An Antibody with Dual Catalytic Activity

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Antibodies raised to the bovine serum albumin conjugate of the adduct of acetoxybutadiene with *N*-(4-carboxybutanoyl)maleimide have been shown to catalyse the Diels–Alder cycloaddition of acetoxybutadiene with *N*-benzyl- and *N*-ethyl-maleimide. For one antibody, designated H11, the reaction was selective for 1-acetoxybutadiene; 1-methoxybutadiene and penta-2,4-diene were not substrates. Product inhibition was not observed but the reaction was found to be pH dependent showing a maximum rate at pH 8.5. Analysis of the products of cycloaddition catalysed by H11 indicated that the expected acetoxy adduct was obtained but underwent further reaction catalysed by H11 to afford the corresponding alcohol. This unexpected discovery of a dual catalytic activity associated with an antibody is discussed in the context of hydrolysis reactions catalysed by antibodies.

The potential of catalytic antibodies to provide novel protein catalysts to a given specification has been widely recognised.[1] With the general acceptance of the value of enzymes to provide homochiral building blocks for organic synthesis,[2] we decided to attempt to obtain a catalytic antibody that would provide a polyfunctional molecule containing several chiral centres of potential use in organic synthesis. The value of catalytic antibodies would be greatest in those reactions for which naturally occurring enzymes are not known. We therefore selected the Diels–Alder cycloaddition for investigation.[3] The target molecule is shown in Fig. 1. Antibody catalysis of the Diels–Alder reaction[4,5] also using maleimides as substrates, has been described by Hilvert's and Schultz's groups.

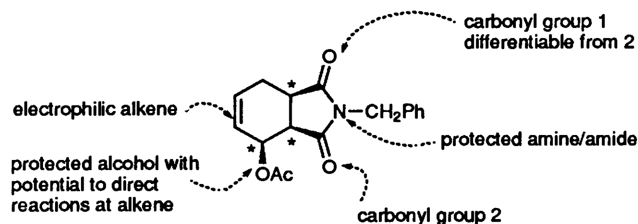

Fig. 1 Potential functionality for synthesis built into the target Diels–Alder adduct

The Diels–Alder reaction has been recognised to proceed through a product-like transition state. If the hapten to be selected for raising antibodies were too closely related to the product of the reaction, the tight binding of the transition state would be reflected in product binding as well as in catalysis and product inhibition would be expected to occur. Both Hilvert's and Schultz's groups designed haptens that differed significantly from the final product. We limited the difference between product and hapten to the *N*-alkyl group of the maleimide through which the hapten was coupled to the immunogenic protein. This strategy is not without risk but, as shown below, was successful.

Preparation of and Characterisation of Antibodies.—Diels–Alder adducts of 1-acetoxybutadiene with *N*-benzyl-, *N*-ethyl-, and *N*-(4-carboxybutyryl)-maleimides **1 a–c** were prepared by heating the reactants in acetonitrile solution (Fig. 2). A molecular mechanics calculation[6] was carried out for the *N*-ethyl adduct which showed that, as expected, the *endo* isomer

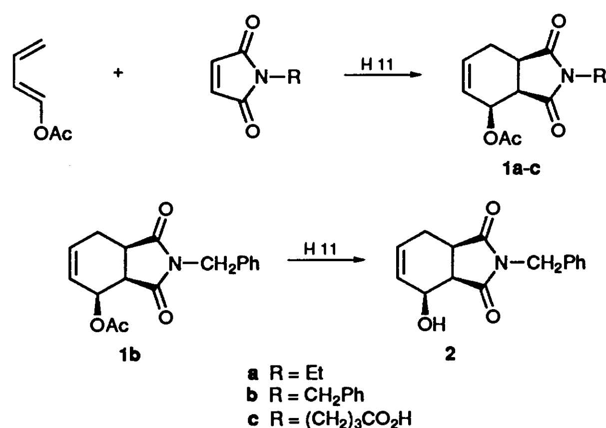

Fig. 2 Reactions catalysed by H11

was favoured. The NMR spectrum of the adduct was consistent with this calculation indicating that the configuration of the acetoxy group was *cis* with respect to the adjacent fused five-membered ring, the coupling constants for the protons at C-2 and C-3 being 9.6 Hz. Thus the hapten **1a** consists of a mixture of two enantiomers each containing three chiral centres. Raising antibodies to this hapten should, in principle, make available catalysts for the preparation of either enantiomer of the adduct. Chiral Diels–Alder reactions have been described in several cases in which the stereochemical course of a reaction is controlled by the presence of a chiral auxiliary[7] or by a chiral metal complex.[8] The effect of some proteins other than antibodies upon the stereochemical course of the Diels–Alder reaction has also been described.[9]

