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The problem of amide hydrolysis using catalytic antibodies was analyzed and attacked. A novel, direct-screening for catalysis method was developed which allows detection of such antibodies in hybridoma supernatants. The method (catELISA) involves a solid-phase substrate and an anti-product ELISA assy; it was found to be general and highly effective using ester-hydrolyzing (anti-phosphonate hapten) antibodies. By employing autoimmune-prone mouse strains, vastly increased numbers of catalytic antibodies for ester hydrolysis were produced. All attempts using catELISA and these exotic mouse strains to obtain amide- or peptide-hydrolyzing antibodies by immunization with simple phosphonate haptens as transition state analogs failed. Therefore, the synthesis of new, more sophisticated transition state analogs as haptens was carried out. 2-Aminoalcohols, aminophosphinates, cyclic phosphinates and phosphonimides were pursued; haptens for hydrolysis of acyl-proline, phenylalanyl amides were prepared. The many hybridomas studied using these haptens failed to produce amide-hydrolyzing catalysts. Our results emphasize the need for additional approaches to attack this challenging problem. However, the results *do* show that powerful antibodies can be produced, at least for ester bonds, and they have made available new technologies that may eventually afford sequence-specific catalytic antibodies.

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Bernard Green 15 April 1995

PI - Signature Date

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INTRODUCTION

When the proposal for which the research described here was written, in 1989, the field of catalytic antibodies was just at its beginning -- despite the number of examples of antibody catalyzed chemical reactions which had already been reported. We set out to perform sequence specific peptide hydrolysis, which may perhaps be termed (still today!) the Holy Grail of catalytic antibody research. The success in the reactions that had been reported by others and ourselves, and the extraordinary leap forward that catalytic antibodies presented, both in concept and in practice, compared to virtually all other attempted approaches to mimic enzymes, gave us great confidence. The choice of the bond to be cleaved, the asp-pro peptide bond, was, as outlined in the original proposal, based on this being the most labile of the peptide bonds and involving a relatively well-understood reaction and one which, in related chemical systems, had been extensively studied. The presence of this bond in a number of important toxins suggested that we had a direct and novel solution to an important problem of detoxification.

In fact, Nature has proven to be far more difficult than we anticipated. We have not yet succeeded in peptide hydrolysis and, indeed, our research has emphasized the exquisite problems involved in achieving peptide hydrolysis, allowing one to admire (as one constantly does in any event!) the power of the naturally-evolved enzymes. On the other hand, this research *did* make path-breaking progress in areas that are intrinsically important and will, we believe, provide the stepping stones that *will* eventually allow us to reach the desired goal: efficient catalytic antibodies for sequence-specific peptide hydrolysis.

In this report we outline the directions we took, the substances that we prepared, and the catalytic antibodies that we prepared. As can be noted in the appendix of this report, six publications, based on this research and acknowledging the support of the USAMRDC, have already been published in first-rate journals; one paper has been submitted for publication and two additional publications are being prepared. These include: the first successful direct screening for catalysis approach (1), which we believe to be milestone in catalytic antibody research (this has now been used successfully by other researchers, as well (2), and is widely quoted; a new, more simple approach to transition state analogs and their protein conjugates (3); the first detailed comparison of a family of catalytic antibodies and the correlation of their biochemical properties with their structures, as provided by the amino acid sequences of their combining sites (4); the exciting finding that autoimmune-prone mice provide vastly increased numbers of catalytic clones compared with normal mice (5), a result which may have importance for autoimmune studies; the production of the most active catalytic antibodies thus far reported, in terms of kcat/Km and in numbers of turnovers (6). Papers in preparation describe the synthesis of the novel haptens, often challenging organic structures, that were prepared and studied in the course of this research.

BODY OF REPORT

Hydrolysis of an unactivated amide or peptide bond represents a reaction proceeding via a high activation energy barrier (the half life of an ordinary peptide bond is about 9 years). This challenge therefore required us to first accomplish a number of hitherto unreported tasks. First, we had to develop an assay which would allow the direct screening of hybridoma supernatants for catalytic activity (in order to be able to readily detect any catalysts that were produced); second, we had to design and synthesize new transition state analogs (TSAs) for amide and peptide hydrolysis, and, examine their ability to elicit catalytic antibodies; third, we chose to investigate new approaches to antibody-catalyzed amide hydrolysis utilizing substrate-assisted or intramolecular catalysis (this was the basic notion of asp-pro cleavage, as outlined in the original proposal); finally, we felt it was important to better understand the function and mechanism of catalytic antibodies and study these aspects as well.

We initially describe what might be considered a *technological* improvement, the invention of an assay which allows the direct screening of hybridoma supernatants for catalytic activity following fusion (Section 1; refs. 1,7). The feasibility of this new approach, which we termed catELISA, was demonstrated by the generation of potent ester-hydrolyzing antibodies by directly screening thousands of individual hybridomas after fusion (Sections 1 and 2). CatELISA replaced existing awkward methods which allowed the screening of only a limited number of hapten-binding clones (after they were individually propagated in ascites and purified). The approach is general and is not limited to a certain type of reaction; it was adopted by us, and by others (2), to screen a variety of cleavage reactions as well as for bimolecular associative reactions (Section 6). CatELISA has made the search for catalytic antibodies less laborious and less risky, enabling us to better examine some fundamental questions of this rapidly evolving, yet still poorly understood field.

Next, the design and synthesis of transition state analogs directed to amide hydrolysis was called for. These compounds represent a challenge in their design; very little was known about the structural requirements for haptens aimed to generate hydrolytic (or any other type of) antibodies. Furthermore, none of the transition state analogs examined thus far, e.g., phosphonates, afforded peptide-hydrolyzing antibodies (see Section 4). We first examined the cleavage of X-Pro bonds because of their low energy of barrier of twisting. The haptens were designed to induce strain and stress on the bond to be cleaved, to mimic the tetrahedral carbon and the oxyanion formed by nucleophilic attack, and to mimic the protonated amino leaving group (see Section 4). The synthesis (Section 9.2 and 9.3) of β-aminoalcohol and aminophosphinate haptens and the attempts to generate antibodies that catalyze the cleavage and the formation of the corresponding X-pro bonds (Section 4) is described. Also described is the design and synthesis of cyclic phosphonamidic acids TSAs aimed at catalyzing the intramolecular deamidation of asparagine and glutamine residues (Section 8.5). Altogether, more than 100 new compounds, haptens, inhibitors and substrates were synthesized, characterized, and conjugated to carrier proteins. In some cases, their synthesis alone are significant achievements (Section 7; ref. 30).

The catELISA approach was adopted for the screening of monoclonal antibodies elicited against each of these haptens. We screened for antibodies that catalyze the cleavage and formation of -X-Pro- (Sections 8.1, 8.3), or for intramolecular, or substrate assisted, deamidation reactions (Section 8.5). Despite these considerable efforts, antibodies that catalyze these reactions have not yet been identified. (The pursuit of amide hydrolyzing antibodies will continue; see Section 10.)

The catELISA technology has provided novel insights to antibody catalysis. Detailed analysis of a large repertoire of ester-hydrolyzing antibodies has generated some new ideas about antibody catalysis. For example, the first evidence that supports the design of expanded TSAs for generating more efficient esterolytic antibodies was provided - a highly efficient and selective p-nitro*phenyl* ester hydrolyzing antibody (with a k_{cat}/Km of 11,000 $s^{-1}M^{-1}$) was elicited against a p-nitro*benzyl* phosphonate hapten (Section 7).

Another interesting aspect regards the possible *in-vivo* significance of catalytic antibodies. It is commonly accepted that the occurrence of catalytic antibodies is generally low, i.e., typically, only a few catalytic clones are obtained from the several dozen that bind the TSA-hapten (1, 8-10). This was further emphasized when, for the first time, the entire repertoire of hybridomas obtained after immunization with a TSA-hapten could be directly examined for catalytic activity. Nine ester hydrolyzing clones were identified from 970 hapten-binding clones (i.e., an occurrence of about 1%; see Section 1). All attempts described thus far to generate catalytic antibodies were performed using normal mouse strains that are routinely used to generate monoclonal antibodies (e.g., Balb/c). We wanted to see whether different repertoires of catalytic antibodies (in number and in type) could be obtained in different strains of mice. The hope was that these different strains would provide amide-hydrolyzing clones that were not found in normal mouse strains. Quite unexpectedly, we found hundreds of esterolytic clones in certain mouse strains (SJL and MRL/lpr; Section 3). A possible link to autoimmunity was suggested by the fact that the appearance of high numbers of catalytic clones is the result of the *lpr* mutation (i.e., a normal, low occurrence is observed in the parental wild type strain, MRL/++) which causes a defect in the Fas-mediated programmed cell death, or apoptosis, during the selection of T- and B-cells.

1. CatELISA - a new route to catalytic antibodies

Catalytic antibodies are elicited against a hapten, typically, a stable synthetic analog of the transition state (TS) of the catalyzed reaction. The repertoire resulting after immunization is immortalized as hybridomas (11) that are then screened to select those clones producing monoclonal antibodies that bind the hapten. The *direct* screening of these hybridomas for antibody catalysis in the growth media was heretofore not possible due to relatively high background reaction, the generally low catalytic efficiency of antibodies, and enzyme contaminations that catalyze the same reaction (7). Therefore, in order to detect catalytic activity, large quantities (usually from ascites) of purified monoclonal antibodies are needed. Only a few, and maybe none, of the dozens of clones that bind a hapten are catalytic; it is

therefore widely recognized that these inefficient and labor-intensive procedures must be replaced by rapid and direct screening procedures (8, 12-14). Novel, non-hybridoma, methodologies, such as combinatorial variable-region cloning in phage (15-17), have also been used to generate antibodies. Yet, as noted (17,18), future applications of these methodologies for obtaining catalytic antibodies also depend upon appropriate screening.

Following an analysis of the basic problems involved in the detection of antibody-mediated catalysis (7), we found that increasing the efficiency of detection (i.e., increasing the antibody-catalyzed signal relative to the noise, or the uncatalyzed background reaction) of catalytic antibodies that are present at low concentrations such as in hybridoma supernatants could *not* be achieved by just using a very sensitive assay for the detection of the product. Efficiency of detection could be maximized by: (i) increasing the affinity to the substrate (i.e., decrease its Km) and, (ii) by reducing substrate concentrations to pseudo-first -order conditions (So <<Km, Fig. 1).

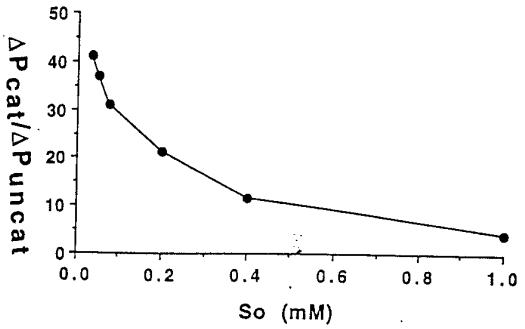


Fig. 1. Signal/Noise ratio ($\Delta P_{cat}/\Delta P_{uncat}$) was determined by measuring initial velocities of the hydrolysis of a p-nitrophenyl ester substrate (0.05-1 mM) in the presence of a catalytic antibody (CNJ157, k_{cat}=2.39 min^{-1}, Km=0.11mM; $[Ab]_o$= 5.5 μM), and a non catalytic antibody under the same conditions.

This analysis led to *catELISA*, an assay based-on a substrate-protein conjugate immobilized on microtiter plates (Fig. 2). The use of a solid-phase substrate meets the two above requirements:(i) the catalytic antibody has increased affinity for the substrate that includes the protein carrier structure (i.e., lower Km values), (ii) the very low concentrations of immobilized substrates ensures that pseudo-first order conditions are maintained (Km>>So). Antibody-catalysed conversion of any "solid-phase" substrate to a product is then detected by ordinary ELISA, using binding, anti-product antibodies.

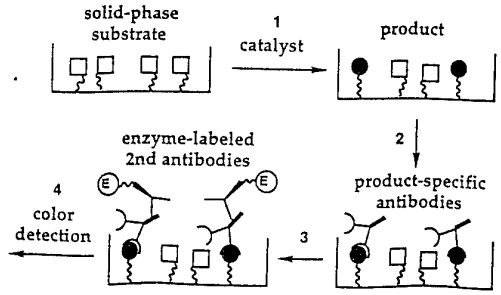


Fig. 2. Schematic presentation of catELISA.

The first system was designed to study a variety of antibody-catalysed hydrolytic reactions. Cleavage of immobilized esters **1a-g**, amide **2** or imide **5** leaves the carboxylic acid product **3** bound to the solid-phase (Fig. 3). Anti-product **3** polyclonal rabbit antibodies which do not cross react with any of these substrate conjugates were used for detection.

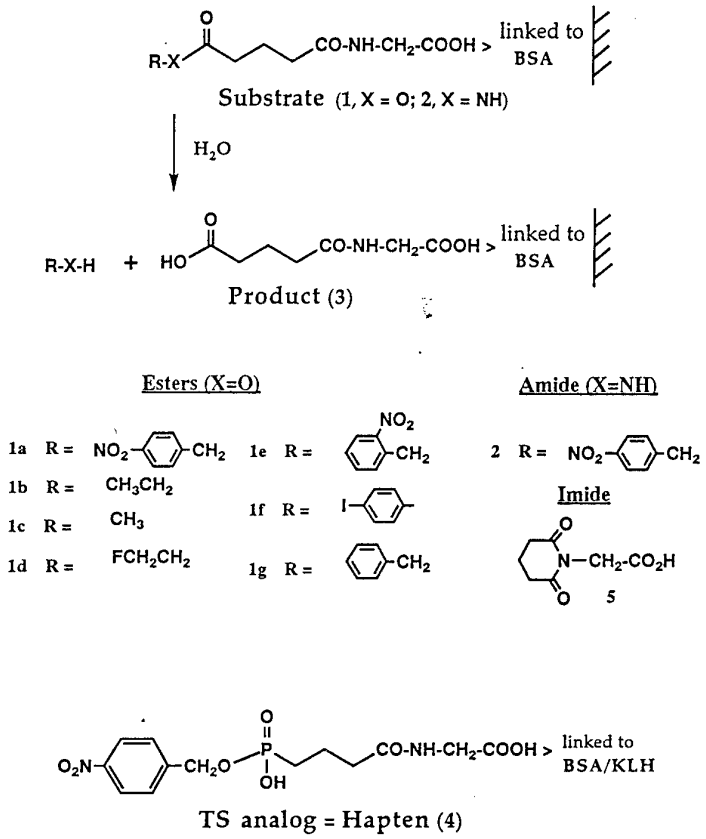


Fig. 3. The DEA system: Structures of substrates; esters **1a-g**, amides **2**, and imide **5**, the product of their hydrolysis, acid **3**, and phosphonate haptens, **4 a-d**. All of these substances were linked to a carrier protein (BSA or KLH) via the carboxyl group; the protein conjugates appear in the text as, for example, **1a-BSA**.

In order to analyse the various parameters of the catELISA we studied the enzyme- or base-catalyzed hydrolysis of esters **1a-d** and imide **5**. The BSA conjugates of these substrates, coated on microtiter plates, were treated with mild base (sodium carbonate, 0.1M, pH 10.9) or pancreatic lipase for various time periods. Formation of the resulting acid product **3** was then determined by conventional ELISA using the rabbit anti-**3** antibodies followed by peroxidase-linked, anti-rabbit antibodies (Fig. 4).

No signal is observed with amides **2a-c** which represent substrates that are stable to base or to lipase-catalyzed hydrolysis. As expected, imide **5** is hydrolyzed in the presence of sodium carbonate ($t_{1/2}$= 45 min compared with 90 min for the methyl ester, **1c**) yet not by the lipase. The rates of hydrolysis of esters **1a-d** in the presence of various dilutions of lipase reach saturation with increasing enzyme concentrations (Fig 4). This is ascribed to the very small amount of substrate that is coated on the solid phase (10-50 picomoles/well). The kinetics of the enzyme- and

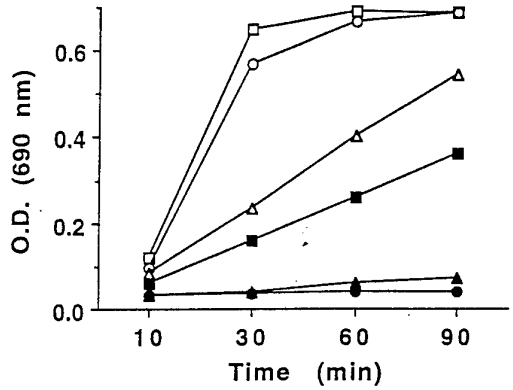


Fig. 4. Base- and enzyme-catalysed hydrolysis of ethyl ester **1b-BSA** was followed by catELISA. Conjugate **1b-BSA**, coated on microtiter plates (0.5 μg/ml) was treated for increasing time periods with: lipase (porcine pancreatic, crude extract, Sigma L-3126: 1.4 mg/ml, □; 0.35 mg/ml, o; 0.07 mg/ml, △) diluted in PBS; 0.1 M Na_2CO_3, pH 10.9,■; TBS, pH 8.25, •, and p-nitrobenzyl ester-hydrolyzing antibody D2.3, 50 nM, ▲. The plates were then washed with phosphate buffered saline (PBS, 10mM sodium phosphate, 0.14M NaCl, pH 7.4) + 0.04% Tween and the rabbit anti-product **3** serum was added (1:5,000 dilution in PBS + 0.04% Tween, 1 hour). After washings and incubation with peroxidase-linked mouse anti-rabbit antibodies (Jackson, diluted 1:5,000 in PBS) the substrate, 2,2'azinodi(3-ethyl benzthiazoline sulphonic acid) (ATBS) was added and the absorbance at 690 nm was measured.

base-catalyzed reactions shows that as little as 5% of substrate conversion to product can be measured. The activity of less than 0.01 units of lipase can be readily detected even though ester substrates such as **1a-d** which are far from optimal structures for this enzyme were used. These studies also indicated that esters **1a-d** are stable in the presence of most esterases that hydrolyze the soluble, unconjugated esters (e.g., acetyl choline esterase, porcine liver esterase). The poor accessibility of solid phase substrates to enzymes (which was found in additional catELISA systems we have established, see Section 6) allows the screening of growth media and other biological fluids which contain enzymes that catalyze the same reaction. These and additional experiments indicated that catELISA is sensitive, selective, and can be applied to directly screen of catalytic antibodies in hybridoma supernatants.

To establish the use of catELISA for the generation of catalytic antibodies we first used phosphonate haptens. These compounds were chosen primarily because of the demonstrated ability of phosphonoesters to mimic the tetrahedral, negatively charged, TS/intermediate of ester hydrolysis and to elicit ester-hydrolysing antibodies.

The first haptens (i.e., TSAs) tested were the alkylphosphonates **4b-d** (structure **4** in Fig. 3, where the R groups replace the p-nitrobenzyl group). The ethyl and fluoroethyl phosphonates (**4b** and **4d**, respectively) failed to elicit any hapten-specific binding antibodies in Balb/c mice (the synthesis of these haptens is described in Section 9.1; see also ref. 3). On the other hand, following the immunization with the methylphosphonate, **4c-KLH**, significant binding activity to the corresponding BSA conjugate was observed in the mice sera. Inhibition of binding by the free, unconjugated, hapten (**4c**) indicated the specificity of the antibodies.

Fusion of the spleen cells of one of the responding mice with NSO myeloma cells followed by screening of the 758 resulting hybridomas for binding of **4c-BSA**

conjugate gave 20 positive clones. However, among these 20 independent clones, only one clone was found to specifically bind the hapten (and not the carrier protein, BSA) but it was, unfortunately (but not unexpectedly, considering the low probability of catalysis), not found to be catalytic.

The results obtained by immunization with alkylphosphonates, which fail to induce a sufficiently strong immune response in the mouse strains we have used, emphasize that highly antigenic TSAs should be used in order to generate a large number of hapten-binding clones, out of which few catalytic ones may be selected (10). The antigenicity of aromatic compounds, and in particular of nitrophenyl derivatives, was widely exploited for the generation of catalytic antibodies. In fact, all but two (19,20), of the haptens used to generate catalytic antibodies thus far included an aromatic residue (21). This point was taken into account in the design of additional haptens targeted for amide hydrolysis (Section 8).

p-Nitro*benzyl* esters, unlike p-nitrophenyl esters, are not activated substrates and are therefore hydrolyzed, under neutral or basic conditions, with rates similar to alkyl esters. Studies of the p-nitrobenzyl ester substrate **1a** (Fig. 2) and amide **2a** indicated that they do not cross-react with the rabbit anti-product **3** serum and that the ester is indeed stable enough to give satisfactory results using catELISA. The corresponding TSA, i.e. p-nitrobenzyl phosphonate **4a**, was prepared and its KLH conjugate (**4a-KLH**) was used to immunize BALB/c mice. As expected, high binding titers were observed as early as 24 days following immunization. Spleen cells from a mouse immunized with the p-nitrobenzyl phosphono ester conjugate, **4a-KLH**, were fused with myeloma cells and the resulting 1570 hybrid clones (970 of which were hapten-binding clones) were assayed by catELISA for their ability to catalyze the hydrolysis of ester **1a** and amide **2a**. Representative data from the screening of the initial hybridoma supernatants is given in Fig. 5. As can be seen, although dozens of clones bind the phosphonate hapten, only two scored positive using catELISA on the microtiter plate illustrated. Altogether, 9 clones that catalyze the cleavage of ester **1a** were identified.

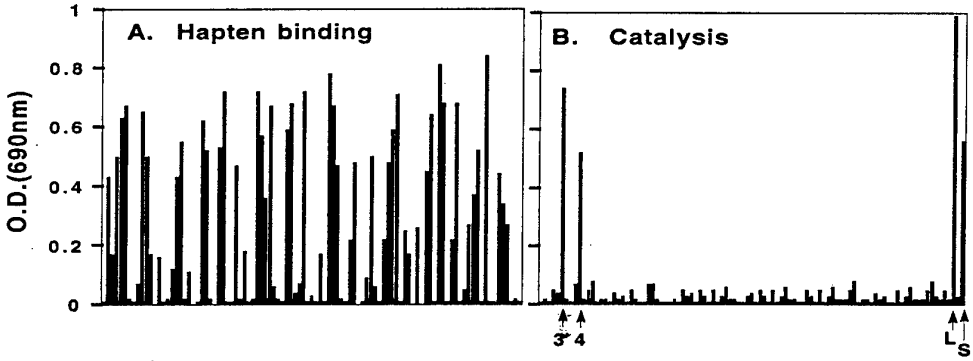


Fig. 5. Results of screening of one microculture plate (96 hybrid clones) for hapten binding,**A**, and for catalytic activity, **B**. Hapten binding (**A**) was determined by ELISA using 50 µl of hybridoma supernatants in microtiter plates coated with **4-BSA** (1µg/ml). CatELISA (**B**): The hybridoma supernatants (50µl) were incubated for 3 hours in microtiter plates (Nunc, Maxisorb) coated with the p-nitrobenzyl ester substrate **1a-BSA** (hapten density =15, 1.0 µg/ml, 1 hour, further blocked with BSA, 1mg/ml, 0.5 hour). Cleavage of ester **1a** was then detected using the rabbit anti-**3** sera (see legend of Fig. 3). The last 3 columns are controls: lipase (50µg/ml, OD = 1.0), **L** ; TBS pH 8.3; and 0.1M sodium carbonate, **S**. Two catalytic clones, D2.3 (**3**) and D2.4 (**4**) were identified from this plate.

Control experiments provided conclusive evidence that the *catELISA* signal observed in the presence of the hybridoma supernatants indeed results from the antibody-catalysed hydrolysis of the p-nitrobenzyl ester substrate, **1a**. None of the selected clones gave a catELISA signal when esters other than the p-nitrobenzyl ester (**1b-d**) were used as substrates (e.g., antibody D2.3 on **1b**, Fig. 3). The affinity pattern of the catalytic site (Fig. 6) is in accord with that expected for an antibody elicited against hapten **4a**, i.e., hapten **4a** > "short" hapten > amide substrate > p-nitrobenzyl alcohol (product). Indeed, the same order of affinity was observed when the inhibition of *binding* of these antibodies to the hapten-BSA conjugate was measured by ordinary competitive inhibition immunoassay (CIEIA; for a similar analysis, see ref. 22). Product inhibition, pH-activity profiles (Fig. 7), substrate specificity and other properties that characterize the overall quality of these antibodies as catalysts were all conveniently determined by catELISA prior to the purification of the antibodies.

