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A Kinetic Analysis of the Reaction of Lysozyme with Oligosaccharides from Bacterial Cell Walls*

David M. Chipman

ABSTRACT: The kinetic behavior of lysozyme-catalyzed reactions of cell-wall oligosaccharides is analyzed. The model considers the various ways in which any oligomer can associate with the enzyme, and assumes that the association constant for any mode depends only on which subsites of the enzyme site are filled. Rates of cleavage of bound substrate to form a glycosyl enzyme intermediate and rates of hydrolysis or transfer of the intermediate to an acceptor are assumed to be the same for any productively bound substrate. The model can be solved by numerical integration with a digital computer, and has been fit to experimental data by a least-squares procedure. Using the parameters so obtained, further reactions can be satisfactorily modelled. The general behavior of the reactions and the significance of the values of the parameters are discussed. Nonproductive binding is seen to be of major importance in the reactions of small oligomers, which are hydrolyzed chiefly *via* pathways in which they react first as transglycosylation acceptors. Nonproductive binding is also seen to have implications for the mechanism of lysozyme action. The model used can be extended to deal with other endocatenases having transferase activity.

The kinetic behavior of lysozyme-catalyzed hydrolyses of oligosaccharides is quite complex, and has a number of peculiar features (Maksimov *et al.*, 1965; Chipman *et al.*, 1968). Reactions of certain oligosaccharides show the pronounced induction periods typical of autocatalytic reactions, and slow down markedly before the oligosaccharide mixture has reached its expected equilibrium composition (which should correspond to nearly complete hydrolysis). During the course of these reactions, oligomers of many different chain lengths appear in the reaction mixtures, and the enzyme synthesizes significant amounts of saccharides of higher degree of polymerization than the starting material by transglycosylation.

Because of these complications, very few true kinetic studies of the action of lysozyme on oligosaccharides have been carried out. Many studies aimed at the elucidation of the molecular mechanism of lysozyme action have utilized poor synthetic substrates (such as phenyl glycosides of mono- or disaccharides) in an attempt to simplify kinetic analyses (Osawa and Nakazawa, 1966; Lowe and Sheppard, 1968; Dahlquist *et al.*, 1968; Raftery and Rand-Meir, 1968; Tsai *et al.*, 1969). We feel that this is an unfortunate situation, as there may be more going on in some of the apparently simple reactions than meets the eye. In addition, we believe the complexities of the lysozyme reactions are intimately related to one of the most fascinating aspects of the molecular mechanism of lysozyme action, the role of "substrate distortion," or "strain." The complex reactions thus have an inherent information content which is unavailable from "simple" reactions.

Chipman *et al.* (1968) carried out a detailed study of the lysozyme-catalyzed reactions of bacterial cell wall oligosaccharides, $(GlcNAc\text{-}MurNAc)_n$,[1] and proposed a simple kinetic mechanism for the enzymic reaction which qualitatively explained all of the observations cited above. According to this mechanism (to be described below in detail), saccharides less than five pyranose units long are very poor substrates because they associate with the enzyme chiefly in nonproductive modes. Since larger oligosaccharides, which can be formed by transglycosylation, are much better substrates, the major reaction of "small" substrates is as acceptors for transglycosylation. Unfortunately, the kinetic mechanism proposed cannot be solved in closed form, and it was not tested mathematically when the above work was reported in 1968.

In order to test the adequacy of the proposed mechanism, and to provide the required tools for the analysis of reactions catalyzed by lysozyme, we have developed a mathematical model for such reactions, and computer programs for the numerical calculation of the time course of lysozyme-catalyzed reactions of oligosaccharides. We have also written a program for obtaining the kinetic parameters giving the best fit to

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[1] Abbreviations used: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid. All oligosaccharides referred to are linked β-(1 → 4), with the reducing terminus written to the right.

experimental data, and tested it on data for the hydrolysis of the bacterial cell wall tetrasaccharide $(GlcNAc\text{-}MurNAc)_2$.

Kinetic Model. The fundamental mechanism on which this analysis is based is a familar one. We assume the enzyme E binds the substrates, S, reversibly to form enzyme–substrate complexes, C. A complex can react to release part of the substrate and form a reactive intermediate A composed of the enzyme and the remainder of the substrate. A may react with water, so that the overall process is hydrolysis, or it may react with any of a number of other acceptors. For lysozyme, the hypothesis of an intermediate is well supported; in particular, retention of configuration at the glycosyl carbon in transglycosylations demands a two-step process (Pollock *et al.*, 1967b; Raftery and Rand-Meir, 1968).

Since lysozyme is an endocatenase (an enzyme which cleaves interior bonds in a polymer), one must consider that any oligomer can be bound in a number of different ways, with each complex having a different bond held at the catalytically active site. Thus, we have eq 1, in which S_i is an oligomer of i units in length, and C_{ij} is the complex of S_i with the enzyme in which the jth susceptible bond from the nonreducing terminus of the oligosaccharide is at the catalytic site. We will assume here that such a reaction is governed by the constant K_{ij}, which may be either a true equilibrium constant or the inverse of a Michaelis constant. Figure 1 shows pictorial representations of some possible binding modes, as well as the catalytic reactions of lysozyme, and is intended to help clarify the notation used in the kinetic scheme.

$$E + S_i \overset{K_{ij}}{\rightleftharpoons} C_{ij}\ (j = 0, \ldots, i + 1) \tag{1}$$

$$C_{ij} \xrightarrow{k_C} A_j + S_{i-j}\ (j = 1, \ldots, i - 1) \tag{2}$$

$$C_{ii} \xrightarrow{k_V} A_i + H_2O \tag{3}$$

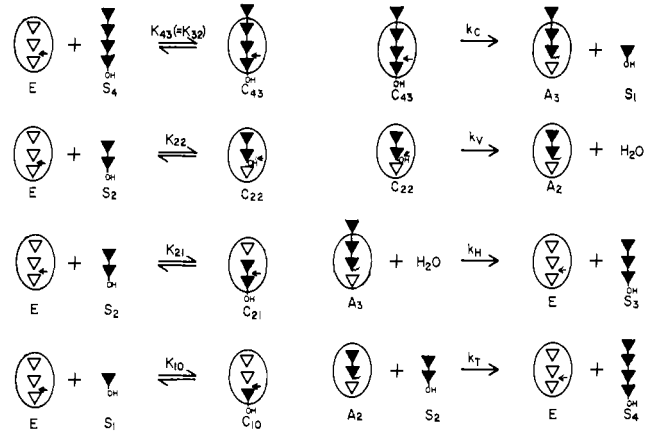
$$A_i(+H_2O) \xrightarrow{k_H} E + S_i \tag{4}$$

$$A_i + S_j \xrightarrow{k_T} E + S_{i+j} \tag{5}$$

Any complex C_{ij}, $j = 1, \ldots i - 1$, may be cleaved by the enzyme to release an oligomer $S_{(i-j)}$ and form the "glycosyl enzyme" intermediate A_i in which a reactive glycosyl group j units long is associated with the enzyme. A major assumption we must make to simplify the analysis of the problem is that any complex with at least one residue bound to the enzyme on each side of the catalytic site is cleaved at the same rate, k_C, regardless of how many additional residues the complex contains (eq 2). For the sake of completeness, we also include a process involving elimination of water from the terminal residue of a substrate molecule, the "virtual" reaction with rate k_V (eq 3).[2] Any complex C_{ij} with $j > i$ or $j < 1$ is assumed to be nonproductive; it has no bonds at the cleavage site.