The bovine serum albumin conjugate of the adduct **1a** was prepared using water-soluble carbodiimide and mice were immunised as described previously.[10] Monoclonal antibodies were obtained using standard hybridoma methodology.[11] Those that bound the hapten were selected by ELISA using the transferrin conjugate of the hapten. Four clones that catalyse the Diels–Alder reaction have been identified of which one, designated H11 (IgG_1), has been studied most extensively. The other clones (D9, E8, and G6) were all of the IgG_{2b} sub-type. Antibodies were purified by ammonium sulfate precipitation followed by ion exchange chromatography on an S-Sepharose Fast Flow column eluting with citrate (10 mmol dm^{-3})/sodium chloride (0.1–1 mol dm^{-3}) buffers. Antibody H11 was found to have an optimal pH for elution of 5.5. The affinity of the

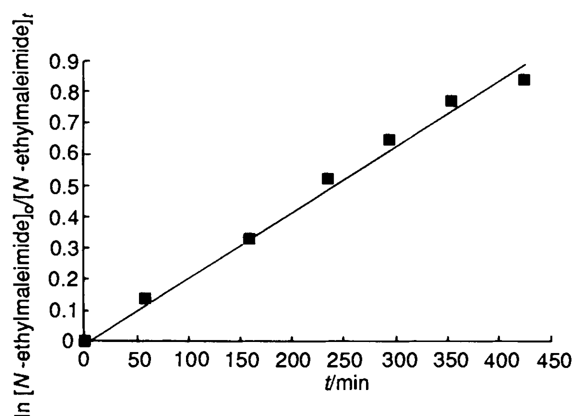

Fig. 3 Pseudo-first-order plot for the uncatalysed addition of *N*-ethylmaleimide to acetoxybutadiene

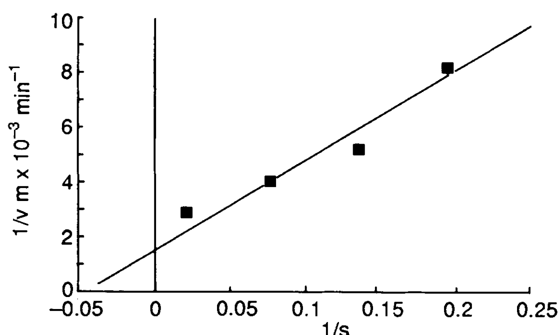

Fig. 4 Michaelis–Menten plot for the addition of *N*-ethylmaleimide to acetoxybutadiene catalysed by H11

transferrin conjugates of the hapten to the strongly binding clones was determined using competitive ELISA.[12] The K_D value determined was 3.99×10^{-8} mol dm^{-3}. The Fab fragment of H11 was prepared by digestion of purified H11 with immobilized papain followed by separation of the Fc and undigested IgG components on a protein A column which binds to the Fc unit (free or intact IgG).

Catalytic Properties of H11.—Control reactions were carried out in the absence of H11 to determine the uncatalysed rate of cycloaddition under conditions appropriate for solubilisation of both reactants and antibodies. The basic reaction medium was pH 8 sodium phosphate buffered saline (10 mmol dm^{-3}) and the reactants were added in acetonitrile–water (1 : 1, v/v). Reactions were followed by the removal of samples (20 mm^3) and analysis by HPLC eluting with acetonitrile–water mixtures. Under these reaction conditions, 1-acetoxybutadiene was found to undergo slow hydrolysis to crotonaldehyde; reaction rates were, therefore, measured by the decrease in concentration of the appropriate maleimide. Reactions were carried out with equimolar concentrations of reactants and under pseudo-first-order conditions with a 10-fold excess of acetoxybutadiene. From these experiments, the uncatalysed rate was determined to be 2.2×10^{-6} mol^{-1} dm^3 s^{-1}, and 3.2×10^{-5} s^{-1} under pseudo-first-order conditions (Fig. 3). As a further control, reactions were carried out in the presence of a non-specific immunoglobulin (mouse IgG) under the same conditions and concentrations as H11 but no enhancement of rate over the background measured above was observed.