Purified antibodies from ascites were assayed by HPLC for their ability to catalyze the hydrolysis of the free, unconjugated p-nitrobenzyl ester **1a**. The results confirmed the catELISA data. The identification of clones exhibiting relatively low rate accelerations (e.g., antibody clone D2.1: k_{cat}/k_{uncat} ~ 700, at pH 9.0), underlines the sensitivity and selectivity of the catELISA; neither "background" hydrolysis of the ester substrate nor the presence of enzymes in the hybridoma supernatants interfered.

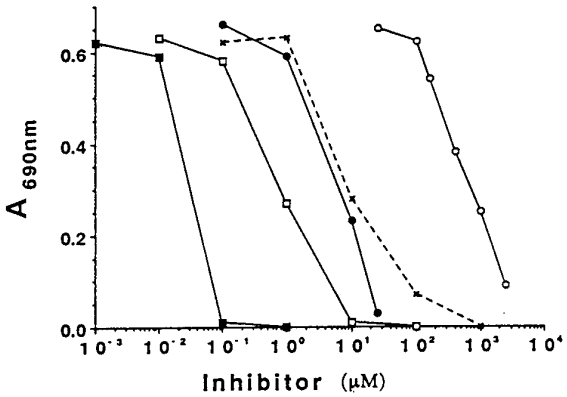


Fig. 6. The inhibition pattern of esterolytic activity of D2.3 was determined by *catELISA* . Antibody D2.3 (protein A purified preparation, 20 nM in TBS pH 8.25) was incubated on mictotiter plates coated with **1a-BSA** in the presence of various dilutions of: phosphonate hapten **4** (4-N-benzylamide), ■ ; a "short" hapten (mono-p-nitrobenzyl methyl phosphonate), ◘ ; amide substrate **2** (2-N-methylamide), • ; and p-nitrobenzyl alcohol, **o**. Inhibition of the signal was also observed when the added rabbit sera (applied after incubation with the catalytic antibody) contained product **3** (3-N-methylamide), x, dashed line.

CatELISA is an efficient and specific detection for catalytic antibodies mainly because antibodies have increased affinity for substrates which include the protein carrier structure (i.e., lower Km values) and because of very low substrate concentrations that ensure pseudo-first order conditions (Km>>So). As predicted, these two factors directly increase the ability to detect antibody-mediated catalysis (23). The signal observed due to antibody catalyzed hydrolysis of ester **1a** (ΔP_{cat}, or $\upsilon_{o\ cat}$) over the background (ΔP_{uncat}, or $\upsilon_{o\ uncat}$; i.e., under the same conditions in the presence of a non-catalytic antibody) is dramatically increased by catELISA (Fig. 8).

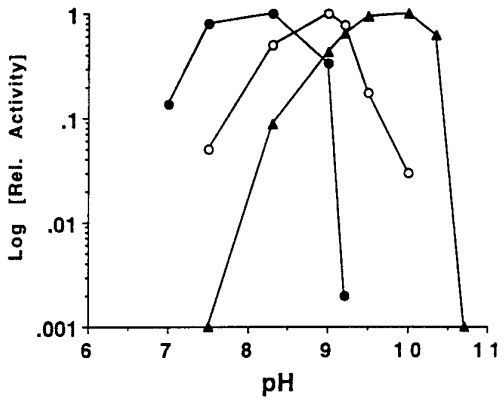


Fig. 7. The pH-rate profiles of antibody D2.3 (•), and of antibody D2.5 (o) and F8.11 (see Section 2.3;), were examined by catELISA. Protein A purified antibodies (20-50 nM) diluted in buffers of various pHs were incubated for increasing time periods in microtiter plates coated with **1a-BSA**. Hydrolysis of ester **1a** was followed as described (legends of Fig. 3 and 4). Relative activities were determined using data taken from initial velocities (t < 15 mins); under catELISA conditions, i.e., S_o<<Km, this activity corresponds to k_{cat}/Km.

For example, catELISA with **1a-BSA** gives a clear and significant signal, both at pH 8.25 and 9.0, using hybridoma supernatants containing antibody D2.5 (Fig 7A, υ_o cat/υ_o uncat = 50 and 80). The rate enhancement observed by conventional assay with the free, unconjugated, **1a**, (using a protein A purified preparation and HPLC for detection and quantitation of the released product) is orders of magnitude smaller (Fig. 7B, υ_o cat/υ_o uncat at pH 8.25 ~ 1.0, and at pH 9.0, ~2.5) even though the concentration of the antibody used was at least 50 fold higher than in the supernatants used for the catELISA. This difference is attributed mainly to the lower Km and So of solid-phase **1a**, and only partially to the higher sensitivity of the ELISA detection; thus, solid-phase substrates are favorable for the screening of antibody-mediated catalysis, regardless of the method used to detect the product of the reaction. Other detection methods, such as measuring the release of a fluorescence, radioactive or biotin-labelled product from an immobilized substrate may also be applied. The generality of the catELISA approach was demonstrated by its application to detect other cleavage (i.e., dissociative) reaction or bimolecular, associative, reactions (Section 6; ref. 2).

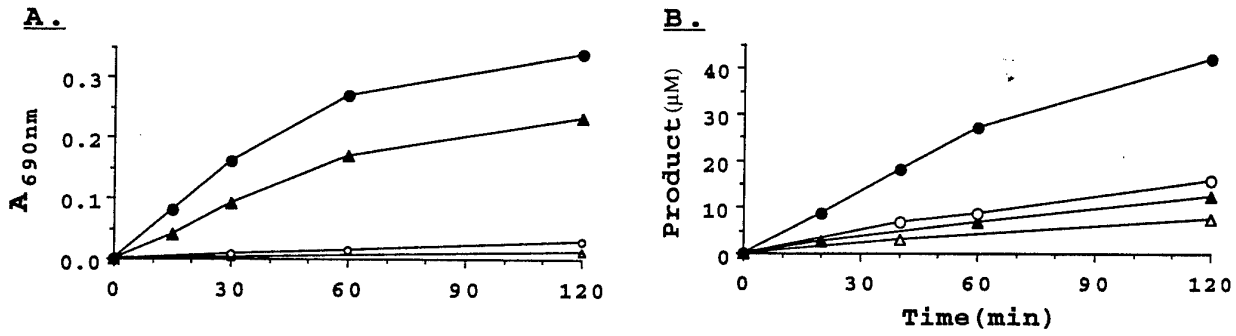


Fig. 8. Rates of hydrolysis of ester **1a** by: catalytic antibody D2.5 (full symbols), and in the presence of a non-catalytic antibody (empty symbols) were assayed by: **A**, catELISA on microtiter plates coated with **1a-BSA** using culture supernatants; and by, **B**, HPLC using **1a**, unconjugated (0.3 mM), and a protein A purified preparation of antibody D2.5 (3.5 μM). The suprnatants of, or purified, antibodies were dialyzed beforehand against 50mM TBS pH 8.3 (▲, △) or pH 9.0 (•, o).

2. The identification of efficient ester-hydrolyzing catalytic Abs

Future application of catalytic antibodies will require more efficient catalysts than are presently available (10). CatELISA allows the facile selection from large repertoires and is therefore an important tool on the way to the identification of truly efficient, enzyme-like catalysts. Antibody D2.3 is the best catalytic antibody for hydrolysis of **1a** identified among the 9 clones obtained from the first fusion described above, it exhibits: *i)* a significant rate enhancement (k_{cat} = 7.35 min^{-1}, Km = 280 μM, k_{cat}/Km = 440 $s^{-1}\cdot M^{-1}$, k_{cat}/k_{uncat} = $2.6x10^5$) with an unactivated ester substrate, and *ii)* multiple turnovers (> 300, see Fig. 9). Compared to all other catalytic antibodies elicited against a TSA-hapten that catalyze the hydrolysis of *unactivated* ester substrates, i.e, benzyl or alkyl esters, including a vinyl or a 2-fluoroalkyl ester, D2.3 exhibits the highest rate acceleration (Table I).

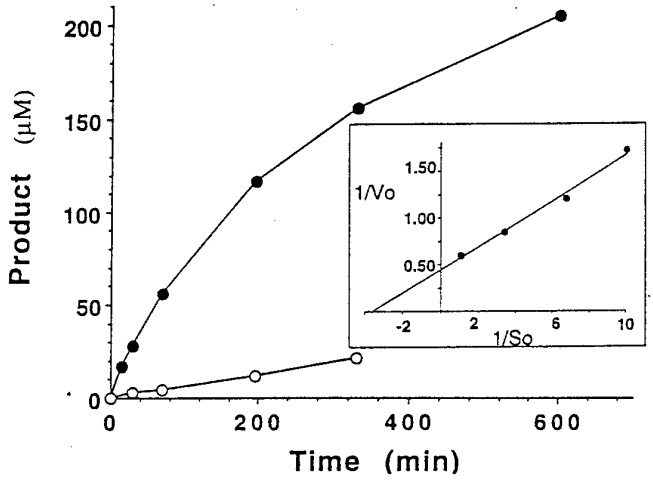


Fig. 9. Hydrolysis of the unconjugated ester substrate **1a** (0.3 mM) by monoclonal antibody D2.3 (0.3 μM) (•) was determined by HPLC;[5] activity is inhibited in the presence of hapten **4** (4-N-benzylamide, 1.0 μM), o. Insert, kinetic parameters determined by Lineweaver-Burk analysis performed with **1a** at pH 8.25 (Ab_o = 0.3 μM, $1/S_o$ is given in mM^{-1} and $1/v_o$ in $min\cdot\mu M^{-1}$).

The advantage of D2.3 is not only in rate enhancement, i.e., k_{cat}/k_{uncat}, but also in k_{cat}/Km, a more significant parameter of enzymic activity (24). Compared to almost every esterolytic antibody described heretofore, the improved reactivity of D2.3 is significant. The differences are even more meaningful when compared with antibodies developed for specific practical applications, e.g., for prodrug release, antibodies 6D9, 49.AG (25, 26) or for cocaine degradation, antibody 3B9 (27).

Turnover, i.e., the ability of a single binding site to repetitively process substrate molecules is the hallmark of any catalyst. Yet, unlike rate-enhancement, turnover is rarely demonstrated in so-called "enzyme-like" catalysts. Moreover, kinetic parameters such as those given in Table I can be obtained even for stochiometric reactions (i.e., for only one turnover). A careful examination of the literature reveals that turnover of catalytic antibodies is often found to be limited by severe product inhibition (for examples, see refs. 28, 29-30). In spite of the presence of the nitrophenyl group, the p-nitrobenzyl alcohol product binds to antibody D2.3 with low enough affinity (K_p= 52 μM) to allow efficient turnover (>300, Fig. 9).

Table I. Comparison of kinetic parameters for different anti-phosphonate esterolytic antibodies.[a]

Ab	substrate(-OR')[a]	k_{cat} (min^{-1})	Km (μM)	k_{cat}/Km ($s^{-1}\cdot M^{-1}$)	k_{cat}/k_{uncat}	ref.
D2.3	**-OCH$_2$PNP**	7.35	280	440	$2.6X10^5$	1
2R3S	**-OCHMeCF**R'	0.94	410	37	n.d.	83
37E8	**-OCHR'CH=C**R"	0.007	177	0.66	88	19
21H3	**-OCHMePh**	0.09	394	3.8	$1.6X10^3$	28
2H6	**-OCHMePh**	4.6	4000	19.2	$8.3X10^4$	28
2H12E4	**-OCHR'CONH-**	0.019	15	21	267	68
6D9	**-OCHPh**R"	0.133	64	34.6	$1.3X10^3$	25
3B9	**-OCHR'**R"	0.11	490	3.7	540	27
49.AG	**-OCH$_2$-**R'	0.03	218	2.3	967	26
1C7	**-OCH$_2$-CH$_3$**	1.97	285	115	n.d.	20

[a] All the antibodies were elicited against phosphonate haptens of the general structure: RP(=O)(-OH)-OR' and catalyze the hydrolysis of the respective substrates: RC(=O)-OR'. The acyl component, R, is an alkyl or a substituted benzyl, the leaving alcohol component, -OR', is noted in the table. The catalytic parametrs of each of the antibodies indicated above were determined at different pHs (7.3-9.0) and temperatures (17-37°C). These differences mainly affect k_{cat}; the effect on the rate acceleration, k_{cat}/k_{uncat}, is less significant.

Thus, D2.3 in comparison with another alkyl or benzyl ester hydrolyzing antibody, e.g., 1C7 (20) (Table I), exhibits not only a higher rate, but is almost unaffected by product inhibition; antibody 1C7 suffers from severe product inhibition (by the carboxylic acid component) so that the catalyzed reaction proceeds with almost stochiometrically. Turnover was observed only with a carbonate, rather than an ester, substrate which is then hydrolyzed to give an alcohol, rather than an acid, product (20). Product inhibition was also observed with antibody 2H6 (28). For most of the other antibodies mentioned in Table I no information regarding product inhibition was published. The only exception, regarding product inhibition, seems to be antibody 6D9 (25); as with D2.3, no product inhibition was observed in spite of the presence of a nitrophenyl group in the substrate and in the product. Still, the rate exhibited by 6D9 is two orders of magnitude less than that of D2.3 (Table I).

Of special interest is the fact that these antibodies, which were elicited against p-nitrobenzyl phosphonate **4a**, are even more efficient catalysts towards the corresponding p-nitro*phenyl* ester. k_{cat}/Km values of up to 11,000 $s^{-1}\cdot M^{-1}$ were measured with practically no inhibition by the p-nitrophenol product.

3. High occurrence of catalytic antibodies in MRL/*lpr* and SJL mouse strains.

With the development of catELISA, screening of the entire repertoire of hybridomas obtained from a number of fusions became practically possible. Results

presented in Section 1 emphasized that antibodies with catalytic activity are generally rare: 9 catalytic antibodies that catalyze the hydrolysis of the respective ester (and none of the respective amide) were identified amongst 970 clones that bound the TSA-hapten. We wished to see whether different repertoires of catalytic antibodies (in number and in features) could be obtained in different strains of mice following immunization with the same hapten. The results of these experiments indicated a dramatic increase in the occurrence of catalytic antibodies in certain mouse strains (SJL and MRL/*lpr*) relative to other strains, e.g., Balb/c, C57BL, NZB/W and MRL/++. Furthermore, the catalytic features of some of the antibodies generated in SJL mice, for example, differ from those generated in Balb/c mice.

Table II summarizes the results of these experiments. Hybridomas were generated from lymphocytes taken from the spleen and lymph nodes of various strains of mice immunized with phosphonate **4a** (Fig. 3). The hybridoma supernatants were screened by catELISA for their ability to catalyze the hydrolysis of p-nitrobenzyl ester **1a** and amide **2a**, and by ordinary ELISA for binding to the corresponding antigen (i.e., to the BSA conjugate of **4a**).

The occurrence, i.e., fraction or percent of wells displaying catalytic activity compared with hapten-binding clones, found in normal inbred mouse strains is generally low, i.e., one to nine clones per fusion, or an average occurrence of < 2% (see first four entries of Table II and Section 1).

A very dramatic difference was observed when we screened hybridomas made of lymphocytes taken from autoimmune-proned mice MRL/*lpr* and SJL (e.g., F9, F18 or F6; Table II). The overall number of binding clones was generally higher compared to normal mouse strains. Yet, the outstanding difference is both in the absolute number of catalytic clones (e.g., 576 in MRL/*lpr* mice, F9) and in their occurrence (75-87% of the hapten-binding clones). None of these clones catalyzed the hydrolysis of the corresponding p-nitrobenzyl amide (**2a**). It is noteworthy that although a high number and occurrence of catalytic antibodies is consistently observed in the *lpr* mice (Table I, F9 & F18), a normal occurrence of catalytic clones was found, in the wild-type , MRL +/+, strain (F11).

A marked increase of catalytic antibodies expression was also found in SJL mice (F6, F8) but not in NZBxNZW mice (F12; or in NZW mice, F10). In both SJL and in MRL *lpr* mice a decline in the appearance of catalytic clones was observed after further immunizations (i.e., F6 vs. F8, or, F9 vs. F14). This decline is not necessarily due to a decrease in the population of anti-hapten clones (e.g., F9 vs. F14). The mechanism for this reduction in catalytic clones is still unclear to us; we believe that this behavior may result from the hypermutation and selection processes occurring during repeated immunization cycles.

The generation of catalytic antibodies in these mice is clearly antigen- and hapten-selected. Esterolytic activity was not generated in non-immunized MRL/*lpr* mice (Table II, F15), or in mice immunized with a TSA-hapten that does not correlate with hydrolysis of ester **1a** (F20) and not even in mice immunized with p-nitrobenzyl amide **2a** (F19), an analog of the substrate rather than of the TS of the reaction.

Table II. Results of screenings for binding and catalytic clones.[a]

Entry	Mouse	Immunization	Total No. of Clones	No. of binding clones	No. of catalytic clones	% of Catalytic binding
F1a	Balb/c	TSA 4; long	1570	970	9	1
F1b	"	" ; long	960	n.d.	3	n.d.
F2a	C57BL/6 (3)	TSA 4; short	576	60	1	1.3
F30	Balb/c (4)	TSA 4; short	990	420	20	5
F6	SJL (4)	TSA 4; short	1200	1128	855	75
F8	SJL (1)	TSA 4; long	1728	162	10	6
F9	MRL/lpr (4)	TSA 4; short	768	640	576	87
F18	MRL/lpr (4)	TSA 4; short	1056	814	630	77
F11	MRL/++ (4)	TSA 4; short	768	104	4	4
F14	MRL/lpr	TSA 4; long	2208	828	15	1.8
F15	MRL/lpr (4)	non-immunized	1440	9	0	0
F19	"	Amide 5; short	1104	1000	0	0
F20	MRL/lpr (4)	DPA 52;[b] short	768	n.d.	0	0
F10	NZW (4)	TSA 4; short	672	252	3	1.2
F12	NZBxNZW (4)	" "	672	91	2	2.2
F21	NZBxNZW (4)	TSA 4; short	664	315	2	0.6

a Long immunization, ca. 20 days; short immunization, ca. 60 days. The following periods (in days; d) and number of injections (inj) were used in those fusions that followed a long immunization: F1a, 45d, 4 inj; F1b, 93d, 5 inj; F8, 106d, 6 inj; F14, 65d, 4 inj. One mouse, or several (for those cases, the number is indicated in parentheses) were used for fusion.

The number of binding and catalytic clones represent results of the intial screening experiments. At this stage, hybridoma growth was apparent at 80-100% of the seeded wells.

From these fusions, a number of randomly chosen positive wells (1-20, in accordance with the total number of catalytic clones) were sub-cloned and and their binding and catalytic properties were studied in more detail. A set of validation experiments, such as those described in Section 1, proved that the catELISA signal is indeed indicative of *antibody* catalyzed hydrolysis of ester **1a**.

b DPA52 is an amino phosphinate hapten (Ph-P(=O)(-OH)-CH_2-L-Pro-Gly-OH) which is structurally unrelated to phosphonate hapten **4** or ester substrate **1**.

Furthermore, because MRL/*lpr* strain is considered a mouse model for lupus (SLE) ,a human autoimmune disease which is characterized by the presence of high

titers of autoantibodies to a number of self antigens including DNA (34-36), we have assayed the DNA-binding activity of the polyclonal sera and of catalytically active monoclonal antibodies isolated from these mice. Hapten binding titers of the immunized mice were 10-50 fold higher than binding titers to single-stranded (ss)- or double-stranded (ds)-DNA. None of the monoclonal antibodies isolated from MRL/*lpr* or SJL mice (F9, F6, see Table II) exhibited significant cross reactivity of binding to ss- or ds-DNA. DNA (20 μg/ml) did not inhibit binding or catalytic activity of these clones. Another known self-antigen, cardiolipin, did not inhibit (at concentrations up to 0.5 mg/ml) the binding nor the catalytic activity.

The isolation of individual hybrid clones after fusion and cloning does not necessarily mean that they in fact originated from different B-lymphocytes or that they secrete *different* monoclonal antibodies. Characterization of the antibodies for their relative affinities to the hapten (**4a**) and its derivatives (i.e., methyl p-nitrobenzyl phosphonate, **6**), the substrate (**1a**), and the product of its hydrolysis (p-nitrobenzyl alcohol, **7**), clearly demonstrates clonal variability. Representative data for three randomly selected clones isolated from MRL/lpr mice (F9) is given in Figure 10. It still has to be determined whether these differences reflect a diverse usage of germ line V-genes, somatic mutations, or both. It should be kept in mind that the generation of *autoantibodies* in the same strain of mice (by a self antigen such as DNA) is antigen-selected and not a result of a non-specific polyclonal activation. Moreover, a highly variable germ line V-gene usage, somatic mutations and affinity maturation were all observed in these mice (34,36).

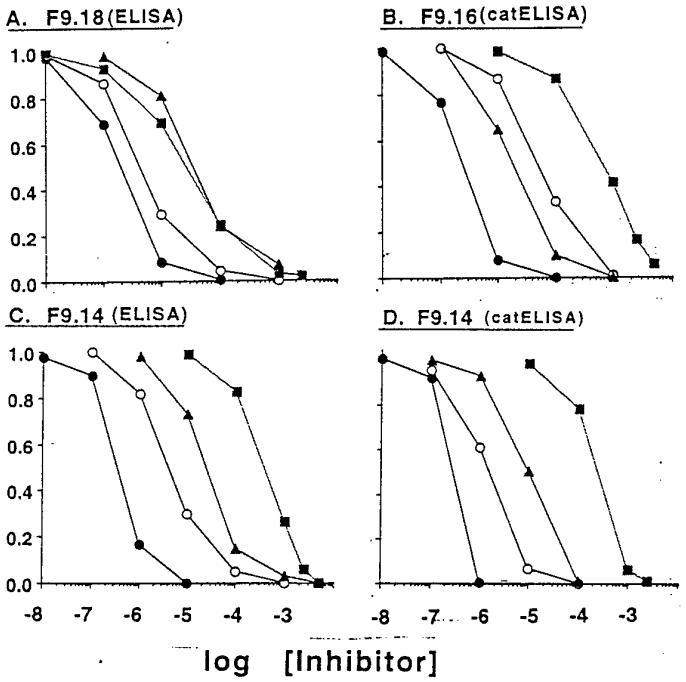


Fig. 10. Affinity patterns of catalytic clones were obtained by competitive inhibition assays of *binding*, by ELISA (A and C), or of *catalytic* activity, by catELISA (B and D). Supernatants of various clones were incubated with increasing concentrations of: TSA-hapten **4** (N-benzyl amide), o; a "short" TSA (mono p-nitrobenzyl methyl phosphonate), o; amide substarte 5 (N-caproyl amide), ▲); p-nitrobenzyl alcohol (**3**), ■. Data for antibodies #18 (**A**), 16 (**B**) and 14, isolated from MRL/*lpr* mice (F9) is presented above; for antibody F9.14, both affinity patterns, obtained by inhibition of binding (**C**) and of catalytic activity (**D**), are presented.

The catalytic efficiency of esterolytic antibodies isolated from MRL/*lpr* and SJL mice (i.e., F6, F9 & F18) was compared to that of antibodies elicited against the same hapten in Balb/c mice (Fig. 11). Rate accelerations (k_{cat}/k_{uncat}) by antibodies isolated from SJL or MRL/*lpr* mice after a short immunization (F6 and F9, respectively) were estimated to be in the range of 50-10^3 and k_{cat}/Km values in the range of 0.1-5 $M^{-1}s^{-1}$. Antibodies obtained from these mice after longer immunization periods (i.e., F8 and F14) exhibited significantly higher rate accelerations (10^3-10^5). The catalytic power of these antibodies is comparable to esterolytic antibodies which were elicited in Balb/c mice after prolonged immunization as well (i.e., F1a).

