ment of AIDS virus infections with cultured $CD8^+$ cells to prevent or delay progression of disease.

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Catalytic Antibodies

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Monoclonal antibodies elicited to haptens that are analogs of the transition state for hydrolysis of carboxylic esters behaved as enzymic catalysts with the appropriate substrates. These substrates are distinguished by the structural congruence of both hydrolysis products with haptenic fragments. The haptens were potent inhibitors of this esterolytic activity, in agreement with their classification as transition state analogs. Mechanisms are proposed to account for the different chemical behavior of these antibodies with two types of ester substrates. The generation of an artificial enzyme through transition state stabilization by antibodies was thus demonstrated. These studies indicate a potentially general approach to catalyst design.

A BASIC PRINCIPLE OF ENZYME CATALYSIS states that strong binding interactions are required by enzymes to reduce the energy barriers along the chemical reaction pathway (*1*). One way in which this may be done is by helping to stabilize the transition state. This insight, attributed to Pauling, foretold that substances that mimic the structure of a transition state in a particular enzymic reaction would be bound tightly to enzymes involved in the reaction (*2*). In fact, the transition state analog concept is now firmly established as a viable approach to the design of enzyme inhibitors (*3*). The converse proposition, that a receptor designed to optimally bind a suitable analog of a transition state would achieve the catalytic function of an enzyme, continues to intrigue experimentalists. The demonstration of this would reinforce the correlation of enzyme function with the principle of active site–transition state complementarity and indicate a powerful approach to devising artificial enzymes.

The immunological reservoir provides the variety of receptor sites with the required specificities for such a study. The combining sites of antibodies have been considered as useful templates for simulating the environment of an enzyme active site (*4*). Previous constructs (*5, 6*), which achieved only limited success, were not based on the Pauling mechanism of binding energy utilization (*7*). Our studies have focused on the use of transition state analogs as the haptens to elicit the desired antibodies (*8*). The hapten would then behave as an inhibitor in the catalytic system. Accordingly, we have demonstrated that such antibodies can exhibit some of the chemical attributes of enzymes (*9*). We now describe how these antibodies are capable of true enzyme catalysis when their proper substrates are identified. These findings portend the emergence of a class of proteins, having the antibody-enzyme dichotomy, for which the term "abzyme" is suggested.

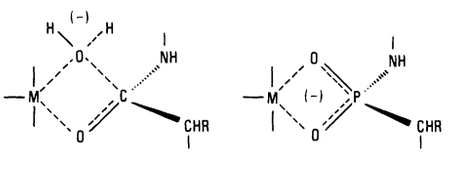

Fig. 1. (A) A possible structure of the transition state in metallopeptidases. The bidentate coordination of the partially hydrated amide to the metal ion is one model for a stabilizing interaction that has been proposed to occur in the mechanism of peptide cleavage by a zinc peptidase (*17*). (B) The interactions of a phosphonamidate analog with the metalloenzyme that allow it to simulate the transition state configuration according to the model shown.

We chose initially to examine a protocol for the hydrolysis of carboxylic esters as an example of a transacylation reaction. Enzymes that catalyze such reactions are expected to bind well those analogs of the substrate having a tetrahedral configuration, thus resembling an intermediate or transition state for nucleophilic addition to the acyl group (*10*). This is true for serine proteases, where a covalent bond between the ligand and the enzyme is formed temporarily (*11, 12*), as well as for metallopeptidases. Metallopeptidases are inhibited by substrate analogs having a tetrahedral phosphoryl or phosphonyl group in place of the cleaved amide unit (*13–15*). A proposed mechanism of peptide bond hydrolysis in metalloenzymes employs the metal ion to either polarize the amide carbonyl by coordination, or deliver a coordinated hydroxide to that group (*16, 17*) (Fig. 1A). Recent structural studies have shown that complexes of enzymes with transition state analogs

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have the phosphorus group as a ligand to the metal ion in the active site (Fig. 1B) (*13, 18*). Therefore, we constructed phosphonate esters **1**–**4** (Fig. 2) to mimic an activated state in ester hydrolysis. The dipicolinic acid–containing ligands **3** and **4** were designed to include a metal ion coordination site by analogy with the metalloenzyme model. Such structures might be recognized immunologically as chelates (*19*). Initially, substance **4** was utilized as a non-chelated hapten along with the simpler phosphonate **2** to obtain specific monoclonal antibodies. Though the role of the picolinyl appendage is not fully understood, only hapten **4** has yielded catalytic antibodies (*20*).

