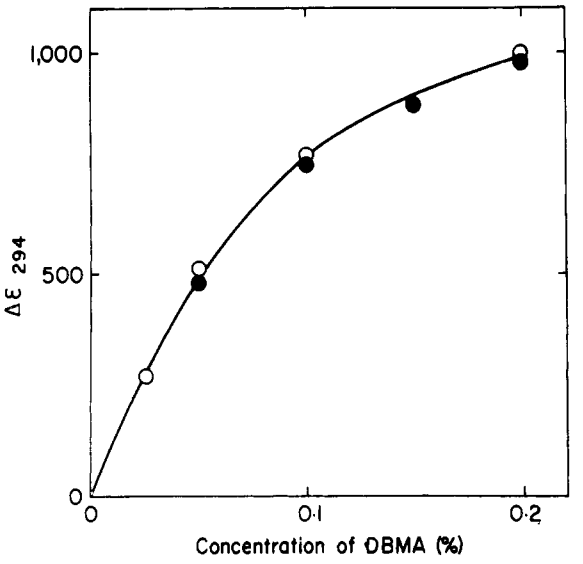

FIGURE 5: The intensity of the difference spectrum at the peak wavelength of acetyltryptophan measured by system 1 at 20° in 0.1 M phosphate buffer (pH 5.6). The concentrations of acetyltryptophan were (○) 4.93×10^{-4} M and (●) 2.01×10^{-4} M.

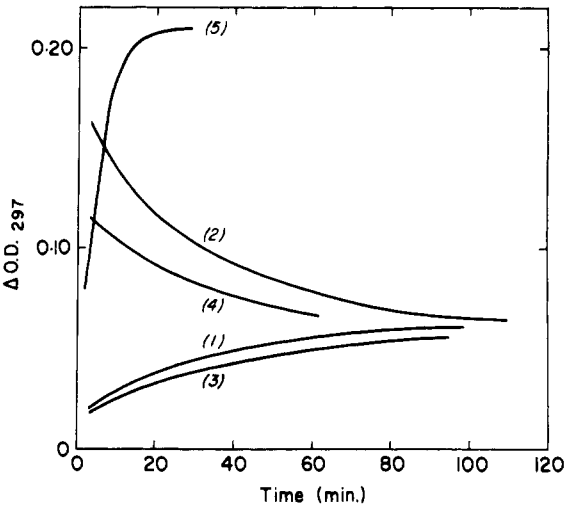

FIGURE 6: The interaction between lysozyme and DMBA in the presence of substrate. The difference spectrum was measured at 30° in 0.1 M phosphate buffer at pH 5.6. The concentrations of lysozyme, substrate, and DBMA were each fixed at 0.1%. For the system used for each curve, see text. When no substrate is present, the result is 5.

up to 0.2% and is seen to reach a value of 4000. These results show that DBMA is interacting with some of the tryptophyl residues in the lysozyme molecule.

The Effect of DBMA on the Spectrum of Acetyltryptophan. The interaction of amino acid chromophores with DBMA was investigated with tyrosine, tryptophan, and acetyltryptophan. Below a concentration of 0.2% DBMA, no difference spectra were observed for tyrosine or tryptophan at pH 5.6, whereas the acetyltryptophan solution at the same pH or tryptophan in alkaline media (pH 9) both showed significant difference spectra. The difference spectrum observed with acetyltryptophan at pH 5.6 had the same shape as that with lysozyme. The difference spectrum of acetyltryptophan had a main peak at 293 or 294 mμ, and the values of $\Delta\epsilon_{max}$ did not change with the incubation time in contrast to that for the lysozyme solutions. In Figure 5, the values of $\Delta\epsilon_{max}$ are plotted as a function of the concentration of DBMA.

Interaction between Lysozyme and DBMA in the Presence of Substrate. The influence of the substrate on the interaction of lysozyme with DBMA was investigated using various arrangements of reagents in the spectrophotometer cells. All of these systems are variants of system 2 in Table I because one of the cells in the reference compartment contains the reaction solution, enzyme plus substrate in buffer, and the other contains detergent in buffer. Thus what is being studied here is the effect of mixing order on the interaction of the substrate and the detergent with the enzyme. The concentrations of E, S, and D are all 0.1% after mixing. The systems used in the spectrophotometer were:

$$[D \longrightarrow (E + S)]/[E + S][D] \quad (1)$$

D is added to a mixture of E and S, and the change in ΔOD_{297} is followed. This is the original system 2 of Table I.

$$[S \longrightarrow (E + D)]/[E + S][D] \quad (2)$$

S is added to a mixture of E and D which had been preincubated for 30 min or more.

$$[E \longrightarrow (S + D)]/[E + S][D] \quad (3)$$

E is added to a mixture of S and D.

$$[(E + D) \longrightarrow (S + D)]/[E + S][D] \quad (4)$$

E, preincubated with D, is added to a mixture of S and D.

