Productive and Unproductive Lysozyme–Chitosaccharide Complexes. Equilibrium Measurements†

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ABSTRACT: A method to determine both productive and unproductive lysozyme–chitosaccharide complexes has previously not been available. The method described in this paper uses a dye, Biebrich Scarlet, which forms a 1:1 complex with only part of the substrate binding site. Complex formation perturbs the spectrum of the compound and thus its dissociation constant can be determined (K_D = 0.13 mM). The dissociation constants for three major enzyme–chitooligosaccharide complexes have also been determined: (1) chitooligosaccharides that bind only to sites A–C of lysozyme perturb the spectrum of the Biebrich Scarlet–lysozyme complex, without affecting the dissociation constant of the dye (K_u = 0.01 mM); (2) chitooligosaccharides that interact with sites D–F displace the dye (K_S' = 5–15 mM); (3) chitohexose forms a complex which involves the whole binding site and, therefore, also displaces Biebrich Scarlet. This complex, with a dissociation constant K_S = 0.03 mM, is considered to be the productive one. The binding mechanism proposed on the basis of the results in this paper differs significantly from those considered previously.

Crystallographic (Blake *et al.*, 1967), ligand binding and transglycosydation experiments (Rupley and Gates, 1967; Rupley *et al.*, 1967; Chipman *et al.*, 1967, 1968) have established that lysozyme can accommodate up to six adjacent pyranose rings of an oligosaccharide chain prepared from chitin or from cell walls of *Micrococcus lysodeikticus*. Each saccharide unit has been assigned a site of interaction on the enzyme, designated sites A–F. Substrates can bind to these binding sites in many ways (Figure 1), but only those molecules whose pyranose rings make contact with sites D and E are catalytically hydrolyzed.

An energetically unfavorable distortion of the pyranose ring is required if site D is to be filled (Blake *et al.*, 1967). Interpretations of mechanistic studies of lysozyme-catalyzed reactions depend on a knowledge of the concentration of the various complexes. In this paper we describe the direct determination of the dissociation constants for three major enzyme–chitooligosaccharide complexes and the methods used in these studies. The principle of the method is illustrated in Figure 1. A preliminary report of part of these data has appeared (Rossi *et al.*, 1969).

Materials and Methods

Twice-crystallized lysozyme (Lots LY 8 HB and LYSF 9FM) was obtained from Worthington Biochemical Corp. The concentrations of enzyme stock solutions were determined spectrophotometrically at 280 mμ using a molecular extinction coefficient of 38,500 M^{-1} cm^{-1} (Sophianopoulos *et al.*, 1962).

The chitooligosaccharides were prepared by hydrolysis of chitin followed by separation and purification on charcoal–Celite columns (Rupley, 1964). Preparations which showed appreciable absorbance above 220 mμ were further purified on a cation exchange resin column, Dowex 50-W-X2. Before use the resin column, 2 cm × 20 cm, was washed with 1 l. each of the solutions of 0.2 N HCl, 0.2 N NaCl, 0.2 N HCl, and with distilled water until the effluent was free of chloride. About 100–200 mg of chitooligosaccharide in water was placed on the column and eluted with water. The homogeneity of the preparations was examined by chromatography on charcoal–Celite columns which were calibrated with homogeneous oligosaccharide standards (Rupley, 1964). Biebrich Scarlet was obtained from Matheson Coleman (Lot BX 620) and was recrystallized from dimethylformamide–ether. Paper chromatography showed that the dye behaves as a single compound (29% aqueous NH_3–H_2O–pyridine–isoamyl alcohol, 1:3:5:5). Buffer solutions containing dye followed Beer's law up to 0.1 mM. The extinction coefficient was 33,000 M^{-1} cm^{-1}. Dried cell-walls of *Micrococcus lysodeikticus* were purchased from Mann Laboratories. All other chemicals were reagent grade.

A Cary Model 14 recording spectrophotometer equipped with a 0–0.1 absorbance unit slidewire was used to measure difference spectra. Difference spectra were measured at 24 ± 1°. The hydrogen ion concentration of each stock solution was determined on a Radiometer pH meter, Type TTTlc, with reference to Matheson Coleman pH 7.0 buffer.

All stock solutions were prepared in sodium–potassium phosphate buffer (pH 7.6), ionic strength 0.1. Saccharide stock solutions containing Biebrich Scarlet were prepared to give the desired concentration on dilution of the saccharide solution. These solutions were 0.02 mM in Biebrich Scarlet but of varying concentrations of oligosaccharides. Binding of saccharide to lysozyme was measured in the following way: 800 μl of identical dye-saccharide solutions was placed in the sample and reference cells (10-mm path length, 1-ml volume) of the Cary spectrophotometer; 200 μl of enzyme in buffer was then placed in the sample cell, and 200 μl of buffer was added to the reference cell. When binding of Biebrich Scarlet alone was investigated, the same procedure was used except that the saccharides were omitted and the concentration of the dye was varied.

