does C5, and with slightly greater efficiency. Kinetic properties for these reactions have not been determined yet, but the rate enhancements above background are not as large as for the original substrate 7. The presence of the amino group opens additional mechanisms for the reaction of 8 and 9 in which the amino group acts as a nucleophile or as a general base. The latter mechanism would parallel that demonstrated for C3 with substrate 7, with a neutral general base acting in place of a presumed anionic base.

Conclusions
The accidental use of hydrolytically labile substrates in evaluating antibodies has led to the discovery of unexpected catalysed reactions. This curiosity may be a flashback to a stage in the evolution of enzymes. As we have argued, the existence of charged groups is to be expected on the surface of water-soluble proteins, and consequently general acid-base catalysis would not be surprising. If a binding site is induced through the antibody-recognition system, it is, perhaps, not surprising that a group capable of providing general acid-base catalysis would coincide. The results of our studies provide some evidence for this potentially general phenomenon. To balance these unexpected results, catalytic antibodies have been found as planned for Diels-Alder cyclo-additions and, after having identified the hapten required, for a human esterase.

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Catalytic antibodies for the hydrolysis of unactivated peptides
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Introduction
Preceding papers in this colloquium have provided a detailed account of the variety of monoclonal antibodies, mAbs, that can be isolated from the mammalian repertoire to effect the catalysis of an amazingly broad spectrum of types of chemical reaction. That repertoire, which is thought to number in the hundreds of billions, is the prime target of current research into catalytic antibodies whose goal is to explore and sift through the entire range of mAbs to identify, express and purify precisely the best catalyst for a particular reaction. It is a goal of future work to modify fragment antibodies, Fabs, arguably by mutagenesis in their six complementarity-determining regions, to discover whether improved catalytic performance can be achieved as a result of modifications in the size, composition and flexibility of the six loops in the critical complementarity-determining region of the antibody variable regions, as discussed by Drs. Janda and Partridge in this colloquium.

The successes of catalytic antibodies are generally to be found either as catalysts of those reactions for which there is a relatively large negative entropy of activation, for which the Claisen rearrangement of chorismic acid into prephenic acid is a prime example [1, 2], or for which there is a rather low free energy of activation, as in the hydrolysis of esters and carbonates, which are the archetypal examples of catalytic antibody activity [3, 4]. Particularly spectacular achievements are the catalysis of reactions for which there is no known enzyme, as in the Diels-Alder [2+2] cyclo-addition reaction [5, 5a], and the catalytic preference for a disfavoured reaction through the selective reduction

Abbreviations used: mAb, monoclonal antibody; TI, tetrahedral intermediate; TS, transition state.
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The nature of the problem
One of the most severe challenges for catalysis by a mAb is that of the hydrolysis of an unactivated amide. Three features of this process merit consideration. Firstly, there is no thermodynamic obstacle to the hydrolysis of an amide, as shown by the free energies of hydrolysis of some standard carboxylate derivatives p-nitrophenyl acetate, -54.64 kJ mol\(^{-1}\); acetyl imidazole, -54.47 kJ mol\(^{-1}\); PhOAc, -43.89 kJ mol\(^{-1}\); ethyl glycinate, -35.28 kJ mol\(^{-1}\); glutmine, -14.28 kJ mol\(^{-1}\); propionamide, -8.82 kJ mol\(^{-1}\) at 25°C [6], all of which are exothermic in hydrolysis at pH 17.

Secondly, aliphatic amides lie midway between carboxylate esters and phosphate diesters in rates of alkaline hydrolysis with a significant decrease of 10\(^b\) between an ester and an unactivated amide (\(k_{OH}\) acetyl imidazole, 316 l\(\text{mol}^{-1}\) s\(^{-1}\); p-nitrophenyl acetate, 15 l\(\text{mol}^{-1}\) s\(^{-1}\); MeOAc, 0.15 l\(\text{mol}^{-1}\) s\(^{-1}\); AcNHMe, 5 \times 10\(^{6}\) l\(\text{mol}^{-1}\) s\(^{-1}\); (MeO)\(_2\)PO\(_2\)-, 6.8 \times 10\(^{-12}\) l\(\text{mol}^{-1}\) s\(^{-1}\) at 25°C). The much slower rates for uncatalysed hydrolysis somewhat converge for compounds with poor leaving groups (acetyl imidazole, 8.33 \times 10\(^{-5}\) s\(^{-1}\); MeOAc, 2.5 \times 10\(^{-7}\) s\(^{-1}\); AcNHCH\(_2\)NO\(_2\), 5.6 \times 10\(^{-8}\) s\(^{-1}\)) [7], while Still's radiochemical analysis of the uncatalysed hydrolysis of a peptide at pH 7 has given a value for the first-order rate constant of 2 \times 10\(^{-4}\) s\(^{-1}\), corresponding to a half-life of some 9 years [8]. Thus it is clear that there is a major kinetic problem to be resolved.