In all of these experiments the difference spectra were the same shape as that observed when no substrate was present, and the major peak of the difference spectra was always at 297 mμ. In Figure 6 values of ΔOD_{max} are plotted as a function of time, and it can be seen that all four systems ultimately attain the same equilibrium value of ΔOD_{max}.

In Figure 7 the time dependence of ΔOD_{max} measured by systems 1 and 2 of Table I is shown. In these experiments detergent is added to the enzyme or the enzyme–substrate mixture, so that the final concentrations of the enzyme and the detergent were both 0.1% while the substrate concentration was varied from 0 to 0.5%. Results similar to those in Figure 7 were observed with a substrate sample of low molecular weight (a sample with average degree of polymerization 11, which had been separated from a partial hydrolysate). On the other hand, glucose, *N*-acetylglucosamine, and glycol cellulose, used instead of substrate up to concentrations of 0.1%, had no effect on the interaction

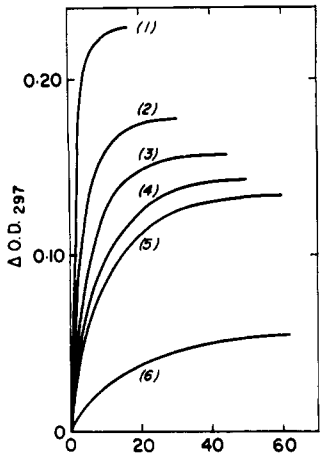

FIGURE 7: The time dependence of intensity measured by system 2. Concentrations of enzyme and DBMA were fixed at 0.1%. ΔOD_{max} was measured at 35° in 0.1 M phosphate buffer (pH 5.6). Concentrations of substrate were (1) 0, (2) 0.025, (3) 0.05, (4) 0.075, (5) 0.1, and (6) 0.5%.

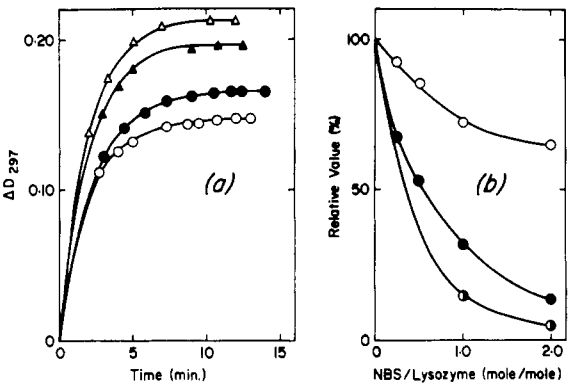

FIGURE 8: The interaction of NBS-treated lysozyme with DBMA. (a) ΔOD_{max} was measured by system 1 at 35°, pH 5.6. The concentrations of NBS-oxidized lysozyme and DBMA were both 0.1%. Lysozyme had been oxidized by various amounts of NBS: (△) 0.25 mole of NBS/mole of lysozyme, (▲) 0.5 mole of NBS/mole of lysozyme, (●) 1.0 mole of NBS/mole of lysozyme, and (○) 2.0 moles of NBS/mole of lysozyme. (b) Relative values of the equilibrium value of ΔOD_{max} (○), the amount of ES complex measured by system 4 (●), and the activity (◐) are plotted as a function of moles of NBS per mole of lysozyme at oxidation.

of the enzyme and detergent. The significance of these results will be dealt with below.

Interaction of NBS-Oxidized Lysozyme with DBMA. This experiment was carried out to investigate whether the tryptophyl residue at position 62 participates in the interaction or not. The interaction with DBMA of lysozyme samples oxidized to various extents by NBS was also studied by spectrophotometry using system 1. When DBMA was added to the solution of NBS-oxidized lysozyme, a difference spectrum, which exhibited the same shape as that of native lysozyme, was observed. It took the same time as the native lysozyme for ΔOD_{max} to reach an equilibrium value, as seen in Figure 8a. However, it is important to notice that the equilibrium value of ΔOD_{max} decreased as the molar ratio of NBS to lysozyme at oxidation increased. In Figure 8b, relative values of the equilibrium ΔOD_{max}, the amount of the enzyme–substrate complex measured by system 4 (Hayashi *et al.*, 1963), and the activity are plotted as a function of the molar ratio of NBS to lysozyme at oxidation.