In order to minimize absorbancy changes due to a volume error, the same 200- or 800-μl pipette was used for all

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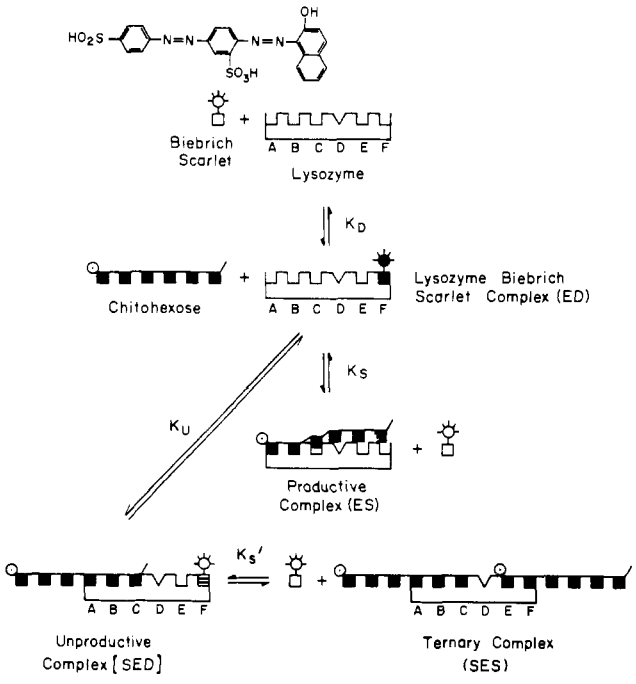
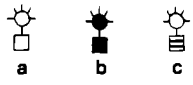


FIGURE 1: A model for multiple binding of chitohexose to the cleft of lysozyme, illustrating the principle of the method for determining the dissociation constants of the various complexes. The major chitohexose–lysozyme complexes shown are the following. A productive complex which competes with the binding of Biebrich Scarlet. This complex involves contacts of the nonreducing end of the saccharide with site A, good contacts with sites B and C, and protrusion of the rest of the saccharide into the D-F region. An unproductive complex which perturbs the absorption spectra of Biebrich Scarlet. This complex involves contact of the reducing end of the saccharide with site C, good contacts with sites B and A, and protrusion of the rest of the saccharide into solvent. A ternary complex in which another chitohexose binds to the unproductive complex and displaces Biebrich Scarlet. The absorption spectra of the free inhibitor (a), of the inhibitor bound to the enzyme (b), and of the inhibitor bound to the unproductive lysozyme–chitohexose complex (c) are different. The binding sites of lysozyme



are indicated by open squares, except for site D which is triangular (to indicate that distortion of the pyranose ring is required for binding to this site). ES* is considered to be the final reactive enzyme–substrate complex in which site D is filled. The dissociation constants of the various complexes are defined in the text.

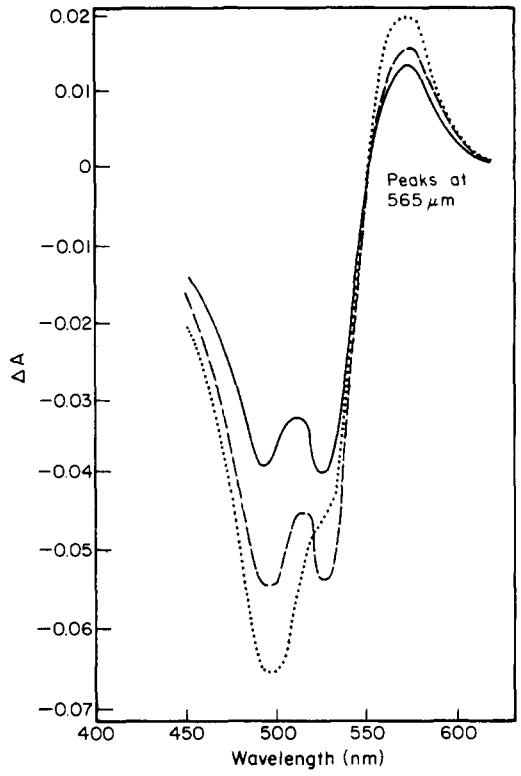


FIGURE 2: Difference spectra, Biebrich Scarlet, lysozyme, and saccharides. Biebrich Scarlet plus lysozyme *vs.* Biebrich Scarlet (. . . .); Biebrich Scarlet plus lysozyme and 3 mM chitotriose *vs.* Biebrich Scarlet plus 3 mM chitotriose (——); Biebrich Scarlet plus lysozyme and 3 mM chitohexose *vs.* Biebrich Scarlet plus 3 mM chitohexose (—). Initial concentrations were 0.177 mM enzyme and 0.016 mM Biebrich Scarlet at pH 7.6 and 24°, sodium–potassium phosphate buffer, ionic strength, 0.1.

additions. Initial concentrations of the reaction mixtures were 0.08–0.37 mM lysozyme, 0.025–16 mM chitotriose, 0.4–16 mM chitotetrose, 2–10 mM chitopentose, 0.1–10 mM chitohexose, and 0.016 mM Biebrich Scarlet. Solutions were mixed rapidly using a Teflon rod and the absorbancy was measured, within 50 sec after the addition of the enzyme. In experiments with chitohexose the absorbance changes were extrapolated to the time of mixing the solutions. Hydrolysis of the other saccharides used is slow compared to the time needed for measurements (Imoto *et al.*, 1972). Difference spectra were measured between 450 and 640 nm. Unless otherwise mentioned, all data refer to differences in absorbance between 495 and 550 nm, an isosbestic point. Saccharide alone at all concentrations used in the experiments had no detectable effect on the absorbance of free dye in the wavelength region used in the experiments. The dissociation constant and molecular extinction of the enzyme–dye complex, the stoichiometry of the dye binding, and the steady-state kinetic inhibition constant of the dye obtained from measurements of the lysozyme-catalyzed hydrolysis of *Micrococcus lysodeikticus* cell walls, were all determined as described previously (Rossi *et al.*, 1969).

The coordinates of the lines in the figures were obtained by the method of least squares using a computer program.