Thirdly, the mechanism of amide hydrolysis is a two-step process. Moreover, it probably involves more than a single tetrahedral intermediate, TI [9]. Consequently, a catalytic antibody must cope equally well with the stabilization of the transition states (TSs) both for formation of the TI and also for its breakdown. This has been achieved in cases when both TSs have the same net charge or where protonation of the leaving group is not critical, as is the case for antibody hydrolysis of esters and carbonates, and for the activated amide, acetyl p-nitroanilide (K. D. Janda, this colloquium) [3, 4, 10]. However, the need for proton transfers to, from and between the TIs places additional requirements on the design for TS analogues that have not yet been met.

Chemical solutions to the problem
Fortunately, we know that this barrier is not insurmountable. Some amides can be hydrolysed readily either as a consequence of chemical structural changes or by means of the catalytic apparatus employed by enzymes. A sizeable part of the impedance to hydrolysis of an amide is the delocalization of electrons from nitrogen into the carbonyl \(\pi\)-system, which can be restricted either by electronic or by steric means. Acetyl imidazole provides a classic example of the former, where alkaline hydrolysis is some 20-fold faster than for p-nitrophenyl acetate, although the leaving group has a p\(K_u\) some 7.5 units greater [9]. This has been explained as arising in part from restricted resonance involving the N-1 and the carbonyl group, leading both to easier nucleophilic attack and to faster C-N bond-breaking [11]. However, torsional rotation of an amide also restricts resonance, as exemplified in a series of bridged amides where the rate of hydrolysis is accelerated some 10\(^6\)-fold as a consequence of steric impedance of resonance ([12, 13] and references therein), and where addition of water to the C=O group is facilitated at pHs where C-N bond-breaking is not rate-limiting [9].

Enzymic hydrolysis of amides
The proteolytic enzymes accelerate amide hydrolysis even faster than the foregoing chemical devices. Such enzymes can be assigned generally to one of three classes according to their primary dependence on covalent catalysis, electrophilic catalysis or general acid-base catalysis, and these classes are typified by the serine and cysteine proteases, the zinc peptidases and the aspartyl proteases respectively. All are very efficient, with turnover numbers [14] of 10\(^{2}\)-200 s\(^{-1}\). Chymotrypsin achieves pseudo-first-order rate constants for some unnatural esters and amides that are 10\(^7\)-10\(^8\) faster than their uncatalysed counterparts (N-acetyltryptophan ethyl ester, 22 s\(^{-1}\); N-acetyltryptophan p-nitrophenyl ester, 31 s\(^{-1}\); N-acetyltryptophan glycaminide, 0.50 s\(^{-1}\); N-acetyltryptophanamide, 0.026 s\(^{-1}\) at 25°C, pH 7.9) [15]. This acceleration is even greater for natural peptides (10\(^9\)-10\(^{10}\)), where the major part of the catalytic enhancement appears to result from the presence of the ubiquitous charge-relay triad, Asp-His-Ser, which accounts for at least 10\(^6\) of the total acceleration [16].

Amide cleavage by antibodies
The four successes of catalytic antibodies achieved so far are significant but slow in comparison.

Firstly, acetyl p-nitroanilide is an activated substrate for which the antibody NPN43C9, identified by a TS analogue approach [10], accelerates
the hydrolysis by a factor of 250,000 over the uncatalysed rate at pH 9.0. This has all the appearances of a system where the TSs for formation and breakdown of the TI are dominantly anionic in character.