An intermediate A_i can react with water to free the enzyme and yield the hydrolysis product S_i at a rate k_H which we again assume to be independent of i (eq 4). It can also react with another molecule of free oligomer, S_j, in a transglycosylation reaction to yield S_{i+j}, with a rate k_T (eq 5). One might

[2] There is no evidence for or against a virtual reaction of lysozyme, and it would in fact be difficult to test the question directly. For the cases we have examined (*vide infra*) the kinetic analysis indicates that k_V is not required, and that it can not be very large.



FGURE 1: Symbolic representation of some of the processes considered in the kinetic analysis. ▼ represents a GlcNAc-MurNAc moiety, with carbon 1 of MurNAc at the lower apex. ▽ represents an empty site on the enzyme capable of binding a GlcNAc-MurNAc moiety. The regions are, from the top, A-B, C-D, and E-F of Phillips (1966). The arrow represents the catalytic site, and a wavy line represents an "active glycosyl" function. The association equilibria and catalytic reactions shown are only representative, and all possibilities are not included.

expect k_T to depend on j if more than one monomer unit of the acceptor S_j interacts with the enzyme. However, for the particular case of the bacterial cell wall oligomers, it is clear that only one GlcNAc-MurNAc moiety in an acceptor can interact with the enzyme (*vide infra*).

One can now write the equations governing the concentrations of species in a reaction mixture. $[\bar{E}]$ and $[\bar{S}_i]$ are the total concentrations (free plus bound) of enzyme and oligomer S_i, respectively. The introduction in these equations of a maximum oligomer length to be allowed, n, is necessary for a finite calculation.

$$[C_{ij}] = [E][S_i][K_{ij}] \tag{6}$$

$$\frac{d[\bar{S}_i]}{dt} = -k_C \sum_{j=1}^{i-1} [C_{ij}] - k_V[C_{ii}] - [S_i]k_T \sum_{j=1}^{n-i} [A_j] + k_H[A_i] + k_T \sum_{j=1}^{i-1} [A_j][S_{(i-j)}] + k_C \sum_{j=i+1}^{n} [C_{j(j-i)}] \tag{7}$$

$$\frac{d[A_i]}{dt} = -k_H[A_i] - [A_i]k_T \sum_{j=1}^{n-i} [S_j] + k_C \sum_{j=i+1}^{n} [C_{ji}] + k_V[C_{ii}] \tag{8}$$

$$[\bar{E}] = [E] + \sum_{i=1}^{n} [A_i] + \sum_{i=1}^{n} \sum_{j} [C_{ij}] \tag{9}$$

$$[\bar{S}_i] = [S_i] + \sum_{j} [C_{ij}] \tag{10}$$

Since the concentrations of the reactive enzyme intermediates $[A_i]$ are always less than the relevant $[S_i]$'s, we can introduce the steady-state approximation for $[A_i]$.

$$\frac{d[A_i]}{dt} \approx 0 \qquad i = 1, \ldots, n$$

TABLE I: Constants Used in the Kinetic Model for Lysozyme Reactions with Cell Wall Saccharides.

Constant	Definition	Value and Error[a]	Comments[a]	Standard Value[b]
K_0	Sum of nonproductive modes of association	$\leq 35\ M^{-1}$	From binding studies. Not very significant	$35\ M^{-1}$
K_{11}	Association in subsites C-D (productive *via* k_V)	$\sim 0.5\ M^{-1}$?	Guess from binding studies. Totally insignificant	$0.5\ M^{-1}$
K_{21}	Association in subsites C-F (productive)	$(6.3 \pm 12.4) \times 10^{-4}\ M^{-1}$	From least-squares fit to reaction curves. Probably a maximum value. See Discussion	$6.3 \times 10^{-4}\ M^{-1}$
K_{22}	Association in subsites A-D (productive *via* k_V)	$2000\ M^{-1}$	Apparent binding constant for (GlcNAc-MurNAc)$_2$[c]	$2000\ M^{-1}$
K_{32}	Association in entire site (productive)	$3.5 \times 10^4\ M^{-1}$	Apparent binding constant for (GlcNAc-MurNAc)$_3$[d]	$3.5 \times 10^4\ M^{-1}$
k_C	Cleavage of productive complex	$1.75 \pm 0.17\ sec^{-1}$	From least-squares fit to reaction curves	$1.75\ sec^{-1}$
k_V	Virtual reaction (H_2O expulsion)	$(4.1 \pm 8.1) \times 10^{-7}\ sec^{-1}$	From least-squares fit to reaction curves. Probably a maximum value See Discussion	0
k_H	Hydrolysis rate (transfer to H_2O)	Unknown, $\geq 1\ sec^{-1}$ $\leq 10^5\ sec^{-1}$	Not rate determining. See k_T/k_H	$100\ sec^{-1}$
k_T	Transglycosylation rate	Unknown, $\geq 10^3\ M^{-1}\ sec^{-1}$	Not rate determining. See k_T/k_H	$2.77 \times 10^5\ M^{-1}\ sec^{-1}$
k_T/k_H	Ratio of transfer to hydrolysis rates	$(2.77 \pm 0.3) \times 10^3\ M^{-1}$	From least-squares fit to reaction curves	

[a] The values listed here are our best estimates of these parameters, with an indication of their reliability. The origin of the value is indicated under comments. Error figures, where given, are standard deviations obtained from the least-squares fit (Wolberg, 1967). [b] Values used in calculation of all the kinetic plots illustrated in this paper. [c] Chipman *et al.* (1967). [d] Pollock *et al.* (1967a).