Two antibodies that were elicited to the larger hapten **4** were shown to react specifically with activated carboxylic esters **5** and **6** which are homologous in the acyl portion with the phosphonyl fragment of the hapten (*9*). These labile esters were useful in detecting low levels of esterolytic activity. The process appears to result in acyl transfer to an essential residue of the antibody combining site, forming a stable acylated antibody. This result was unexpected since the bound phosphonate group should not resemble a transition state in which a covalent bond to the antibody is formed. However, we believe that the binding interaction directed to the phosphonate moiety helps to stabilize a transition state or tetrahedral intermediate of this reaction. The observed mechanism could represent a deviation from the expected pathway that is a result of the particular choice of substrate. Since histidine may be a crucial residue in the combining site, we reasoned that the imidazole group may participate in either nucleophilic or general base catalysis. This is known to occur in the catalysis of ester hydrolysis by imidazole (*21*). In the context of an active site, where the imidazole group is provided by the protein structure, this alternative would be manifested as covalent or noncovalent catalysis by the enzyme. We proposed that the functional groups in the active site allow two alternate mechanisms for transacylation to compete and that the prevailing pathway would change with the choice of substrate.

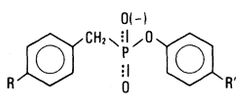
Testing this idea required the modification of the ester substrate to change the basicity of the phenolate expelled in the reaction while retaining the structure needed for binding. The study of these chemically reactive monoclonal antibodies with more stable substrates has now revealed that a highly specific esterase activity is expressed. Carboxylic esters that are similar to the structure of the hapten used are accepted by the antibody in a catalytic process that exhibits many of the characteristics of an enzyme, including the specific inhibition by the transition state analog. The leaving group structure suggested by the hapten is the disubstituted phenolate with some abbreviated para-substituent to occupy the site of the coupling appendage (*22*). The acetamide group was used to replace this linkage in the free haptenic inhibitors **1** and **3**. The analogous substrate would have the 2-picolinylcarboxamidomethyl - 4 - acetamidophenol as the alcohol portion of the ester. As a first approximation of this we prepared the 4-acetamidophenyl ester **7**, which corresponds to the phosphonate **1**. The absence of the structurally significant ortho substituent might diminish the potential binding of the ligand but should not drastically affect the chemical reactivity of the ester bond (*23*).

A mixture of ester **7** (5 μ*M*) and the monoclonal antibody from hybridoma 6D4 (0.1 u*M*) in phosphate buffer (50 m*M*, *p*H 8.0, 23°C) was analyzed over time by high-performance liquid chromatography (HPLC). The accelerated hydrolysis of the ester was apparent from the decrease in size of its peak and the concurrent increase in two new peaks that corresponded with the expected products. Under these conditions the ester was completely consumed in 60 to 80 minutes during which time the background rate accounted for about 12 to 16% hydrolysis (Fig. 3). The chromatographic profile was the same as that produced by treatment with hog liver esterase. Nonspecific antibodies or antihapten antibodies that were inactive in the stoichiometric reaction with coumarin ester **5** did not have this ability. The succinylated ester **8** was hydrolyzed by the antibody at a somewhat slower rate than the corresponding acetamide **7**. This rate difference was also seen with hog

Table 1. Hydrolysis of carboxylic esters by monoclonal antibody (from hybridoma 6D4) and by hog liver esterase (Sigma, EC 3.1.1.1) determined by HPLC on an analytical RP-C18 column (Vydac 218TP54) with isocratic elution (65:35 water:acetonitrile; 0.1% trifluoroacetic acid) at a flow rate of 1.0 ml/min and detector set to 245 nm. The initial substrate concentration was 5 μ*M* and that of internal standard (acetophenone) was 10 μ*M* in 50 m*M* phosphate buffer at *p*H 8.0. The retention times (minutes) were as follows: acetophenone, 5.0; **7**, 8.3; **8**, 6.7; **9**, 4.1; **10**, 11.1 (40% acetonitrile elution); **11**, 8.2. The antibody concentration was 15 μg/ml (0.1 μ*M*) and that of esterase was 5.5 μg/ml. The reaction mixtures were kept at 23°C and aliquots were analyzed at intervals of 2 to 20 minutes. Three or more determinations were used to plot a curve from which the half-life of the reaction was estimated (see Fig. 3).