Secondly, the challenge of mimicry of a serine protease appears for the present to be unattainable by a catalytic antibody. The probability of finding the correct juxtaposition of an Asp–His–Ser catalytic triad in mAb seems just too slender, even allowing for the size of the antibody repertoire, and it seems likely that this catalytic structural unit may be achieved in an antibody only as a result of site-directed mutagenesis. By contrast, cysteine proteases use a less complicated zwitterionic mechanism [17, 18] and this has been mimicked by a chemical model system using 2-mercaptomethyl-1-methylimidazole on a torsionally distorted amide ([13] and references therein). It is thus very encouraging that Janda has reported results recently using an antibody where the presence of a thiolate residue is first identified through screening for binding and then ingeniously pursued by screening for catalysis, leading to an antibody counterpart of catalysis by a covalent peptidase (K. D. Janda, this colloquium).

Thirdly, there is one extant example of the use of electrophilic catalysis to complement the approximation benefits of antibody catalysis. Iverson [19] has employed a kinetically stable complex between Co(III)(trien) and an α-aminocarboxylic acid to isolate antibody 28F11. This mAb then uses Zn(II)(trien) to achieve hydrolysis of an unactivated peptide bond with a turnover number of $6 \times 10^{-4}$ s$^{-1}$, corresponding to an enhancement ratio of $3 \times 10^3$. However, there is a disconcerting and unexplained feature of this otherwise significant result: cleavage of the peptide substrate occurs at the Gly–Phe amide bond in the substrate, which is one frameshift removed from the amide adjacent to the metal-binding site in the hapten. A possible explanation is that this switch might occur as a consequence of the absence of a tetrahedral centre in the hapten, which has reactant-like structure [19] rather than having a TS geometry.

Lastly, Paul has described the isolation of an IgG from a human subject that can cleave the vasoactive intestinal peptide between Gln$^{16}$ and Met$^{17}$ with a $k_{cat}$ of 0.26 s$^{-1}$. The moderately slow turnover is compensated by relatively tight vasoactive intestinal peptide binding ($K_m$ 37.9 nM) [20]. It is hoped that further characterization of this antibody will reveal details of its mode of action.

**Solutions to the problem**

The activation of amides by the location of electron-withdrawing groups in the carbonyl function has hitherto passed unattended in the search for catalytic antibodies. Yet there is ample evidence to show that α-halogenation of the acyl group can provide a factor of over $10^4$ in rate enhancement of ester and amide hydrolysis [7, 21] (ethyl acetate, $k$, $2.5 \times 10^{-7}$ s$^{-1}$ l.mol$^{-1}$ s$^{-1}$; chloroacetate, $5.5 \times 10^{-6}$ s$^{-1}$; trichloroacetate, $2.7 \times 10^{-5}$ s$^{-1}$; and trifluoroacetate, $2.5 \times 10^{-4}$ s$^{-1}$; $k_{OH}$ AcNMePh, $1 \times 10^{-4}$; CICH$_2$CONMePh, $6.6 \times 10^{-4}$ l.mol$^{-1}$ s$^{-1}$; F,$_3$CHCONMePh, $0.17$ l.mol$^{-1}$ s$^{-1}$; and F,$_3$CCONMePh, $2.45$ l.mol$^{-1}$ s$^{-1}$). In particular, this means that greater attention can be given to the design of a TS analogue with potential for protonation of the nitrogen leaving group in the breakdown of the TI.

One candidate for such hydrolysis is the dichloracetamide function of the antibiotic chloramphenicol (I) (Figure 1). Moreover, antibody-catalysed hydrolysis of this species might open the way for positive screening for the turnover of antibodies cloned in to a bacterium to be grown in a

![Figure 1](image-url)

Some TS analogues (2–4) for the hydrolysis of chloramphenicol (1)
culture containing limiting amounts of chloramphenicol. We have approached this by the successive development of TS analogues. Initially, we have used the robust sulphonamide (2), linked to a carrier protein by the reduction of its nitro group and acylation with the classical glutarate spacer. As described earlier (L. J. Partridge, this colloquium), this enterprise has provided monoclonal proteins by both hybridoma and pComb3 technologies that have a submicromolar affinity for the hapten and substrate. We therefore progressed initially to the phosphonamide TS analogue (3a). In theory, this could have a zwitterionic character (3b) but, in practice, we found that migration of phosphorus from N to O was too facile for this species to be employed as a hapten. We have thus turned to the expanded TS analogue (4a), in which the zwitterionic character of the Ti± is conserved without the destabilization of a P-N bond. Results on the use of this hapten will be reported elsewhere. Design considerations encourage conjecture about the response of the immune system to a hapten that contains a further cationic centre, as in (4b). Such a TS analogue might be employed as a bait for an antibody that has two aspartate residues and the capacity to recognize a tetrahedral centre in its combining site. However, the immune response might respond simply to the sum of the separate charges in (4b) and complement them with an antibody having a single anionic centre. A key component of future hapten design hinges on the question: 'Do multiply charged haptens elicit a complementary microscopic read-out in an Fab, or will they only result in a net charge complementarity?'