To avoid having an intractable number of disposable parameters, one must make further assumptions about the K_{ij}'s. It seems chemically reasonable to assume that these constants depend only on the part of the substrate molecule in contact with the enzyme. A large number of parameters can then be replaced with a few, each characteristic of a given mode of occupation of the active site. The case of lysozyme acting on saccharides derived from the repeating GlcNAc-MurNAc copolymer of *M. lysodeikticus* cell walls is particularly simple. Since lysozyme cleaves these oligosaccharides only at the glycosyl bonds of the *N*-acetylmuramic acid residues (Sharon *et al.*, 1966; Sharon, 1967), the monomer unit for the purposes of a kinetic analysis can be considered to be the disaccharide GlcNAc-MurNAc. Lysozyme, with six monosaccharide-binding subsites, thus has only three subsites for GlcNAc-MurNAc (Phillips, 1966; Blake *et al.*, 1967). Only one of these (composed of the regions designated E and F by Phillips) is an acceptor or leaving-group site, while two more subsites (in the regions A and B, and C and D) are on the other side of the cleavage point. The three subsites can only be occupied in a limited number of ways, and only two of these modes (K_{32}, K_{21}) are productive, *i.e.*, put a glycosidic bond at the cleavage point. Two other modes can conceivably lead to the "virtual reaction" (K_{22}, K_{11}). In addition, there are some completely nonproductive binding modes, but since none of these involves interaction of more than one monomer unit, they can be subsumed under a single constant K_0. These assumptions are defined in eq 11–15, and the constants are also further clarified in Table I.

$$K_{ij} = K_{32} \quad \text{For } j \geq 2,\ i \geq j \tag{11}$$

$$K_{ij} = K_{21} \quad \text{For } j = 1,\ i > j \tag{12}$$

$$K_{ii} = K_{22} \quad \text{For } i \geq 2 \tag{13}$$

$$K_{ii} = K_{11} \quad \text{For } i = 1 \tag{14}$$

$$K_{i0} + K_{i(i+1)} = K_0 \quad \text{For all } i \text{ (nonproductive)} \tag{15}$$

One additional change in notation considerably simplifies the computations required. Rather than keeping track of each $[C_{ij}]$, we define

$$[\bar{C}_i] = \sum_{j=0}^{i+1} [C_{ij}] \tag{16}$$

$$\bar{K}_1 = K_0 + K_{11} \tag{17}$$

$$\bar{K}_i = K_0 + K_{22} + K_{21} - (i-2)K_{32} \text{ for } i = 2, \ldots, n \tag{18}$$

so that

$$[\bar{C}_{ij}] = [\bar{C}_i]K_{ij}/\bar{K}_i \tag{19}$$

The combination of eq 6–19 leads to the final set of eq 20–26. Equations 20 and 21 are the differential equations for the change in total concentration of an oligomer S_i with

time; they depend on concentrations determined by the steady-state equations 22–26. With or without the steady-state approximation, such a set of nonlinear differential equations can not be solved in closed form. It can be integrated numerically, however, and the forms of eq 20–26 are those we have found most efficient for this.

$$\frac{d[\bar{S}_1]}{dt} = -k_V K_{11}[\bar{C}_1]/\bar{K}_1 - k_T[S_1]\sum_{j=1}^{n-1}[A_j] + k_H[A_1] + k_C\left(K_{21}[\bar{C}_2]/\bar{K}_2 + K_{32}\sum_{j=3}^{n}[\bar{C}_j]/\bar{K}_j\right) \quad (20)$$

$$\frac{d[\bar{S}_i]}{dt} = -[k_V K_{22} + k_C K_{21} + (i-2)k_C K_{32}][\bar{C}_i]/\bar{K}_i - k_T[S_i]\sum_{j=1}^{n-i}[A_j] + k_H[A_i] + k_T\sum_{j=1}^{i-1}[A_j][S_{(i-j)}] + k_C[K_{21}[\bar{C}_{i+1}]/\bar{K}_{i+1} + K_{32}\sum_{j=i+2}^{n}[\bar{C}_j]/\bar{K}_j] \text{ for } i = 2, \ldots, n \quad (21)$$

$$[E] = [\bar{E}]\Big/\left[1 + \left(\sum_{i=1}^{n}[A_i] + \sum_{i=1}^{n}[\bar{C}_i]\right)\Big/[E]\right] \quad (22)$$

$$[\bar{C}_i] = [E]\bar{K}_i[S_i] \qquad i = 1, \ldots, n \quad (23)$$

$$[S_i] = [\bar{S}_i]/(1 + [E]\bar{K}_i) \qquad i = 1, \ldots, n \quad (24)$$

$$[A_1] = \frac{k_V K_{11}[\bar{C}_1]/\bar{K}_1 + k_C K_{21}\sum_{j=2}^{n}[\bar{C}_j]/\bar{K}_j}{k_H + k_T\sum_{j=1}^{n-1}[S_j]} \quad (25)$$

$$[A_i] = \frac{k_V K_{22}[\bar{C}_i]/\bar{K}_i + k_C K_{32}\sum_{j=i+1}^{n}[\bar{C}_j]/\bar{K}_j}{k_H + k_T\sum_{j=1}^{n-i}[S_j]} \quad (26)$$

for $i = 2, \ldots, n$

Numerical Integration. There are many numerical techniques available for solving ordinary differential equations; the most appropriate ones for kinetic problems are essentially more accurate elaborations of the approximation $X_{t+h} = X_t + (h\,dX/dt)$ (Ralston and Wilf, 1960). The choice of a technique usually involves a compromise between accuracy and speed, and which particular method is best depends on the problem at hand. We tested both the fourth-order Runge–Kutta routine and Hamming's modified predictor–corrector method (using double precision routines DRKGS and DHPCL, respectively, from the IBM Scientific Subroutine Package (International Business Machines Corporation, 1968)), and found the latter to be about twice as fast for a comparable level of accuracy. The predictor–corrector method used has the advantage over much simpler methods of estimating the local truncation error at each step, and choosing and changing the integration step size h during the integration to keep the truncation error within given limits.

The numerical integration routine requires a function subroutine to evaluate the derivatives $d[\bar{S}_i]/dt$ at each step. The subroutine we have written calculates the steady-state concentrations $[A_i]$, $[\bar{C}_i]$, and $[E]$ from current values of $[\bar{S}_i]$ (eq 22–26) by an iterative process starting from the previous set of steady-state values. After the first integration step, only one or two iterations are required for convergence to 0.03%. The derivatives can then be calculated using eq 20 and 21.