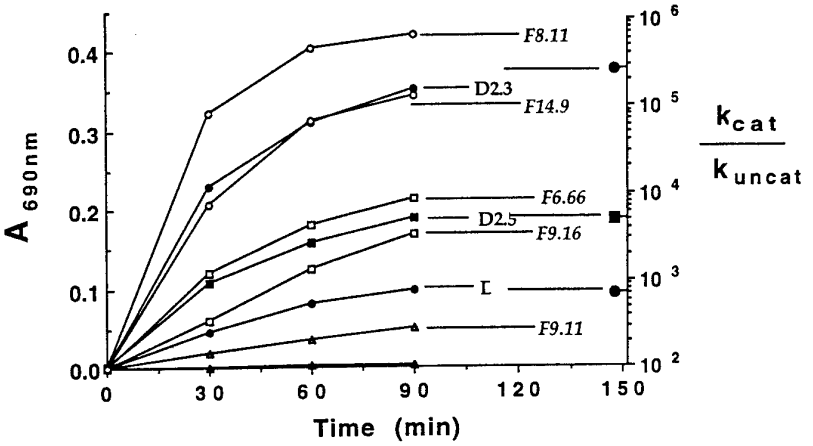


Fig. 11. Rates of esterolytic activity of clones isolated from different mice strains were compared by catELISA. Supernatants of active clones isolated from MRL/*lpr* (F9.11, F9.16 and F14.9) and SJL (F6.66 and F8.11) autoimmuned mice (empty symbols), or a Balb/c mouse (F1a; full symbols), and of a non-catalytic antibody (▲), were incubated with immobilized ester **1a** in microtiter plates for increasing time periods (0-90 mins). Kinetic parameters of the antibodies isolated from Balb/c mice (F1a) are given for comparison: D2.3, k_{cat}/Km= 440 $s^{-1}\cdot M^{-1}$, k_{cat}/k_{uncat}= 260,000; D2.5, k_{cat}/Km= 6.9 $s^{-1}\cdot M^{-1}$, k_{cat}/k_{uncat}= 5,000; D2.1, k_{cat}/Km= 1.1 $s^{-1}\cdot M^{-1}$, k_{cat}/k_{uncat}= 700.

In summary, a dramatic increase in the number and in the occurrence of esterolytic antibodies in SJL and MRL/*lpr* mice immunized with transition state analog **4a** was observed following short immunizations. The generation of catalytic antibodies in these mice strains was found to be antigen- selected. Esterolytic antibodies were found neither in non-immunized MRL/*lpr* mice, nor in mice immunized with a haptens that do not mimic the TS of the hydrolysis of ester **1a**. Immunological and biochemical analysis of the selected antibodies show their specificity to the antigen. Significant cross reactivity with major self-antigens, e.g., DNA and cardiolipin, was not found. Variability was observed among the tested clones (randomly selected from all the active clones): different antibodies displayed different affinity patterns; similarly, their binding parameters such as K_S/K_{TSA} or K_P/K_S, catalytic efficiencies isotypes and other features differ considerably.

One aspect of these observations may apply to the future generation of catalytic antibodies. The use of various mouse strains may allow us to expand the repertoire of catalytic clones elicited against a certain TSA-hapten. We (4), and others (37,38), have found that all the catalytic antibodies generated from a given fusion share significant primary structural and biochemical identity. Thus, new antibody-catalysts that are too rare to be found easily or that have different features than

those found in ordinarily used mouse strains, might be isolated. For example, some of the esterolytic antibodies obtained in SJL or MRL/*lpr* mice, in particular after prolonged immunizations, exhibit catalytic efficiencies similar to those elicited in Balb/c mice but differ in their substrate specificities.

It is especially intriguing to consider the possible link between autoimmunity and catalytic antibodies. The *lpr* mutation in mice was found by Nagata and coworkers to correlate with defects in Fas antigen that mediates programmed cell death, or apoptosis, during T- and B- cells development (39). These interesting findings led us to examine the response both in *lpr* mutant and in the MRL/++ wild type. The expression of the *lpr* mutation is known to cause a massive poliferation of T cells (34-36). Indeed, a relatively large number of hapten-binding clones is observed in the mutated mice (640 vs. 104 in MRL/++). Still, it is not only the absolute number of catalytic clones that is dramatically elevated in the mutant mice over its wild type (576 vs. 4 in MRL/++) but more significantly, their fraction in the overall repertoire of hapen-binding clones (87% vs. 4% in MRL/++).

Tolerance and negative selection eliminates anti-self T- or B- cells; these results may indicate that in the absence of such selection, as observed in the *lpr* phenotype for example, a marked and preferential increase in the expression of particular V-region genes that encode for catalytic antibodies is observed. The above may evoke some intriguing speculations: is there normally a negative selection *against* the expression of certain variable genes encoding antibodies with catalytic activity ? Is the frequent appearance of catalytic clones linked to an autoimmune disorder?And, do catalytic antibodies play a physiological role in some autoimmune diseases (40)?

Sporadic observations support the proposed linkage between autoimmunity and the appearance of catalytic antibodies. Two cases of naturally occurring catalytic antibodies have been thus far reported. Both support our notion: Paul *et al* described cleavage of a peptide (vasointestinal peptide, VIP) by polyclonal antibodies isolated from the sera of an asthma patent (41); Shuster *et al* identified DNA-hydrolyzing autoantibodies in the serum of a systemic lupus erythematosus (SLE) patient (42). In both cases, similar catalytic activity was not found in the sera of healthy individuals. In addition, we have noted a V-region sequence homology between groups of catalytic monoclonal antibodies obtained by immunization of normal mouse strains with a TSA-hapten, and antibodies having different specificities generated in mouse strains known to develop autoimmune diseases (4).

These studies delt with a model antigen and a certain reaction of no apparent *in-vivo* significance. Important questions still remained unresolved, e.g., how clones that exhibit catalytic activity are deferred from ordinary binding clones selected for the same antigen? Or, what mechanism leads to the decline in the occurrence of catalytic antibodies after prolonged immunizations? More conclusions are expected from the amino acids sequence of the series of catalytic and binding antibodies obtained from SJL mice (after a short and a long immunization).

4. Phosphonate haptens did not generate amide-hydrolyzing antibodies

CatELISA was developed for the isolation of catalytic antibodies that catalyse more demanding and mechanistically more complex reactions such as the

hydrolysis of an amide bond. Using the system described above we have examined two issues related to the aims of the proposed research (see also Section 5):

First, is the repertoire of antibodies elicited against a single hapten sufficiently diverse so that making haptens that accurately mimic the transition state is unnecessary? Almost all the hybrid clones elicited against p-nitrobenzyl phosphonate hapten (**4a-KLH**), both in normal and in autoimmune mice, were screened for the hydrolysis of both the respective ester (**1a**) and amide (**2**) substrates. None of the thousands of hybridoma clones, out of which hundreds of ester-hydrolyzing antibodies were identified, yielded antibodies which cleave the amide substrate (Section 3, Table II). Other attempts to use phosphonates to generate peptide hydrolyzing antibodies were in general unsuccessful(for examples, see refs. 43,44).

We conclude that generating antibodies that catalyze the hydrolysis of an amide bond requires a better mimic of the TS than a phosphonoester structure. Amide hydrolysis proceeds via several intermediates which are significantly different from that of ester hydrolysis and include, in the rate-determining step, protonation of the amino leaving group. An amide hydrolyzing antibody should cope equally well with stabilization of the TSs both for the formation and for the breakdown of these intermediates (Section 8 and Fig. 18). Phosphonoesters mimic only the first intermediate which is formed by a nucleophilic attack on the carbonyl.

For simple reactions, such as hydrolysis of an activated ester or amide (e.g., a p-nitrophenyl ester or anilide, careful design of the TSA-hapten seem to be less important (45). For more demanding reactions, the design and synthesis of appropriate TS analogs is probably the key to successful attempts to generate efficient catalytic antibodies.

5. Can a substrate induce the formation of antibodies that catalyze its cleavage?

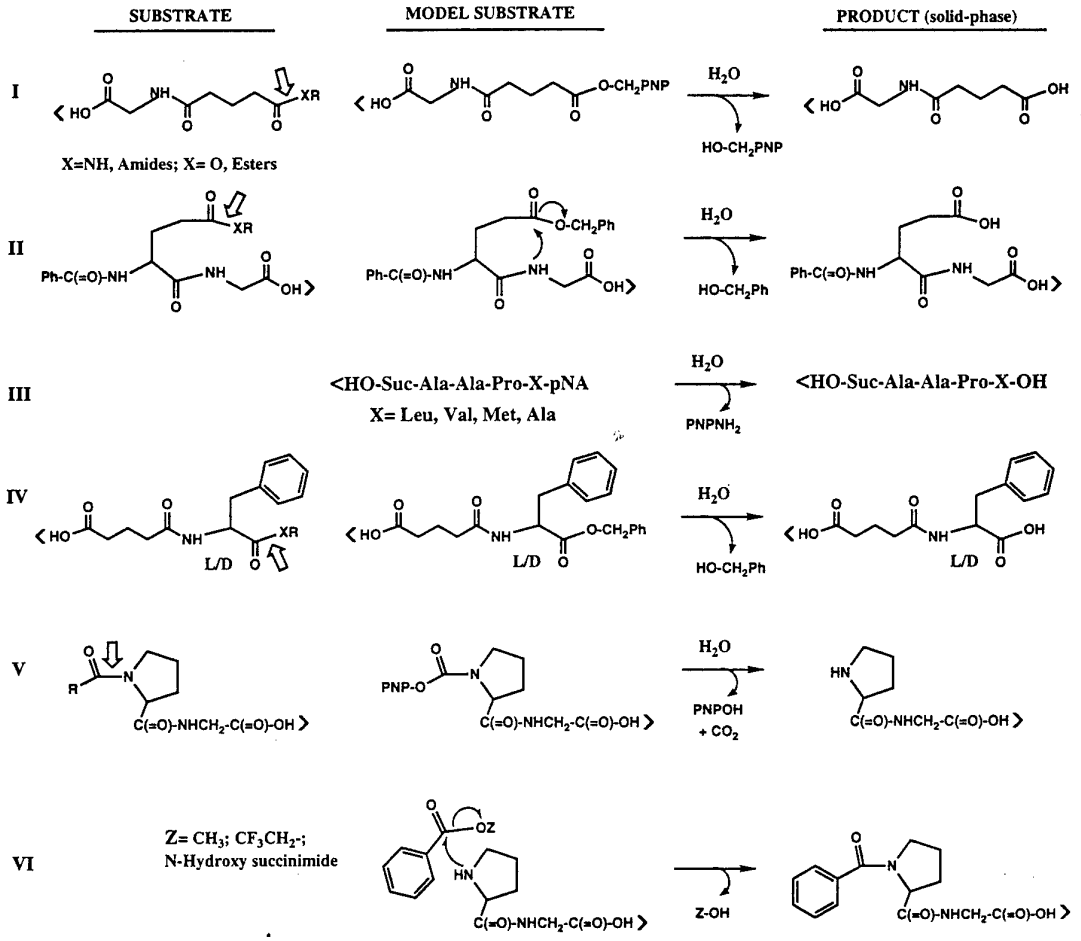
Traditionally, catalytic antibodies are elicited against a transition state analog (TSA). It is the complementary of the active site to the transition state, rather than to the substrate, that provides the decrease in the activation energy of the reaction and thereby to the acceleration of its rate. An interesting question is, whether a substrate (or an analog of the ground state substrate rather than of the transition state) can induce antibodies that catalyze its own cleavage (1,41,46)? Following immunization with amide **2**, a stable analog of ester substrate **1a** (which is too labile to be used for immunization) we could not detect any antibodies that catalyze the hydrolysis of ester **1a**, or of amide **2** itself using both normal mice and MRL/*lpr* autoimmune mice (together, more than 1,200 clones that bound **2a** were examined).

It has been reported that a peptide substrate (VIP) can induce polyclonal and monoclonal antibodies that catalyzed its cleavage (46). However, we find that ground state molecules (i.e., substrates, or substrate analogues) did not select for antibodies having catalytic properties. Even under conditions in which catalytic antibodies readily appear (i.e, in MRL/*lpr* autoimmune mice), selection of the proper antibody-forming B cells by a transition state analog is necessary.

6. The establishment of additional CatELISA systems

The catELISA approach is not only facile but general: it is not limited to a certain type of reaction or substrate. Indeed, we have been able to readily expand it to other reactions and products (Table III). catELISA systems were developed for dissociative reactions (amide, ester and carbamate-hydrolysis, Table III, **I-V**) and associative reactions (amide formation, **VI**).

Table III. CatELISA detection systems.[a]



[a] PNP= p-nitrophenyl, Ph= phenyl.

Linkage site of these substrates/products (by an amide bond to the ε-amino groups of lysines) to the carrier protein, BSA or KLH, is marked by < sign. Model substrates were used to optimize the catELISA assay by applying chemical catalysis. Conditions used for each of these hydrolyses reactions are: **I, II** and **IV**, 100 mM carbonate buffer pH 10.8, 1-5 hrs; **III**, 0.4-1.0 M NaOH, 3-20 hrs; **V**, 100 mM carbonate buffer pH 10.8, 20-48 hrs. L/D indicates both enantiomers of phenylalanine; seperate detection antibodies were prepared for each enantiomer.

The preparation of the protein conjugates of all those products having a free carboxyl or amino group (Table I, **I-IV** & **V**, respectively) was performed by introducing a temporary protection group which was removed after conjugation. This approach has several distinct advantages. It allows selective conjugation via a single site only, and provides an easy way to determine the degree of conjugation,

or hapten density, (by measuring the concentration of the released protective group). Furthermore, in most cases, the same deprotection reaction was performed *in-situ* on microtiter plates and was used to examine the catELISA and the anti-product antibodies; these reactions are designated "model substrates" in Table III .

For all of the reactions described in Table III, polyclonal anti-product sera elicited in rabbits were used for detection. Thus, the laborious generation of product-specific *monoclonal* antibodies could be avoided. Best results were obtained by immunizing rabbits with product-KLH conjugates having moderate hapten densities (Hd; i.e., 10-20 product molecules per KLH molecule) administered in relatively low doses (<100 μg/rabbit/injection). In most cases, the first bleedings (i.e., after 25-35 days and two injections with Complete Fruend's adjuvant) exhibited high enough binding titers to the product and very low, if any, cross reactivity with the substrates. In some cases, the same rabbit sera could be used, under appropriate conditions, for several different substrates which afforded a similar solid-phase product. For example, rabbit sera elicited against the product of **I**, was used for detection of a variety of carboxylic acid products, e.g., **II** and **III** (Table III). In other cases, **IV** for example, polyclonal sera against both the L and the D sterioisomers had to be separately elicited. These polyclonal antibodies specifically bound to each of the enantiomeric product and exhibited no cross reactivity.

For the successful application of catELISA, conditions under which a significant reaction signal was observed (relative to the background signal obtained in the presence of buffer or a non-relevant hybridoma supernatant) had to be optimized. Chemically catalyzed reactions of a model substrate were used for optimizing assay conditions. Parameters that affect Signal/Noise ratio are:

(i) Loading of the *BSA-substrate conjugate*, i.e., number of substrate molecules per BSA (generally, hapten densities of 10-20 were used) and the concentration of substrate-BSA used for coating of the microtiter plates (Fig 12A).

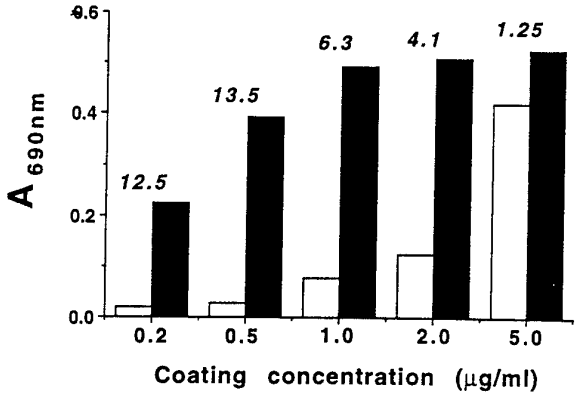
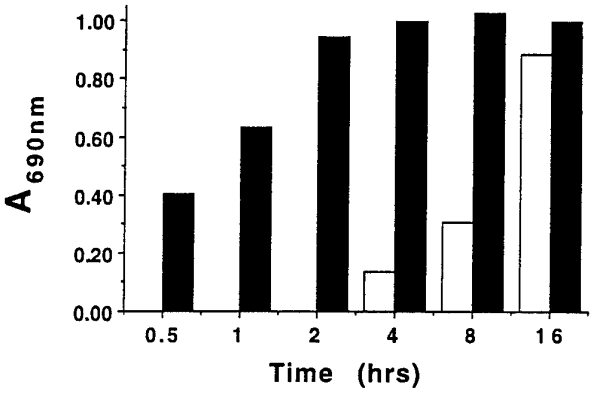
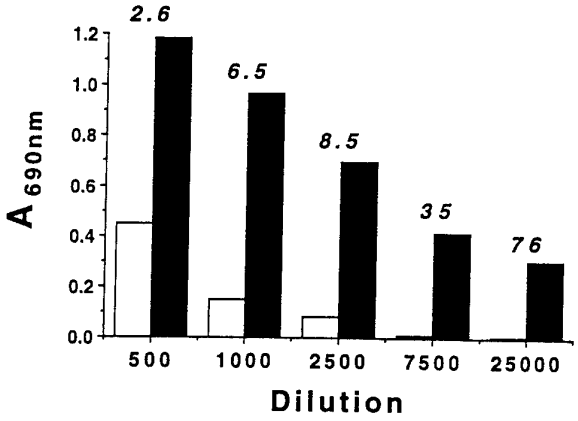


Fig. 12. A typical sets of catELISA optimization exeperiments.
12A. Microtiter plates were coated (1 hr; r.t.) with BSA conjugates of ethyl ester of **IV** (D- isomer; **35D**, see Fig. 24) at different concentrations (0.2-5 μg/mL). After blocking with BSA (1 mg/mL, 30 mins, r.t.), the plates were incubated (3 hrs, r.t.) with TBS pH 8.3 (*background*, open bars) or, 0.1M carbonate pH 11 (*cleavage-signal*, solid bars). The plates were washed, and the rabbit anti-product **IV** serum (D-isomer) was added (1:1,000 dilution in PBS) followed by peroxidase-linked mouse anti-rabbit antibodies and then the substrate (ATBS). Results are given in absorbance at 690 nm. Signal/Noise ratios for each of the entries are given above each of the pairs of bars.



12B. Microtiter plates were coated **1a-BSA** (Hd= 16; 1 μg/ml) were incubated with supernatant of a catalytic antibody, D2.3 (see Section 2.1; *signal*, full bars), or, a non-catalytic antibody (*background*, open bars) for various time periods (0.5-24 hrs); at ambient temperature. The plates were developed using rabbit anti-product 3 serum (1:5,000 dilution in PBS + 0.04% Tween). Results are given in absorbance at 690 nm.



12C. Microtiter plates were coated with the BSA conjugate of prolyl p-nitrophenyl carbamate (Table III, **V**; 5 μg/mL) and then incubated with TBS pH 8.3 (*background*, open bars) or, 0.1M carbonate pH 11 (*cleavage-signal*, solid bars) for 6 hrs at room temperature. The plates were washed and incubated with the rabbit anti-product serum at different dilutions (1:500 - 1: 25,000) in PBS. After washings, the peroxidase-linked mouse anti-rabbit antibodies were added and then the substrate (ATBS). Results are given in absorbance at 690 nm. Signal/Noise ratios for each of the entries are given above each of the pairs of bars.

(ii) *Reaction conditions*, i.e., time, temperature and pH of incubation of the antibody supernatants. Substrate stability is the major factor here; ester substrates were usually incubated with the assayed supernatants only for several hours (Fig 12, B), whereas, amide substrates, which are far more stable, could be incubated for 24-72 hrs at 37°C and at basic or acidic pH (see, Footnote no. 6).

(iii) The *anti-product antibody*, i.e., dilution and binding buffer (Fig 12C).

The *chemical* models developed for each of these systems have provided a routine "positive control" and a facile way for developing the assay (see Fig. 12 A-C) and for preparing the product conjugates. This is probably the more general approach since in only one case, was an enzyme found that catalyzes the cleavage of

the *immobilized* substrate. Examples for the results of such model experiments of an *associative* reaction (Table I, **VI**) are given in Figure 13.

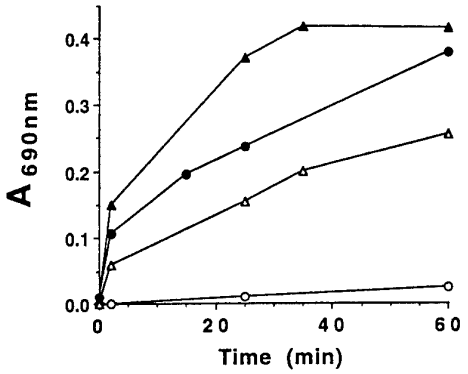


Fig. 13. Microtiter plates coated with prolyl-glycyl-BSA (**VI**) (full symbols; Hd~ 30; 5 μg/mL; overnight, 4°C) were incubated with benzoyl N-hydroxy succinimide ester (0.1 mM), diluted in PBS pH 7.4, for different time periods. As control, plates coated with unconjugated BSA (empty symbols; 1 mg/mL) were similarly treated with benzoyl N-hydroxy succinimide ester. All the plates were then washed and incubated with rabbit anti-product (**10g**, see Fig. 23) serum, first bleeding (●, ○) or third bleeding (▲, △; both at 1:4,000 dilution in PBS + 0.04% Tween-20). After washings, the peroxidase-linked mouse anti-rabbit antibodies were added and then the substrate (ATBS). Results are given in absorbance at 690 nm.

Acylation of the solid-phase prolyl amine by activated benzoyl esters, e.g., N-hydroxy succinimide or trifluoroethyl esters served as the chemical model. A concentration- and time-dependent signal was observed with the catELISA (Fig. 13). In this case, detection conditions were optimized so that benzoylation of the free ε-amino group of lysine side chains (of the BSA carrier) would not give a positive signal (e.g., compare first bleeding vs. third bleeding of the rabbit sera).

Following recent reports on polyclonal catalytic antibodies (47,48) we examined whether catELISA could be used to screen sera or polyclonal antibodies. If so, this would allow pre-evaluation of the best immunization protocol and selection of the best mouse for fusion. Sera taken from mice immunized with **4a-KLH** (including sera of those mice that eventually yielded catalytic monoclonal antibodies) were tested for catalytic activity using catELISA with **1a-BSA**. No signal was observed at any serum dilution; furthermore, the esterolytic activity exhibited by supernatants of the catalytic clones was competitively inhibited by dilution with an excess of the same sera. Thus, the presence of substrate-binding, non-catalytic antibodies in the serum interfere with the catELISA detection by competing for the immobilized substrate and thus inhibiting its cleavage by the catalytic antibodies.

The low reactivity of enzymes with immobilized substrates was found both in the first system established (see Section 1) and in other systems as well. For example, the p-nitroanilide peptides **III** (Table III) are specific substrates of elastase. When conjugated to BSA, this substrate reacts 50 fold slower compared to an equivalent concentration of unconjugated **III**. After immobilization, the substrate is completely unreactive with elastase; i.e., no catELISA signal is observed after prolonged incubations (48 hrs) with high concentrations of pancreatic elastase (1-30 units/mL). Several of the immobilized L-phenylalanyl esters and amides **IV** (XR=

OEt, OCH_2Ph and NH_2, see also Section 8.4) are cleaved on catELISA by α-chymotrypsin. However, the rate of hydrolysis is lower than observed with similar soluble substrates.

In general, for many of the reactions we wish to study (Table I, **I-IV**) relevant enzymatic activities (i.e., esterases and proteinases) appear in the biological media assayed (e.g., supernatants, ascites, bacteria growth media). Nevertheless, owing to the poor accessibility of most enzymes to the conjugated and immobilized substrates, no background signal resulting from enzymic contaminants was observed with catELISA on immobilized substrates. This feature is unique to the catELISA; the problem of enzymatic background activity is not addressed in the other direct approaches suggested for screening of catalytic antibodies (49-51).

7. Studies of anti-phosphonate esterolytic antibodies

Further analysis of the esterolytic antibodies first identified by catELISA (Sections 1 and 2) led to several interesting findings. Unexpectedly, we found that at least 3 of the clones elicited against **4a**, efficiently catalyze the hydrolysis of not only the corresponding nitrobenzyl (NB)-ester **1a** but also of the p-nitrophenyl (NP) ester **1p** (Fig. 14).