Substrate	$t_{1/2}$(min)		k_{uncat}‡ (10^5 sec^{-1})
	Antibody (6D4)	Esterase	
7	16 ± 3	4 ± 1	2.8
8	55 ± 5	52 ± 5	3.8
9	*	4 ± 1	0.25
10	*	<2†	1.63
11	*	5 ± 1	6.10

*The ester was not consumed faster than the background rate of hydrolysis. †The reaction was too rapid to be measured accurately by HPLC. ‡The rate constants were determined spectrophotometrically (at 245 nm) by measuring initial rates at five concentrations of ester.



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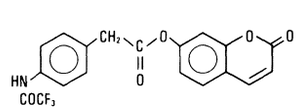

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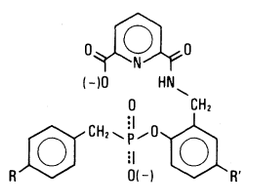

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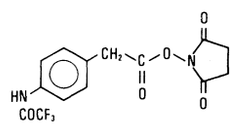

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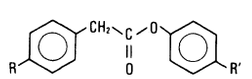

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Fig. 2. Haptens and substrates used in the production and assay of monoclonal antibodies with esterolytic properties. The identity of substituents R and R′ are as follows: (**1**, **3**, and **7**) R = $NHCOCF_3$, R′ = $NHCOCH_3$; (**2** and **4**) R = $NHCOCF_3$, R′ = $NHCO(CH_2)_4COON(COCH_2)_2$; (**8**) R = $NHCOCF_3$, R′ = $NHCO(CH_2)_2COOH$; (**9**) R, R′ = $NHCOCH_3$; (**10**) R = $NHCOCF_3$, R′ = H; (**11**) R = $NHCOCH_3$, R′ = $NHCOCF_3$.

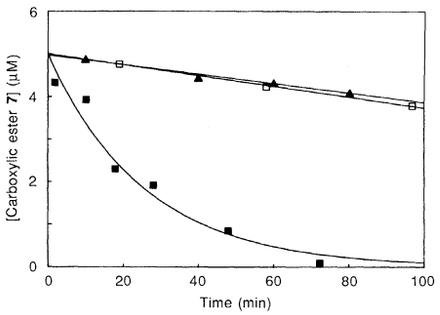

Fig. 3. Rate of hydrolysis of carboxylic ester 7 determined by HPLC under the conditions described in Table 1 (50 m*M* phosphate buffer, *p*H 8.0, 23°C). (▲) Uncatalyzed (background) rate of hydrolysis. (□) Effect of 0.5 μ*M* non-specific monoclonal immunoglobulin G. (■) Monoclonal antibody (0.1 μ*M*) from hybridoma 6D4 against **4** (anti-**4**). The superimposed curve represents a theoretical exponential decay that fits the data points.

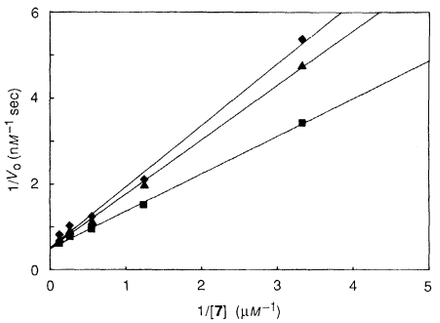

Fig. 4. Lineweaver-Burk plot for hydrolysis of substrate 7 by anti-4. Velocities were determined spectrophotometrically by measuring initial rates during the first linear portion of the reaction as described in Table 2. The substrate concentrations were corrected for amounts consumed during initial equilibration. (■) No inhibitor present. (▲) Inhibited by 50 n*M* phosphonate **3**. (◆) Inhibited by 100 n*M* **3**.

liver esterase (Table 1). It may represent the unfavorable electrostatics of the charged succinate interacting with the protein, or the disadvantage of a hydrophilic ligand binding to a hydrophobic active site.