H11 accepted both *N*-ethyl-and *N*-benzyl-maleimides as substrates. H11 displayed saturation kinetics (Fig. 4) and the constants $k_{cat} = 0.055$ s^{-1}, K_m (*N*-ethylmaleimide) = 8.3 mmol dm^{-3} were obtained. As mentioned above, acetoxybutadiene underwent slow hydrolysis to afford crotonaldehyde under the

Table 1 Properties of Antibody H11 as a function of pH

pH	Cycloaddition relative rate	Hydrolysis relative rate	Fluorescence enhancement
4.0	0.16	—	—
5.0	0.14	0.37	0.83
6.0	0.20	0.62	0.81
7.0	0.31	1.00	1.00
8.0	1.00	0.72	0.92
9.0	—	0.73	0.75
9.5	0.86	—	—

reaction conditions and a value for K_m was consequently not obtained. From these values $k_{cat}/K_m = 6.6$ mol^{-1} dm^3 s^{-1} and the rate enhancement was approximately 1700-fold comparing the pseudo-first-order rate constant with the catalysed rate. H11 can thus be seen to be a moderately effective catalyst for the Diels–Alder reaction of 1-acetoxybutadiene with *N*-ethylmaleimide.

The selectivity of H11 towards substrates was investigated with a short series of compounds. Both *N*-ethyl- and *N*-benzylmaleimides were substrates. However H11 failed to catalyse the reaction of the intrinsically more reactive diene, 1-methoxybutadiene, with the *N*-alkylmaleimides. Not surprisingly, it also had no effect on the rate of reaction of the non-activated diene, penta-1,3-diene. A further question that required investigation was the possibility of substrate inhibition. Strategies for avoiding product inhibition in the catalysis of Diels–Alder reactions have been ingeniously derived by other workers[4,5] and this question was investigated for H11 using three compounds, the acetoxybutadiene adducts of *N*-ethyl-, *N*-benzyl-, and *N*-butyryl-maleimide. In the presence of 42 μmol dm^{-3} H11, 0.45 mmol dm^{-3} *N*-ethylmaleimide, and 0.41 mmol dm^{-3} *N*-ethylmaleimide adduct **1a**, the rate of cycloaddition of *N*-ethylmaleimide and acetoxybutadiene was not measurably reduced. Similar behaviour was found at higher concentrations of **1a** (0.81 mmol dm^{-3} and 1.6 mmol dm^{-3}) and for the other two adducts **1b** and **1c**.

Two unexpected features were also found in studying H11. First, the reaction was found to be pH dependent (Table 1); it was extremely slow at pHs < 7 but the rate increased rapidly between pH 7 and 8. A pH dependence is not expected for neutral substrates in the Diels–Alder reaction although it is well known that the reaction is catalysed by Lewis acids. Two explanations seemed possible for this observation. One possibility is that the antibody undergoes a significant conformational change at pH < 7 moving from an unfavourable to a favourable conformation. To examine this possibility, the CD curves of H11 were measured at 260 and 320 nm over the pH range 5–9 but no significant changes were observed. On the other hand, a change in the intrinsic fluorescence of H11 over this pH range was observed (Table 1). This probably reflects the behaviour of an exposed tryptophan residue that undergoes local changes in its environment as a function of pH. These results do not support a substantial conformational change that might influence catalysis but do not rule out subtle changes involving specific regions of the antibody. A second possibility is that the deprotonation of an acidic group is required for catalysis to occur; in the protonated form, this group could be interacting with acetoxybutadiene reducing its reactivity by protonation. Although such a group has not yet been identified, its presence is consistent with the second unexpected feature of H11, namely catalysis of hydrolysis (Table 1).