Results

Difference Spectra. A Biebrich Scarlet–lysozyme complex measured against Biebrich Scarlet exhibits a difference spectrum due to a red shift of the absorbance maximum from 505 nm for the free dye to 510 nm when bound to the enzyme. The difference spectrum (Figure 2) has a small maximum at 565 nm, a negative shoulder at 525 nm, a large minimum at 459 nm and an isosbestic point at 550 nm. The extinction difference coefficient for the minimum at 495 nm was obtained from the intercept of a Hildebrand–Benesi plot (Benesi and Hildebrand, 1949) (Figure 3a) and has a value of 6900 M^{-1} cm^{-1}. Addition of chitosaccharide to solutions of lysozyme and Biebrich Scarlet produce a number of changes in the difference spectra (Figure 2).

Characterization of the Lysozyme–Biebrich Scarlet Interaction. The dissociation constant and the molar absorbance difference coefficient, 550 nm − 495 nm ($\Delta\epsilon_{M550-495}$), for the binding of Biebrich Scarlet to lysozyme were determined according to the method of Benesi and Hildebrand (1949). The difference in absorbance between a solution of the dye alone and one of an equal amount of Biebrich Scarlet but varying concentrations of lysozyme is defined

$$\Delta A = [D_0]\epsilon_{M_D} - ([D]\epsilon_{M_D} + [ED]\epsilon_{M_{ED}}) \quad (1)$$

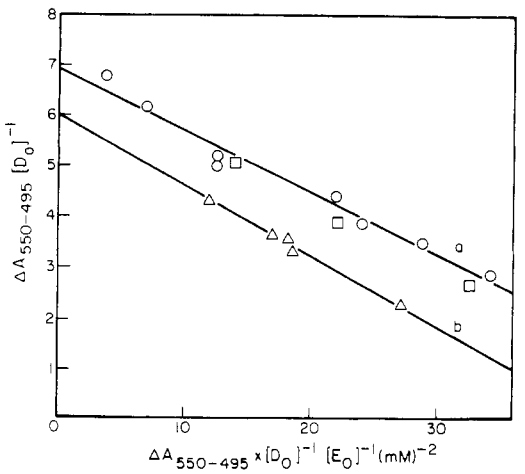


FIGURE 3: Binding of Biebrich Scarlet to lysozyme and to lysozme–chitotriose complex. Difference in absorbancy at 495 nm between a solution containing Biebrich Scarlet and a solution containing an equal concentration of Biebrich Scarlet but varying amounts of lysozyme (line a), or of a lysozyme–chitotriose complex (line b). The absorbancy measurements are in reference to the isosbestic point of the difference spectra at 550 nm. The data were plotted according to eq 4. The coordinates of the line were obtained by the method of least squares and using a digital computer. Sodium–potassium phosphate buffer (pH 7.6), ionic strength, 0.1, 24°. (a) Lysozyme, 0.085–1.8 mM; Biebrich Scarlet, 0.016 mM (□), 0.023 mM (○). (b) Lysozyme, 0.086–0.36 mM; Biebrich Scarlet, 0.016 mM (△). The $\Delta A_{550-495}$ values used in this portion of the graph were obtained from experiments in which lysozyme and Biebrich Scarlet concentrations were constant and chitotriose concentrations were varied (Figure 6a). In order to obtain these $\Delta A_{550-495}$ values, the ΔA *vs.* chitotriose values which were obtained at high concentrations of sugar were extrapolated to zero saccharide concentration (dotted line in Figure 6a). The $\Delta A_{550-445}$ values used in this figure represent these extrapolated values obtained in experiments with different concentrations of lysozyme.

$[D_0]$ represents the total molar dye concentration, and [D] and [ED] the molar concentration of free dye and of the enzyme–dye complex, respectively. ϵ_{M_D} and $\epsilon_{M_{ED}}$ are the molar absorbance coefficients of the dye and of the enzyme–dye complex at a particular wavelength. $\Delta\epsilon_M = \epsilon_{M_D} - \epsilon_{M_{ED}}$.

$$[ED] = \Delta A[\Delta\epsilon_M]^{-1} \quad (2)$$

When $[E_0] \gg [D_0]$, the dissociation constant of the enzyme–dye complex may be defined as

$$K_{ED} = [E_0]([D_0] - [ED])/[ED] \quad (3)$$

If eq 2 is substituted in eq 3 and rearranged, a linear plot of ΔA *vs.* $\Delta A\ [E_0]^{-1}$ can be drawn, from which K_{ED} and $\Delta\epsilon_M$ can be evaluated. The data in Figure 3 are plotted according to eq 4. The dissociation constant of the Biebrich Scarlet–lysozyme complex, calculated from the slope of line (a), was found to be 0.13 ± 0.005 mM. The molar difference coefficient, $\Delta\epsilon_{M550-495}$, obtained from the intercept of the same line (a) in Figure 3, has a value of 6900 M^{-1} cm^{-1}. The concentrations of chitotriose in the experiments in curve (b), Figure 3, were sufficient to ensure the formation of a 1:1 complex with the enzyme (Rupley *et al.*, 1967; Blake *et al.*, 1967). Lines (a) and (b) are parallel within experimental error. This indicates that the dissociation constant of the lysozyme–Biebrich Scarlet complex is not measurably affected by formation of the lysozyme–chitotriose complex. The difference in $\Delta\epsilon_M$ obtained in the two experiments represented by curves (a) and (b) in Figure 3 is due to the perturbation of the dye spectrum by the chitosaccharide.