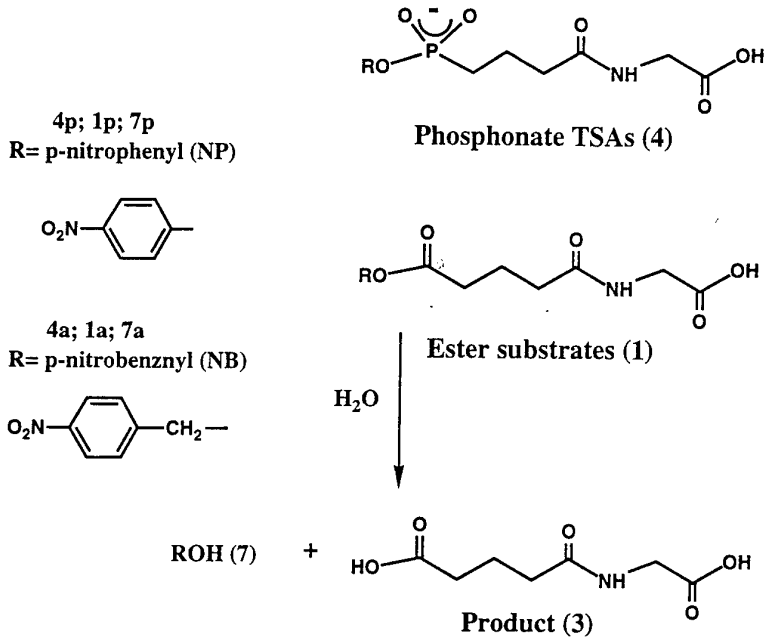


Fig. 14. Structure of the nitrobenzyl (NB) and nitrophenyl (NP)-phosphonate TSAs (**4a** and **4p**, respectevely), ester substrates (**1a**, **1p**) and the products of their hydrolyses (**7a**, **7p**).

Even more surprising was the observation that activity with the NP-ester **1p** is not only higher in terms of the turnover number (k_{cat}) but also more favorable in terms of *specificity* (Km and k_{cat}/Km) and product inhibition. The kinetic parameters of the 3 antibodies with both the NB and NP ester substrates are given in Table IV.

Table IVa. Kinetic parameters of the catalytic antibodies for hydrolysis of p-nitrobenzyl (NB)-ester **1a**.[a]

Ab	$k_{cat(min^{-1})}$	Km(μM)	k_{cat}/k_{uncat}	$k_{cat}/Km_{(M^{-1}\cdot s^{-1})}$	$K_{p(\mu M)}$[b]
D2.3	7.3	280	$2.6X10^5$	440	50
D2.4	2.05	300	$7.3X10^4$	114	70
D2.5	0.14	340	$5X10^3$	6.9	n.d.

[a] Kinetic pararametrs were determined by an HPLC assay with protein A purified antibodies.

[b] Kp is given for p-nirobenzyl alcohol (**7a**), the acidic component, **3**, did not exhibit any inhibition of catalytic activity at concentrations as high as 10 mM.

Table IVb. Kinetic parameters of the catalytic antibodies for hydrolysis of p-nitrophenyl (NP)-ester **2p**.

Ab	$k_{cat(min^{-1})}$	Km(μM)	k_{cat}/k_{uncat}	$k_{cat}/Km_{(M^{-1}\cdot s^{-1})}$	$K_{p(\mu M)}$[b]
D2.3	9.2	57	$1.93X10^4$	2690	>5
D2.4	12.7	19	$2.67X10^4$	11,100	>500[a]
D2.5	2.95	22	$0.62X10^4$	1,340	n.d.

[a] Kinetic pararameters were determined with protein A purified antibodies by measuring the release of p-nitrophenol (absorbance at 405 nm).

[b] Kp is given for p-nirophenol (**7p**). No inhibition of hydrolysis of **1p** (0.05-0.3 mM) was observed at p-nitrophenol concentrations up to 0.1 mM ; higher concentrations increased the absorbance of the reaction mixture (at 405 nm) to non-linear ranges. In addition, competitive inhibition of the hydrolysis of the immobilized **1a-BSA**, measured by catELISA, showed that IC_{50} for the inhibition by p-nitrophenol (**7p**) is at least 10 fold higher than p-nitrobenzyl alcohol (**7a**). The acidic component, **3**, did not exhibit any inhibition of catalytic activity at concentrations as high as 10mM.

Reactivity towards a substrate having a more labile leaving group, such as NP-ester, is commonly found in hydrolytic enzymes. In the case of catalytic antibodies, a more distinct substrate specificity is generally observed (10), even though, cases in which a higher rate, i.e., higher k_{cat}, were observed with a more labile ester substrate (relative to the "original" substrate that corresponds to the haptenic structure) were described (52). However, determination of the kinetic parameters of these antibodies with NP-ester **1p**, and comparison to data with the NB-ester **1a**, revealed that the NP-ester is a far better substrate for these antibodies. A meaningful difference in the k_{cat}/Km values observed with the two esters is observed, e.g., for D2.4, k_{cat}/Km for the NB-ester **1a** is 114 $M^{-1}\cdot s^{-1}$, and for the NP-ester **1p**, 11,100 $M^{-1}\cdot s^{-1}$. Higher k_{cat}/Km values reflect not only a higher selectivity towards the NP substrate but also a better fit of the antibody active site to the corresponding transition state (52). Moreover, comparison of the kinetic parameters (k_{cat}/k_{uncat} and k_{cat}/Km) of D2.4 with other p-nitrophenyl ester and carbonate hydrolyzing antibodies raised against *NP*-phosphonate haptens illustrates the superiority of

D2.4 which was elicited against a NB-phosphonate hapten (21,22). In fact, this is one of the highest k_{cat}/Km values observed with any catalytic antibody elicited against a TSA (esterolytic or others; see also Section 2 and ref. 21).

Of no less interest is the fact that the p-nitrophenol product (**7p**) released by the hydrolysis of NP-ester **1p**, does not inhibit to any measurable extent the activity of D2.4 (Figure 15), or of the other two antibodies. This is in contrast to p-nitrobenzyl alcohol (**7a**) which does inhibit the action of these antibodies (Table IV). Thus, antibodies D2.3 and D2.4 exhibit more than 1000 turnovers with ester **2p** . Hydrolysis of the p-nitrophenyl ester **1p** is neither inhibited by acylation of the antibody by the activated substrate (as previously observed with antibodies elicited against a NP-phosphonate hapten, see ref. 22).

In light of these results we examined the activity of these antibodies towards other esters (Table V). All 3 antibodies described above, D2.3, D2.4 and D2.5, are characterized by their notable substrate specificity. The reactivity of these antibodies towards different benzyl esters reveals that substrate specificity is derived from the haptenic structure (**4a**) and can be ascribed to two independent sub-sites. The first sub-site recognizes the p-nitrobenzyl moiety (i.e., no catalysis is observed with the benzyl or o-nitrobenzyl esters (**1g** and **1e**, respectively); the second recognizes the acyl part of the substrate (and of the hapten): the k_{cat}/Km value for NB-acetate (**8a**) is less than 10 $S^{-1}M^{-1}$, compared to 114 with NB-O-glutaryl-N-glycine (**1a**). It was therefore unexpected to find that all three antibodies efficiently catalyze the hydrolysis of p-nitrophenyl(NP)-ester **1p** (Tables IV & V).

Table V. k_{cat}/Km for the hydrolysis of various ester substrates, R-C(=O)-OR' by catalytic antibody D2.4.[a]

Substrate	R =	R' =	$k_{cat}/Km_{(M^{-1}\cdot s^{-1})}$
1a	HO_2C-CH_2NH-$CO(CH_2)_3$-	p-Nitrobenzyl	114
1g	" "	Benzyl	n.r.
1e	" "	o-Nitrobenzyl	n.r.
8a	CH_3-	p-Nitrobenzyl	<10[b]
1p	HO_2C-CH_2NH-$CO(CH_2)_3$-	p-Nitrophenyl	11,100
1h	" "	o-Nitrophenyl	n.r.
8p	CH_3-	p-Nitrophenyl	<10[b]

[a] n.r., non-reactive. The specificity pattern of the two other catalytic antibodies, D2.3 and D2.5, observed with these substrates is essentially the same as obseved with D2.4; k_{cat}/Km for **1a** and **1p**, that are the only substrates for these antibodies, are given in Table IV.

[b] With both NP- and NB- acetates (**8a** and **8p**) a very low rate enhancement was observed in the presence of 1-10 μM antibody and 100-300 μM substrate ($\upsilon o_{cat}/\upsilon o_{uncat} < 2$; relative to a non-catalytic antibody); this rate enhancement corresponds to a k_{cat}/Km value smaller than 10 $M^{-1}\cdot s^{-1}$.

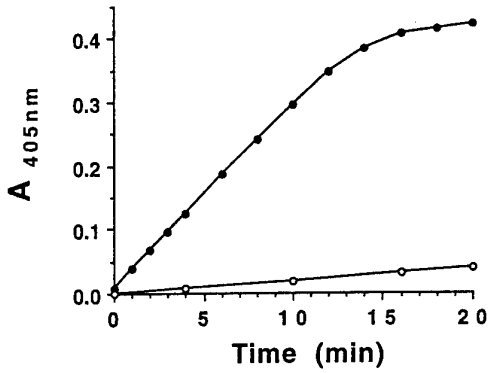


Figure 15. Hydrolysis of p-nitrophenyl ester **1p** (0.3 mM; pH 8.3) in the presence of antibody D2.4 (1 μM, •) and, inhibition of catalytic activity in the presence of PNB-phosphonate **4b'** (**4b**-N-benzyl amide, 2.5 μM, o)

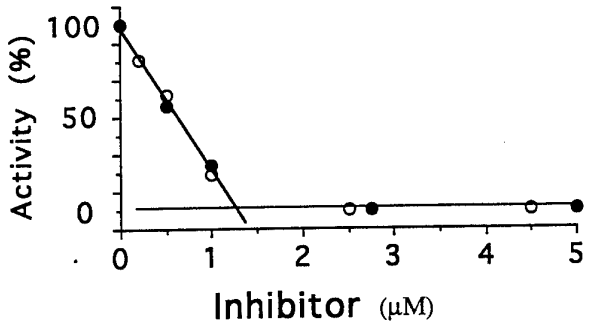


Figure 16. Esterolytic activity of antibody D2.4 (0.65 μM) with NP-ester **1p** (o) and NB-ester **1a** (•) was titrated in the presence of various concentartions of the hapten, **4a**-N-benzyl amide. Initial velocities observed with these substrates (**1p**, 0.1 mM; **1a**, 0.3 mM) are presented as relative activities (υ_o at zero inhibitor concentrations = 100%).

Evidence that hydrolysis of both NB and NP esters occurs at the same active site comes from titration curves in the presence NB phosphonate **4a**-N-benzyl amide, a hapten derivative which has sub-nanomolar affinity to these antibodies). Titration of the esterolytic activity of D2.4 towards both the NB-ester and the NP-ester gives essentially identical curves (Figure 16). The same results were obtained with antibodies D2.3 and D2.5. In addition, the activity towards these ester substrates is mutually exclusive; for example, hydrolysis of the NP-ester **1p** (0.1 mM) catalyzed by D2.4 (1 μM) is competitively inhibited by NB-ester **1a** (at [**1a**]=0, υ_o= 3.52 μM·min^{-1} (100%); at [**1a**]=0.3 mM, υ_o= 1.78 μM·min^{-1} (50%); at [**1a**]=1.0 mM, υ_o=0.65 μM·min^{-1} (20%)).

Competitive inhibition immunoassay (CIEIA), under conditions where hydrolysis of the substrates is extremely slow (pH 6.0), was used to estimate the affinities of these antibodies towards the NP-substrate and phosphonate-TSA. The BSA conjugate of NB-phosphonate **4a** immobilized on microtiter plates, was used as the solid-phase for these assays. A typical pattern obtained with antibody D2.4 is

shown in Figure 17. The differential affinities, K_S/K_{TSA}, extrapolated from CIEIA, for all 3 antibodies are given Table VI.

Table VI. Differential affinities, K_S/K_{TSA}, measured for catalytic antibodies D2.3, D2.4 and D2.5 with NB and NP substrates (**1a**, **1p**) and phosphonates (**4a**, **4p**), are compared to the rate enhancements, k_{cat}/k_{uncat}, and to Km/k_{cat} values for the antibody-catalyzed hydrolysis of these substrates.

	p-Nitrobenzyl (NB)			p-Nitrophenyl (NP)		
Ab	K_S/K_{TSA}	k_{cat}/k_{uncat}	k_{cat}/Km	K_S/K_{TSA}	k_{cat}/k_{uncat}	k_{cat}/Km
D2.3	$1.1X10^5$	$2.6X10^5$	440	$1.8X10^3$	$1.93X10^4$	2690
D2.4	$3.3X10^4$	$7.3X10^4$	114	$2.8X10^2$	$2.67X10^4$	11,100
D2.5	$1.3X10^5$	$5X10^3$	6.9	$2.0X10^2$	$6.2X10^3$	1,340

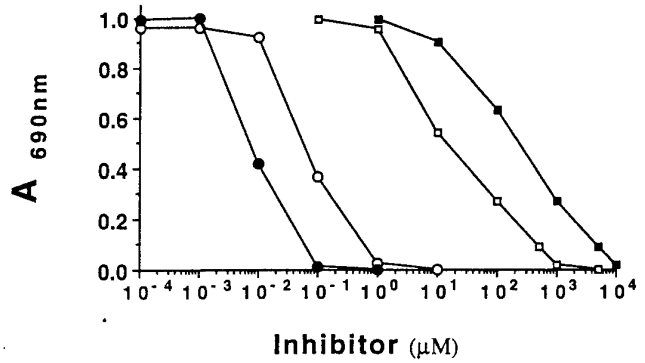


Figure 17. Relative Affinities of antibody D2.4 to the NB and NP phosphonates (**4a**, ●; **4p**, o) and substrates (**1a**,■; **1p**,□) were determined by competative inhibition immunoassay (CIEIA).

Analyzing the data presented in Table VI and Fig. 17 leads to several conclusions. First, relative affinities of D2.4, and of the other two antibodies, measured by CIEIA indicate that the differences observed in Km values for the two substrates may be correlated to differences in binding affinities (e.g., for D2.4, Ks^{1a}/Ks^{1p} = 15, Figure 4, and Km^{1a}/Km^{1p}= 15, Table IV & VI). This data is important because differences in Km observed with different substrates do not necessarily reflect differences in *affinity* of the enzyme to these substrates. A well known example is the lower Km and higher k_{cat}/Km values in the activity of serine proteinases with ester vs. amide substrates (54). This difference is attributed to the change of the rate determining step of the enzyme-catalyzed reaction rather than to differences in affinities toward these substrates. Second, as expected, all three antibodies exhibit higher affinity to the NB-phosphonate-hapten **4a** which was used to generate them, rather than to the NP-phosphonate **4p**. This order of affinities is opposed to that observed with the respective substrates (**1a** & **1p**).

The application of phosphonoesters as TS analogs, both with enzymes (as potent competitive inhibitors of several proteinases (55,56)) and for the generation of ester-hydrolyzing antibodies (8-10,21) is well-documented. Thus, phosphonates, more than other TSAs, may allow us to draw some general conclusions.

These results support the use of expanded TSA (57), i.e., it is shown here that highly effective p-nitro*phenyl* ester hydrolyzing antibodies can be elicited using a p-nitro*benzyl* phosphonate hapten. Practical uses of catalytic antibodies demand the generation of more effective catalysts in terms of k_{cat}/Km as well as the minimalization of product inhibition to allow efficient turnover (10). Severe product inhibition is often observed in catalytic antibodies (Section 2 and refs. 10,28-33). It is suggested here that including in the design of the hapten expanded elements to mimic those bonds that are being cleaved, or formed, in the transition state of the reaction to be catalyzed (such as demonstrated here for a phenyl ester substrate using a benzyl phosphonate hapten) may provide more efficient, enzyme-like, catalytic antibodies. The fact that several of the antibodies elicited against NB-phosphonate **1b** were found to react so well with NP-ester **2p** and that none of these antibodies is inhibited by the p-nitrophenol product suggests that expanded TSAs may be generally useful.

8. Towards antibody-mediated peptide hydrolysis

The uncatalyzed hydrolysis of a peptide proceeds at pH 7 with half life of 7-9 years (58). Rates of hydrolysis of ordinary, unactivated amides (under neutral or basic conditions) is 10^6-10^8 slower than that of esters. Thus, a major kinetic problem (i.e., a high activation energy barrier) has to be solved. In addition, amide hydrolysis is a multi-step process (Fig. 20A); the catalyst (antibody or enzyme) must efficiently stabilize the TSs leading to the formation as well as the breakdown of both $\mathbf{I_1}$ and $\mathbf{I_3}$. The last step ($\mathbf{I_3}$) requires protonation of the leaving amino group and is most critical; otherwise, the reversed reaction, back to $\mathbf{I_1}$ and to the substrate takes over.

Enzymes cope very well with all these demands; utilizing general-base, covalent and electrophilic catalysis for the first step, and highly efficient general-acid catalysis for protonation of the leaving amino component in the next step. Consequently, proteolytic enzymes exhibit remarkable rate accelarations (i.e., k_{cat} of up to 200 s^{-1} and k_{cat}/k_{uncat} of up to 10^{10} !) for the hydrolysis of a variety of peptides and other unactivated amides. Can an antibody achieve such demanding reactions? On the basis of the catalytic antibodies described thus far the answer, for the time being, is not yet!

Relative to enzymes, antibody-mediated catalysis is still very modest (10); the largest turnover number, k_{cat}, reported for a catalytic antibody is 20 s^{-1} (59), while k_{cat} values as high as 10^5 s^{-1} are not uncommon for enzymes. The catalytic power of antibodies is generally presented in terms of k_{cat}/k_{uncat}, i.e., the rate enhancement of the catalyzed reaction, rather than the absolute rate, k_{cat} (turnover number), commonly used to describe enzymatic activity. Rate enhancement values for catalytic antibodies are generally in the range of 10^2-10^5 which are still much lower than those for enzymes (where rate enhancements are as high as 10^{14}).

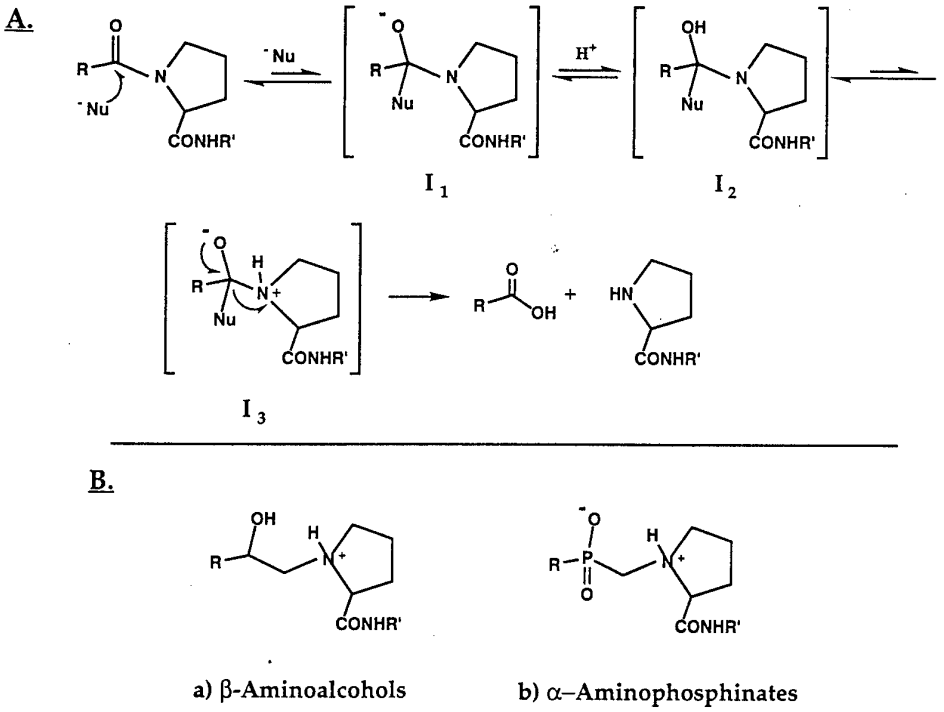
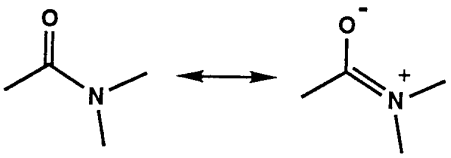


Fig. 20. A. Schematic mechanism of the hydrolysis of an acyl-prolyl bond. Nucleophilic attack on the acyl carbonyl leads to the first intermediate ($\mathbf{I_1}$). Cleavage of the bond occurs mainly through intermidiate $\mathbf{I_3}$ in which the proline nitrogen is protonated. This intermediate is thermodynamically disfavored (i.e., both $\mathbf{I_1}$ and $\mathbf{I_2}$ dissociate more readily to the starting material rather than to cleavage products). Thus stabilization of $\mathbf{I_3}$ should significantly affect the rate of hydrolysis.
B. Proposed analogs for intermediate $\mathbf{I_3}$. Structures appear as their ionic forms at pH 7.4.

In addition, the nature of all of the reported antibody-catalyzed reactions is such that they proceed with measurable rates even in the absence of any catalyst, i.e., the reactions have a relatively low activation energy barrier. This point can be readily illustrated with hydrolytic reactions. Most of the antibody-catalyzed hydrolytic reactions involve esters, and in particular phenyl esters (which are relatively labile); alkyl or benzyl ester hydrolyses were also described, yet these usually proceed with much lower rates. Carbamates, having medium stability (i.e., between esters and amides) have not yet been reported to be hydrolyzed by antibodies. The hydrolysis of amides, which are orders of magnitude more stable than esters, was reported for highly activated amide substrates (i.e., a p-nitroanilide, (60)) or when intramolecular deamidation could occur (61), or with a metal complex (62). Martin et al, described an antibody (elicited against a methylphosphinate hapten) that catalyzes the hydrolysis of a dansylated D-phenylalanine amide (63); however, the activity is extremely weak ($k_{cat}=1.65\times10^{-7}$ s^{-1}; $k_{cat}/k_{uncat}=132$) and, control experiments to eliminate the possibility of enzyme contaminants are not fully convincing.

Our attempts to achieve antibody catalyzed amide hydrolysis include the design of novel transition state analogs for cleavage of -X-Pro and Phe-X bonds (Section 8.4) as well as for intramolecular, or substrate assisted reactions (Section 8.5). The choice of X-Pro- bonds has been primarily made in order to exploit their relatively low energy barrier of twisting of the amide bond. The stability of amide bond relatively to esters, is mainly attributed to delocalization of the nitrogen lone pair, which also results in the planarity of the amide group (Scheme 1).



Scheme I

Twisting of an amide bond causes the loss of its delocalisation energy (18-21 Kcal/mole). Thus, distorted amides were shown to adopt reaction patterns and rates of hydrolysis similar to those of esters, i.e. protonation of the leaving amine component, which generally determines the rate of bond cleavage, is highly facilitated in the twisted form and so is the nucleophilic attack on the carbonylic center (64). -X-Pro- bonds are unique in their significantly low free energy barrier of cis-trans isomerisation, i.e., activation energy of the twisted state is relatively low (65). The binding energy of an antibody can be utilized to induce formation of that high-energy twisted transition state at the binding site, thus leading to bond cleavage. Induction of strain and stress were found to play an important role in the catalytic mechanisms of various enzymes, including proteinases (66), but were not yet used for antibody-mediated catalysis.

Our TSA-haptens were therefore designed to: (*i*) induce strain and stress on the bond to be cleaved, and (*ii*) to mimic the partially protonated leaving amino component of the tetrahedral intermediate/TS $\mathbf{I_3}$ (Fig. 20.A), that leads to the cleavage of the amide bond. Thus, in the TSA-haptens designed for our study: *i*) bond angles, and lengths, around the C-N bonds significantly differ from the planar ground state amide substrate; and *ii*) the proline component is protonated and positively charged at physiological pH (Fig. 20.B). These elements are absent in simple phosphonoesters such as **4a** (see Section 2.4) and in almost all other haptens used thus far to elicit amide-hydrolyzing antibodies. The oxyanion portion of the transition states is mimicked in these haptens by different elements, i.e., by a hydroxyl group (**a**) or by an oxy-phospho group (**b**), Fig. 20.B. The synthesis of these haptens is described in Sections 9.2 and 9.3.