The strict substrate specificity was indicated by the inability to detect accelerated hydrolysis with aryl esters having diverse substituent variations in the aromatic rings (Table 1). Ester **9** was not accepted as a substrate, which demonstrates the absolute requirement of the trifluoromethyl group (as was also observed in the stoichiometric reaction). The phenyl ester **10** was of approximately the same reactivity as 4-acetamidophenyl ester 7 and is more congruous with the haptenic structure than the coumarin ester **5**; yet here again, the antibody had no effect on the hydrolytic rate. Ester **11**, in which the trifluoroacetyl and the acetyl groups of structure 7 are interchanged, would allow an inverted orientation of the ester bond in the binding site, if the similarities of the phenolic and benzylic moieties allow this kind of interchange. However, accelerated hydrolysis of this ester was not observed. On the other hand, the hydrolysis of all these esters was accelerated by the indiscriminate esterase from hog liver. The chemical selectivity of this catalytic antibody may be considered a reflection of the binding specificity of immunological recognition.

It was desirable to have substrate **8** to ascertain that the saturation velocity observed with substrate 7 was not a consequence of its limited solubility in aqueous buffer solutions. The succinylated substrate **8** was freely soluble at concentrations up to 100 μ*M* in phosphate buffer (50 m*M*, *p*H 8.0), while solutions of ester 7 became slightly turbid at concentrations above 15 μ*M*. Reaction kinetics were measured spectrophotometrically by following the absorption change at 245 nm. The pseudo first-order rate showed enzyme-like saturation and the phosphonate ligands behaved as competitive inhibitors in Lineweaver-Burk analysis (Fig. 4). Kinetic parameters obtained with these substrates are tabulated along with the inhibition constants found with phosphonate **3** (Table 2). Under these conditions, the acceleration above the background rate was about 960-fold for substrate 7 and about 210-fold for substrate **8** (corrected for the background hydrolysis rate). The *p*H at which these measurements were made is probably not optimal. Preliminary indications suggest that this reaction is more sensitive to *p*H than the previously reported transacylation with activated esters. The catalytic reaction was nearly undetectable at *p*H 7.0. A greater rate difference may be expected for an ester containing all the epitopes of the hapten including the picolinyl group.

Indications that a histidine is critical to the activities of the esterolytic antibodies provided the earliest clue regarding the mechanism of transacylation. Despite the nucleophilic character of imidazole, there is no evidence that enzymes use the imidazole group of histidine for nucleophilic catalysis, but it is often implicated in general acid-base catalysis (*24*). The dual role of imidazole as a nucleophilic and general base catalyst in ester hydrolysis is well established (*21*). The transition between these mechanisms is determined by the relative rates of formation and breakdown of the two possible tetrahedral intermediates: that derived from addition of imidazole to the acyl group versus that from hydroxide addition. The relatively labile coumarin ester **5** may form an imidaz-