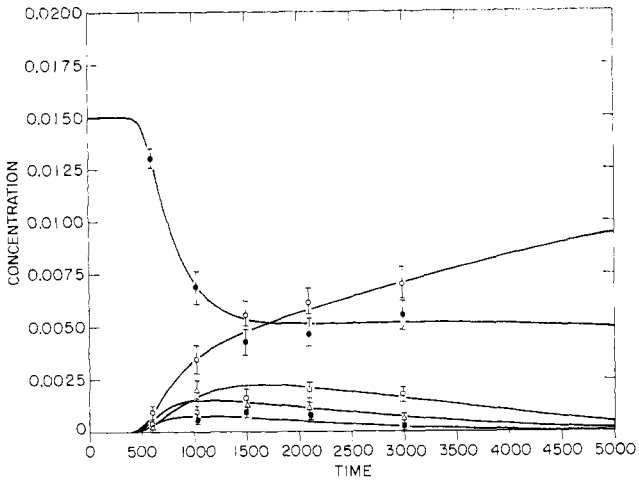


FIGURE 2: Course of the reaction of 7.5×10^{-3} M (GlcNAc-MurNAc)$_2$ with 2.07×10^{-5} M lysozyme at pH 5.25, 37°. Ordinate is in concentration of GlcNAc-MurNAc units (normalities). Abscissa is time in seconds. Points are experimental values obtained using labeled saccharides (Chipman *et al.*, 1968): (●) monomer, (○) dimer, (□) trimer, (△) tetramer, and (■) pentamer. Lines are calculated as described in text, using constants listed in right-hand column of Table I, and traced from CalComp Plotter output. Concentrations of oligomers larger than S_5 are not shown.

Figure 2 is a typical plot obtained using the kinetic program.[3] The value of n, the maximum oligomer length considered, was 9 in this calculation, and the results for S_1 through S_7 were unaffected by setting n greater than 9. The truncation error limits were also such that further restriction had no effect (to three significant figures) on the results. The results, using our current "best values" of the rate and equilibrium constants (Table I) fit the experimental data quite well.

Fitting Experimental Data. While it is useful to have a program for calculating the concentration *vs.* time relationships predicted by a kinetic model, it is desirable to be able to find the best parameters to fit experimental data. When no analytical integration of the kinetic equations is possible, and the data are such that direct differentiation of the data is unsatisfactory, this becomes a difficult problem. Himmelblau *et al.* (1967) have proposed a general technique for such cases, but it depends on the availability of experimental values of *all* the variables at each time, and is awkward to use with steady-state intermediates. A remaining alternative which we have chosen is to use a general nonlinear least-squares method (Wolberg, 1967). The method involves starting with an initial set of values for the parameters and calculating successive corrections which improve the fit of the calculated kinetics to the experimental data. Our program was written to allow treating any desired group of kinetic constants as parameters to be varied to obtain the best fit, while the remaining ones are held fixed.

This least-squares fitting routine was applied to the experimental data for the reaction of 7.5×10^{-3} M (GlcNAc-

[3] The programs described here were written in FORTRAN IV and run on the M.I.T. Information Processing Center's IBM System 360/65/40 computer. Graphs were automatically plotted on the Center's CalComp 565 Plotter. Some have been redrawn to allow inclusion of other data.

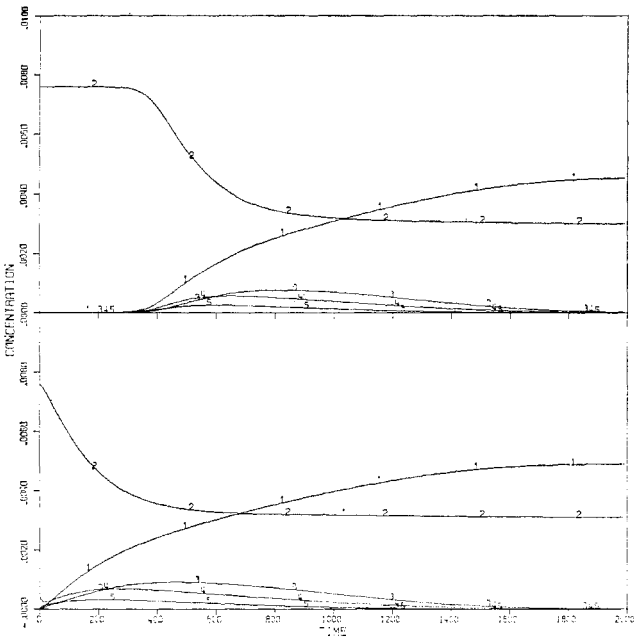


FIGURE 3: Calculated effect of addition of higher oligomer on reaction of 3.8×10^{-3} M S_2 with 2×10^{-5} M enzyme, plotted by CalComp Plotter. Upper plot: no added higher oligomers. Lower plot: 1×10^{-4} M S_4 added. Lines are calculated as described in text, using parameters listed in right-hand column of Table I. Ordinate is in concentration of GlcNAc-MurNAc units (normalities), and abscissa is time in seconds. Numbers on lines serve as identification only; 1 is calculated concentration of S_1, 2 of S_2, etc.

MurNAc)$_2$ (generally labeled with ^{3}H) with 2.07×10^{-5} M lysozyme (at 37°, pH 5.25), the conditions for which we have the best data (Chipman *et al.*, 1968). The 24 data points shown in Figure 2, taken at five different times, were used.

The nine parameters for the reaction are listed in Table I. For these particular reaction conditions, many of the constants are not significant; the calculated course of the reaction is essentially unaffected by allowing K_{11} and/or K_0 to be zero. The calculation is somewhat insensitive to the values of K_{22} and K_{32} as well, and we have chosen to use the experimental apparent binding constants for the dimer and trimer, respectively, for these parameters (Chipman *et al.* 1967; Pollock *et al.*, 1967a). These values are reliable, and there is good reason to believe that they correspond to K_{22} and K_{32} as we have defined them. Attempts were therefore only made to fit the remaining parameters with the fitting routine.

A complete fit of the data varying all five parameters simultaneously could not be made, however. When changes in two different parameters have essentially the same effects on the calculated kinetics, the set of simultaneous equations to be solved to obtain corrections to the parameters is not linearly independent. The matrix which must be inverted to solve them becomes singular, and no unique solution is possible. We could solve simultaneously for the best values of three parameters: k_C, k_T/k_H, and *either* K_{21} or k_V, with the remaining rate and equilibrium terms as constants. The problems encountered in trying to fit K_{21} and k_V simultaneously, or k_T and k_H simultaneously, are informative and important to our understanding of the system.

The parameters k_V and K_{21} are apparently both small, and their only significant role in the kinetics is in the induction period in the reaction of the dimer, when only S_2 is present in solution. Before higher oligomers have been formed, there are only two reactions S_2 can undergo: (a) cleavage to form A_1 and release S_1 (rate = $k_C K_{21}[S_2][E]$ M sec^{-1}), and (b) expulsion of water to form A_2 (rate = $k_V K_{22}[S_2][E]$ M sec^{-1}). Once either process occurs, however, A_1 or A_2 can react with additional S_2 to form higher oligomers. Oligomers three or more units long are good substrates; they fill the entire enzyme site and are nearly always bound productively. They can be lengthened by further cleavage and transfer to S_2, and, if they are six or more units long, can be cleaved to form two molecules of "higher" oligomers. The major reaction in solution rapidly becomes a branching chain reaction, with S_3 and larger saccharides acting as chain carriers and S_2 being consumed almost entirely as an acceptor for transglycosylation.