An appropriate catELISA system to screen for antibodies that catalyse the hydrolytic cleavage of -X-Pro- bonds was designed. Rabbit polyclonal antibodies, elicited against the KLH conjugate of prolylglycine (**11**) (Fig. 21A), are used for detection (Section 6). The feasibility of detection by catELISA was demonstrated by the base-catalysed hydrolysis of the p-nitrophenyl and phenyl carbamates (**10c** and **10i**, respectively; Fig. 21). With all the substrates, amides and carbamates, no background signal was observed even after prolonged incubation (>24 hrs at 37^{o}c, pH 5-9) with hybridoma supernatants, sera and diluted ascites fluid. Hydrolysis was observed only with carbamates **10c** and **10i** under basic conditions (Section 6).

Spleen, or draining lymph node, lymphocytes taken from mice immunized with haptens **12a-c** and **13** were fused with myeloma cells and the resulting hybridomas were screened by catELISA for the production of antibodies catalyzing the hydrolysis of the corresponding substrates (**10a-f** and **10g-i**,

respectively, Fig. 21). A large number of monoclonal antibodies generated against three different aminoalcohol haptens (Section 8.1) and an aminophosphinate hapten (Section 8.2) did not afford even one clone that scored positive for the hydrolysis of nine related -X-Pro- amide and carbamate substrates. We tentatively conclude that if antibodies that catalyze the cleavage of X-Pro- bonds are produced, they are extremely rare. Alternatively, they must be selected by immunization with completely different TSA-analogs than those described here.

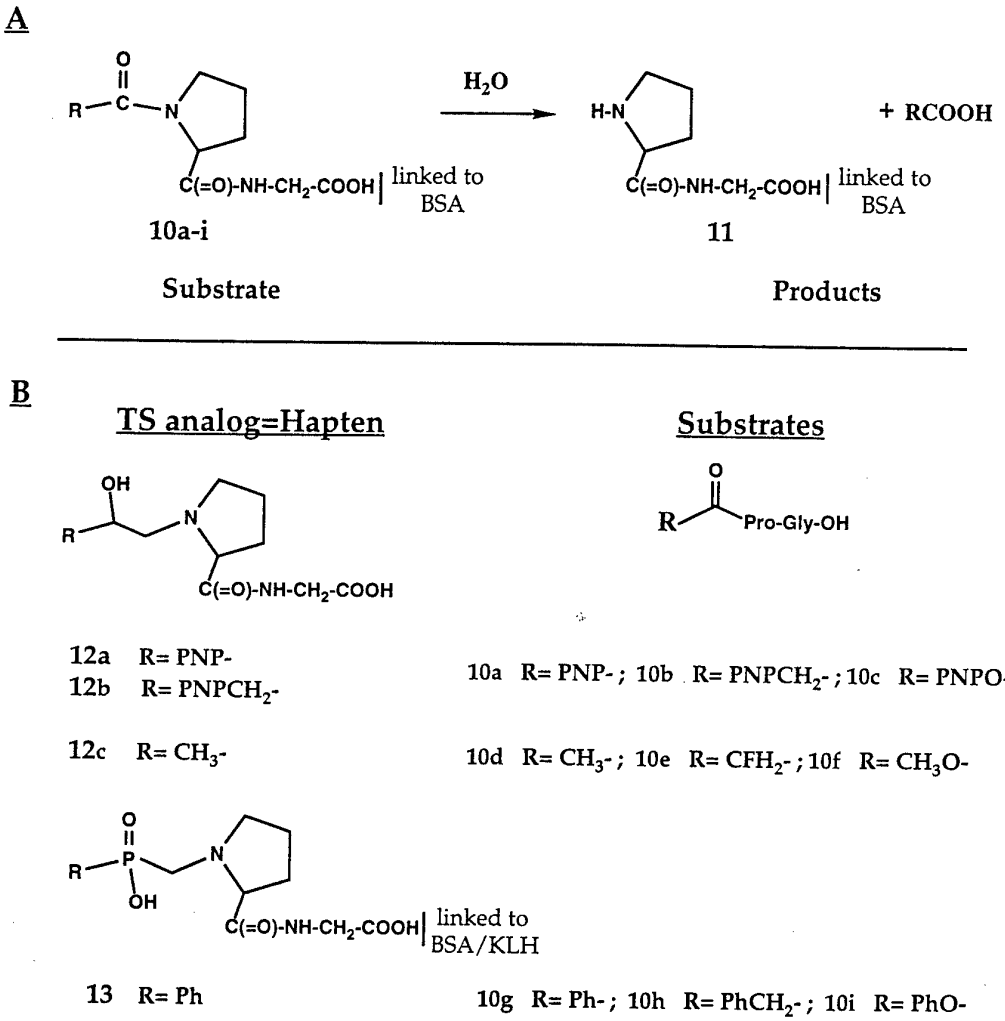


Fig. 21. The DPA system (All the proline residues introduced in both the haptens and the substrates are L-prolines): **A.** Hydrolytic cleavage of the immobilized substrates, **10a-i**, leaves the prolylglycine amino component, **11**, bound to the "solid-phase". Detection of that product was performed by catELISA using anti-**11** rabbit antibodies.
B. Stucture of TS analogs used as haptens, **12a-c** and **13**, and, the corresponding substrates, **10a-f** and **10g-i**, respectively.

It is also possible that -X-Pro bonds have intrinsic problems and were not a good choice as a target for antibody catalysis. Enzymes that catalyze the hydrolysis of *endo* -X-Pro bonds are extremely rare (actually, the only known example is the HIV-proteinase). There are only a few known *exo* peptidases with -X-Pro specificity, i.e., where X is the amino terminus (67). In fact, this was one consideration in our choice of endo -X-Pro bonds; screening complications due to enzyme contaminants

would be avoided, and, if successful, novel catalysts for reactions for which enzymes with similar substrate specificities do not exist would be obtained. The reason for the rarity of enzymes with -X-Pro cleaving activity is not known. Perhaps this restricton holds for catalytic antibodies as well. The fact that we were not able to identify antibodies that catalyze the *formation* of -X-Pro bonds (Section 8.3) via amidation of the respective esters (a facile reaction, of the type for which catalytic antibodies were already produced) supports this notion.

Efforts to isolate antibodies that catalyze both the cleavage and the formation of X-Pro bonds merits more effect in the future. New transition state analogs, based on the same design as in hapten **13**, directed for the cleavage of other peptide bonds (e.g., Phe-X bonds, Section 8) have been synthesized. Another possibility is to synthesize and immunize with the D-enantiomer of hapten **13** (or of **12a-c**). Racemic TSAs, i.e., a mixture of enantiomers that correspond to both the L and D amino acid substrates, were used in literature reports. Analysis of the resulting monoclonal antibodies showed that catalytic antibodies were generated only against the D enantiomer and were specifically active towards the respective D ester or amide substrate (for examples, see refs. 63 and 68). This phenomenon cannot be explained by the higher antigenicity of D amino acid derivatives. A possible explanation is that selection *against* antibodies that catalyze the cleavage of natural peptide substrates exists. It should be interesting to see whether D-haptens, unlike the L-haptens, will generate antibodies that hydrolyze -X-Pro bonds (of -X-D-Pro substrates).

8.1. Cleavage at X-Pro: β-Aminoalcohol Haptens

β-Aminoalcohols were used in the first attempts to generate antibodies that catalyse amide hydrolysis. Three differently substituted β-amino alcohols of proline (**12a-c**, Fig. 21) were prepared and conjugated to carrier proteins (see, section 9.2). All 3 haptens **12(a-c)-KLH**, including the methyl substituted one, **12c**, proved to be highly immunogenic. High binding titers (> 10,000) to the corresponding BSA conjugates were observed in the sera of immunized mice even after two immunizations. Inhibition of binding by the free (unconjugated) haptens, indicated hapten specificity of the sera.

Spleen, or lymph node, lymphocytes taken from mice immunized with **12 (a-c)-KLH** were fused with NSO myeloma cells. After two weeks, the resulting hybrid cells were screened by catELISA for catalyzing the hydrolysis of the corresponding substrates (**12 a-f**, Fig. 21). Among a considerable number of hapten binding clones (Table VII), no antibody-catalyzed hydrolytic signal was detected by the catELISA. More detailed studies with the hapten-binding clones elicited against **12(a-c)** indicated that all the secreted antibodies bind the corresponding substrates. Furthermore, a considerable number of clones exhibited differential binding affinity in favor of the TSA compared to the substrate (K_S/K_{TSA}), as expected for a catalytic antibody. Immunization of different strains of mice, including autoimmune-prone mice also failed to yield any catalytic clones.

Table VII. Results of screening of clones generated against amino alcohol haptens **12a-c.**[a]

			Number of clones	
Hapten	Mice	Immunization[b]	Total	Hapten-binding
12a	5X Balb/c	2 / 21 days	1248	27
12a	1X Balb/c	4 / 45 "	1176	175
12b	3X C57/BL	2 / 21 "	288	96
12b	3X Balb/c	2 / 21 "	672	175
12c	1X Balb/c	4 / 46 days	1056	134

[a] All haptens were conjugated to KLH (hapten densities: **12a**, 14; **12b**, 13; **12c**, n.d.) Hapten binding was determined by ELISA using the corresponding hapten-BSA conjugates and by, inhibition of binding by the free, unconjugated haptens (as N-methylglycyl amides, > 85% at 1 mM). CatELISA with the corresponding substrates (see Fig. 21) was performed under the conditions described for amide **2a**.

[b] Immunization details: number of injections /total immunization period (for typical immunization protocols, short protocol (2/21 days), and a long protocol (4/45 or 5/80 days).

8.2 Aminophosphinate Haptens

Aminophosphinates contain both an oxyanion and a protonated amine component; they are therefore a closer mimic of the intermediate $\mathbf{I_3}$, leading to cleavage of the amide bond (Fig.18A). After several unsuccessful attempts, a convenient route for the synthesis of these compounds was found. The phenyl phosphinate **13**, together with the corresponding substrates, **10g-i** (see Fig. 21) were synthesized and conjugated to carrier proteins.

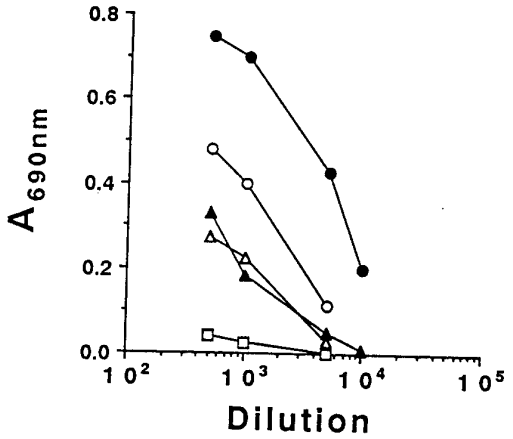


Fig. 22. Binding activity of polyclonal serum antibodies of a mouse immunized with **13-KLH**. Five Balb/c mice were immunized with **13-KLH** (50 μg/mouse emmulsified in CFA). After 14 days a boost was administered (50 μg/mouse in INCFA). Serum taken 10 days later was diluted in PBS (1:500-1:10,000) and assayed, by ELISA, for binding to **13-BSA**, ●, **10g-BSA**,○,**10i-BSA**,△, and BSA,□(1μg/ml of these conjugates, in PBS, were used for coating). Results presented above are of serum taken from mouse #3 that exhibited the highest titer. Competitive inhibition of binding to **13-BSA** was observed in the presence of free, unconjugated **13** (13-N-glycylmethyl amide), 5μM, ▲, (and, > 95% inhibition at 0.1mM). Binding to **13-BSA** was inhibited in the presence of substrates as well, yet with a significantly higher I_{C50} (e.g., **10g**, I_{C50} = 2mM, at 1:2,500 serum dilution).

As in the case of the aminoalcohol haptens (**12a-c**), amino phosphinate **13** gave a strong, specific binding response in Balb/c mice (Fig. 22). Immunization of additional strains of mice, e.g., SJL, C57BL, or C3H, resulted in even higher binding titers in the sera. Hybrid clones obtained by fusion of lymphocytes taken from these mice with NSO cells were screened for binding of the hapten, and by catELISA for cleavage of the corresponding substrates **10g-i** (Fig. 21). These experiments are summarized in Table VIII; none of the hundreds of clones gave a positive catELISA signal using the amide substrates **10g** and **10h**. No catELISA signal was even found with the less stable carbamate, **10i**.

Table VIII. Results of screening of clones generated against amino phosphinate hapten **13.**[a]

			Number of clones	
Entry	Mice	Immunization[b]	Total	Hapten-binding
1	BALB/c	5/46 days	1112	34
2	C57/BL	4/46 "	520	32
F1	" "	6/68 "	768	38
F3	SJL/J	4/44 "	1728	1690
F4	C3He/J	4/44 "	1100	86
3	3X MRL/*lpr*	2/21 "	768	n.d.

[a] Hapten binding was determined by ELISA using the hapten-BSA conjugates and by, inhibition of binding by the free unconjugated haptens (**13**-N-methylglycyl amide, > 85% at 0.1 mM). CatELISA with the corresponding substrates (see Fig. 21) was performed under the conditions described for amide **2a**.

[b] Immunization details: see Table VII.

8.3 Attempts to identify antibody-catalyzed formation of a -X-Pro bond

Unlike amide hydrolysis, the formation of amide bonds by amidation of carboxylic esters, proceeds at measurable rates even in the absence of a catalyst. Antibodies that catalyze similar reactions were generated before our work had begun (69) and two examples of antibodies that catalyze peptide bond formations were published soon after (70,71). The cleavage and the formation of an amide bond proceed via the same transition states. Thus, the system designed for the identification of -X-Pro bond cleavage was adapted to search for antibodies that catalyze the amidation of benzoyl esters to synthesize -X-Pro bonds (Fig. 23).

Immobilized prolylglycyl conjugates (**11-BSA**; previously used as the product of the cleavage reaction, see Fig. 21) were reacted with benzoyl esters (**16a-d**) to give benzoyl-prolylglycine (**10g**). Conjugates of **10g-BSA** that were previously used as substrates for cleavage were used here as the product conjugates; polyclonal anti-product serum was elicited in rabbits and used for detection by catELISA (Section 6). Antibodies elicited in mice against phosphonate hapten **13**, and against hapten **15** (for synthesis, see section 9.3), were assayed for catalyzing the amidation reaction described above. The results of these experiments are summarized in Table IX;

none of the ca. 3,300 clones elicited against these haptens afforded antibodies that catalyze the amidation of benzoyl esters **16b-d** to give **10g**, as tested by catELISA.

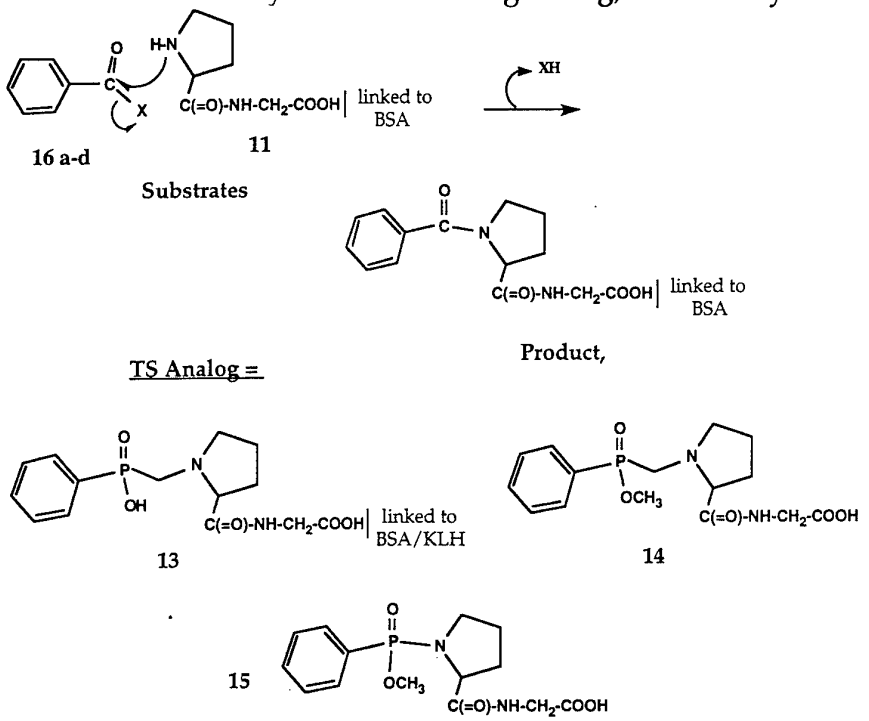


Fig 23. The experimental system applyed for the identification of antibodies that catalyze the formation of -X-Pro bonds. Antibodies elicited against haptens **13-15** were screened by catELISA for catalyzing the acylation of immobilized prolylglycyl conjugates (**11**) by benzoyl esters (**16a-d**), using rabbit polyclonal antibodies for product **10g**.

Table IX. Results of screening of clones generated against haptens **13** & **15**.[a]

			Number of clones	
Hapten	Mice	Immunization[b]	Total	Hapten-binding
13	1X SJL	4 / 50 days	864	864
15	1X Balb/c	4 / 46 "	1152	240
15	5X MRL/lpr	2/ 21 "	1344	806

[a] Hapten-KLH conjugates were used for immunizations; for typical immunization protocols see Footnotes nos. 2,3 & 7. Hapten binding was determined by ELISA using the corresponding hapten-BSA conjugates and by, inhibition of binding by the free, unconjugated haptens.

[b] Immunization details: see Table VII.

8.4 Cleavage at -Phe-X-

In the previous sections the (as yet) unsuccessful attempts to generate antibodies that catalyze reactions at -X-Pro bonds were described. Thousands of clones that bound seven different haptens did not afford a single antibody that catalyzes any of the reactions examined, i.e, hydrolysis or formation of a variety of

-X-Pro bonds. The possibility exists that the repertoire of antibodies that catalyze reactions at -X-Pro bonds is particularly restricted. It was therefore decided to investigate a new series of TSA-haptens that are based on sequences without proline.

Phenylalanine was chosen mainly to take advantage of the antigenecity of the aromatic side chain. A catELISA system was established to allow detection of the carboxylic moiety of a phenylalanine residue which results from the cleavage of any Phe-X bond (Fig. 24 and Section 6). The first hapten to be tested was the aminophosphinate **31,** using morpholine as a model rather than introducing a complete amino acid sequence. The same methodologies used for the synthesis of hapten **13** were applied here (see Section 9.3). The corresponding morpholine amides (**32**) and primary amides (**33**) were used as substrates for screening. In addition, unlike the situation with X-Pro cleavage, hydrolysis of the related ester substrates can also be examined (Fig. 24). Hapten **31** (unlike the proline haptens **12** & **13**) was prepared and injected as a racemate (a mixture of both phosphinate analogs of L and D phenylalanine); both enantiomers (L and D) of the corresponding substrates must be assayed.

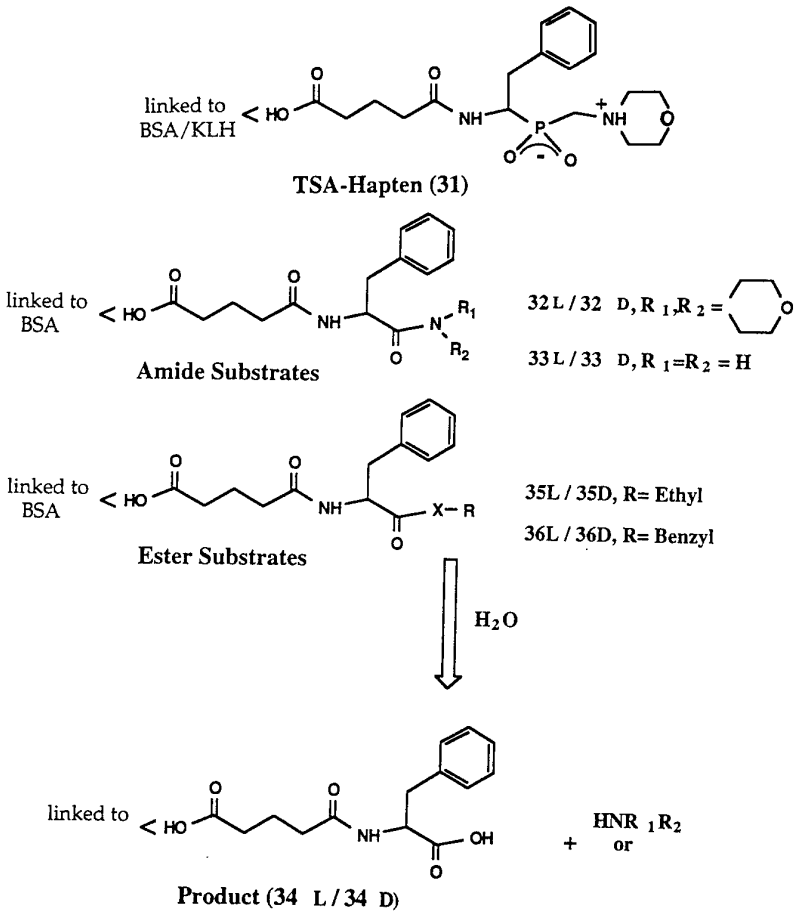


Fig. 24. The CLA system: Structures of the substrates, amides **32** & **34**, and esters **35** & **36**, the product of their hydrolyses, **34**, and of the aminophosphinate hapten **31**. Hapten **31** was prepared and used as a racemic mixture while for all substrates and products, both enantimers, L and D, were separately synthesized and conjugated. Substances were linked to carrier proteins (BSA or KLH) via the carboxyl group of the glutaryl linker. Ester substrates (**35** & **36**) were also used for preparation of the product conjugates and to model the catELISA detection (Section 6).

Different mouse strains were immunized with TSA-hapten **31** and satisfactory serum titers (1:5,000 - 1:20,000) were obtained. Fusions using Balb/c and SJL mice afforded relatively low numbers of hapten-binding clones, but the MRL/*lpr* strain (short immunization, 21 days) gave hundreds of binding clones. Three clones gave positve catELISA signals with D-phenylalanine morpholine and the primary amides (**32D** and **33D**); surprisingly no activity with the ester substrates was found with any of the clones. The clone with the highest activity (F24.19) was subcloned and further studied. The properties of F24.19 are consistent with it being a catalyst: differential binding of TSA-hapten relative to substrate ($K_S/K_{TSA} > 10^3$); loss of hapten binding at pH > 9, as expected for morpholine deprotonation; pH-independent binding to substrates **32D** and **33D**; inhibition of catELISA signal by 31-N-benzylamide. However, competitive inhibition shows that the antibody is severely product inhibited, binding the product as strongly as the hapten. Much more work is needed to better understand the behavior of F24.19. Perhaps we have an amide-hydrolyzing antibody that is product inhibited; perhaps we have a stoichiometric reaction between antibody and substrate to give a covalent acyl-antibody adduct which is stable under the catELISA conditions. This is one of the most tantalizing results that we are left with at this stage.

8.5 Intramolecular catalysed amide hydrolysis

Intramolecular catalysed reactions are well studied models for proximity and orbital steering effects in enzyme catalysis (72-74). Numerous reactions were shown to proceed with large rate enhancements (10^2-10^{13}) when two reacting groups, e.g., a catalytic residue and a "substrate", are situated near on the same molecule. Intramolecular, or substrate assisted, mechanisms were found to occur in enzymes as well, e.g., ribonuclease (75).

Intramolecular catalysed amide hydrolysis was studied in various synthetic structures (mainly with hydroxyl and carboxyl groups as nucleophiles) and is also observed in peptides and even in natural proteins. Two well known examples are: (i) deamidation of asparagine, which occurs most often at Asn-Gly sequences, to give α or β aspartate (76-79); and (ii) cleavage at Asp-Pro bonds (which proceeds readily at mild acidic conditions (80)) (Fig. 25). In the case of Asp-Pro cleavage, it is evident that the steric properties of the proline largely contributes to the susceptibility of this bond (81). The mechanisms of deamidation reactions were studied in detail and were shown to proceed via cyclic intermediates (77, 78). The nature of these intermediates, and of the transition states leading to their formation, can also be deduced from related intramolecular reactions that were studied in detail (82).