During experiments to isolate the product of the reaction of acetoxybutadiene with the *N*-benzylmaleimide adduct **1c** it was found that the expected ester was difficult to isolate; the major

product after an incubation of several hours was identified as the hydroxy product **2** by HPLC and ^{1}H NMR spectroscopy in comparison with an authentic sample. Control reactions showed the acetoxy adduct **1c** to be completely stable under the reaction conditions. Kinetic experiments then established that H11 was capable of catalysing the hydrolysis of the Diels–Alder adduct with the following kinetic constants $k_{cat} = 9.2 \times 10^{-4}$ s^{-1} and $K_m = 1.1$ mmol dm^{-3}. Thus, the rate of hydrolysis was approximately 1/70 of the rate of cycloaddition. To correlate this observation with the unexpected pH dependence of the Diels–Alder reaction, the pH dependence of hydrolysis was also measured and a very similar profile emerged but with a maximum at pH 7 (Table 1).

The relationship between the hydrolysis and cycloadditions was investigated further by testing the ability of the initial reactants to inhibit hydrolysis using *N*-benzylmaleimide. *N*-Benzylmaleimide (32 mmol dm^{-3}) was found to inhibit essentially completely the hydrolysis of the corresponding acetoxybutadiene adduct **1c** (0.5 mmol dm^{-3}) in the presence of H11; similarly, in the presence of **1c** (2 mmol dm^{-3}), the concentration of *N*-benzylmaleimide required for 50% inhibition of hydrolysis was approximately 30 mmol dm^{-3}. A further extensive kinetic analysis of the relationship between hydrolysis and cycloaddition awaits the production of further supplies of H11. These results suggest that we have the first example of a catalytic antibody with dual catalytic activity. Because of the hydrolysis reaction and the instability of the derived alcohol to modification by chiral reagents, we have been unable to determine the chirality of the products. Attempts at chiral chromatography have also been unsuccessful so far. We are investigating alternative, more stable substrates, to address this question.

Catalysis by Fab Fragment of H11.—If catalytic antibodies are to become truly useful proteins, it is important that catalysis should be exhibited by the antigen binding fragment alone. There are several examples of antibodies in which Fab fragments or smaller fragments of the antibody are capable of catalysing reactions.[13] The ability of H11-Fab to catalyse both cycloadditions and hydrolyses was investigated. H11-Fab was equally catalytically competent as the intact antibody. We are currently developing molecular biological methods to obtain further and smaller fragments of H11 to investigate their catalytic properties.

Discussion

Catalysis of the Diels–Alder reaction has been demonstrated by several methods. Lewis acid catalysis is well known[14] and the effect of inclusion compounds in particular cyclodextrins has been investigated.[15] In the latter context, it was recognised that Diels–Alder reactions in aqueous solution are favourable. The major effect of catalysis by proteins would be expected to be entropic. Also, the chiral environment of the protein might be expected to lead to asymmetric induction in the reaction. So far, stereoselective catalysis has not been observed with isolated proteins although there are some reports of asymmetric induction using yeast to mediate the reaction.[9] It is notable that products do not cause substantial inhibition of the cycloaddition catalysed by H11. The lack of a significant effect may reflect the weak binding of substrate and product compared with protein-conjugated product (the hapten) which differ by the order of 10^5. It is well recognised that high affinity antibodies will not make good catalytic antibodies because they will bind the molecules too tightly for turnover. H11 probably represents a fortuitous outcome of the screening methods used in this case. The data available so far for catalysis by H11 does not so far allow a detailed description of the mechanisms of catalysis of cycloaddition. Significant features include the selectivity for 1-acetoxybutadiene compared with penta-1,3-diene and 1-methoxybutadiene, the pH dependence of the cycloaddition and the observation of hydrolysis. It may be that all three phenomena result from the same functional groups of the antibody H11 (Fig. 5). Thus the non-reactive dienes may not be substrates