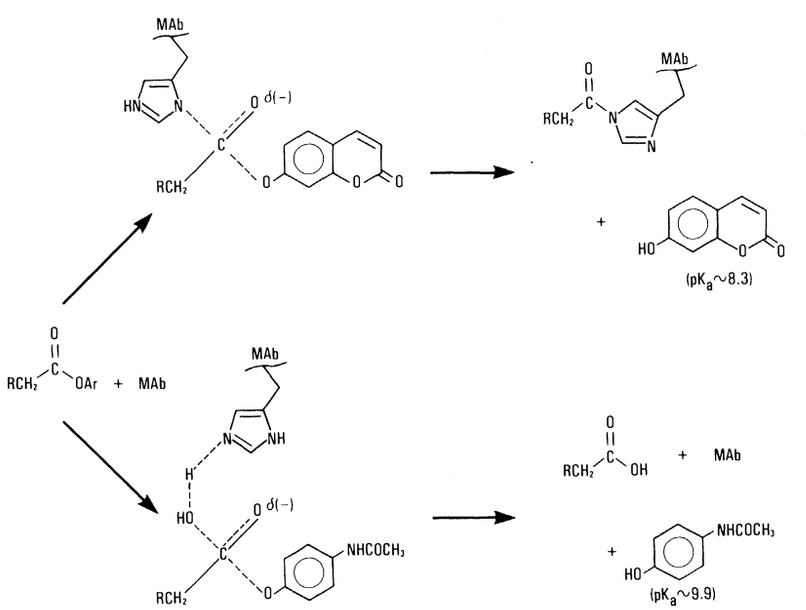

Fig. 5. A proposed scheme to account for the divergent chemistry observed in the reaction of an anti-4 monoclonal antibody (Mab) with carboxylic esters **5** and 7. A histidine residue in the combining site is presumed to act as a nucleophilic (upper pathway) or general base (lower pathway) catalyst during the formation and breakdown of a tetrahedral intermediate. The ester with a good leaving group reacts by the upper pathway since the rate-limiting step, formation of the intermediate, is facile. This pathway cannot be used by the ester with a poor leaving group since the rate-limiting step, breakdown of the intermediate, is not catalyzed relative to the analogous step in the lower pathway, which may be general-base catalyzed.

Table 2. Kinetic parameters for hydrolysis of esters 7 and **8** by monoclonal antibody. A Perkin-Elmer lambda 4B spectrophotometer, equipped with thermostatted cell holder, was used to measure absorption changes at 245 nm. Cells containing the substrate at concentrations of 0.5 to 50 μM in phosphate buffer (50 mM, pH 8.0) were preequilibrated at 25°C. The concentration of active immunoglobulin G in a stock solution was found by reacting with coumarin ester **5** and measuring the yield of hydroxycoumarin by fluorescence (*9*). The kinetic run was initiated by addition of an aliquot of the antibody stock solution (in 50 mM phosphate buffer, pH 8.0) calculated to give 100 nM immunoglobulin G. The mixture was allowed to equilibrate for 2 to 3 minutes and the rate was then measured during the subsequent 10 minutes. The absorption change for complete hydrolysis ($\Delta\epsilon$ 4500) was determined by treatment with esterase. Kinetic parameters were obtained from Lineweaver-Burk plots (Fig. 4). Inhibition constants were determined from a plot of the slopes with at least four concentrations of **3**. The data were analyzed by linear regression.

Substrate	K_m ($10^6 M$)	K_i ($10^7 M$)	V_{max} ($10^9 M$ sec^{-1})	k_{cat} (10^2 sec^{-1})	k_{cat}/k_{uncat}
7	1.90 ± 0.20	1.60 ± 0.40	2.2 ± 0.2	2.7 ± 0.2	960
8	0.62 ± 0.05	0.65 ± 0.25	1.0 ± 0.1	0.8 ± 0.1	210

ole adduct that could readily collapse to the acyl imidazole intermediate by loss of the coumarin alkoxide. This step would be more difficult with poor leaving groups that form less stable alkoxides. The 7-hydroxycoumarin (pK$_a$ ~8.3) is a substantially better leaving group than 4-acetamidophenol (pK$_a$ 9.9). The 4-acetamidophenyl ester 7 may, therefore, form a tetrahedral adduct with water or hydroxide and the breakdown of this is presumably catalyzed (Fig. 5). As evidence for the existence of separate mechanisms, we found that the product of the reaction of the antibody with ester **5** was not an intermediate in the catalytic reaction with 7. Indeed, **5** acted as a specific inactivator of the catalyst when it was added to a mixture of the antibody and ester 7. The two esters were distinguished with considerable fidelity, as the antibody was observed to turn over several hundredfold with substrate 7 without noticeable inactivation. Catalysis by the antibody through both mechanisms implies that the binding interactions can stabilize either transition state in these two-step processes. However, only the general base process (rate-limiting breakdown to products) is relevant to the transition state defined in our experiment.