$$\Delta A = \Delta\epsilon_M[D_0] - (\Delta A/[E_0])K_{ED} \quad (4)$$

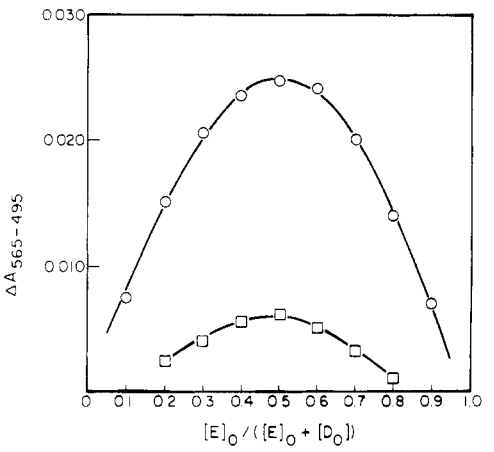


FIGURE 4: Job plot of the difference in absorbancy, $\Delta A_{565-495}$, for solutions containing equal concentrations of Biebrich Scarlet in the presence or absence of lysozyme. In an experimental series in which the sum of the enzyme and Biebrich Scarlet concentrations was kept constant, ΔA was plotted *vs.* molar fraction of enzyme (Job, 1928). The total concentration of enzyme plus Biebrich Scarlet was 0.035 mM for the upper curve and 0.015 mM for the lower curve. Points represent the average of three to four determinations. Conditions were pH 7.6 at 24°, sodium–potassium phosphate buffer, ionic strength, 0.1.

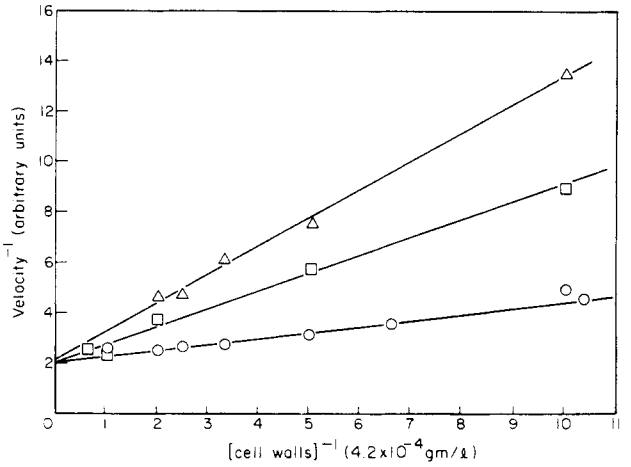


FIGURE 5: Inhibition of the hydrolysis of cell walls of *Micrococcus lysodeikticus* at 0 (○), 0.3 mM (□), and 0.6 mM (△) concentration of Biebrich Scarlet. The rate of hydrolysis was followed at 700 nm, pH 7.6, 24°, sodium–potassium phosphate, and ionic strength, 0.1. Initial concentrations were 0.18 μ M lysozyme and 0.042–0.7 mg/ml of cell walls. The data were plotted according to the method of Lineweaver and Burk (1934). The dissociation constant for the enzyme–inhibitor complex was evaluated from the slopes of the lines to be 0.15 mM.

The data shown in Figure 4 were used to determine the stoichiometry of the enzyme–dye complex. $\Delta A_{565-495}$ was plotted against the molar fractions of enzyme, with the sum of concentrations of enzyme and dye kept constant (Job, 1928). The data are not as good as one might expect since the limbs of the Job plot for the lower curve do not go through the points $f = 0$ and $f = 1$, and the A_{max} agrees within only 20% of the expected value of $A_{M565-495}$ of approximately 11,000 M^{-1} cm^{-1}. The symmetry of the curve and the maximum at 0.5 molar fraction of enzyme do suggest that 1 mol of dye binds/mol of enzyme in the concentration region investigated.

In Figure 5, the observed velocity of lysozyme-catalyzed hydrolysis of cell walls in the absence and presence of Biebrich Scarlet is plotted according to Lineweaver and Burk (1934). The common intercept with the ordinate for the different lines, each representing a series of experiments using

different concentrations of Biebrich Scarlet, is generally interpreted to be indicative of competitive inhibition.

Binding of Chitooligosaccharides to Lysozyme. In the experiments shown in Figure 6, the difference in absorbancy between solutions containing Biebrich Scarlet and solutions containing equal concentrations of enzyme and varying concentrations of the saccharides were measured at three wavelengths. It should be noticed in Figure 6a that at the minima of the difference spectra (Figure 2) at 525 and 495 nm, ΔA is dependent on saccharide concentration at each concentration, but at the maximum at 565 nm, ΔA becomes independent of chitotriose concentrations higher than 1 mM.

At all wavelengths examined, extrapolation of the data obtained at high chitosaccharide concentrations to zero chitosaccharide concentrations gives an ordinate intercept which is different from the experimentally observed value at zero chitosaccharide concentrations. The experiments presented in Figure 3 indicate that this is due to a perturbation of the absorption spectrum of the enzyme–Biebrich Scarlet complex when at low chitosaccharide concentrations sites A–C are filled, but Biebrich Scarlet is not displaced.

Binding of Chitooligosaccharides to the High Affinity Site of the Enzyme. Analysis of the concentration dependence of the absorbancy changes observed at low saccharide concentration (Figure 6b) indicates that the binding of chitosaccharides to the high affinity site of the enzyme, sites A–C (Blake *et al.*, 1967; Rupley, 1967), is being measured.