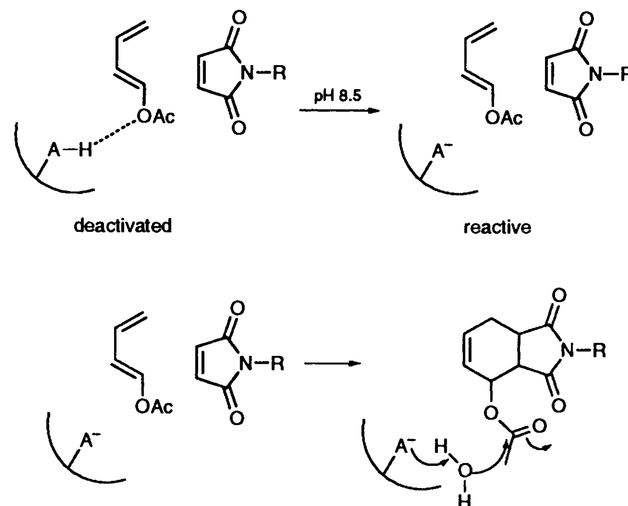

Fig. 5 Rationalisation of the pH dependence of cycloaddition catalysed by H11 and its subsidiary hydrol activity.

because they are unable to take advantage of binding to the putative functional group that can also be associated with the pH dependence of cycloaddition and the concomitant hydrolysis. It is also possible that several amino acid residues are responsible for the observed behaviour.

The binding site of small, polar haptens is likely to be on the surface of the protein. Inspection of the surfaces of all water-soluble globular proteins reveals a large number of charged and polar residues as required for solvation. There is a high probability, therefore, that there will be at least one such charged group in the vicinity of the binding site of the antibody. Any of the charged residues could, in principle, contribute to general base catalysis in a hydrolysis (Fig. 5) either as a general base (CO_2^-) or as a counter ion (NH_3^+). Thus, if binding is strong enough, hydrolysis of suitably reactive substrates could easily ensue, as we found with the general base-catalysed hydrolysis of a 4-nitrophenyl ester by an antibody.[16] These results suggest that the discovery of hydrolysis by antibodies should, therefore, be relatively common. Indeed Paul[17] has recently reported observations to suggest that some human autoantibodies to vasointestinal peptide catalyse its hydrolysis with some selectivity; similar observations that antibodies catalyse hydrolyses had also been made 10 years ago[18] but their significance was not clear. These observations of hydrolysis, and ours too, have little to do with the original concept of transition state analogues promoting the formation of a catalytically active site in an abzyme; if this hypothesis is correct, it will be an intrinsic property of antibodies, and indeed all proteins, provided that there is sufficiently potent binding.

Experimental

Preparation of Diels–Alder Adducts—The relevant diene and dienophile were heated under reflux in acetonitrile solution for 6 h; the solution was cooled and the solvent removed under reduced pressure. The residue was purified either by chromatography on silica gel or by recrystallisation. In this way, the following compounds were prepared.

*4-Acetoxy-2-ethyl-*3a,4,7,7a*-tetrahydroisoindole-*1,3*-dione* **1a**. This compound, purified by chromatography and eluting with 2:1 hexane–ethyl acetate, was obtained in 42 % yield, m.p. 84–86 °C, (Found: C, 61.1; H, 6.6; N, 6.0. $C_{12}H_{15}NO_4$ requires C, 60.8; H, 6.3; N, 5.9 %); δ_H($CDCl_3$, 250 MHz) 1.15 (t, 3 H), 2.03 (s, 3 H), 2.50 (m, 2 H), 3.08 (m, 1 H), 3.38 (dd, 1 H), 3.54 (q, 2 H), 5.42 (m, 1 H) and 6.06 (m, 2 H).

*4-Acetoxy-2-benzyl-*3a,4,7,7a*-tetrahydroisoindole-*1,3*-dione* **1b**. This compound was purified by chromatography and eluting with 1:1 hexane–ether, was obtained in 39% yield, m.p. 79–81 °C, (Found: C, 67.7; H, 5.5; N, 4.4%; M, 299.115 76. $C_{17}H_{17}NO_2$ requires C, 68.2; H, 5.7; N, 4.7%; *M*, 299.115 74); δ_H($CDCl_3$ 250 MHz) 1.74 (s, 3 H), 2.52 (m, 2 H), 3.33 (dd, 1 H), 4.62 (s, 2 H), 5.42 (m, 1 H), 6.04 (m, 2 H) and 7.30 (m, 5 H).