The contribution of binding to catalysis by this general base mechanism is illustrated best by the different behavior of 4-acetamidophenyl ester 7 and phenyl ester **10**. Phenol as a leaving group (pK$_a$ 9.89) is equivalent to 4-acetamidophenol, yet the hydrolysis of **10** was not catalyzed by these antibodies. Neither was a stoichiometric reaction apparent, although **10** has the correct structure for the acyl group. Therefore, though this ligand may bind to the protein, the interaction is not proper for the expression of the inherent esterase function. The effect of binding to the acetamide group of 7 appears to be sufficient to stabilize the rate-limiting transition state. Further refinement of the substrate structure, as in the addition of the picolinate substituent, will reveal the full extent of the binding interactions in catalysis. In this system, there is the disadvantage that the low K_m values may eventually overwhelm the contribution of additional binding interactions to catalysis. The transition state complementarity criterion can assure that k_{cat}/K_m will tend to be maximized, while at a given value of k_{cat}/K_m the maximization of the rate (k_{cat}) depends on the poor binding of substrate (high K_m) (*1*). A future challenge may be to determine how to further differentiate between transition state and substrate in immunological binding.

The transition state analog designation is accepted cautiously in the study of enzymes (*12, 25*) since neither the catalytic mechanisms nor the mode of interaction of enzymes with their ligands is understood well in most cases. By eliciting the expected chemical activity in an immunological receptor, the phosphonate structure behaves in agreement with its role as a transition state analog, providing persuasive evidence for the theoretically derived principle of enzyme-transition state complementarity. That ability is unique and independent of the physiological origins of enzymic catalysis.

The direction of further work is not dependent on a detailed understanding of existing enzymes. The catalysis of any reaction for which a credible mechanism can be formulated may be given attention. However, information from the study of enzyme mechanisms has been useful in designs for artificial catalysis. Enzymes in their incipient form are nothing but specialized protein molecules, as are monoclonal antibodies of unique specificity. Proteins alone cannot exhibit all the chemistry of life processes. Organisms have, therefore, evolved to import extracellular components that, along with proteins, form the vast array of enzymic activities. Similarly, it will be necessary to involve cofactors in order to increase the range of chemical reactions accessible to "abzymes"; one possibility would be to include metal chelation in transition state binding. Cofactors found in nature should be useful models for defining systems in which immunological binding may be brought to bear on the catalytic reactions.

Techniques for obtaining antibodies of desirable specificities have led to a broad range of applications in medicine and biology. These all use the common function of antigenic recognition in some coupled fashion to associate other activities or properties with the antibody-antigen complex. The simple binding interaction is thought to be an invariant property of antibodies. In these studies, we demonstrate how knowledge of chemical mechanisms can be used to harness the potential energy of antibody-antigen binding to perform a new, kinetic function. The success of this basic inquiry should encourage the application of mechanism-based design to evoke more interesting and perhaps useful proteins from the immune system (*26*). For example, the ability to impart hydrolytic activity to anti-peptide antibodies of predetermined specificity would provide site-specific reagents or catalysts for protein chemistry. As the implications of binding energy in catalysis become widely appreciated, the extraordinary properties of antibodies will continue to gain prominence in fundamental and applied research efforts.

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Selective Chemical Catalysis by an Antibody

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The immunoglobulin MOPC167, which binds the transition state analog *p*-nitrophenylphosphorylcholine with high affinity, catalyzed the hydrolysis of the corresponding carbonate 1. MOPC167 catalysis displayed saturation kinetics with catalytic constant (k_{cat}) = 0.4 min^{-1} and Michaelis constant (K_m) = 208 μ*M*, showed substrate specificity, and was inhibited by *p*-nitrophenylphosphorylcholine. The rate of the reaction was first order in hydroxide ion concentration between *p*H 6.0 and 8.0. The lower limit for the rate of acceleration of hydrolysis by the antibody above the uncatalyzed reaction was 770. This study begins to define the rules for the generation of catalytic antibodies.