The dissociation constants of chitosaccharides for the 1:1 lysozyme-chitosaccharide complex were determined as follows (inset Figure 6a). The ΔA value obtained at a particular wavelength in the difference spectrum between lysozyme plus Biebrich Scarlet *vs.* Biebrich Scarlet is termed ΔA_0. Equal concentrations of saccharide are then added to the two cell compartments. The line defined by ΔA values obtained at high saccharide concentrations is extrapolated to the ordinate to give the intercept value, ΔA_{SED} (inset, Figure 6a). ΔA_{SED} is the difference in absorbancy at a particular wavelength between Biebrich Scarlet and solutions containing dye and the high affinity 1:1 lysozyme-saccharide complex. Δ_0 is the difference between the extrapolated line and the ΔA_0 value, and for any particular low saccharide concentration is referred to as Δ_{obsd} (inset Figure 6a). In order to compare results from different experiments we used the normalization procedure (eq 5 and 6). If the absorbancy changes we observed at low saccharide concentrations (Figure 6a) are a measure of the formation of the 1:1 lysozyme-saccharide complex, the concentration of the complex, [SE], is given by (β[E$_0$]).

$$\Delta_0 = \Delta A_{SED} - \Delta A_0 \quad (5)$$

$$\beta = (\Delta_0 - \Delta_{obsd})/\Delta_0 \quad (6)$$

The dissociation constant, K_u, of the complex may then be defined by

$$K_u = \frac{([E_0] - [SE])([S_0] - [SE])}{[SE]} = \frac{1 - \beta}{\beta}([S_0] - \beta[E_0]) \quad (7)$$

K_u can be evaluated from a linear form of eq 7

$$(1 - \beta)^{-1} = [S_0]\beta^{-1}K_u^{-1} - [E_0]K_u^{-1} \quad (8)$$

In Figure 6b the data shown in Figure 6a are plotted according to eq 8. The value for the dissociation constant for the 1:1 chitotriose–lysozyme complex, 0.01 ± 0.002 mM, is in good agreement with the value obtained by other methods (Imoto *et al.*, 1972).

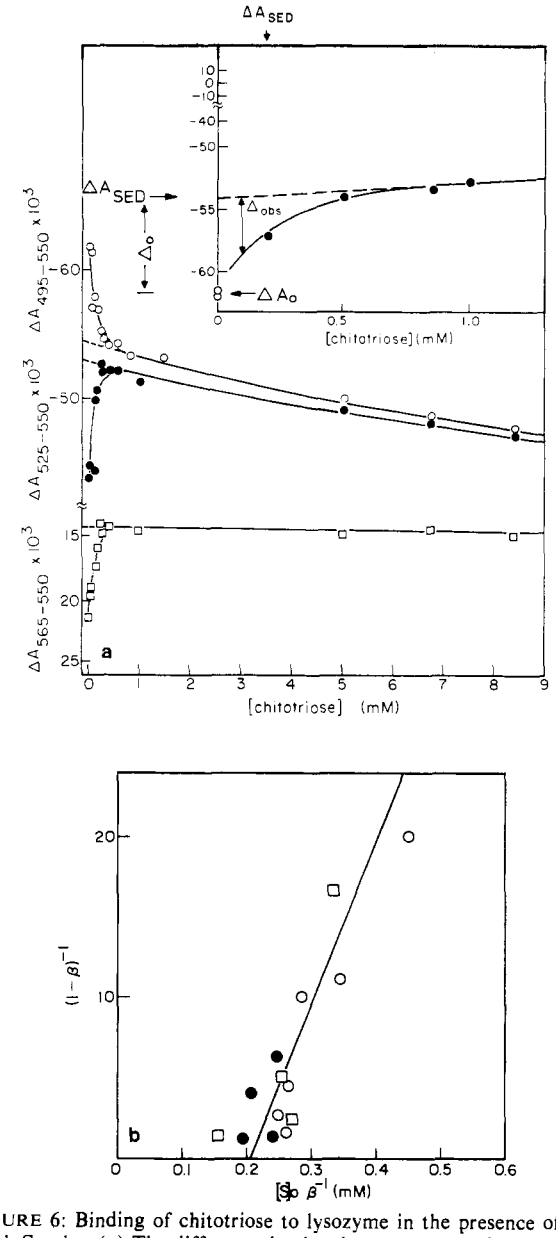


FIGURE 6: Binding of chitotriose to lysozyme in the presence of Biebrich Scarlet. (a) The difference in absorbancy, measured at 495, 525, and 565 nm, between a solution containing Biebrich Scarlet and solutions containing an equal concentration of Biebrich Scarlet, a constant concentration of lysozyme, and varying concentrations of chitotriose. All absorbancy measurements are in reference to the isosbestic point of the difference spectra at 550 nm. (○) $\Delta A_{495-550}$; (●) $\Delta A_{525-550}$; (□) $\Delta A_{565-550}$. Initial concentrations of lysozyme were 0.18 mM, and of Biebrich Scarlet 0.016 mM. Sodium-potassium phosphate buffer (pH 7.6), ionic strength, 0.1, 24°. Inset: The first portion of the $\Delta A_{495-500}$ *vs.* chitotriose curve is redrawn to indicate which measurements are used to analyze this portion of the curves, and the symbols used which correspond to these measurements. To avoid overcrowding the graph, some of the experimental points are not shown. (. . .) Extrapolation of the $\Delta A_{494-500}$ values obtained at high saccharide concentrations; (○) values obtained in the absence of saccharide; (●) values obtained in the presence of saccharide. (b) The determination of the dissociation constant of the high affinity lysozyme-chitotriose complex. The ΔA values obtained at three wavelengths at low chitotriose concentrations as shown in Figure 6a are plotted according to eq 8. (○) 495 nm; (●) 525 nm; (□) 565 nm.