*4-Acetoxy-2-(butyryl)-*3a,4,7,7a*-tetrahydroisoindole-*1,3*-dione* **1c**. This compound, purified by recrystallisation from ether, was obtained in 40% yield (m.p. 121–122 °C); δ_H[$(CD_3)_2SO$ 250 MHz] 1.65 (m, 2 H), 1.93 (s, 3 H), 2.17 (m, 2 H), 2.40 (t, 2 H), 2.50 (m, 2 H), 5.33 (m, 1 H), 6.02 (m, 2 H) and 10.74 (s, 1 H).

*2-Benzyl-4-hydroxy-*3a,4,7,7a*-tetrahydroisoindole-*1,3*-dione* **2**. The corresponding acetoxy ester **1b** (100 mg, 0.33 mmol) was added to methanolic potassium carbonate (saturated 5 cm^3); and the solution stirred at room temperature for 30 min. The progress of the reaction was followed by HPLC on a reverse phase column (octadecylsilyl) eluting with acetonitrile–water (1:2, v/v). After consumption of starting material, the solution was evaporated to dryness and the required alcohol separated by repeated injections on a semi-preparative HPLC column (Hichrome ocatadecylsilyl silica). At a flow rate of 2.7 cm^3 min^{-1} the alcohol **2** had a retention time of 11 min; δ_H($CDCl_3$ 400 MHz) 2.22 (m, 1 H), 2.67 (m, 1 H), 3.10 (m, 1 H), 3.30 (dd, 1 H), 4.44 (m, 1 H), 4.65 (s, 2 H), 5.80 (m, 1 H) and 6.01 (m, 1 H).

Preparation of Hapten-protein Conjugates.—*N*-Hydroxysuccinimide (4 mg, 3.5×10^{-5} mol) in dimethylformamide (100 mm^3)* was added to a solution of the hapten **1c** (10 mg, 3.5×10^5 mol) in dimethylformamide (100 mm^3) together with a solution of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (7 mg, 3.5×10^{-5} mol) in dimethylformamide (100 mm^3). The solution was stored in the dark for 16 h. Transferrin (10 mg) was dissolved in aqueous saline (0.9%; 2 cm^3) and the activated hapten solution added slowly to it with stirring. Stirring of the mixture was continued for a further 4 h at 4 °C. The precipitated material was removed by centrifugation and the supernatant applied to a PD 10 Sephadex column. Fractions (1.5 cm^3) were collected and the absorbance of the fractions at 280 nm monitored. Fractions with absorbances > 0.5 were pooled and dialysed against phosphate buffered saline (10 mmol dm^{-3}; pH 7.4) with several changes of buffer. An identical procedure was used with bovine serum albumen in place of transferrin.

Studies of Cycloaddition Catalysed by H11.—*Rate of cycloaddition.* Prior to investigating catalysis, solutions of H11 (4.02×10^{-5} mol dm^{-3}) were dialysed overnight against pH 8 phosphate buffered saline (10 mmol dm^{-3}) at 4 °C. To portions of this solution (0.3 cm^3) a solution of the two reactants (6 mm^3) was added to give a final concentration of the maleimide of 9.4×10^{-4} mol dm^{-3}. The substrate solution was prepared by dissolving the maleimide (*ca.* 6 mg) and 1-acetoxybutadiene (5 mm^3) in water–acetonitrile (1:1, v/v; 1 cm^3). The reaction was allowed to proceed at 18 ± 2 °C and was followed by removing samples (20 mm^3) and analysis by HPLC on reverse phase C_{18} Hichrome with a flow rate of 1 cm^3 min^{-1} eluting with water–acetonitrile (1:1, v/v) for *N*-ethylmaleimide and water–acetonitrile (3:1, v/v) for *N*-benzylmaleimide. To obtain the pseudo-first-order rate constant for the reaction in the absence of antibody, reaction was carried out under the same conditions but using 50 mm acetoxybutadiene.