Since K_{21} and k_V are significant only in the initiation processes in this scheme, they have identical effects on the overall reaction, and a simultaneous solution for both constants is impossible. Assuming $k_V = 0$, the value of K_{21} is found to be $(6.3 \pm 12.4) \times 10^{-4}$ M^{-1}. Assuming $K_{21} = 0$, the value of k_V is $(4.1 \pm 8.1) \times 10^{-7}$ sec^{-1}. With either assumption, the values obtained for the remaining parameters are the same. This sort of ambiguity is characteristic of reactions which are autocatalytic because of a branching-chain mechanism. Since a single molecular event initiating one chain could in principle account for all the propagating chains in such a reaction, one can never determine the nature of the initiation reaction by kinetic means. This dilemma has been pointed out by Breslow (1959) for the case of the formose reaction. Because of this ambiguity, the values of K_{21} and k_V derived by curve fitting must be considered maximum values only. What is clear, however, is that the sum of the turnover numbers for *all* reactions of S_2 in the absence of higher oligomers (*e.g.*, $k_C K_{21}/\overline{K}_2$) must be less than 10^{-6}–10^{-4} sec^{-1} (the higher number being based on the most conservative estimate of the statistical significance of our values for K_{21} or k_V).

An attempt to solve for k_C, k_T, and k_H at the same time also fails, apparently because k_T and k_H are not rate determining. Examination of reactions of higher oligosaccharides under low concentration conditions where transglycosylation plays a minor role indicates that k_H must be at least of the order of 0.5 sec.$^{-1}$ For the dimer reaction being considered, in which acceptor concentrations are high, it thus turns out that the breakdown of active intermediates *via* k_H and k_T is much faster than their formation from enzyme–substrate complexes *via* k_C. The absolute values of k_H and k_T cannot be obtained; therefore, we can only arbitrarily set k_H and solve for the ratio k_T/k_H.

The fit to the experimental data using the final set of parameters is seen in Figure 2. The fitting routine converges to the same solution independent of the initial values for the parameters to be fit so long as the calculated reaction using the initial guesses does in fact proceed to some extent. The standard deviations for the parameters k_C and k_T/k_H are quite good, and while the standard deviation in K_{21} (or k_V) is large, this value is still informative.

General Behavior of the Kinetic Model. If the kinetic model we have suggested is adequate, it should be possible to correctly predict the course of a lysozyme-catalyzed reaction with different starting conditions using the same set of constants. We have used the model to examine three further aspects of the reaction of S_2: the effect of added small amounts of higher oligomers, the effects of initial S_2 concentration, and inhibition by S_1.

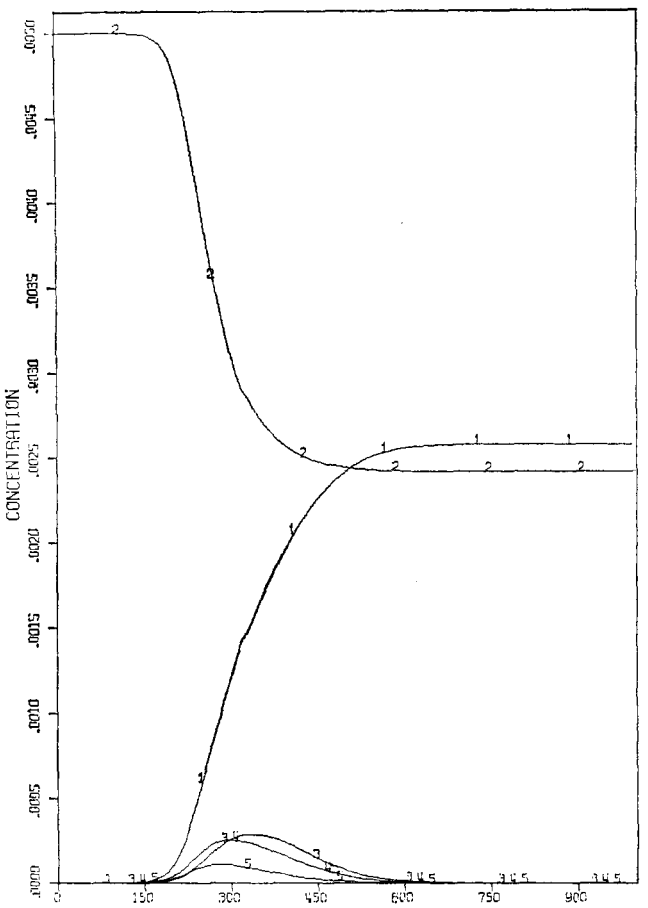


FIGURE 4: Calculated reaction of 2.50×10^{-3} M S_2 with 4×10^{-5} M enzyme. See legend to Figure 3.

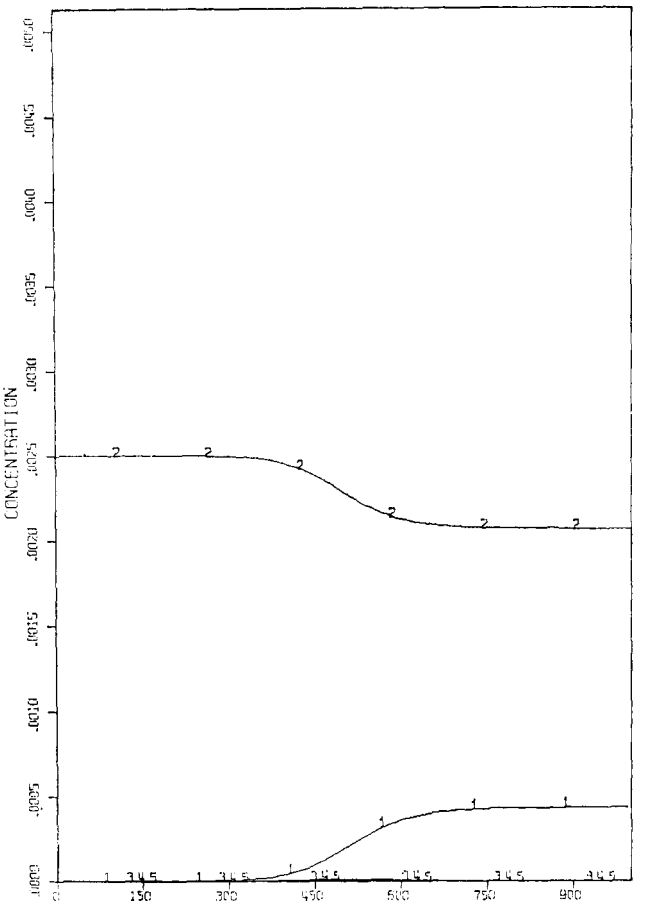


FIGURE 5: Calculated reaction of 1.25×10^{-3} M S_2 with 4×10^{-5} M enzyme. See legend to Figure 3.