Binding of Chitooligosaccharides to the Productive Binding Site of the Enzyme. The change in absorbancy of

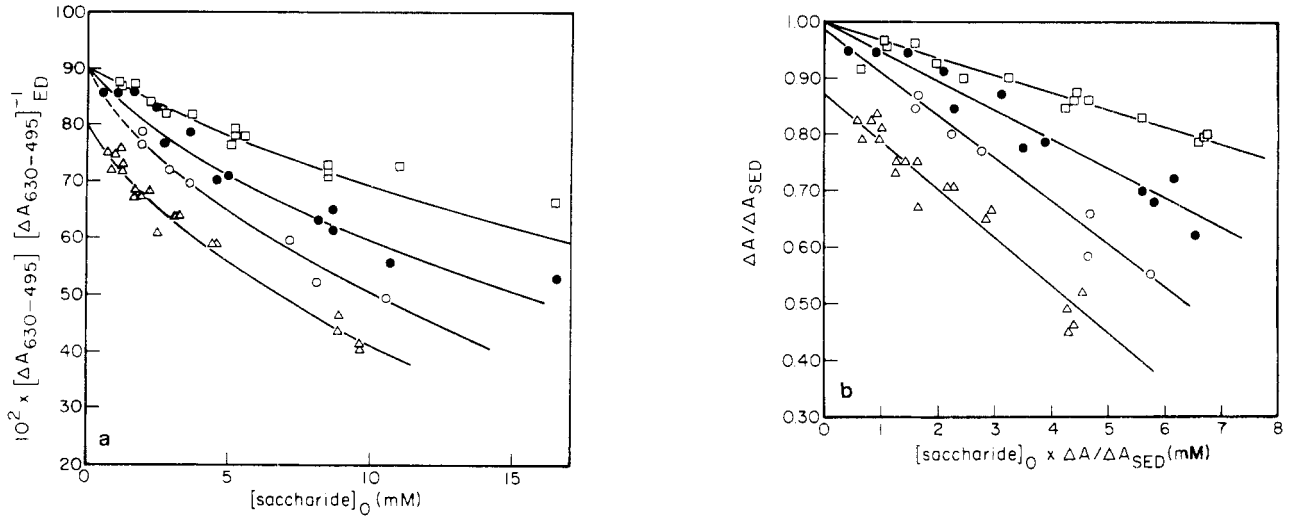
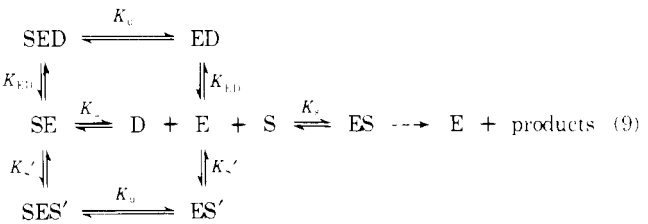


FIGURE 7: Binding of chitooligosaccharides at high concentrations to lysozyme in the presence of Biebrich Scarlet. (a) Difference in absorbancy, $\Delta A_{495-630}$, recorded as a function of chitooligosaccharides concentrations. Difference in absorbancy was measured at 495 nm between a solution containing Biebrich Scarlet and solutions containing an equal concentration of Biebrich Scarlet, a constant concentration of lysozyme, and varying concentrations of chitooligosaccharide. In order to normalize the data, the ΔA values in the presence of sugars were divided by ΔA_0, the absorbance difference of the lysozyme-dye complex (see Figure 6a inset). The following chitooligosaccharides were used: chitotriose (□), chitotetrose (●), chitopentose (○), and chitohexose (△). Sodium-potassium phosphate buffer (pH 7.6), ionic strength, 0.1, 24°. Initial concentrations were 0.015 mM Biebrich Scarlet and 0.15 mM lysozyme. The lines were computed on the basis of eq 11 and using the dissociation constants for the binding of the chitosaccharides to lysozyme (Tables I and II). (b) The data from (a) were plotted according to eq 15 in order to test the applicability of the binding modes of chitooligosaccharides proposed in eq 9 and to determine the dissociation constants K_S and K_S' pertaining to this model. The symbols ΔA and ΔA_{SED} are defined in the text.

the difference spectra at high concentration of various oligosaccharides is shown in Figure 7a. The lines are extrapolated to the ordinate at low saccharide concentrations as indicated by the dashed lines in Figure 6. It should be noticed in Figure 7a that the absorbancy difference decreases with increasing saccharide concentrations. Evaluation of the data indicates a complete elimination of the difference spectra at high saccharide concentrations as a result of displacement of Biebrich Scarlet from the catalytic site of the enzyme. Limited solubility of the saccharides prevents direct experimental verification of this complete dissociation. The solid lines in Figure 7a are computed on the basis of this mechanism which is discussed below.

When the ΔA values obtained for chitohexose are extrapolated to the ordinate they give an intercept about 10% less (Figure 7a) than the one observed for chitotriose and chitotetrose. The suggestion that this is due to displacement of Biebrich Scarlet by even low concentrations of hexamer is incorporated in the proposed mechanism shown in eq 9. In



(9)

eq 9, the addition of dye or substrate has been omitted from some steps for the sake of clarity. All the chitosaccharides investigated bind to lysozyme, without interaction with Biebrich Scarlet, to form complex SE, which is characterized by a dissociation constant K_u (Figures 6 and 7). This involves the binding site which does not catalyze the bond-breaking step (Blake *et al.*, 1967; Rupley, 1967). All the chitooligosaccharides investigated also bind to the catalytic site to form complex ES′, characterized by a dissociation constant K_S'. Finally, the chitohexose and chitopentamer can bind to the noncatalytic and catalytic sites simultaneously to form complex ES, characterized by a dissociation constant K_S. ES is considered to be a productive complex in the catalytic reaction. Biebrich Scarlet competes with the formation of productive complexes by both cell walls of *Micrococcus lysodeikticus* and chitohexamer, and it is, therefore, assumed that it binds to the catalytic site of the enzyme. The formation of ES accounts for the changes in the difference spectra observed at low concentrations of chitohexamer. When $[S_0]$, $[E_0]$, and $[D_0]$ represent the initial concentrations of oligosaccharide, enzyme, and dye respectively, the concentration of the lysozyme-Biebrich Scarlet complexes in the presence of oligosaccharides is given by eq 10, providing $[S_0] > [E_0] > [D_0]$. When eq 10