In a somewhat related enterprise, Fujii has characterized an antibody 6D9 that catalyses the hydrolysis of a 4-trifluoroacetamidobenzoate ester of (1) to release chloramphenicol [23]. This system acts as a model for pro-drug activation [24] that can be screened by using *Bacillus subtilis* as the indicator strain.

Finally, we turn to the question of electrophilic catalysis of antibody hydrolysis of an amide. The basics for such metal-ion catalysis have been described well by Buckingham [25]. They are founded on an initial discovery of Kroll [26], who identified the capacity of Cu(II) and other metal ions to catalyse the hydrolysis of esters of α-amino acids. A detailed analysis of the hydrolysis of esters shows that both neutral and alkaline processes are accelerated, and the mechanism varies according to the reaction type [27]. Most spectacularly, there is a gain of $10^6$ in the rates of water hydrolysis, and of up to $10^7$ in the hydroxide reactions of the isopropyl esters of glycine and of β-alanine when coordinated to Co(en)$_2$$^{3+}$.

In a more recent endeavour, Chin has shown that the efficiency of the catalyst, especially for Co(III), depends on the nature of the ligand and that hydrolysis of a phosphate diester can be accelerated by a huge amount as a result of tuning the geometry of the metal ligand (Figure 2) [28].

While the most widely studied zinc peptidase is carboxypeptidase A [29], other zinc proteases such as angiotensin-converting enzyme, collagenase, enkephalinase and subtilisin have been targets for drug design. Nonetheless, we have directed our efforts in TS analogue design towards the aminopeptidases. Many examples of this family of enzymes are known, where either zinc or cobalt electrophilic catalysis is observed, and where both mononuclear and binuclear metal complexes are active [30].

**Figure 2**

Hydrolysis rates for bis-p-nitrophenyl phosphate (BNPP) and its complexes with Co(III)(trpn) and Co(III)(tren). X-ray analysis indicates that (trp)Co is able to stabilize a four-membered ring for TS stabilization better as a result of increasing the bond angle opposite the four-membered ring [28].

![BNPP: [(trpn)Co(OH$_2$)$_3$]$^{3+}$](image1.png)  ![BNPP: [(tren)Co(OH$_2$)$_3$]$^{3+}$](image2.png)  ![BNPP](image3.png)
The core of the design of TS analogues to generate mAbs to mimick aminopeptidases is the placement of a tetrahedral phosphoryl centre at the projected point of amide cleavage. To that end, we have prepared a variety of phosphonate esters and phosphinic acid species (Figure 3, 5-8) which, as their benzyloxycarbonyl-protected derivatives, are candidates for use as haptens in their own right. Following removal of the Z-protecting group, the α-aminophosphoryl species form good complexes with Co(II) as its (trien) chelate (Figure 3, 10-12).

We have found these complexes to be sufficiently stable for chromatographic purification on silica and for dialysing as BSA or keyhold limpet haemocyanin conjugates and they are typified by a >40 p.p.m. downfield shift of the phosphonate (phosphinate) $^{31}$P n.m.r. signal. However, we perceive such systems to have two inherent deficiencies. Firstly, they include at least eight different stereoisomeric forms of the octahedral complexes, whose existence will inevitably impair catalytic efficiency. Secondly, the outer surface of the (trien) ligand would appear to offer little functionality to stimulate the antigenic response of the immune system.

We have made complexes accordingly using the tris(2-pyridylmethyl)amine complex [31] of Co(III) (13) (Figure 3), which appears to overcome both of these problems and also offers scope for the fine tuning of the four nitrogen ligands to gain the advantages of the (trpn) system. Our results on these and on related systems will be reported in due course.

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