Discussion

It has been shown by polarimetry that DBMA does not cause lysozyme to undergo any gross changes in molecular structure (Table II). Nevertheless this detergent certainly interacts with the enzyme molecule, as shown by its effect on the enzymatic activity and the ultraviolet spectrum.

As Figure 1 shows, a concentration of 0.05% DBMA almost completely eliminates lysozyme activity at pH 5.6 with the glycol chitin substrate. This apparently comes about because the detergent competes with the substrate for the active center (Figures 2 and 6). Thus spectral investigation of the interaction of lysozyme and DBMA can be expected to provide information about chromophoric groups in the active center.

DBMA certainly causes a marked red shift in the ultraviolet spectrum when it is added to lysozyme or acetyltryptophan (Figures 3a,b). The shape of the difference spectra, and the fact that they are the result of a red shift in the spectrum are consistent with the interpretation that tryptophyl residues are being buried in a nonpolar region (Bigelow and Geschwind, 1960; Yanari and Bovey, 1960). This could, of course, be caused by a conformation change in the enzyme molecule, brought about by interaction with detergent molecules, or it could be caused by binding of the detergent molecules to regions of the enzyme surface containing exposed tryptophyl residues. We believe that the latter explanation is the more reasonable one, because as mentioned above, the polarimetric measurements give no reason to think a conformation change is occurring.

The observed spectral effect is therefore probably due to the specific interaction of some detergent molecules with the enzyme molecule. It is certainly not due to a solvent effect, that is, it is not due to changing the solvent composition, because solvent effects on protein spectra are typically smaller by several orders of magnitude then the spectral shifts we have observed here (Bigelow and Geschwind, 1960). Nor is the observed spectral effect likely to be due to interaction with the cationic charge of the detergent molecules, because interaction of aromatic chromophores with positive ions causes blue shifts of small magnitude (Hermans and Scheraga, 1960). All of these facts lead to the conclusion that the hydrocarbon moieties of the detergent molecules are associating with the indole rings of several tryptophyl residues in the enzyme molecule.

The effect of some other detergents on the spectrum of lysozyme was also studied. It was found that lysozyme gave a difference spectrum with trimethyldodecylammonium chloride, but not with dimethyl-2-hydroxyethylhexadecylammonium chloride, tetramethylammonium bromide, or dodecylammonium chloride. It is

difficult to see any real pattern in these results, though both of the interacting detergents have one chain of at least 12 carbon atoms and have quaternary nitrogen atoms.

Spectral studies with model compounds showed that DBMA interacted with tryptophan only at a fairly high pH, where its amino group would be uncharged. *N*-Acetyltryptophan, however, interacted with DBMA across the pH range. These results indicate that a positive charge close to the chromophore will prevent interaction with the cationic detergents used here. But if the charge is removed, the indole ring is sufficiently nonpolar that it will associate with detergent molecules. This is important supporting evidence for the conclusion reached above that the detergent is interacting directly with tryptophyl residues in the protein molecule.

Experiments carried out on the lysozyme–DBMA interaction in the presence of substrate showed that the substrate interfered with the interaction (Figures 6–7). This leads to the conclusion that DBMA acts as a competitive inhibitor of lysozyme. One concludes from this that both detergent and substrate bind to the active center of the enzyme molecule. If this is correct it becomes of considerable importance to examine the effect of the detergent on the spectrum of an inactive lysozyme derivative, previously prepared (Hayashi *et al.*, 1965) in which one tryptophyl residue has been oxidized by NBS. This was done and the results are shown in Figure 8.

Figure 8b shows that when the molar ratio of NBS to lysozyme is raised to 1, a derivative of lysozyme can be prepared which gives about two-thirds of the spectral shift observed when DBMA interacts with native lysozyme. This derivative forms almost no enzyme–substrate complex, and is therefore almost completely devoid of activity. If one assumes that three tryptophyl residues contribute equally to the difference spectrum of the native molecule, the lower value observed in the derivative is conveniently interpreted as being due to the oxidation of one of them (because the oxidation of tryptophan by NBS is accompanied by a loss of the spectral peak at 280 mμ so the oxidation product will not contribute to a difference peak at 297 mμ). Previous work (Hayashi *et al.*, 1965) showed that the tryptophyl residue at position 62 is preferentially oxidized by NBS. We therefore reach the conclusion, not only that the tryptophyl residue at position 62 is essential for the enzyme's activity (Hayashi *et al.*, 1965), but also that it is one of probably three which interact with the detergent DBMA. These conclusions are in excellent agreement with the crystallographic studies of Phillips and his colleagues (Blake *et al.*, 1967a,b) which also show that three tryptophyl residues (those at positions 62, 63, and 108) are implicated in binding various substrates and substrate-like compounds to the active site of the enzyme.

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