$$[ED] + [SED] = \frac{[E_0][D_0]}{[E_0] + K_{ED}\left\{1 + \frac{[S_0]}{K_u} + \frac{[S_0]}{K_S} + \frac{[S_0]}{K_S'} + \frac{[S_0]^2}{K_S' K_u}\right\} \times \left(1 + \frac{[S_0]}{K_u}\right)^{-1}} \quad (10)$$

is used to evaluate only the displacement of Biebrich Scarlet, that is at high oligosaccharide concentrations, the following approximations can be made: (1) SED ≫ ED, and the concentration of ED can be neglected; (2) the value for K_u determined in these and previous experiments is about 10^{-5} (Rupley, 1967; Holler *et al.*, 1969). In the substrate concentration range of interest, $[S_0] > 0.5$ mM, $([S_0]K_u^{-1}) \gg 1$. The simplified equation becomes

$$[SED] = \frac{[E_0][D_0]}{[E_0] + K_{ED}\left(1 + \frac{K_u}{K_S} + \frac{K_u}{K_S'}\right) + \frac{K_{ED}}{K_S'}[S_0]} \quad (11)$$

Table I: Binding of Biebrich Scarlet to Lysozyme and Lysozyme-Chitotriose Complex at pH 7.6 and 24°, Phosphate Buffer Ionic Strength 0.1.[a]

K_{ED} (mM)	K_{SED} (mM)	K_I (mM)	ϵ_{M_D}	$\epsilon_{M_{ED}}$	$\epsilon_{M_{SED}}$ (10^4 M^{-1} cm^{-1}, λ 495 nm)
0.13 ± 0.005	0.14 ± 0.01	0.15 ± 0.01	3.30	2.61[b]	2.69[b]

[a] K_{ED} and K_{SED}, the dissociation constants for the binding of the dye to the enzyme and enzyme-saccharide complex, respectively, were obtained from equilibrium measurements (Figure 3). K_I is the dissociation constant of the enzyme-inhibitor complex evaluated for the cleavage of cell walls from *Micrococcus lysodeikticus* (Figure 5). ϵ_{M_D}, $\epsilon_{M_{ED}}$, and $\epsilon_{M_{SED}}$ are the molar extinction of free Biebrich Scarlet, lysozyme-Biebrich Scarlet complex, and lysozyme-Biebrich Scarlet-chitotriose complex, respectively. [b] These values were obtained by subtraction of the molar difference coefficient, obtained from the intercepts of the plots of Figure 3, from the molar absorption coefficient of the dye at 495 nm.

Table II: Binding of Chitosaccharides to Lysozyme at pH 7.6 and 24°, Phosphate Buffer Ionic Strength 0.1.

	Slope (mM^{-1})	Intercept	K_u[a] (mM)	K_S'[c] (mM)	K_S[c] (mM)
Chitotriose	0.031	1.0	0.01	15	
Chitotetrose	0.052	1.0	(0.02)[b]	9	
Chitopentose	0.075	0.99	(0.02)[b]	6	
Chitohexose	0.084	0.87	(0.02)[b]	5	0.03

[a] K_u is the dissociation constant for noncatalytic binding at sites A, B, and C. The value for chitotriose has been evaluated from the initial drop in absorbance at low saccharide concentrations (Figure 6a). [b] The values of K_u for the higher oligomers have been determined as a preequilibrium dissociation constant in kinetic experiments (Holler *et al.*, 1970). [c] The absorbance difference as a function of high saccharide concentration was evaluated using the slopes and intercepts of lines determined by the data in Figure 7. The dissociation constants K_S and K_S' refer to binding to the catalytic portion of the active site of lysozyme defined by the models shown in eq 9 and Figure 1 and the equations pertaining to this model (eq 9–17).

In a linear form

$$[SED] = [E_0][D_0]\Phi - [SED][S_0]\frac{K_{ED}}{K_S'}\Phi$$

and

$$\Phi^{-1} = [E_0] + K_{ED}\left(1 + \frac{K_u}{K_S} + \frac{K_u}{K_S'}\right) \quad (12)$$

The concentration of SED can be determined if the values of ΔA and $\Delta\epsilon_{M_{SED}}$ are known. Since under the conditions of the experiments all the enzyme is in the form of the high affinity saccharide-lysozyme complex, and the formation of this complex perturbs the absorbancy of the enzyme-dye complex, the appropriate $\Delta\epsilon_{M_{SED}}$ can be found from eq 13. ΔA_{SED} is the difference in absorbance between the free

$$\Delta\epsilon_{M_{SED}} = \Delta A_{SED}/[ED] \quad (13)$$

Biebrich Scarlet and the Biebrich Scarlet-lysozyme-saccharide complex in which the saccharide occupies the high affinity unproductive site. This value is obtained as shown in Figure 6. Expressing [ED] in terms of $[E_0]$, $[D_0]$, and K_{ED} we obtain

$$[SED] = \frac{\Delta A}{\Delta\epsilon_{M_{SED}}} = \frac{\Delta A}{\Delta A_{SED}}\frac{[E_0][D_0]}{[E_0] + K_{ED}} \quad (14)$$