Owing to their "internal reactivity", intramolecular reactions are attractive candidates for antibody-catalysed amide hydrolysis. Antibody catalysis should be designed to provide TS stabilization as well as "orbital steering", i.e., to create a molecular conformation of the peptide such that the catalytic group is correctly oriented.

Eliciting antibodies that catalyze intramolecular reactions such as those described in Fig. 25 obviously requires cyclic haptens. Unfortunately very little information exists about stable compounds, neither phosphonates nor others, that could mimic such cyclic transition states. Considerable efforts were therefore invested in studying the chemistry of several cyclic phosphonates and phosphinates that might serve as haptens for eliciting antibodies expected to catalyze intramolecular, or substrate-assisted, amide hydrolysis (see section 9.4). These efforts yielded, so far, the cyclic phosphonoimide hapten **51** (Fig. 26).

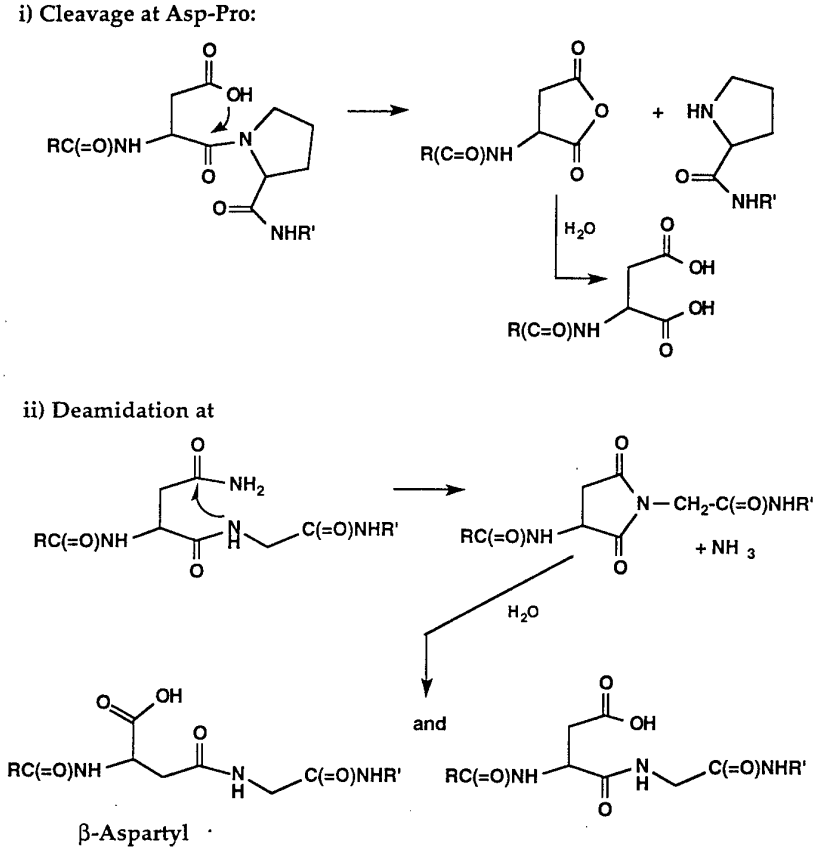


Fig. 25. Intramolecular catalyzed amide hydrolyses observed in proteins: (i) cleavage at Asp-Pro bonds; and (ii), deamidation of asparagine, which occurs most often at Asn-Gly sequences, to give α or β aspartate.

Phosphonoimide **51** is a stable analog of the intermediates $\mathbf{T_1}^-$ and $\mathbf{T_2}^-$ that lead, respectively, to the intramolecular deamidation of amide **2d** to give imide **5**, and the base-catalyzed hydrolysis of the latter to yield acid **3**. This reaction serves as a model for the intramolecular deamidation of glutamine and asparagine in peptides.

The intramolecular reactivity of succinyl derivatives is significantly higher than that of the glutaryl ones (at least 70 fold (76)); yet the corresponding TSA, i.e., a 5-membered phosphonoimide, is highly unstable (Section 9.4). Nevertheless, the 6-membered TSA seems to be close enough to mimic the TS of the succinyl derivatives as well; therefore the succinamide and succinimide derivatives (**2e** and **5a**; Fig. 24) were prepared. CatELISA with N-glycine-succinimide conjugated to

BSA, using sodium carbonate to catalyze its hydrolysis, indicated that rabbit anti-acid **3** antibodies cross-react with the resulting N-glycyl-succinic acid, **3a**. Thus, deamidation of succinamide **2e** and the hydrolysis of the resulting imide, **5a**, have been tested for in the very same system.

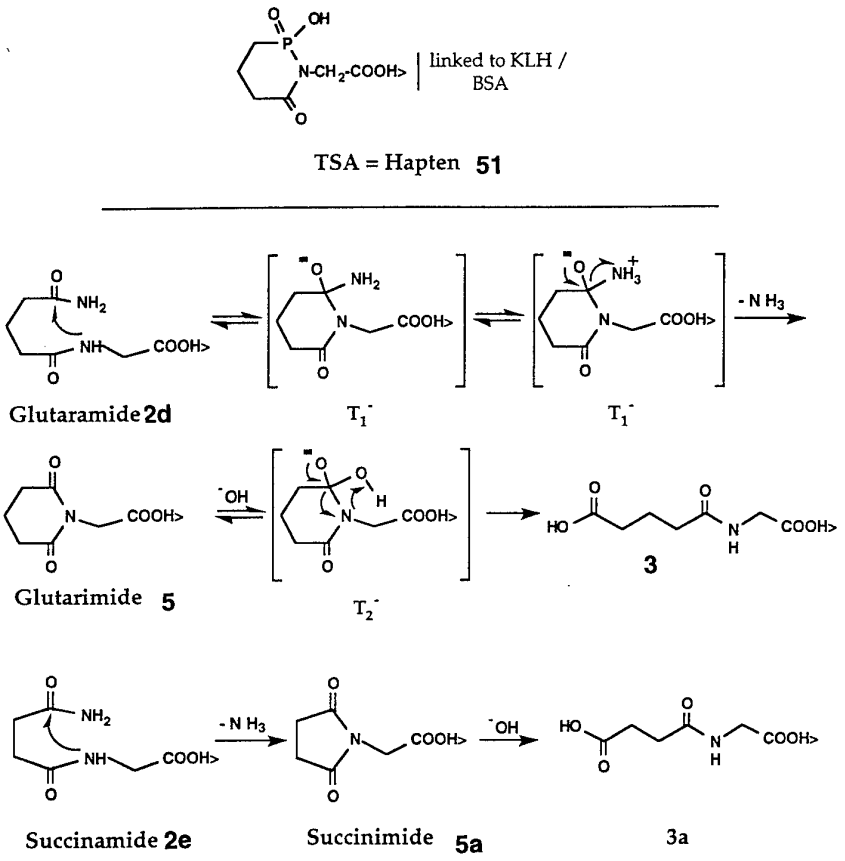


Fig. 26. The DCA system: Deamidation of glutaramide **2d** via intermediates T_1 leads to cyclic imide **5**. Hydrolysis of the relatively labile imide bond, via T_2, gives acid **3**. TSA **51**, conjugated to KLH, was used as hapten. The resulting antibodies were also assayed for catalyzing the deamidation of the succinamide derivative **2e** and the corresponding imide, **5a**. Detection of both acid products, **3** and **3a**, was performed by catELISA using anti-**3** rabbit antibodies. Substances were immobilized by covalently linking the glycyl carboxyl to the ε-amino of lysine side chains of a carrier protein, BSA or KLH. The synthesis of hapten **51** and of all the substrates is described in Section 9.4.

Balb/c mice immunized with **51-KLH** exhibited a very low hapten-specific response; only after repetitive immunizations could a significant binding titer to **51-BSA** be detected in the sera. Much higher binding titers were observed in the sera of C57BL mice immunized with the same hapten after only two injections (SJL mice, on the other hand, exihibited no response towards this hapten). In both cases, inhibition of binding in the presence of the free, unconjugated, hapten (**51**-benzyl ester, Section 9.4) indicated hapten specificity (Table X). Three separate fusions, each with the spleen lymphocytes taken from a single Balb/c or C57BL mouse gave a total of 3088 hybrid clones, out of which 51 hapten-binding clones were identified (Table X). None of these clones was found to catalyze the deamidation of

glutaramide **2d** and succinamide **2e** or the hydrolysis of imides, **5** and **5a** using catELISA.

The 51 hapten-binding clones were further examined by competitive inhibition ELISA (CIEIA) to determine their specificity pattern (Fig. 27). Binding to **51-BSA** was inhibited in the presence of the hapten, IC_{50} = 1- 50 μM (The benzyl ester of **5**, as well as the benzyl esters of the other substances were used for CIEIA). Most of the clones (~85%) bind, although with significantly lower affinity (IC_{50} > 100 μM), the ethyl-phosphono derivative of the hapten (**54c**, see Section 9.4, Scheme XXII). Thus, the antibodies selected for hapten **51** not only recognize the cyclic conformation but are also capable of stabilizing the negatively charged TSs of the reaction (for example see, Fig. 27). However, none of these clones exhibited any measurable binding affinity to any of the relevant substrates.

Table X. Results of screening of clones generated against haptens **51**.[a]

			No. of clones	
Strain	Immunization	Serum titers	Total	Hapten-binding
Balb/c	4 / 62 days	1: 1,000 (90%)	840	-
Balb/c	5 / 85 "	1: 12,500	1400	7
C57BL/6	4 / 62 "	1: 5,000 (95%)	848	44

[a] Fusions were performed with a mouse chosen from the 3-5 immunized, exhibiting the highest binding serum titer. Binding titers were determined by ELISA using **51-BSA**; also given, inhibition by the unconjugated hapten (**51**-benzyl ester, 0.1 mM) determined at the titer dilution (in parenthesis). Cross reactivity with BSA determined by ELISA at the titer was less than 10%.

This observation was confirmed by ELISA using the substrate-BSA conjugates (**2d-BSA** and **5-BSA**), coated on microtiter plates: no *binding* signal (using peroxidase-linked goat anti-mouse immunoglobulins antibodies) was observed with any of the 51 hapten-binding monoclonal antibodies.

Rabbits immunized with **51-KLH** indicated the same pattern: high binding titers (>1: 50,000) to the TSA-hapten were observed in the sera of these rabbits, but no binding to the respective substrates. Catalytic activity, assayed by catELISA, was also not found. Since the formation of an antibody-substrate complex, as with enzyme-substrate complexes, is a critical initial step for any catalytic process, it is clear that antibodies with insufficient affinity towards the relevant substrates cannot be suitable. It may be noted that cross reactivity of antibody binding of polyclonal or monoclonals, elicited against a TSA-hapten to the corresponding substrates was observed in all the other systems that we have studied thus far (see, for example, antibodies elicited against a p-nitrobenzyl-phosphonate, Fig. 6, or a phenyl amino-phosphinate hapten, Fig. 22).

These results lead us to conclude that cyclic phosphonimidic acids are suitable haptens, yet that some modifications of the haptenic structure of **51** are required. Difficulties in obtaining high binding titers in the sera and the resulting low number of hapten-binding clones (<20/mouse compared with 300, on the average, clones/mouse obtained with aromatic haptens, **4a** or **13**) indicates poor antigenicity

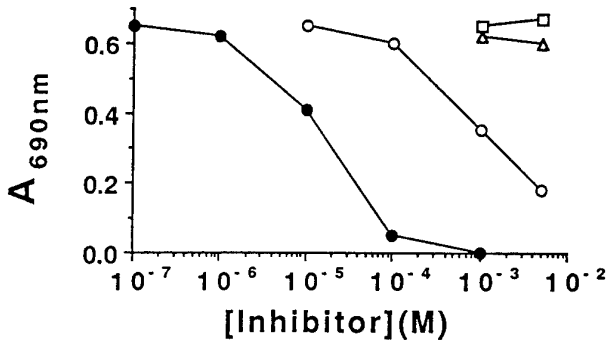
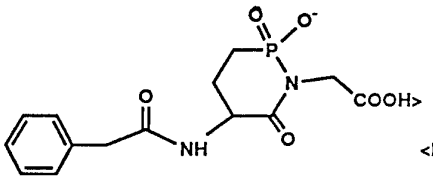
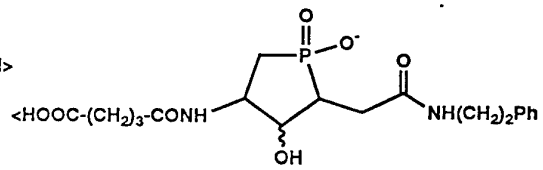


Fig 27. The affinity pattern of antibody DCA.2.1.48 was characterized by competitive inhibition immunoassay (CIEIA). Supernatants (Ab ~10nM) were incubated (1 hr, 37°C) on microtiter plates coated with **51-BSA** (1μg/ml) in the presence of various dilutions of the glycyl-benzyl esters of: hapten-bezyl ester (**55**), ● ; the P-ethoxy derivative (**54c**), o; imide **5**, □, and, amide **2d**,Δ. After washings with PBS/T and incubation with peroxidase-linked goat anti-mouse antibodies, the substrate (ATBS) was added and absorbance at 690nm was measured. Similar patterns were observed with all the other clones obtained from the same fusion.

of **51**. We have therefore designed hapten **70** with the benzamido moiety; this should not only increase the antigenicity of the hapten, but should also suffice for the required affinity to the substrates: N-benzoyl-glutaminyl-glycine (Bz-Gln-Gly-OH) and the corresponding imide intermediate. Hapten **70** is a TSA for the deamidation reaction of a glutamine, or asparagine, peptide rather than that of a model "peptide-like" substrate, such as **2d** (Scheme II); its synthesis is not simple and has not been completed (Section 9.4).



TSA-Hapten 70



Gibbs et al., 1992

Scheme II

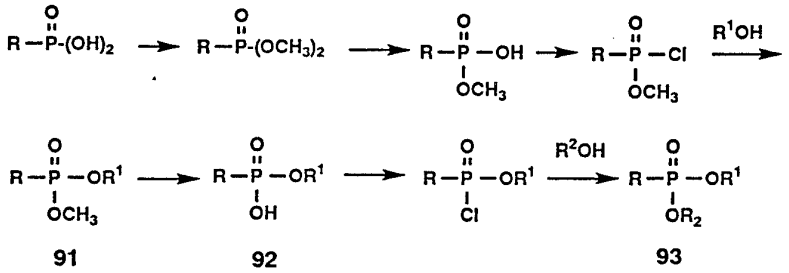
Generating antibodies that catalyze the deamidation of asparagine residues was investigated by others as well. During the course of these studies, the group of Benkovic described the generation of antibodies that mediate the deamidation of an Asn-Gly peptide (61). The hapten used is a 5-membered ring phosphinate (Scheme VII); the resulting antibodies are very weak catalysts, k_{cat}/k_{uncat} ~ 70 at pH 9.0. Thus, efforts to complete the synthesis of hapten **70** is important for any future follow-through of this project.

9. Chemical Synthesis

9.1 DBU-Mediated Transesterification of p-Nitrophenyl Phosphonates - A Novel Route to Phosphonoesters haptens.

A novel approach, based-on DBU (1,8-diazabicyclo[5.4.0]-undecene)-mediated transesterification of p-nitrophenyl phosphonates was developed for the preparation of a series of phosphonoester transition state analogues **4a-e** that were used as haptens for the generation of catalytic antibodies.

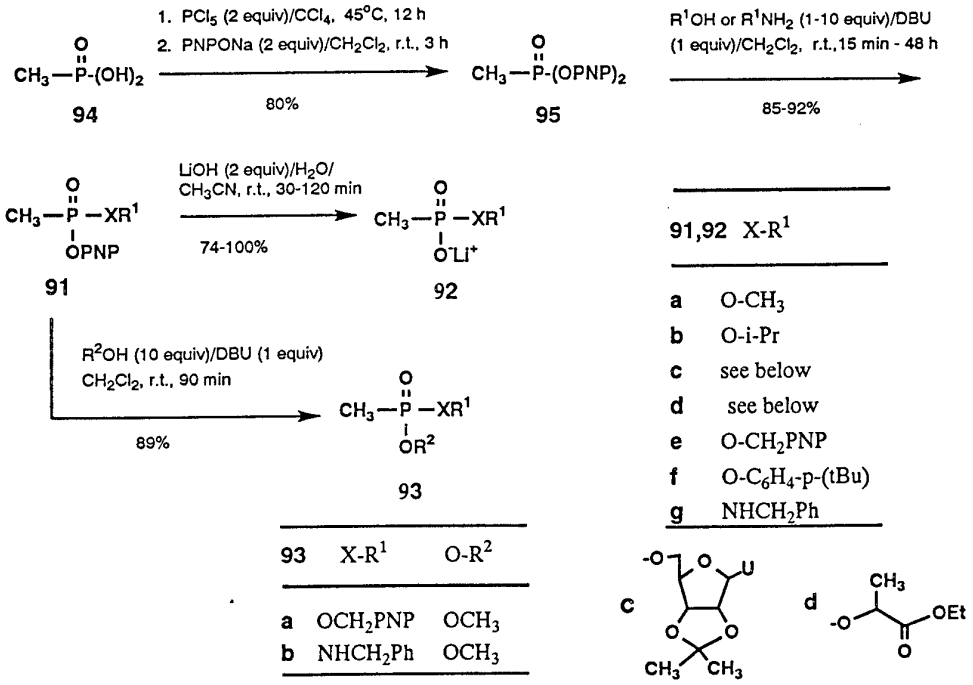
Phosphono and phosphoro esters and amidates, as transition state analogues, are potent inhibitors of many hydrolytic enzymes (55,56) and are recently being used to elicit catalytic antibodies (8-10,13,21,82; see also Sections 1 and 7). The synthesis of mono and differently disubstituted phosphonates from phosphono acids is difficult since the displacement of phosphonoacid dichloridates by alcohols is generally too rapid to allow selective mono esterifications (84). Of the various methods that afford selective esterification of phosphonic acids, the methodology most commonly used today for the preparation of phosphonate enzyme inhibitors (55,85,86) and haptens (for examples see refs. 19,26,28,60,87) from phosphono acids is summarized in Scheme III.



Scheme III

We developed a facile route to phosphono esters based on the transesterification of p-nitrophenyl (PNP) phosphonates by alcohols in the presence of 1,8-diazabicyclo[5.4.0]-undecene (DBU) (Scheme IV). The effectiveness of DBU was not matched by the other bases we tested (potassium tert-butoxide, sodium hydride, triethyl amine, and N-ethyl morpholine). The DBU-mediated reactions proceed rapidly and with high yield; in the presence of one equivalent of DBU, a period of 10 minutes-3 hours, depending on the excess of alcohol, is required to complete the exchange of one PNP ester; the rate of reaction of the second PNP ester (Scheme IV, **91**) is significantly slower than that of the bis-PNP starting material.

^{31}P NMR spectroscopy of the reaction of bis-PNP methylphosphonate (**95**, δ=25.3) in the presence of one equivalent of DBU and 5 equivalents of ethanol indicates that formation of monoethyl mono-PNP methylphosphonate (Scheme VIII, **91**: $XR^1=OCH_2CH_3$; δ=28.0) is complete within 30 minutes. The bis-ethyl phosphonate (**93**, $XR^1=OR^2=OCH_2CH_3$; δ=30.0) appears only after 12 hours, and more than 3 days were required to complete the reaction. Each ester appears as the



Scheme IV

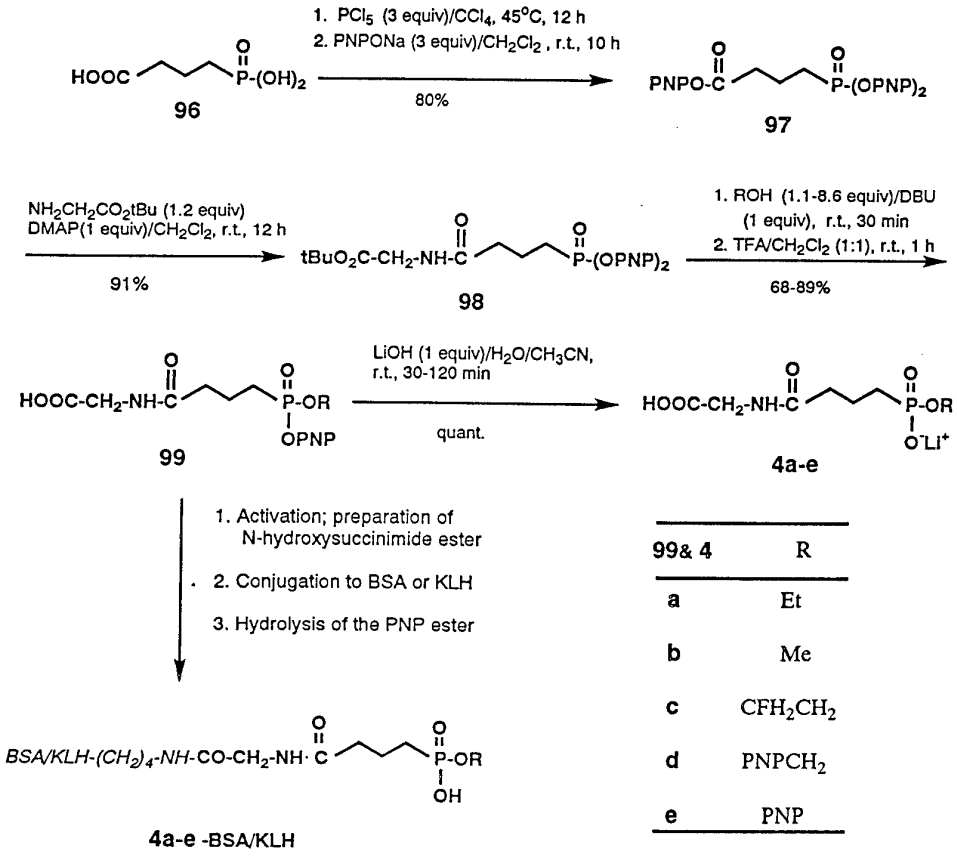
sole product in NMR spectra run after 30 min and > 3 days. Rapid rates and high yields were found using both primary and secondary alcohols and with phenols (Scheme IV); however, with tert-butanol no significant transesterification was observed. The amidation of PNP phosphonates was also found to be enhanced by DBU, although at a much slower rate than that observed with alcohols.

Various mechanisms have been proposed for nucleophilic substitutions on phosphorus (88). Transesterifications of p-nitrophenyl*phosphates* were shown to proceed via a concerted SN2-like mechanism, involving a single transition state (89,90). The DBU-mediated transesterification of bis-PNP phosphonates is probably also the result of nucleophilic displacement by the phenoxide or alkoxide formed in the presence of DBU base. The relative rates of displacement by various types of nucleophiles, i.e. ArOH > AlkylOH > $(Alkyl)_2$-CHOH > RNH_2 , and the rate dependence upon nucleophile concentration, as well as the accelerated rate with alcohols having a lower pKa which is closer to the pKa of the DBU base (e.g. p-nitrobenzyl alcohol vs. ethanol), are all in accordance with an SN2-like mechanism.

The resulting monoalkyl/aryl mono-PNP phosphonates (**91**, Scheme IV) can be further reacted with another alcohol (in a "one-pot" reaction) to give the corresponding disubstituted phosphonates **93**, or selectively hydrolysed to yield the monoalkyl/aryl phosphonic acids, **92**. An additional equivalent of DBU was added to mediate the second transesterification that otherwise proceeds too slowly. When the mono-PNP phosphonate group introduces a second chiral center, it was possible to separate the diastereomers (e.g., **91d**). This may find use since the second exchange step of PNP esters is stereoselective (91).

Hydrolysis of the monoalkyl/aryl mono-PNP phosphonates in the presence of two equivalents of lithium hydroxide was shown by ^{31}P and ^{1}H NMR, to proceed without formation of side products to give the corresponding phosphono acid monoesters (Scheme IV, **92**). Further hydrolysis of the monoalkyl or even of the mono aryl phosphonates to methyl diphosphonic acid does not occur under these conditions. The phosphonic acid monoester can be isolated from the reaction mixture by acidification and extraction with an organic solvent (e.g., **92e**) The isolation of the free mono phosphonoacids (which is sometimes difficult) is not always necessary; for most biochemical purposes, such as inhibition of enzymes or catalytic antibodies, the presence of the p-nitrophenol does not affect the binding properties of the phosphonates and need not be eliminated.