A KEY ELEMENT IN THE SYNTHESIS of enzymatic catalysts is the rational design of highly selective substrate binding sites. Current efforts in this regard focus primarily on two strategies: the synthesis of cavity-containing organic compounds and the modification of enzyme specificities by oligonucleotide-directed mutagenesis. With the advent of monoclonal antibodies, homogeneous ligand-binding sites with enzyme-like binding affinities and specificities can be generated for most biologically active macromolecules as well as smaller synthetic molecules. This property has made antibodies one of the most important classes of receptors in biology and medicine today, finding applications in diagnostics, drug delivery, and protein and nucleic acid purification and characterization. The development of strategies for introducing catalytic activity into the combining sites of immunoglobulins could potentially provide general routes for the construction of enzymatic catalysts with tailored specificities and catalytic properties.

One mechanism whereby enzymes are believed to act as highly specific catalysts is by providing an environment complementary in structure and electronic distribution to a rate-limiting transition state of a given reaction. This hypothesis is supported by crystal structures of a number of hydrolytic enzymes as well as by studies of enzyme inhibition by transition state analogs (*1, 2*). One might then expect that the highly specific binding interactions of antibodies could be exploited to catalyze chemical reactions by a similar mechanism. An antibody elicited to a haptenic group resembling the presumed transition state of a given reaction should lower the free energy of activation by stabilizing the transition state relative to reactants or products (*3, 4*). Such an approach, in addition to affording selective catalytic antibodies, may provide additional insight into mechanisms of enzyme catalyzed reactions.

In order to determine the viability of this strategy, we and others (*5, 6*) have begun to investigate the catalytic properties of antibodies specific to transition state analogs for the hydrolysis of esters and carbonates. We chose these well-characterized reactions in order to simplify mechanistic studies of ligand binding and catalysis and because tetrahedral phosphonates and phosphonamidates have been reported to act as transition state analog inhibitors for enzymatic peptide hydrolysis (*7–10*). Our initial studies have therefore focused on immunoglobulins specific for phosphonate and phosphate tetrahedral transition state analogs. We have succeeded in characterizing two catalytic antibodies, one that hydrolyzes *p*-nitrophenyl *N*-trimethylammonioethyl carbonate and a second that hydrolyzes methyl-*p*-nitrophenyl carbonate. The latter catalytic antibody was generated by specifically eliciting monoclonal antibodies to the corresponding *p*-nitrophenyl phosphonate transition state analog (*6*). However, to begin to understand in detail the mechanism of antibody catalysis so that we can develop rules and guidelines for generating more sophisticated catalysts, we have focused our attention on the immunoglobulin A (IgA) MOPC167 (*11, 12*).

MOPC167 binds nitrophenylphosphorylcholine, a transition state analog for the hydrolysis of *p*-nitrophenyl *N*-trimethylammonioethyl carbonate chloride **1** with association constant (K_a) = 1.4×10^6 M^{-1} (*13*) (Fig. 1). MOPC167 is a member of a well-characterized class of antibodies specific for phosphorylcholine (PC) mono- and diesters (*14–16*). These antibodies share a high degree of sequence homology among amino acids in the heavy chain (*16–18*), show a high affinity for phosphorylcholine and related ligands (*19, 20*), and can be obtained easily in large quantities. More-

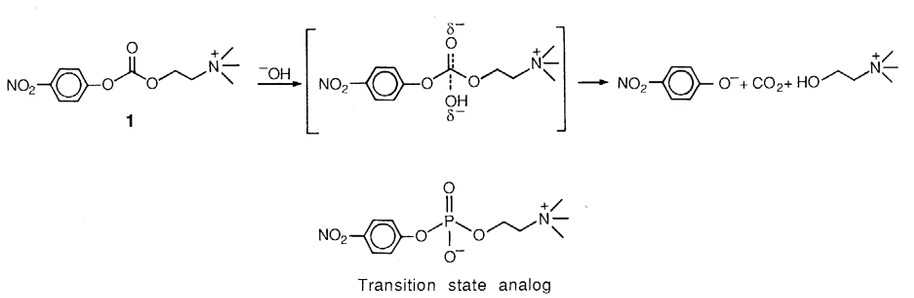

Fig. 1. Transition state analog for aqueous hydrolysis of carbonate **1**.

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