Polarimetric measurements and semiquantitative experiments with labeled saccharides[4] indicate that the addition of a small quantity of S_2 or S_4 completely abolishes the induction period in the hydrolysis of S_2, as expected. This experimental observation is also reproduced by the kinetic model, and a pair of plots illustrating this point is given in Figure 3.

The hydrolysis of S_2 is quite concentration dependent between 1×10^{-3} M and 5×10^{-3} M in substrate, even though the apparent dissociation constant for S_2 is 5×10^{-4} M. This phenomenon, characteristic of many lysozyme-catalyzed reactions, is reproduced by the kinetic model. Figures 4 and 5 show the calculated behavior of 1.25×10^{-3} M and 2.5×10^{-3} M S_2, respectively, with 4.0×10^{-5} M lysozyme. The difference between these reactions is extraordinary, but it should be noted that although higher oligomers never account for more than 1% of the saccharide in the reaction at lower concentration, here too the hydrolysis occurs essentially entirely *via* the transglycosylation pathway. When calculations are carried out for even lower initial S_2 concentrations—say, 5×10^{-4} M—the reaction hardly proceeds at all. Even with addition of 2 mole % of S_3, no more than a per cent or two or the S_2 is hydrolyzed before the higher oligomer pool decays to zero.

[4] The experimental behavior of lysozyme-catalyzed reactions of cell wall saccharides has been studied by radiochemical techniques (Chipman *et al.*, 1968), as well as by colorimetric and polarimetric techniques (Sharon, 1967, and unpublished work by N. Sharon, J. J. Pollock, D. M. Chipman, and V. Grisaro). Much of this work is only semiquantitative, and a statistical comparison with calculation is not meaningful.

This phenomenon can be qualitatively understood by considering what is necessary for the chain reactions of the transglycosylation pathway to be self-sustaining. Chain termination occurs if, for instance, S_4 is cleaved to form A_2 and S_2, and A_2 is hydrolyzed to a second molecule of S_2. Chain branching, *i.e.*, the formation of two reaction chains from one, can occur if a large oligomer is split into two molecules which are each three or more units long. However, the formation of two molecules of S_3 from one requires at least three consecutive steps in which intermediates are transferred to acceptor saccharides rather than to water. Since each such step involves a competition between water and saccharide, this aspect of the mechanism introduces a dependence of chain growth on something like the third power of the S_2 concentration when $[S_2] \times k_T/k_H$ is less than one.

S_2 is never completely hydrolyzed by lysozyme, because the hydrolysis product, S_1, inhibits the reaction. Inhibition can also be seen directly in reactions in which S_1 is included in the starting reaction mixture, and the kinetic model reproduces this behavior, as illustrated in Figure 6. This observation is again related to the propagation of kinetic chains in the transglycosylation pathway. S_1 competes with S_2 for active intermediates, retards oligomer lengthening, and thus inhibits kinetic chain branching.

Cell wall oligosaccharides three or more GlcNAc-MurNAc units long are rapidly hydrolyzed by lysozyme to mixtures of dimer and monomer, with no induction period. Figure 7 shows the computer-plotted course of the reaction of 3.0×10^{-4} M S_3 with 2.0×10^{-6} M enzyme, together with experi-

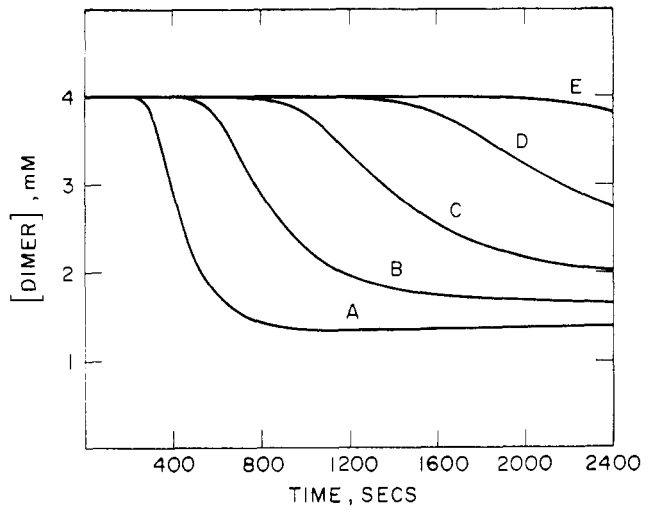


FIGURE 6: Calculated effect of addition of S_1 on the disappearance of S_2, in reaction of 4×10^{-3} M S_2 with 4×10^{-5} M enzyme: curve A, no added monomer; B, $+4 \times 10^{-3}$ M S_1; C, $+8 \times 10^{-3}$ M S_1; D, $+1.2 \times 10^{-2}$ M S_1; E, $+1.6 \times 10^{-2}$ M S_1. Constants in right-hand column of Table I were used.

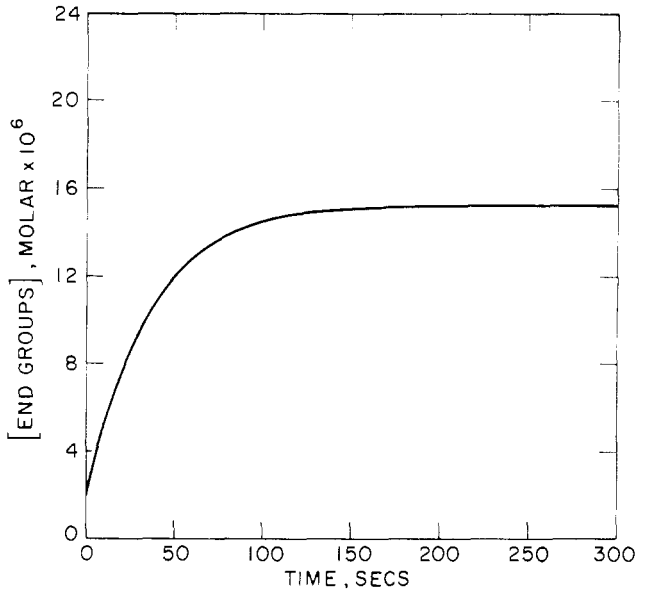


FIGURE 8: Calculated increase in end group concentration in the hydrolysis of 2×10^{-6} M S_{12} by 5.0×10^{-7} M lysozyme. Constants in right-hand column of Table I were used.

mental data for such a reaction (the data for this reaction is less reliable than that used for the least-squares fit, largely because it proceeds much faster). At this substrate concentration, transglycosylation is still significant, as indicated by the growth and decay of concentrations of higher oligomers. One consequence of this is that the final reaction mixture consists of S_1 and S_2 in a 1.6:1 molar ratio (0.8:1 in normalities), rather than in the 1:1 molar ratio expected for direct hydrolysis of S_3 to S_1 and S_2. A calculation for the reaction of S_4 is also in good agreement with experiments.