We applied this approach for the preparation of a series of phosphono ester haptens; Scheme V:



99& 4	R
a	Et
b	Me
c	CFH_2CH_2
d	$PNPCH_2$
e	PNP

Scheme V

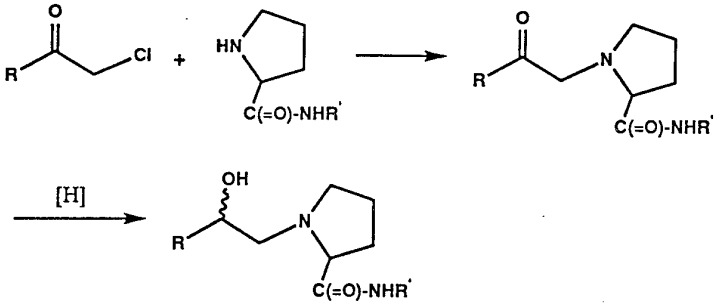
Tris-PNP ester **97** was obtained by chlorination of phosphonobutyric acid **96** and esterification with sodium p-nitrophenolate. The carboxylic PNP ester was then amidated by tert-butylglycinate to give **98**. (It is noteworthy that amidation of **97** in the presence of DMAP proceeds solely via the carboxylic PNP ester, whereas the bis-PNP phosphonate is hardly affected. Transesterification of **98** with various alcohols and deprotection of the carboxylic moiety afforded the phosphonates **99a-d**.

Activation (using carbonyldiimidazole or the N-hydroxysuccinimide ester) allowed the conjugation of these PNP-phosphonatesto the carrier proteins BSA (bovine serum albumin) and KLH (keyhole limpet hemocyanin) via the ε-amino groups of the lysine side chains. The PNP esters were then hydrolysed *in-situ* on the protein, to give the desired monoalkyl/aryl phosphonate hapten-protein conjugates (**4a-e**). This approach avoids the use of a carboxy-phosphonic acid hapten, e.g., (19,26,28,60,83,87), a substance which is not only more difficult to synthesize and isolate but may also conjugate improperly, e.g., link to the protein via the phosphonic acid (92).

The phosphonoester haptens **4a-d**, were used for immunization and the generation of catalytic antibodies (Section 1). The ester and amide substrates used for the study of the antibodies elicited against these phosphonoester haptens (Section 1, Fig. 3) were prepared by routine methods used in peptide synthesis.

9.2 β-Amino alcohols

Published procedures for the synthesis of β-prolylalcohols, and of other β-aminoalcohol peptide derivatives, proceed mainly via alkylation of the amine by the corresponding haloketone (Scheme VI; (93)). Trying to follow the same route we prepared tosyl-glycine chloromethylketone, and then reacted it, under conditions described in the cited literature, with prolyl peptides. The ketoamine was isolated in poor yield; attempts to reduce the crude reaction mixture to the amino alcohol, as previously described (93) did not improve the yield.

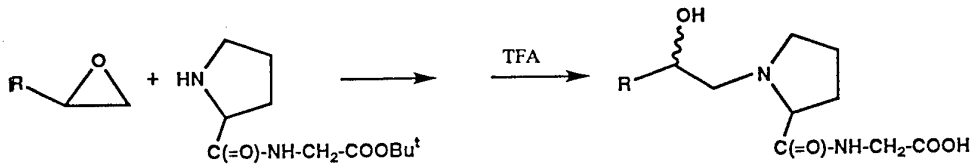


Scheme VI

We therefore tried an alternative route via epoxides, which are known to react with primary and secondary amines to give β-aminoalcohols (94). p-Nitrophenyl oxirane was prepared and reacted with H-Pro-Gly-OBu^t. High yields (70-85%) of the aminoalcohol were obtained at 70°C in the presence of triethylamine. Purification on a silica column (using methanol-dichloromethane solvent gradients) and removal of the tert-butyl ester afforded the desired hapten (Scheme VII, **12a**).

The reaction of epoxides with amines may result in two different regioisomers, the desired 1-hydroxy-2-amino compound (e.g., **12**) which is usually

the main product of such reactions, and, the 1-amino-2-hydroxy isomer which is unsuitable as a hapten.



12 a-c

12a R= p-Nitrophenyl
12b R= CH_3
12c R= p-Nitrobenzyl

Scheme VII

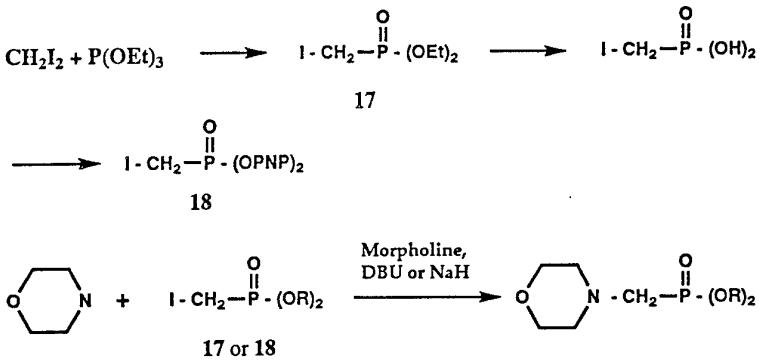
NMR data for **12a** is rather complex due to the presence of the two diastereoisomers. In order to confirm the structure, we prepared the analogous aminoalcohol with morpholine by two routes: (a) by reaction of the bromomethylketone with morpholine, followed by reduction to the alcohol (which can give only the 1-hydroxy-2-amino isomer), and, (b) via the epoxide. Both routes yielded the same isomer whose NMR data confirms the proposed structure of **12a**.

Following the same route we also prepared the methyl derivative (Scheme VII, **12c**) from propylene oxide. Reaction was performed in sealed glass tubes (due to the low boiling point of the epoxide), at 80°C, for 24 hours; followed by purification on a silica column and removal of the tert-butyl ester. The p-nitrobenzyl derivative (**12b**) was prepared following the same procedures described for the preparation of the p-nitrophenyl and methyl aminoalcohols (**12a** and **b**).

The substrates, **10 a-d** (Fig. 21), were prepared by acylation, using the corresponding acid chlorides, of prolylglycine tert-butyl or benzyl ester, followed by removal of the protective ester (the other substrates, **10 e-i**, were prepared by the same method). The product conjugates were prepared by introducing specific protecting group (on the prolyl amine) that were removed in-situ on the protein after conjugation.

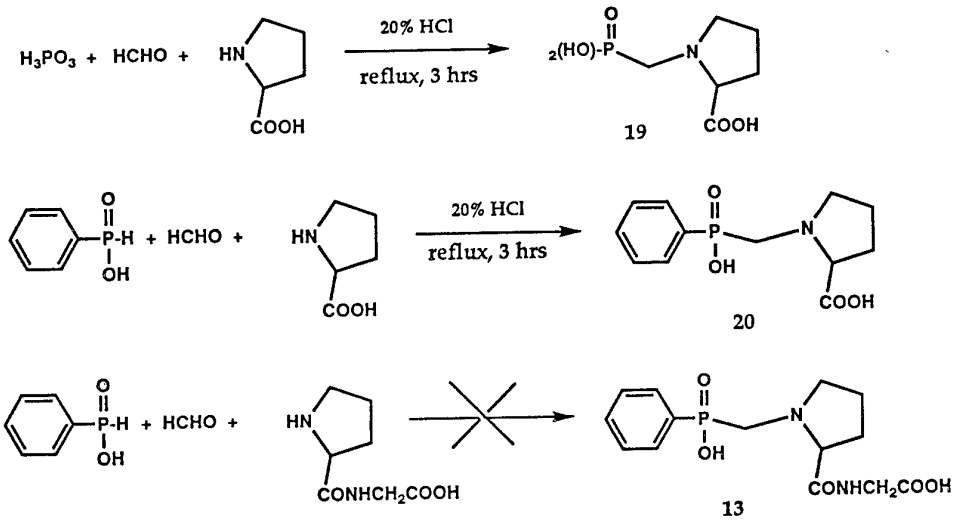
9.3 α-Aminophosphinates

The failure of the β-aminoalcohols to induce the generation of amide hydrolyzing antibodies promoted the use of aminophosphinates having the general structure: R-P(=O)(OH)-CH_2-Pro-Gly-OH (Fig. 21). The use of a N-(phosphino-methyl) proline was once reported in an attempt to prepare potent HIV-proteinase inhibitors (95), but no details regarding the synthesis were available. Preliminary attempts to synthesize model compounds via N-alkylation of iodomethylene phosphonates **17** and **18**, gave only poor yields (Scheme VIII).



Scheme VIII

An alternative route to such compounds is a Mannich reaction between formaldehyde, an amine, and an hydrogen phosphonate or phosphinate. For example, Maier reported the preparation of phosphonate **19** by the acid catalyzed Mannich reaction of phosphorus acid, formaldehyde and proline (Scheme IX, (96)). Under the same conditions we obtained phosphinate **20** in high yields. However, condensation of phenyl phosphinic acid, formaldehyde and prolylglycine proceeds with a slower rate, so that the desired phosphinate **13** is formed together with side products resulting from the cleavage of the prolyl-glycine peptide bond due to the strongly acidic conditions. Using milder acidic conditions very poor yields were obtained (Scheme IX).

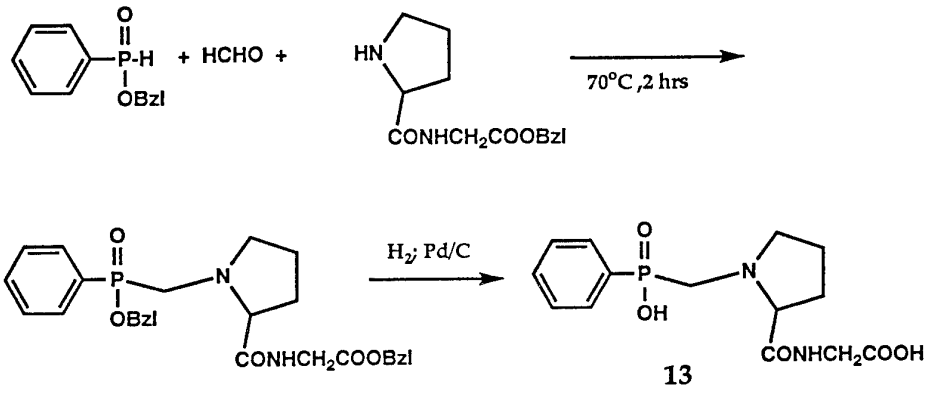


Scheme IX

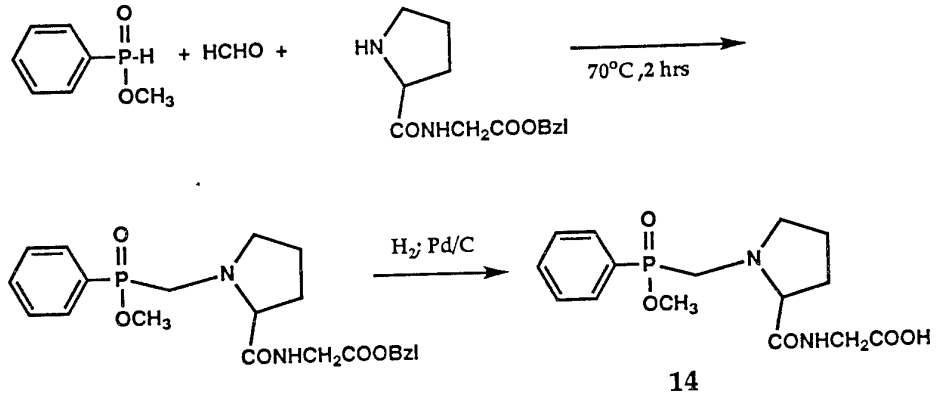
Mannich reactions with active hydrogen compounds, e.g., with ketones, are generally performed under acidic conditions (e.g., by using the hydrochloride salt of the amine); the acid-catalyzed reaction probably involves an iminium ion intermediate as the electrophile. Still, base-catalyzed Mannich reactions were also described in the literature (97), as well as condensations of various hydrogen-

phosphonoesters with aldehydes and amines (98). Condensation under basic conditions, followed by the removal of the benzyl ester protective groups afforded phenyl phosphinate **13** (Scheme X). The corresponding substrates, **10e-i**, were prepared as described for substrates **10 a-c** .

It is noteworthy that the preparation of an N-(phosphinomethyl)proline, analogous to **13**, was recently described, and the compound is a potent HIV-proteinase inhibitor (99). (Interestingly, this work was done at the Scripps Research Institute by a group working in the field of catalytic antibodies.) The Scripps inhibitor was prepared by: (i) Arbuzov reaction of the phosphinic acid trimethyl silyl ester with formaldehyde, (ii) triflation of the resulting hydroxymethylene phosphinate using CF_3SO_2Cl, and, (iii) alkylation by the prolyl peptide. This route required three steps, compared with only one step (Scheme X), to achieve the amino phosphinate skeleton in our approach. (The use of this HIV-proteinase inhibitor for the generation of catalytic antibodies has not been reported.)



Scheme X

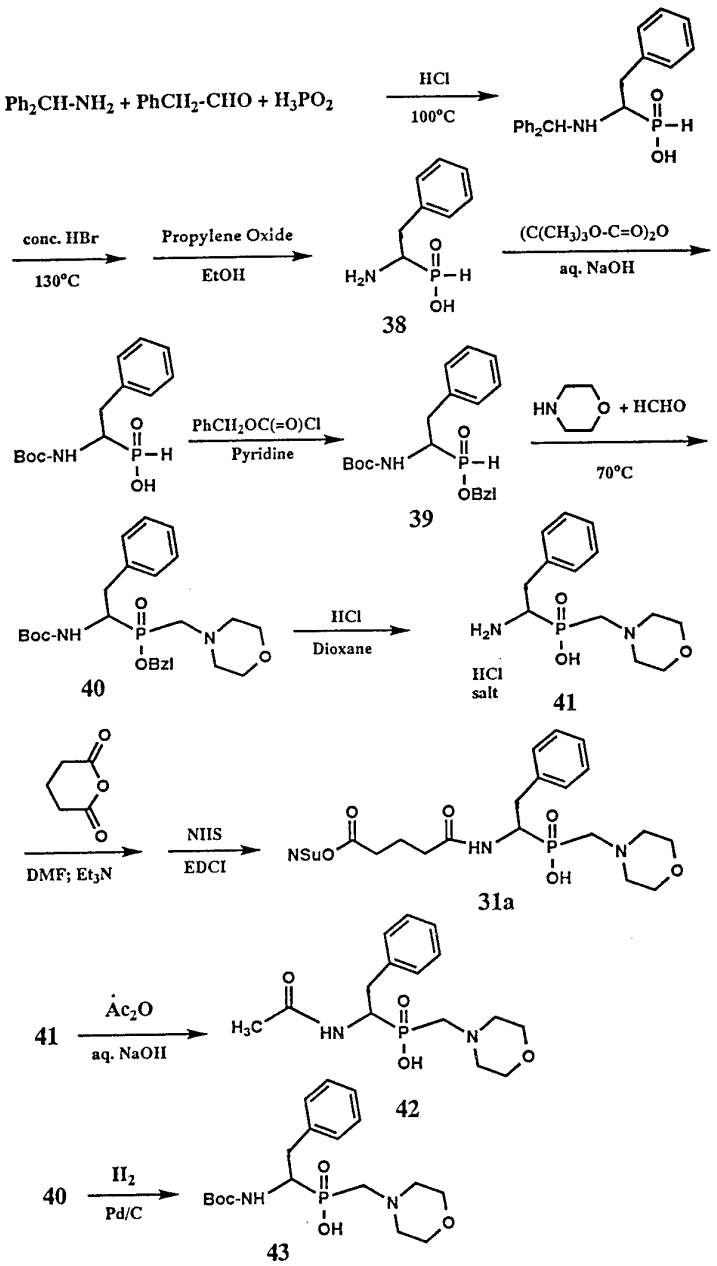


Scheme XI

The Mannich-like condensation of phosphonus acids was succesfuly applied for the synthesis of other haptens as well. Aminophosphinate hapten **14**, was prepared for the generation of antibodies that catalyze the *formation* of acyl-prolyl bonds (Section 8.3; Scheme XI).

Haptens designed for cleavage of Phe-X- bonds (Section 8.4) were also prepared by the same methodology (Scheme XII). The phosphonous acid analog of phenylalanine (**38**) was prepared by modificaton of a reported method (100). This reaction also involved condensation of an amine (protected by a diphenylmethine group), an aldehyde and phosphonus acid under acidic conditions.

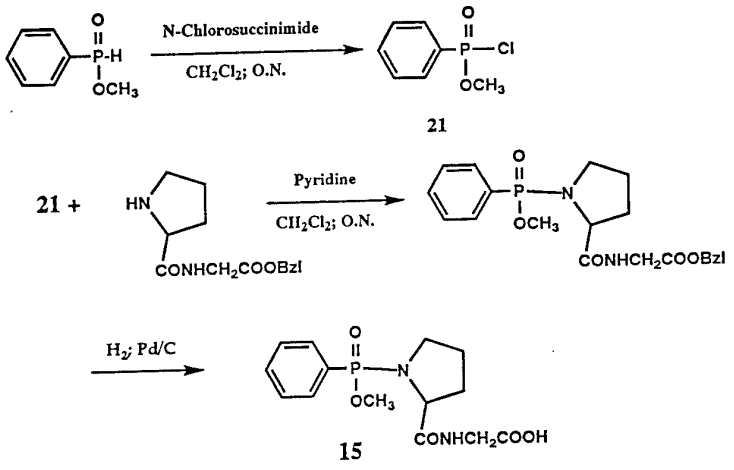
Optimizing the conditions originally described (100) gave acid **38** with > 85% yield (the original yield was 26%). The amino group was then protected by a tert-butyloxy carbonyl group (Boc), and the phosphonus acid by a benzyl group. Condensation (under basic conditions) of **39** with formaldehyde and morpholine gave **40** in good yields. Unexpectedly, the removal of the Boc protection by acidolysis (trifluoroacetic acid or HCl in dioxane) was accompanied by the removal of the benzyl protection of the phospinic acid. This imposed difficulties on the isolation



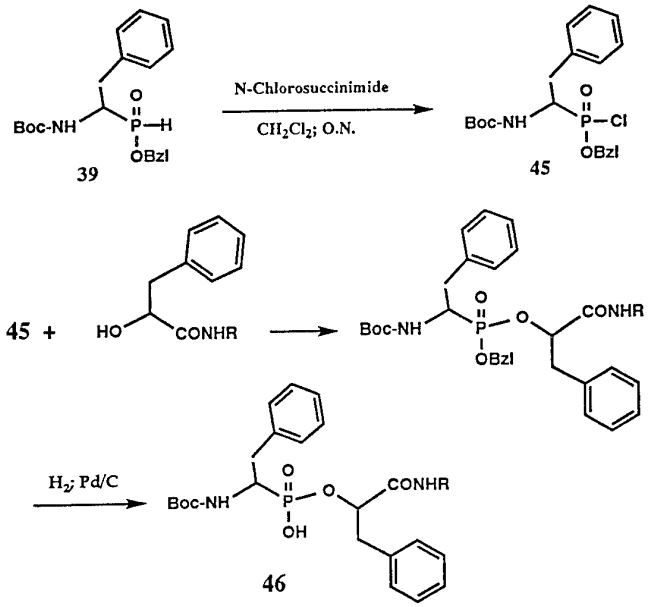
Scheme XII

in the further steps. Thus, the aminophosphonous acid **41** (as hydrochloride salt) was reacted in the presence of triethylamine with glutaric anhydride followed by N-hydroxysuccinimmide and DCC, to give the activated hapten **31a** that was used for conjugation. ^{31}P NMR indicted that both of these reactions has proceed with >95% yield and that no side reactions involving the phosphonous acid has occurred. Selective removal of the benzyl protection by catalytic hydrogenation gave phosphonous acid **43**. This compound, and compound **42** (obtained by acetylation of **41**, see Scheme XII), were both used as competitive inhibitors of antibodies elicited against the KLH conjugate of **31**.

Phosphonous acids can also be used as starting materials for the sythesis of phosphonoesters and amidates by Todd's chlorinative oxidation. This route was used for the synthesis of hapten **15** prepared for the generation of antibodies that catalyze the *formation* of acyl-prolyl bonds (Section 8.3; Scheme XIII).



Scheme XIII

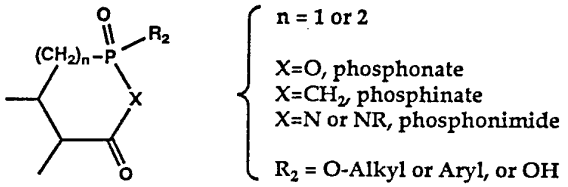


Scheme XIV

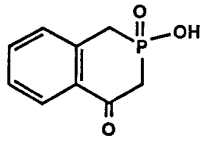
A similar route can be applied for the synthesis of phosphonoester or amidate haptens for cleavage of Phe-X- bonds. Oxidative chlorination of **39**, under similar conditions to those applied for the phenyl phosphonous chloride **21**, gave chloride **45**, which can then react with alcohols or amines (e.g., with the hydroxy derivative of phenylalanine; Scheme XIV) to give, after removal of the benzyl protection, phosphonoester **46**.

9.4 Cyclic phosphinates and phosphonimides

Eliciting catalytic antibodies that catalyze intramolecular reactions, such as cleavage of aspartyl-proline bonds or deamidation of asparagine and glutamine residues, requires stable analogs of the cyclic transition states/intermediates via which these reactions proceed (Section 8.5). We examined several cyclic phosphonates and phosphinates of the general structure **51**, that might serve as haptens for eliciting antibodies that catalyze intramolecular, or substrate assisted, amide hydrolysis (Scheme XV).



51



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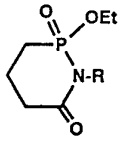
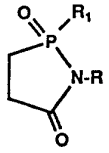


53



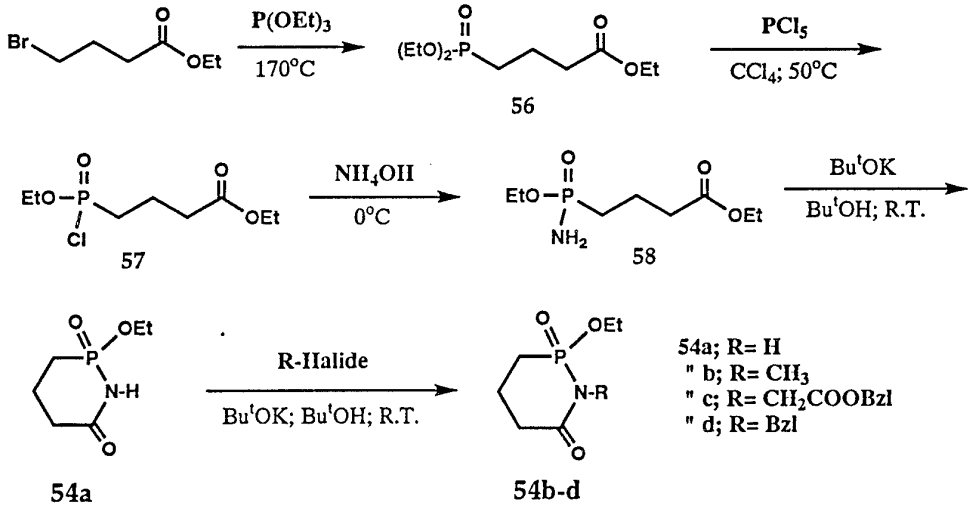
54

Scheme XV

Various cyclic 1,3-oxo phosphonates derived from carboxy-phosphonic acids (i.e., **51**, X=O) are described in the literature (101). However, as expected, these mixed anhydrides are highly labile in the presence of any nucleophile including water and are therefore unsuitable as haptens. A thorough literature search revealed only one example of a cyclic 1,3-oxo-phosphinic acid (i.e., **52**, X=CH_2), **53** (102), and two phospono-imides (**53**, X=N- and **54**; (103,104)).