Substituting eq 14 in eq 12 we now have

$$\frac{\Delta A}{\Delta A_{SED}} = ([E_0] + K_{ED})\Phi - \frac{\Delta A}{\Delta A_{SED}}[S_0]\frac{K_{ED}}{K_S'}\Phi \quad (15)$$

In Figure 7b the data shown in Figure 7a are plotted according to eq 15 and an excellent fit is observed. The values of K_S and K_S' can be evaluated from the slope and intercept of the lines, using the independently determined values of K_{ED} = 0.14 mM and K_u = 0.01 mM. The various parameters determined in these studies are listed in Tables I and II. In view of the complexity of the binding reactions and the number of independent measurements which were made to determine the parameters, we do not attach any significance to the variation of K_S' values for the various chitosaccharides (Table II).

$$K_S' = \frac{\text{intercept}}{\text{slope}}\frac{K_{ED}}{[E_0] + K_{ED}} \quad (16)$$

$$\frac{1}{K_S} + \frac{1}{K_S'} = \frac{[E_0] + K_{ED}}{K_{ED}K_u}\left(\frac{1 - \text{intercept}}{\text{intercept}}\right) \quad (17)$$

Discussion

Biebrich Scarlet forms a 1:1 complex with lysozyme (Figure 4). Binding of chitotriose in concentrations sufficient to saturate the A–C sites of the enzyme does not affect the dissociation constant of the lysozyme-Biebrich Scarlet complex (Figure 3), but perturbs the absorption spectrum of the inhibitor. The experiments which show that Biebrich Scarlet is a competitive inhibitor of cell wall hydrolysis (Figure 5) indicate that the compound binds to the catalytic part of the active site from which it can be displaced by chitosaccharides which interact with this site. These properties of the Biebrich Scarlet-lysozyme interactions allow evaluation of three major chitosaccharide complexes, those involving only sites A–C, those involving sites C–D, and, in the case of chitohexose, of one which is considered to involve the total binding site of the enzyme.

The model in eq 9 is based on the following binding modes: (I) All the oligosaccharides investigated bind to sites A–C with their reducing end in site C and the rest of the molecule, if longer than three carbon atoms, protruding into the solvent. These complexes are designated SE in eq 9 and the dissociation constant K_u for this unproductive mode is about 10 μM (Table I). This binding mode is illustrated in Figure 1. (II) The oligosaccharides investigated can form ternary complexes involving sites A–C, and E–F simultaneously, as is depicted in Figure 1. (III) The data indicate that alternative complexes to the one mentioned above exist for the hexamer and by implication for the chitopentamer and chitotetramer. In this binding mode, chitohexamer protrudes into sites D–F and displaces Biebrich Scarlet (Figure 1). This complex is designated ES in eq 9 and is characterized by a dissociation constant K_S. The K_S value for the chitohexamer is 30 μM (Table II), which is close to

the K_u value of 10 μM determined for binding mode I (Figure 1). This binding mode of chitohexamer accounts for the large initial change in absorbancy of the lysozyme–Biebrich Scarlet complex observed at low concentrations of chitohexose (Figure 7). This particular binding mode is considered in the quantitative treatment of the dependence of the absorbance changes of the lysozyme–oligosaccharide complexes (Table II).

It should be noted from the data in Figure 7 that only a fraction of the inhibitor is displaced at low concentrations of chitohexose. One explanation of this is that binding mode I is more favorable than the binding mode just discussed, $K_S > K_u$ (eq 9). The factor of 3, experimentally observed, is sufficient to account for the data. It is possible that chitohexose forms complexes which place the nonreducing end of the sugar into solvent and which cannot, therefore, completely fill the D–F region. In this binding mode Biebrich Scarlet may not be displaced. The experiments of Rupley (1967) suggest that such complexes, if formed, are weak and of minor importance, since the lysozyme-catalyzed hydrolysis of chitohexose leads to the exclusive formation of chitotetrose and chitobiose.

The sharp initial absorbancy change of the lysozyme–Biebrich Scarlet complex observed with chitohexose is not observed at low concentrations of chitotetrose or chitopentose. A possible interpretation, consistent with kinetic experiments and other data discussed below, is that Biebrich Scarlet interferes mainly with binding to the E site of the enzyme. This binding mode of Biebrich Scarlet would be competitive with the lysozyme-catalyzed hydrolysis of cell walls (Figure 5) and chitohexose but not with those of chitopentose or chitotretose at low concentrations of these saccharides. The steady-state kinetic experiments of Rupley and Gates (1967) are consistent with this interpretation. These authors report the same apparent K_m for both chitohexose and chitopentose and their value is similar to the K_S value obtained from the displacement of Biebrich Scarlet from lysozyme by chitohexose.

Previous mechanisms of lysozyme-catalyzed reactions considered the existence of only a single unstable reactive complex (Imoto *et al.*, 1972). The binding model proposed on the basis of our results differs in an important way from this mechanism. The data indicate the formation of stable productive complexes. It appears unlikely that this stable complex involves distortion of the pyranose ring of the substrate making contact with site D, because of the free energy requirement of such a distortion (Imoto *et al.*, 1972). It is suggested, therefore, that the stable productive complexes which we observe are transformed into reactive complexes (Figure 1). The existence of such unstable reactive complexes is suggested in all chemical reactions. In lysozyme-catalyzed reactions when either chitopentamer or chitohexamer is the substrate, the apparent dissociation constants are similar, but the rates of hydrolysis of the two substrates are different (Imoto *et al.*, 1972; Banerjee *et al.*, 1973). A possible explanation of this observation is that the concentration of those reactive chitopentamer or chitohexamer complexes which do not contribute to the stability of substrate binding is different for the two substrates.

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