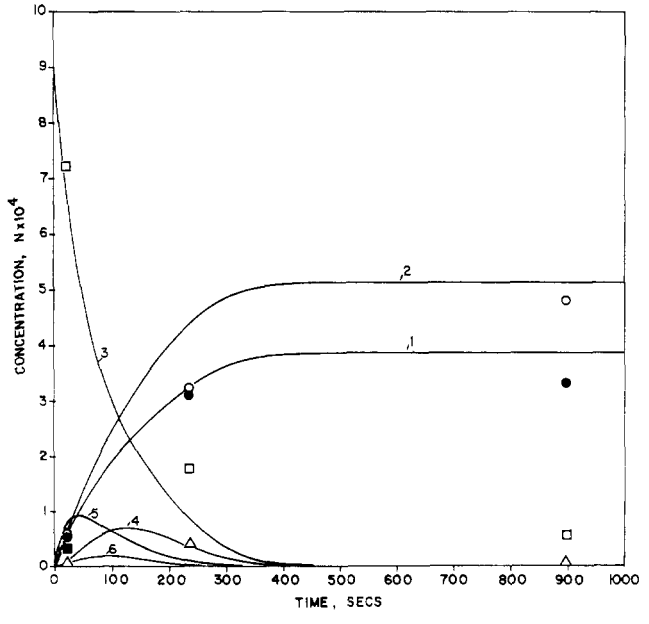


FIGURE 7: Course of the reaction of 3.0×10^{-4} M S_3 with 2.0×10^{-6} M lysozyme. Ordinate is in terms of GlcNAc-MurNAc units (normalities). Lines are calculated as described in text, using constants from right-hand column of Table I. Points are experimental values, from reaction of labeled saccharide at pH 5. 25,37°: (●) S_1, (○) S_2, (□) S_3, (△) S_4, (■) S_5. Error analysis for data was not carried out, since reaction between sampling and separation (see Chipman *et al.* 1968) is probably a major systematic error source.

The kinetic plotting program can also be used to calculate the course of reactions of much larger saccharides, for which no direct experimental data is available. Figure 8 shows the concentration of end groups (*i.e.*, of total individual molecules) as a function of time, in the hydrolysis of 2×10^{-6} M S_{12} by 5.0×10^{-7} M lysozyme. This reaction is similar in the concentration of enzyme and of lysozyme-susceptible bonds to a typical whole bacterial cell experiment with 0.25 mg/ml of dry *M. lysodeikticus* and 7.5 mg/ml of enzyme. The calculated kinetics are exponential, with a half-time of 25 sec, in surprisingly good accord with the experimental exponential clearing of the turbidity of the cell wall suspension with a half-time of about 1 min. The calculated final composition of the reaction mixture is about 58 mole % S_2 and 42% S_1, close to the ratio of these compounds isolated from lysozyme digestion of cell walls.

Conclusions

The kinetic model developed here for the reactions of bacterial cell wall oligosaccharides with lysozyme appears to be an adequate one. A good fit of the model to quantitative data for the reaction of the dimer was obtained by the nonlinear least-squares technique, and with the constants thus obtained, the behavior of many other reactions, for which less accurate data were available, was satisfactorily reproduced. The success of this model justifies drawing from it some conclusions about the mechanism of lysozyme action.

A striking result of the kinetic fit is that the dimer S_2 is a poorer substrate than any larger oligomer by a factor of some 10^4–10^6, largely because productive binding of S_2 is not favorable.[5] Its hydrolysis by the enzyme depends almost entirely on the transglycosylation pathway, without which the turnover number for the reaction of the dimer would be

[5] It is possible that the rate of cleavage of the productive complex of S_2 is not equal to k_C for all other complexes as assumed initially. Examination of the differential equations (20–26) shows that this in no way affects the kinetic calculations, since K_{21} always appears in a product term $k_C K_{21}$. This also is irrelevant to the discussion which follows.

about 1 mole per mole of enzyme per week. Whatever the nature of the direct reaction of S_2, such a reaction must have an activation energy five to eight kilocalories per mole higher than the cleavage of S_3; yet the only difference between S_2 and S_3 is that the latter has an additional GlcNAc-MurNAc unit which can interact with the enzyme. Lysozyme must therefore have some way of utilizing association energies to lower the activation energy for glycoside bond cleavage. There are a number of conceivable ways in which an enzyme can do this, all closely related to conformational changes in enzyme, substrate, or both (Jencks, 1969). Phillips has suggested on the basis of crystallographic studies of lysozyme (Phillips, 1966; Blake *et al.*, 1967) that substrates must be distorted towards the half-chair conformation expected for the transition state in glycoside cleavage in order to fit the enzyme active site. Other evidence for such distortion has since been brought forward, and attempts have been made to estimate the magnitude of the effect (Rupley and Gates, 1967; Chipman *et al.*, 1967; Johnson *et al.*, 1968; Chipman and Sharon, 1969). The value of 5–8 kcal per mole suggested above is one of the least equivocal estimates available for the contribution of conformational effects to the lowering of the activation energy in lysozyme-catalyzed reactions.

One can estimate from our results that the activation energy for glycoside cleavage catalyzed by lysozyme is some 12–13 kcal per mole less than that for the nonenzymic reaction. The rate of cleavage of a productive enzyme–substrate complex, about 1.75 sec^{-1}, is about 10^9 times faster than the expected rate of hydrolysis of di-*N*-acetylchitobiose in water at pH 5.25 and 37°, extrapolated from Bruice's data on the acid-catalyzed reaction (Piszkiewicz and Bruice, 1968).

While it is clear that lysozyme has a very strong tendency to transfer glycosyl residues to saccharides, rather than to water, it has not been simple to estimate the ratio k_T/k_H. The least-squares fit now gives us a reliable estimate for k_T/k_H for acceptors of the form $(GlcNAc\text{-}MurNAc)_n$, of about 2.8×10^3 M^{-1}. The simplest explanation for this remarkable specificity of the active intermediate is that the GlcNAc-MurNAc moiety of an acceptor can be strongly bound in subsites E and F of the glycosyl enzyme intermediate, with $K_d = 3.5 \times 10^{-4}$ M. This interpretation requires that the association equilibrium be established faster than the intermediate can react with water. If the association occurs with a rate constant typical of protein–small molecule interactions, 10^6–10^8 M^{-1} sec^{-1} (Hammes, 1968), the requirement can be met with a glycosyl enzyme intermediate with a life-time of 10^{-3}–10^{-5} sec in water.