The fact that phosphonamidic acids (i.e., R'-P(=O)-(OH)-NHR") are rapidly hydrolyzed even under neutral conditions and that the corresponding phosphonoacids derived from phosphonoimides, such as **53** or **54** (i.e., **20**, X=NH, R_2=H), were not previously described led us to try first to synthesize phosphinate **52**. This compound was obtained, although in very low yields, by Friedel-Crafts acylation of benzyl-carboxymethylene-phosphinic acid as described (102). Alternative routes by acylation of the isopropylphosphonate-carboxylic acid, or chloride, all failed.

Difficulties in the synthesis of **53**, and the limitation of this synthetic route, i.e., Friedel-Crafts acylation of aryl-phosphinic acids to the preparation of model aromatic systems only, prompted the synthesis and the study of cyclic phosphonoimides. Preliminary work shown that **54a** can be prepared, as previously described (103), starting from ethyl-4-(diethylphosphono) butyrate (Scheme XVI).



Scheme XVI

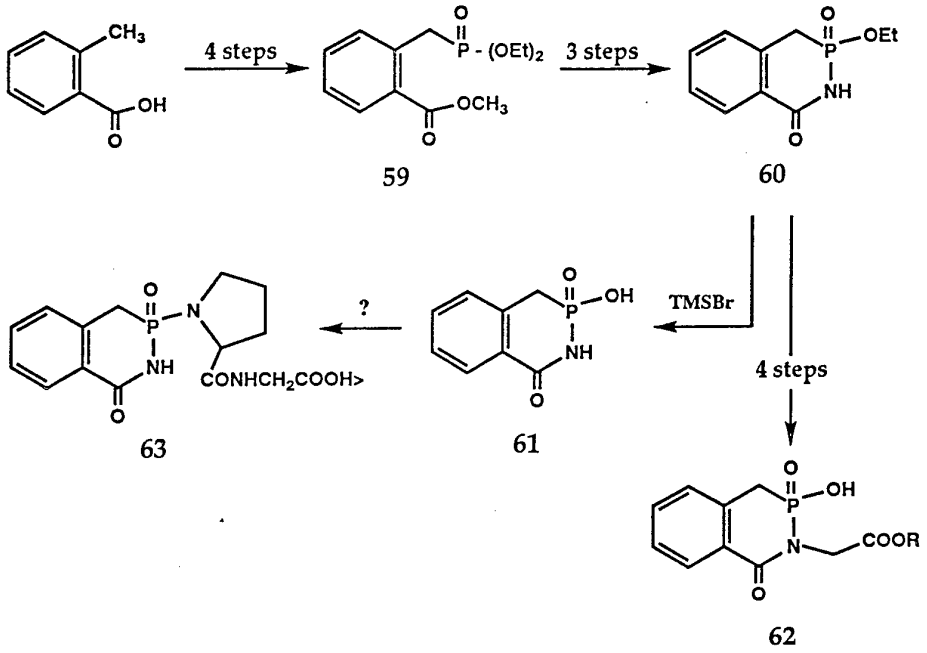
Using ^{31}P NMR, conditions were optimized so that cyclization of phosphonamide **58** to **54a** proceeds with >90% yield (compared with 55%, (103)). Alkylation of **54a** with methyl iodide or benzyl bromide to give **54b** and **54d** were performed as described (103); under similar conditions, **54a** was alkylated using benzyl bromoacetate to give the N-(carboxymethyl) imide derivative **54c** in high yield.

The synthesis of cyclic phosphonoimides by cyclization of the corresponding carboxy-phosphonamides was found to be a general route; it was readily applied for the preparation of **29** (Scheme XVII). Phosphonoimide **60** was then used for the first attempt to prepare a cyclic phosphonimidic acid (**61**). The acid was readily obtained under mild conditions by the reaction of trimethylsilyl bromide and ethylphosphonate **60**.

The stability of ring systems **60** and **61** under acidic and basic aqueous conditions was then studied. Phosphonamidic acids are rapidly hydrolyzed even under mild acidic conditions (85); it was therefore encouraging to find that phosphonimidic acid **61** is relatively stable to acid catalysed hydrolysis. The lower basicity of the imidic nitrogen of **60** or **61**, compared with the amidic nitrogen of phosphonoamidates, probably accounts for the difference. Both **60** and **61** are stable under basic conditions.

Attempts to synthesize a 5-membered cyclic phosphonimide analogous to **54** by base-catalyzed ring closure of the corresponding phosphonamide, or carboxy-amide,

were unsuccessful. This was not unexpected since phosphorus containing 5-membered rings are known to be far less stable than the 6-membered analogs (88).



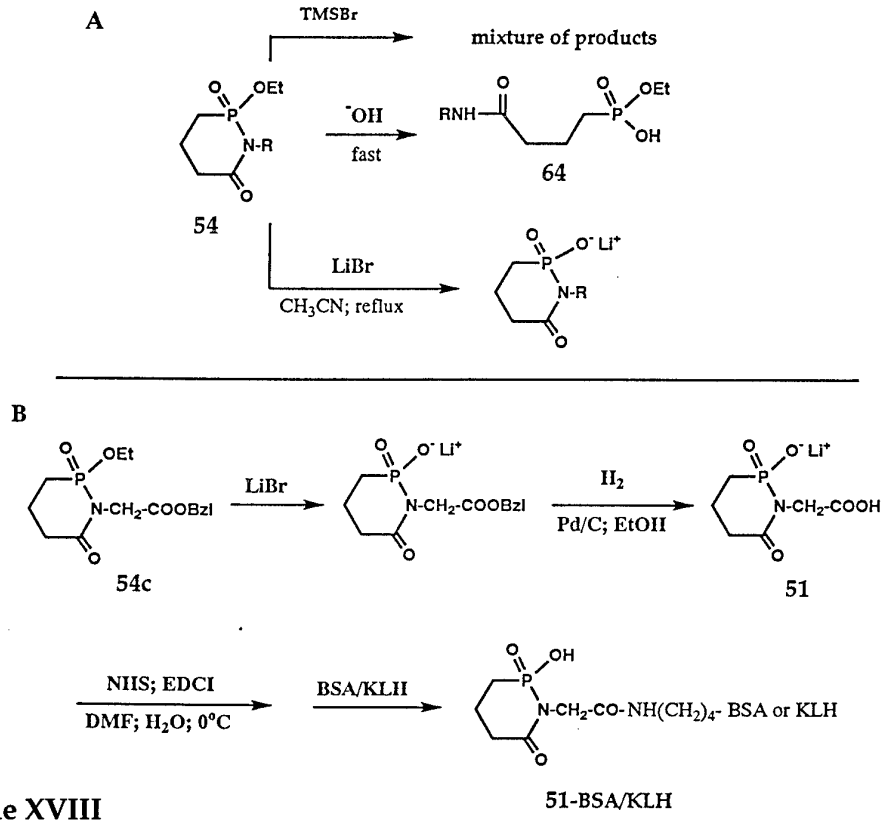
Scheme XVII

These results led to the preparation of **62** (Scheme XVII), a stable hapten representing a TSA of a model deamidation reaction. The stability of phenyl esters to cleavage by halosilanes provided a direct route to the preparation of the hapten as an active ester (**62**, R= p-nitrophenyl) for conjugation to a carrier protein via the glycyl carboxyl. We have also used **62** as a model compound to examine routes for preparation of **63**, a model TSA for the intramolecular cleavage reaction of Asp-Pro bonds. Attempts to directly amidate phosphonimidic acid **61** using various coupling reagents were not succesful. It was also found that chlorination of **61** proceeds with side products due to the imidic nitrogen; thus, it was concluded that an appropriate protective group that can be removed after chlorination and amidation, e.g., N-benzyl, must be used.

We also prepared a TSA-hapten that could be used with the DEA catELISA detection system that was already available (Section 1, Fig. 3). Cyclic phosphono-imide **51** (Scheme XVIII) was designed for the deamidation reaction of N-glycyl-glutarimide (**2d**), via imide **5**, to give N-glycyl-glutaric acid (**3**) (Section 8.5, Fig. 26).

Attempts to follow the same route used for the preparation of phosphonimidic acid **61** from the ethyl ester **60** (Scheme XVII) encountered difficulties; cleavage of the ethylphosphono ester of **54c**, or **54a**, using trimethylsilyl bromide gave a mixture of products. It was also found that unlike aryl-phosphonoimide **60**, the alkyl derivative, **54** (**a** or, **c**) is rapidly and cleanly hydrolyzed under mild basic conditions at the P-N bond to give the corresponding phosphonic acid **64** (Scheme XVIII.A). The lability of this bond in a 6-membered ring system, compared with similar acyclic systems (105), remains unexplained. Finally the phosphonoethyl

ester was cleanly removed by dealkylation using lithium bromide; removal of the benzyl glycine ester by catalytic hydrogenation gave phosphonocarboxy acid **51** which was then coupled to carrier protein BSA or KLH via an N-hydroxy-succinimide ester (Scheme XVIII.B). The stability of hapten **51** was examined both under acidic and basic conditions; it was found that this ring system, although less stable than the aromatic derivative **61**, is still stable enough to be used as a hapten.



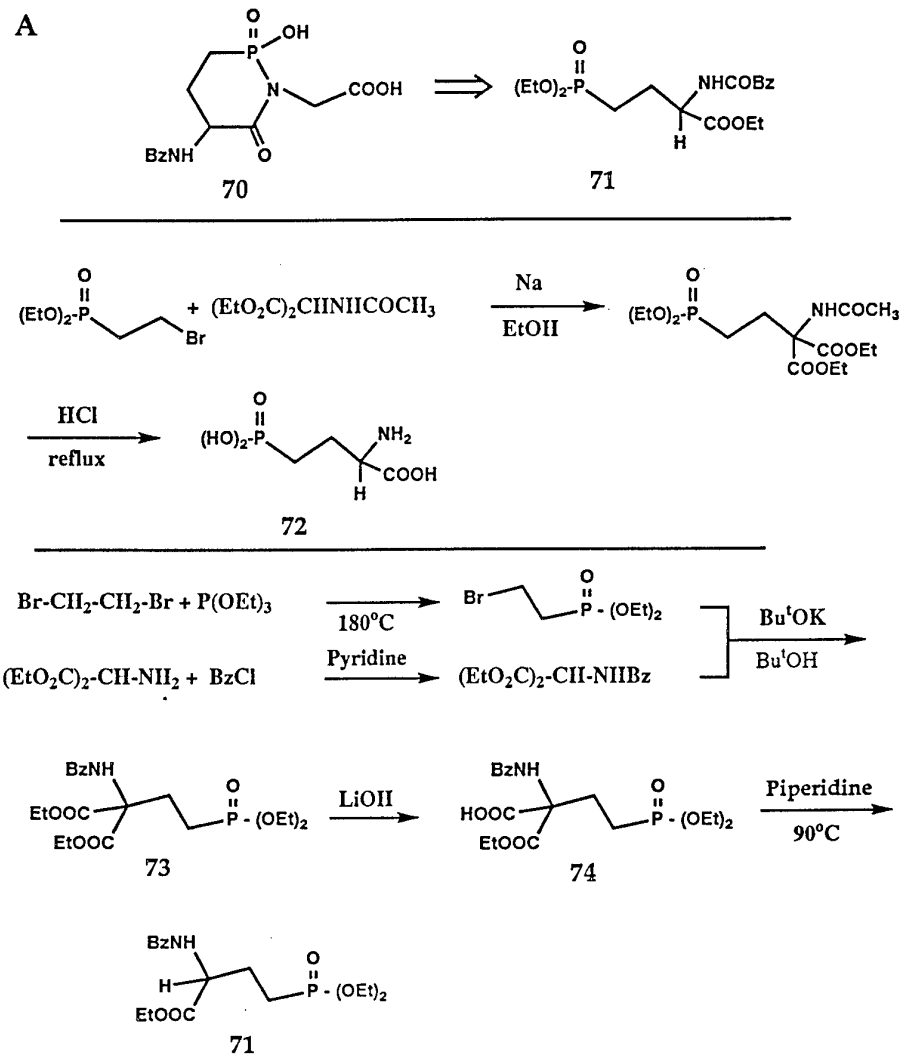
Scheme XVIII

The attempts to generate catalytic antibodies that catalyse the deamidation of glutaramide using hapten **51** are summarized in Section 8.5. The results indicate that this substance is stable enough to be used as a hapten and may generate specific antibodies; yet, those monoclonal antibodies that bind the TSA-hapten did not exihibit any binding affinity to the corresponding substrates, **2d** or **5**.

Extensive work should therefore be directed towards the preparation of hapten **70** (Scheme XIX); the benzamido moiety should increase the antigenicity of the hapten and lead towards the required affinity to the corresponding substrates.

Retrosynthetic analysis (Scheme XIX.A) suggested that ethyl-4-(diethyl phosphono)-3-(benzamido)butyrate (**71**) could lead to **70** by the same sequence of reactions applied for the synthesis of **6** from ethyl-4-(diethyl phosphono)butyrate (see Schemes XVI and XVIII).

2-Amino-4-phosphonobutyric acid (**72**) is a known NMDA antagonist (106); it is prepared by a malonic ester synthesis that was widely used for the preparation of other amino acids (Scheme XIX.B). Benzoylation of **72**, by a Schotten-Boumann



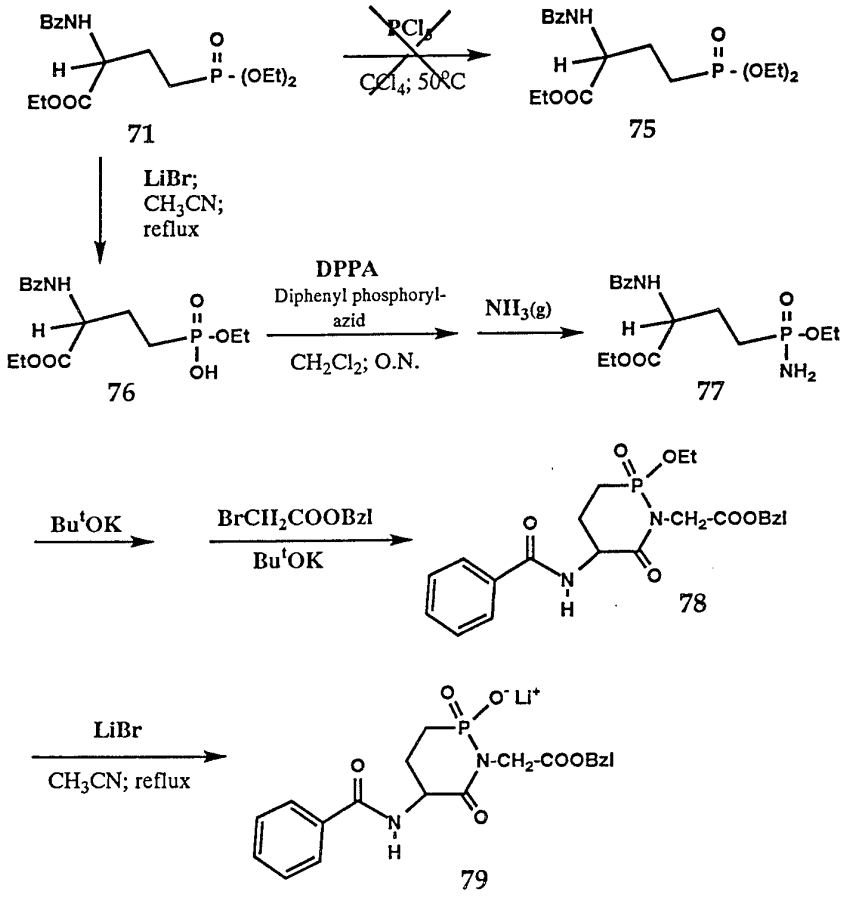
Scheme XIX

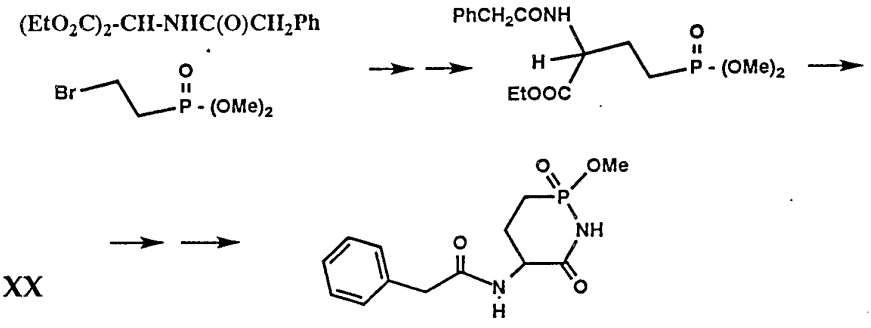
reaction in aqueous base, was found by ^{31}P NMR to procceed in reasonable yields, yet the extraction of the carboxy-phosphonic acid product from the aqueous reaction media was found to be a tedious process. An alternative route, described in Scheme XIX.C, was developed. The malonic condensation product, **73**, was selectively hydrolyzed in the presence of one equivalent of lithium hydroxide to give the monoethyl ester derivative **74**; decarboxylation gave **71** as a sole product. Conditions under which all these steps proceed in very high yields were found.

Direct chlorination of **71** by PCl_5 or PCl_3 to give the monochloro monoethyl phosphonate **75** (as performed with diethylphosphonate **56**, Scheme XVI) gave complex mixtures of products (Scheme XX). This is probably due to the reactivity of the benzamido nitrogen with chlorinating reagents. For the same reason, the monoacid **76** (obtained by dealkylation of **71** with LiBr) could also not be cleanly chlorinated. Thus, we have applied the method of Bartlett and Acher (107), using diphenylphosphoryl azide followed by ammonia, to obtain phosphonoamidate **77**. Cyclization of **77**, followed by alkylation with benzyl bromoacetate gave **78** (Scheme

XX); these reactions were performed under similar conditions used for the cyclophosphonoamidates **54a-c** (Scheme XVI), although in much lower yields (10-20%). Unexpectedly, the last step, i.e., removal of the phosphono ethyl ester to give phosphonimidic acid **79** proceeded with difficulties. Much stronger conditions than those applied for the analogous ester **54** (Scheme XVIII) had to be applied and a number of side products were observed.

We therefore decided to change the phosphono ethyl esters into methyl esters, which are much easier to remove (by hydrolysis or by dealkylation), and the benzoylamide into a phenylacetamide group (to allow chlorination without side products which are typical to the benzoylamido group). Using the same route described in Scheme XIX, we have been able to obtain thus far the cyclic methyl phosphonoamidate **80**.





Scheme XX

10. Future plans

The tremendous efforts put forward during the course of this research grant -- in the organic synthesis, in the antibody production, in the development of new techniques and new protocols, in the repeated searches for a catELISA signal indicating amide-hydrolyzing clones, in the exhaustive control experiments that would unambiguously rule out non-antibody sources -- without being able to yet report sequence specific peptide (or even unactivated amide) hydrolysis, all this still leaves us undaunted! We feel that the progress that has been made will allow us, and/or others, to achieve this goal, the Holy Grail of catalytic antibody research, as we termed it in the Introduction. The tools that we have made available (catELISA for rapid and effective catalyst detection, autoimmune-prone mouse protocols for producing large numbers of catalytic antibodies, the synthesis of sophisticated haptens which continue to look very promising as transition state analogs) plus the conclusions that we may draw from the unsuccessful attempts (the need for antigenic haptens, low Ki values, and subtrates with good antibody affinity, low Km values) will allow, we are confident, the eventual application of antibody-based catalysts in chemistry, biology, and medicine.

There are a number of aspects of this research which merit much further attention. A number of the haptens that were studied using only Balb/c mouse strains should be examined using the autoimmune-prone strains. The very promising preliminary results described in Section 8.4 (Fig. 24) using hapten **31** require repeated attempts and further study and characterization of the interesting clone, F24.19, that appears to be, at the very least, a first step towards our goal.

Since such a large proportion of enzymes contain metal ion cofactors, we feel that future efforts should also include metal ions within the antibody binding sites (there has thus far been only a single report involving antibody-catalysis utilizing a metal ion (62) and the absence of follow-up suggests that there may have been problems in reproducing those results). The combination of an antibody directed to a TS analog and the presence of a metal ion for substrate and/or water activation should be a powerful combination.

We and others (108-111) have recently reported on crystal structure analysis of catalytic antibody Fab fragments complexed with the haptens used to elicit the antibody. These studies are, we feel, going to provide valuable insights into the mechanism(s) of reaction and details concerning the environment that a substrate experiences in an antibody binding site. Comparison of catalytic antibody structures with enzyme crystal structures is fascinating in that we find some elements in common and others that are quite different. We believe we are witnessing elementary catalytic processes that may have been tried and discarded along the evolutionary pathway to the efficient enzymes we see today; we suggest that only a limited number of ways apparently exist for a protein to catalyze a given chemical transformation (without added metal ion or other cofactors) and that convergent catalytic mechanisms are being revealed in these antibodies (112). Also, from the results in hand we see that certain amino acids, especially tyrosine, are invariably involved in hapten binding and in transition state (oxyanion) stabilization; exploitation of this information will be an important future direction.

CONCLUSIONS

Catalytic antibodies remain, far and away, the vehicle that has come most close to mimicking enzymes. The demand for multidisciplinary groups and dedicated personnel willing to take upon themselves risky and highly demanding research, often without pre-established methods and approaches, makes this field a particularly narrow one in practice, even though it attracts so much attention in reviews and conferences.

As outlined in the Future plans section above, we are optimistic about the future of this field in general and for the eventual success of reaching the goal of sequence-specific peptide-hydrolyzing catalytic antibodies, in particular. Our research has made available, as indicated in the detailed report and in the published papers that have resulted, techniques and tools that can be used (and some already have been) by others.

The number of laboratories producing catalytic antibodies is still relatively small (about 10) and more effort must be made to nurture the field both by encouraging new groups to get involved and by supporting the groups now active. We have shown here that, for ester-hydrolysis, at least, catalytic activities can reach very respectable levels; the extension of antibody-catalyzed activity to amide and other reactions can be visualized by continuing along the lines we have taken (new haptens and different ways of eliciting antibodies) and by new directions (e.g., incorporating metal ions).

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APPENDIX: PUBLICATIONS RESULTING FROM RESEARCH GRANT

Published papers (not including abstracts and presentations at conferences) that acknowledge support by US Army Medical Research and Development Command (Grant DAMD 17-99-Z-0010)

D.S. Tawfik, B.S. Green, R. Chap, M. Sela, and Z. Eshhar
catELISA - a new route to catalytic antibodies.
Proc. Nat. Acad. Sci., U.S. **90**, 373-377 (1993).

D.S. Tawfik, Z. Eshhar, A. Bentolila, and B.S. Green
DBU catalyzed transesterification of p-nitrophenyl phosphonates - a novel route to phosphonoester transition state analogues and haptens.
Synthesis 968-972 (1993).

R. Zemel, D.G. Schindler, D.S. Tawfik, Z. Eshhar and B.S. Green
Differences in the biochemical properties of esterolytic antibodies correlate with structural diversity.
Molecular Immunology **31**, 127-137 (1994).

D.S. Tawfik, Z. Eshhar, and B.S. Green
Catalytic antibodies: a critical assessment.
Molecular Biotechnology **1,** 87-103 (1994).

D.S. Tawfik, R. Chap, B.S. Green, M. Sela, and Z. Eshhar
Unexpectedly high occurrence of catalytic antibodies in MRL/*lpr* and SJL mice immunized with a transition state analog. Is there a link to autoimmunity?
Proc. Nat. Acad. Sci., U.S. **92**, 2145-2149 (1995).

D.S. Tawfik, A. B. Lindner, R. Chap, S.-H. Kim, B.S. Green and Z. Eshhar
catELISA: ELISA-based detection of catalytic antibodies and enzymes.
Immunology Methods Manual (Ed. Lefkovits) in press (1996).

[Submitted for publication]

D.S. Tawfik, Z. Eshhar, R. Chap, A. Lindner and B.S. Green
Efficient and selective p-nitrophenyl ester hydrolyzing antibodies elicited by a p-nitrobenzyl phosphonate hapten
Europ. J. Biochem. accepted for publication (1996).

[In preparation for publication]
Two papers are in preparation dealing with the details of the syntheses of the haptens and substrates; these are all novel substances and of interest in their own right as well as for the future preparation of catalytic antibodies. These papers will also, of course, acknowledge support from the USAMRDC.

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