Product inhibition. Reactions were carried out as described above using a stock substrates solution of *N*-ethylmaleimide (6 mg) and acetoxybutadiene (5 mm^3) in water–acetonitrile (1:1, v/v; 1 cm^3) and product solution of the *N*-ethylmaleimide adduct **1a** (10 mg) in acetonitrile (1 cm^3). To the standard solution of H11 in phosphate buffered saline (pH 8) samples of substrate (3 mm^3) were added to give a final concentration of substrate of 4.7×10^{-4} mol dm^{-3}. The reaction was followed as before. The reaction was repeated in the presence of **1a** at 8.1×10^{-4} mol dm^{-3} and 1.6×10^{-3} mol dm^{-3} but no difference in the rate of reaction was detected. Similar experiments using *N*-benzyl- and *N*-butyryl-maleimides also showed no inhibition of the cycloaddition as measured by the disappearance of the maleimide.

pH *Dependence.* The procedures used were the same except that the solutions of H11 were prepared by dialysis against a saline buffered with phosphate to the required pH. Samples were prepared at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.50.

CD and fluorescence studies. Sample solutions of H11 at pH 5.0, 6.0, 7.0, 8.0 and 9.0 were prepared by addition of H11 solution prepared above (2 cm^3) to sodium phosphate buffer solutions at the appropriate pH (0.02 mol dm^{-3}). The pH of the solution was checked before the spectra were recorded. CD spectra were recorded courtesy of Dr. N. Price (University of Stirling) over the range 260–320 nm but no significant change was observed. Fluorescence spectra were measured at 20 °C exciting at 295 nm and observing the emission at 342 nm.

Isolation of products. A solution of H11 (2.8×10^{-5} mol dm^{-3}; 2 cm^3) was prepared by dialysis and diluted with phosphate buffered saline (1 cm^3). A substrate solution was prepared from *N*-benzylmaleimide (7 mg) and acetoxybutadiene (12 mm^3) in aqueous acetonitrile (1:1, v/v ; 1 cm^3). The reaction was carried out using H11 solution (6 cm^3) and substrate solution (124 mm^3) to give a final concentration of 1.87×10^{-5} mol dm^{-3} H11 and 7.7×10^{-4} mol dm^{-3} *N*-benzylmaleimide. The reaction was followed by HPLC at 45 min intervals and was carried out over a period of *ca.* 4 h. After this time, the antibody was filtered (Amicon filter) and the low molecular weight products isolated from the filtrate. Filtration was slow (2 h) and the reaction was presumably continuing during the filtration process. The products were separated using a reverse phase C_{18} semipreparative Hichrome column eluting with water–acetonitrile (2:1, v/v) at a flow rate of 2.7 cm^3 min^{-1}. Products were detected by UV absorption at 210 nm. Retention times were: *N*-benzylmaleimide 25 min, acetoxybutadiene 24 min, and the adduct **1b** 40 min. When the reaction products were separated, a new peak retention time 11 min was isolated. This peak was shown to be the hydrolysis product **2** from further reaction of **1b** by NMR and comparison with a sample prepared by non-abzymic hydrolysis (see above). A further peak retention time 18 min was isolated but the product was not characterised.

Hydrolysis Catalysed by H11.—Hydrolysis of the benzylmaleimide adduct **1b** was studied using both H11 and the H11 Fab fragment. A solution of H11 (4.8×10^{-5} mol dm^{-3} 350 mm^3) in phosphate buffered saline pH 8 (10 mmol dm^{-3}) was prepared and to this solution a solution of the adduct **1b** (6 mg) in acetonitrile (0.5 cm^3) was added to give a concentration of adduct of 1 mmol dm^{-3}. The reaction was followed by HPLC eluting with water–acetonitrile (2:1, v/v). An identical experiment was carried out using H11 Fab fragment and this protein was used to determine the kinetic constants for the

* 1 mm^3 ≡ 1 μl

reaction. For the determination of the rate, the H11 Fab concentration was 7.9×10^{-5} mol dm^{-3}. No hydrolysis was observed using phosphate buffered saline in the absence of H11 or of H11 Fab.

Preparation of H11 Fab Fragment.—Fab fragments were prepared from purified H11 antibody using the Pierce ImmunoPure Fab kit (Pierce and Warriner, Chester).

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