The vital role of transglycosylation pathways in the reactions of poor substrates (*i.e.*, those which are short and are bound chiefly in nonproductive modes) is a very important consideration in the analysis of reactions of simple, synthetic substrates. As seen in the example illustrated in Figure 5, a reaction may be occurring nearly exclusively *via* transfer pathways even though higher oligomers never reach concentration levels which would be experimentally detectable. Great care should be taken in drawing conclusions from experiments which may involve such complications.

Finally, one can use the various kinetic constants to estimate the value of the equilibrium constant for hydrolysis of a glycosidic bond in a cell wall saccharide.

$$K = \frac{[S_2][S_1]}{[S_3][H_2O]} = \frac{K_{32}k_C k_H}{K_{22}k_V k_T \times 55} \geq 10^2$$

This constant is somewhat larger than that for hydrolysis of simple alkyl glycosides, presumably because of the significant steric crowding in the (1 → 4) linkage.

The general technique described here for the analysis of lysozyme reactions can clearly be extended to the analysis of reactions of other oligomers and other endocatenases. The replacement of eq 11–15 with the appropriate set of equations based on the active site model in question is necessary, and the problem may become somewhat more complex than the case we have treated. For an analysis of the reactions of chitin oligosaccharides (oligomers of *N*-acetylglucosamine only) with lysozyme, for instance, one would have to consider the enzyme as having six subsites, and as many as two dozen parameters might be of importance. The general behavior of lysozyme reactions with cell wall saccharides is clearly fundamentally related to the arrangement of lysozyme's subsites: the enzyme has one subsite for a GlcNAc-MurNAc unit on the leaving-group (or acceptor) side of the catalytic site and two on the other side. No choice of constants in a calculation based on this model could lead, for instance, to the prediction that S_2 will be the exclusive end product of hydrolysis of a large saccharide. An enzyme which does lead to such a result must have a differently arranged active site (an even number of subsites would in fact be required). This kind of insight suggests promising approaches to the study of other endocatenases with transferase properties, and we hope to apply such approaches to other enzyme systems in the near future. We are now gathering data on the reactions of bovine testicular hyaluronidase, and hope to use the techniques described here to analyze such data.

Acknowledgments

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Independent and Cooperative Reactions of Myosin Heads with F-Actin in the Presence of Adenosine Triphosphate*

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ABSTRACT: A myosin solution partially inactivated with 6-mercapto-9β-ribofuranosylpurine 5′-triphosphate (SH-ATP) (an analog of ATP) is a mixture of molecules having zero, one, and two of their "heads" enzymatically inactivated [concentrations: $(1 - p)^2C_t$, $2p(1 - p)C_t$, and p^2C_t, respectively, where p = fraction of myosin reacted, C_t = total concentration of myosin]. To discover whether the cooperative reaction of the two heads of myosin is required for contraction, the partially reacted system was compared to a control system consisting of native myosin and myosin totally inactivated with SH-ATP (concentrations: C_n and C_i, respectively; $C_n + C_i = C_t$) with respect to *extent* and *rate* of superprecipitation (E_{spptn} and $\bar{V}_{spptn}$) and actin-enhanced ATPase rate ($\bar{V}_{ATPase}$) in the presence of molar excess actin. E_{spptn}'s reached by reacted systems (p = 0.2, 0.3, and 0.5; C_t = 0.1 mg/ml) and control systems ($C_n + C_i$ = 0.1 mg/ml) are approximately identical (control experiments show $E_{spptn} \propto C_t$). A double-log plot of $\bar{V}_{spptn}$ of the reacted systems *vs.* $(1 - p)^2C_t$ is essentially superimposable on that of $\bar{V}_{spptn}$ of the control systems *vs.* C_n, while a plot of $\bar{V}_{ATPase}$ of the reacted systems *vs.* $(1 - p)C_t$ is superimposable on that of $\bar{V}_{ATPase}$ of the control systems *vs.* C_n. The performance ratio of $\bar{V}_{spptn}$ of a reacted system (p = 0.4, C_t = 0.1 mg/ml) to a control system (C_n = 0.04 mg/ml, C_i = 0.06 mg/ml) observed experimentally (0.61 at 4.6×10^{-5} M ATP) is approximately identical with that calculated (0.73) assuming that the only effective molecules in superprecipitation are those with two native heads and considering that the ratio is equal to the ratio of effective molecules in these two systems raised by power 3 (since control experiments show $\bar{V}_{spptn} \propto C_n^3$ at 4.6×10^{-5} M ATP). However, the observed performance ratio of $\bar{V}_{ATPase}$ (1.4 $\sim$ 1.6) is best explained by considering that all unreacted heads participate in ATP hydrolysis [$(1 - p)^2C_t + 0.5 \times 2p(1 - p)C_t/C_n$ in control system = 1.5]. These results suggest that in catalyzing ATP hydrolysis there is no cooperative reaction between "heads" of the same molecule, but, in order to cause structural change with actin, a myosin molecule must have both of its two heads undamaged.

There is growing evidence that myosin is a duplex molecule with two globular "heads," attached to a rodlike tail, and that each head carries one ATPase site and one actin binding site (Lowey *et al.*, 1967, 1969; Slayter and Lowey, 1967; Schliselfeld and Bárány, 1968; Nauss *et al.*, 1969; Murphy and Morales, 1970). However, the "purpose" of these two heads in muscle contraction has not been examined. In the present work, in order to learn whether the concerted action of these two heads is required in muscle contraction, we attempted to prepare molecules, one head of which is enzymatically inactive.

An ATP analog, 6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate (SH-ATP),[1] is a specific affinity label for the ATPase sites of myosin; 2 moles of this analog attach to 1 mole of myosin, and myosin ATPase activity is thereby reduced to zero. Furthermore, the two ATPase sites of myosin alone appear to be equivalent and noninteracting (Murphy and Morales, 1970). Therefore, a myosin solution partially reacted with SH-ATP will be a mixture of three species of molecules: (1) molecules having two native heads, (2) molecules having one native and one reacted head, and (3) mole-

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[1] Abbreviations used are: SH-ATP, 6-mercapto-9β-ribofuranosylpurine 5′-triphosphate; PK–LDH enzymes, mixed pyruvate kinase and lactic dehydrogenase enzymes; PEP, phosphoenolpyruvate; NEM, *N*-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate.