A polyclonal antibody preparation with Michaelian catalytic properties

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1. 4-Nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I), an amide conjugate (XI) involving the carboxy group of 4-nitrophenyl 4'-carboxymethylphenyl phosphate and an amino group of keyhole-limpet haemocyanin, and a fluorescein derivative (XVII) were synthesized. 2. The conjugate (XI) was used as an immunogen with which to raise polyclonal antibodies in multigeneration cross-bred sheep; the fluorescent derivative (XVII) was used for the initial assessment of the antisera via binding assays monitored by fluorescence polarization; the carbonate ester (I) was used as a chromogenic substrate for the investigation of catalytic activity. 3. The IgG from the antiserum of sheep no. 270 was isolated by Na_2SO_4 precipitation and chromatography on Protein G–Sephadex. 4. This preparation of IgG catalysed the hydrolysis of the carbonate ester (I); the catalysis at pH 8.0 and 25 °C obeyed Michaelis–Menten kinetics with at least 25 turnovers, $K_m = 3.34 \mu\text{M}$, and lower limits for k_{cat} of 0.029 s^{-1} and for k_{cat}/K_m of $8.77 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, on the unlikely assumption that the concentration of catalytic antibody is provided by twice the total IgG concentration (two sites per molecule); probable estimates of the fraction of the total IgG that is anti-haptenic IgG and of the fraction of this that is catalytically active suggest that the values of k_{cat}/K_m are actually very much larger than these lower limits. 5. The failure of the antibody preparation to catalyse the hydrolysis of the isomeric 2-nitrophenyl carbonate (II), which differs from compound (I) only in the position of the nitro substituent in the leaving group, compels the view that catalytic activity is due to antibody rather than contaminant enzyme; this conclusion is supported by (a) the failure of the following to discriminate effectively between the isomeric substrates (I) and (II): pig liver carboxylesterase, rabbit liver carboxylesterase (collectively EC 3.1.1.1), whole serum from a non-immunized sheep and whole serum from a sheep immunized with a derivative of 3-O-methylnoradrenaline and (b) the lack of catalytic activity in IgG preparations from sheep immunized with sulfoxide or sulphone analogues of immunogen (XI). 6. The various parameters used for the comparison of the kinetic characteristics of hydrolytic catalytic antibodies are discussed. 7. The characteristics of hydrolysis of compound (I) catalysed by the present polyclonal antibody preparation are shown to be substantially better in most respects than those of analogous reactions of two other carbonate esters catalysed by monoclonal antibodies.

INTRODUCTION

The design and production of molecules that provide specific and efficient catalysis under the mild conditions associated with enzyme action is of growing interest. Their investigation promises advances in understanding molecular recognition, and the nature of transition states in both enzymic and non-enzymic reactions. Advances may be expected also in the design of tailor-made catalysts with highly specific recognition features and valuable potential applications in biotechnology and medicine. Catalytic systems on which considerable attention is being focused include (a) synthetic polymers (reviewed by Klotz, 1987) where, in some cases, cavities of defined geometry may be created (Pike *et al.*, 1978), (b) other cavity-containing compounds such as crown ethers (reviewed by Stoddart, 1987) and cyclodextrins (reviewed by Bender, 1987), (c) enzymes modified by site-directed mutagenesis (reviewed by Leatherbarrow & Fersht, 1987) and (d) catalytic antibodies (reviewed, e.g., by Schultz & Jacobs, 1988; Blackburn *et al.*, 1989; Powell & Hansen, 1989; Schultz, 1989; Kang *et al.*, 1990).

There is growing awareness that in enzyme catalysis catalytic-site chemistry might be partly determined by interactions involved

in the molecular recognition process (Knowles, 1987; Brocklehurst *et al.*, 1988a,b; Kowlessur *et al.*, 1990). It may be necessary, therefore, to arrange for such interdependence in some of the non-enzymic catalysts, rather than for ligand recognition and rate acceleration as separate events, if the catalytic efficiency associated with enzymes is to be realized. Antibody-mediated catalysis might be expected to bring about rate enhancement by mechanisms that depend critically on binding. Differential binding of ligand species along the reaction pathway in which the transition state is stabilized relative to the reactant state is predicted to lead to rate enhancement (Pauling, 1946). The substantial structural and electronic changes that acyl compounds and carbonates undergo during hydrolysis suggested the possibility of exploiting differential binding effects leading to catalysis by appropriate antibodies (Jencks, 1969), and a number of monoclonal catalytic antibodies for such reactions have been reported (see, e.g., Schultz & Jacobs, 1988).

Essentially all of the many publications on catalytic antibodies for a wide range of reaction types (see, e.g., Blackburn *et al.*, 1989) have reported the production and characterization of monoclonal antibodies. Early attempts to produce polyclonal catalytic antibodies failed (Raso & Stollar, 1975), and Schultz

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(1988), who has successfully produced many monoclonal catalytic antibodies, reported that his group had been unsuccessful in generating polyclonal catalytic antibodies. At about the same time Schultz & Jacobs (1988) reported that after extensive purification of a polyclonal antiserum from a rabbit catalytic activity was found in one fraction eluted from an affinity column with 6 M-guanidinium chloride, but the catalytic characteristics of this preparation were not reported. More recently Shokat & Schultz (1990) suggested that the early lack of success in producing some catalytic antibodies may have been due to the use of polyclonal rather than monoclonal antibodies. It may be that these reports have discouraged investigation of the possibility of producing polyclonal catalytic antibodies, although other reports suggest that this objective may be worth pursuing. Thus polyclonal antibodies have been shown to act as a template in a photochemical reaction (Balan *et al.*, 1988), although catalysis was neither claimed nor investigated. Also, IgG from a human subject expressing autoantibody to vasoactive intestinal peptide has been reported to catalyse the hydrolysis of vasoactive intestinal peptide (Paul *et al.*, 1989).

It seemed important to investigate the possibility of producing polyclonal catalytic antibodies for several reasons: (i) the relative simplicity of producing polyclonal as against monoclonal antibodies, (ii) the possibility of extending the catalytic antibody field to include investigation of catalytic antibody responses in animals, (iii) the possibility that if polyclonal antibody preparations that obey the Michaelis-Menten equation could be prepared, despite the overall structural heterogeneity of polyclonal IgG, they would provide a ready means of investigating a wide range of kinetic characteristics before the detailed structural studies that would necessarily demand use of monoclonal antibodies, (iv) the potential value of polyclonal catalytic antibodies as catalysts for technological applications, and (v) the potential value of catalytic antibodies produced by immunization in the development of novel therapies. The production of polyclonal antibodies that bind small molecules has been extensively investigated (Landsteiner, 1947; Pauling & Pressman, 1945), and it is possible to produce polyclonal antibodies with particular recognition features by careful design of the immunogen employed for antibody production and of the ligands involved in subsequent binding interactions (Bauminger *et al.*, 1974; Gallacher *et al.*, 1988).

In the present paper we report for the first time details of the production and characterization of a catalytically competent polyclonal antibody preparation from a sheep (sheep 270). This preparation of IgG catalyses hydrolysis of the mixed aryl 4-nitrophenyl carbonate (I) [but not of the isomeric 2-nitrophenyl carbonate (II)]. The catalysed reaction obeys Michaelis-Menten kinetics. This demonstrates that, whatever structural heterogeneity exists in the IgG, the catalytic characteristics of the active IgG species are all sufficiently similar that differences between them are not readily detected as deviations from a single-site saturation model.

Even if the concentration of catalytic antibody is assumed to be equal to twice the total IgG concentration, the value of $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ (the pH-corrected value of $k_{\text{cat.}}/K_m$; see the Results and discussion section) for the polyclonal-antibody-catalysed hydrolysis of the 4-nitrophenyl carbonate ester (I) is 27 times larger than the value of $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ for the hydrolysis of the 4-nitrophenyl carbonate ester (III) and 800 times larger than the value of $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ for the hydrolysis of the 4-nitrophenyl carbonate ester (IV), in both cases catalysed by monoclonal antibodies (Pollack *et al.*, 1986; Jacobs *et al.*, 1987). On the more reasonable, though still conservative, assumption that only about 10% of the IgG is catalytic antibody, the corrected values of both $\tilde{k}_{\text{cat.}}$ and $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ would be correspondingly larger. Various ways of comparing the

catalytic effectiveness of different catalytic antibody preparations are discussed. A preliminary account of some related work has been presented (Gallacher *et al.*, 1990).

MATERIALS AND METHODS

Materials

Carboxylesterases (EC 3.1.1.1) from pig liver and rabbit liver were the purified products of Sigma Chemical Co. (Poole, Dorset, U.K.).

Solvents (AnalaR) and aluminium-backed silica gel 60 t.l.c. plates (Merck 5554) and silica gel 60 for flash column chromatography (Merck particle size 0.040–0.063 mm) were obtained from BDH Chemicals (Dagenham, Essex, U.K.). Protein G-Sepharose (4 Fast Flow) was from Pharmacia-LKB (Uppsala, Sweden) and keyhole-limpet haemocyanin was from Calbiochem-Behring (San Diego, CA, U.S.A.). All other chemicals were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Extraction of aqueous systems with organic solvents

After extractions of aqueous systems, organic solutions were dried with anhydrous MgSO_4 .

Synthesis of the carbonate substrate, 4-nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I) (Scheme 2a)

To a solution of 4-hydroxyphenylacetic acid (VI) (152 mg, 1 mmol) and *N*-hydroxysuccinimide (130 mg, 2 mmol) in acetonitrile (2 ml) was added 1-dimethylaminopropyl-3-ethylcarbodi-imide (190 mg, 1 mmol). The reaction mixture was stirred at room temperature (approx. 22 °C) for 1 h, after which time t.l.c. monitoring [developing solvent chloroform/methanol (6:1, v/v)] showed that the reaction was incomplete. Another 190 mg of the carbodi-imide was added and stirring was continued. After 15 min t.l.c. monitoring showed complete formation of the activated ester, and excess butylamine (150 μl) was then added. The reaction mixture was stirred at room temperature for 1 h, diluted with ethyl acetate (50 ml), washed sequentially with aq. 0.5 M- NaHCO_3 (20 ml), and 1 M-HCl (20 ml), dried and concentrated *in vacuo*. The residue was purified by flash chromatography [eluting solvent ethyl acetate/cyclohexane (9:1, v/v)] to give the pure 4-(3-aza-2-oxoheptyl)phenol (VII) (110 mg, 53% yield; R_f 0.75 in ethyl acetate) as white crystals, m.p. 66–67 °C (found: m/z 207; $\text{C}_{12}\text{H}_{17}\text{NO}_2$ requires $M_r = 207$). A portion of this phenol (VII) (92 mg, 0.44 mmol) was dissolved in acetonitrile (2 ml). To the resulting solution was added triethylamine (92 μl , 0.66 mmol) and 4-nitrophenyl chloroformate (98.5 mg, 0.49 mmol), and the reaction mixture was stirred for 1 h at room temperature. Volatile material was removed *in vacuo* and the residue was mixed with ethyl acetate (5 ml). After filtration the filtrate was evaporated under reduced pressure to give the crude product as a white solid. Purification by preparative t.l.c. [developing solvent ethyl acetate/cyclohexane (2:1, v/v); R_f 0.42] gave the pure carbonate (I), which was then recrystallized from ethyl acetate/cyclohexane (1:1, v/v) to give the carbonate as white crystals (107 mg, 64% yield), m.p. 117–119 °C. ^1H N.m.r. δ (p.p.m.) (C^2HCl_3) 8.35 (2H, d, HArNO_2), 7.50 (2H, d, HArNO_2), 7.35 (2H, d, ArH), 7.30 (2H, d, ArH), 5.40 (1H, s, $-\text{NH}-$), 3.60 (2H, s, $-\text{PhCH}_2\text{CO}-$), 3.25 (2H, t, $-\text{NHCH}_2-$), 1.45 (2H, quin, $-\text{NHCH}_2\text{CH}_2-$), 1.25 (2H, m, $-\text{CH}_2\text{CH}_3$) and 0.90 (3H, t, $-\text{CH}_3$).

Synthesis of the isomeric carbonate, 2-nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (II) (Scheme 2a)

4-(3-Aza-2-oxoheptyl)phenol (VII) (100 mg, 0.48 mmol) prepared as described above was dissolved in acetonitrile (1 ml). 2-

Nitrophenyl chloroformate (106 mg, 0.53 mmol) and triethylamine (67 μ l, 0.48 mmol) were added to the resulting solution. The mixture was stirred for 1 h at room temperature, after which time the volatile material was removed *in vacuo*. The residue was dissolved in ethyl acetate, then filtered, and the solvent was removed to give crude product. Preparative t.l.c. (silica, eluting solvent ethyl acetate) gave the isomeric carbonate (II) (R_f 0.30) (124 mg, 69 % yield), m.p. 115–116 °C, which was then crystallized from ethyl acetate/cyclohexane (1:1, v/v). I.r.: ν (cm^{-1}) (CHCl_3) 3450 (NH), 1780 (carbonyl, carbonate) and 1665 (CO amide). N.m.r.: δ (p.p.m.) (C^2HCl_3) 8.20 (1H, dd, $J = 6.25$ Hz, 1.60 Hz, ArH , *o*- NO_2), 7.73 (1H, td, $J = 7.81$ Hz, 1.60 Hz, ArH , *p*- NO_2), 7.45 (2H, m, td overlapping dd, ArH , *m*- NO_2 and *o*-OCO), 7.65 (4H, s, ArH), 5.35 (1H, s, NH), 3.55 (2H, s, ArCH_2CO), 3.20 (2H, q, NHCH_2CH), 1.40 (2H, m, NHCH_2CH_2), 1.30 (3H, m, CH_2CH_3) and 0.85 (3H, t, CH_3).

Synthesis of the phosphate immunogen (XI) (Scheme 2b)

Preparation of 4-nitrophenyl 4'-(3-oxa-2-oxobutyl)phenyl hydrogen phosphate (IX). A solution of methyl 4-hydroxyphenylacetate (VIII) (300 mg, 1.8 mmol) in acetonitrile (2 ml) was added dropwise to a stirred solution of 4-nitrophenyl phosphorodichloridate (512 mg, 2 mmol) in pyridine (4 ml) at 0 °C and the mixture was stirred at this temperature for 30 min. Water (800 μ l) was then added and the reaction mixture allowed to come to room temperature over 2 h. Solvents were removed *in vacuo*, and the residue was dissolved in aq. 0.5 M- NaHCO_3 (15 ml) and extracted with diethyl ether (2 $\times$ 15 ml). The aqueous solution was acidified with HCl and extracted with ethyl acetate (3 $\times$ 25 ml). The combined ethyl acetate extracts were dried and concentrated *in vacuo* to give the crude product (550 mg, 75 % yield) as a yellow oil [R_f 0.5 with developing solvent methylene dichloride/methanol (3:1, v/v)]. This was purified by flash chromatography [eluting solvent methylene dichloride/methanol (3:1, v/v)] to give the product (IX) as a colourless oil. I.r.: ν (cm^{-1}) (neat) 3500 (broad) and 1725 (sharp). ^1H n.m.r. (250 MHz): δ (p.p.m.) ($[\text{D}_6]\text{dimethyl sulphoxide}$) 3.32 (s, 3H, CH_3), 3.59 (s, 2H, CH_2), 7.05 (d, 2H, $J = 8.8$ Hz, H_A of ABq, $\text{HArCH}_2\text{CO}_2$), 7.12 (d, 2H, $J = 8.8$ Hz, H_B of ABq, $\text{HArCH}_2\text{CO}_2$), 7.40 (d, 2H, $J = 9.2$ Hz, H_A of ABq, HArNO_2) and 8.17 (d, 2H, $J = 9.2$ Hz, H_B of ABq, HArNO_2).

Preparation of 4-nitrophenyl 4'-(carboxymethyl)phenyl hydrogen phosphate (X). Aq. 1 M- NaOH (500 μ l) was added to a stirred solution of the above methyl ester (IX) (180 mg, 0.49 mmol) in tetrahydrofuran (3 ml) and water (2 ml). After 5 min a second 500 μ l was added and the reaction mixture was stirred at room temperature for 1 h. The mixture was then acidified with HCl and extracted with ethyl acetate (3 $\times$ 20 ml). The combined ethyl acetate extracts were dried and concentrated *in vacuo* to give the crude product [R_f 0.7 with developing solvent methylene dichloride/methanol/acetic acid, 50:50:1, by vol.) as a pale-brown solid (140 mg). Recrystallization from ethyl acetate/light petroleum (b.p. 40–60 °C) gave the pure product (X) (50 mg, 29 %) as white crystals. ^1H n.m.r. (250 MHz): δ (p.p.m.) (D_2O) 3.54 (s, 2H, CH_2CO_2), 6.99 (d, 2H, $J = 8.5$ Hz, H_A of ABq, ArCH_2CO_2), 7.10 (d, 2H, $J = 8.5$ Hz, H_B of ABq, ArCH_2CO_2), 7.18 (d, 2H, $J = 9.3$ Hz, H_A of ABq, ArNO_2), 0.08 (d, 2H, $J = 9.3$ Hz, H_B of ABq, ArNO_2).

Preparation of the immunogen (XI). 1,1-Dimethylaminopropyl-3-ethylcarbodi-imide (100 mg, 0.52 mmol) was added portion-wise over 5 h to a stirred solution of 4-nitrophenyl 4'-(carboxymethyl)phenyl hydrogen phosphate (X) (45 mg, 0.13 mmol), *N*-hydroxysuccinimide (100 mg, 0.87 mmol) and keyhole-limpet haemocyanin (60 mg) in water (4 ml) and pyridine (100 μ l). The reaction mixture was stirred at room temperature for a further

16 h, then dialysed against running tap water for 3 days, and the dialysis residue was freeze-dried to give the immunogen (XI) as an off-white powder (50 mg).

Synthesis of the fluorescent label (XVII) (Scheme 2c)

Preparation of 4-(4-aminophenyl)butanol (XIII). $\text{SnCl}_4 \cdot 2\text{H}_2\text{O}$ (6.0 g, 26.6 mmol) was dissolved in conc. HCl (10 ml) and the solution was cooled to 0 °C. To the cold stirred solution was added 4-(4-nitrophenyl)butanol (0.866 ml, 1.0 g, 5.13 mmol) and the mixture was stirred at 0 °C for 90 min. At the end of this time ethyl acetate (50 ml) was added and the aqueous phase of the resulting biphasic mixture was basified by cautious addition of solid NaHCO_3 (to pH 8–9). The layers were then separated, the organic phase being retained, and the aqueous phase was extracted twice more with ethyl acetate (2 $\times$ 50 ml). The organic layers were combined and dried. The solvent was removed *in vacuo* and the yellow-brown solid residue was crystallized from ethyl acetate/light petroleum (b.p. 40–60 °C) to give the product (XIII) (550 mg, 65 % yield) as yellow crystals.

Preparation of 4-(4-chloroacetamidophenyl)butanol (XIV). 4-(4-Aminophenyl)butanol (XIII) (10 g, 6.06 mmol) was dissolved in a mixture of methylene dichloride (4 ml) and pyridine (2 ml) and the solution was cooled to 0 °C. To the cold stirred solution was added dropwise a solution of chloroacetyl chloride (1.5 ml, 2.73 g, 78.8 mmol) in methylene dichloride (2 ml) that had been cooled to 0 °C. The solution became orange in colour and pyridinium hydrochloride was precipitated. The reaction was complete [as tested by t.l.c. with ethyl acetate/methanol (10:1, v/v)] after 15 min. To the resulting cold stirred solution was added a 1:1 (v/v) mixture of triethylamine/water (10 ml), followed by sufficient methanol (8 ml) to keep the solution homogeneous. The solution was allowed to warm to room temperature and was stirred at this temperature overnight. Solvent was then removed *in vacuo* and the residue was redissolved in methylene dichloride; this was repeated several times. The solid residue was then partitioned between water (30 ml) and ethyl acetate (30 ml). The aqueous layer was again extracted with ethyl acetate (2 $\times$ 30 ml). The organic phases were combined, concentrated *in vacuo* and dried by employing a water/methanol azeotrope. The residue was then crystallized from ethyl acetate to yield the product (XIV) as pale-yellow crystals (770 mg, 53 % yield).

Preparation of the *N*-protected phosphate (XV). 4-(4-Chloroacetamidophenyl)butanol (XIV) (200 mg, 0.83 mmol) was dissolved in a 2:1 (v/v) mixture of pyridine and acetonitrile (2.4 ml), and the solution was cooled to 0 °C. To the cold stirred solution was added 4-nitrophenyl phosphorodichloridate (600 mg, 2.34 mmol), and stirring was continued at 0 °C for 1 h. Water (2 ml) was then added, and precipitation of a pale-yellow solid began immediately. The mixture was allowed to warm to room temperature and stirring was continued for 3 h. At the end of this time solvent was removed *in vacuo* and the residue was partitioned between aq. 0.5 M- NaHCO_3 (25 ml) and diethyl ether (25 ml). The aqueous layer was then washed again with diethyl ether (25 ml) and the organic layer was discarded. The aqueous layer was acidified by cautious dropwise addition of conc. HCl (to pH 1–2) and extracted with ethyl acetate (5 $\times$ 30 ml). The organic extracts were combined, concentrated *in vacuo* and subjected to flash column chromatography on silica [gradient elution with ethyl acetate/methanol (10:1, v/v)–ethyl acetate/methanol (3:1, v/v)] to provide the product (XV) as an off-white solid (785 mg, 50 % yield).

***N*-Deprotection.** The *N*-protected phosphate (XV) (140 mg, 0.32 mmol) and thiourea (140 mg, 1.84 mmol) were dissolved in methanol (3 ml), and the solution was heated under reflux for 3 h. At the end of this time t.l.c. [with methylene dichloride/

methanol (3:1, v/v)] showed that the reaction was complete. The solvent was then removed *in vacuo* and the residue was subjected to flash column chromatography on silica [gradient elution with methylene dichloride/methanol (5:1, v/v)–methylene dichloride/methanol (3:1, v/v)] to afford the *N*-deprotected phosphate product (XVI) (80 mg, 70% yield) as a yellow oil.

Preparation of the fluorescent label (XVII). To a solution of the *N*-deprotected phosphate (XVI) (3.6 mg, 0.01 mmol) in methanol (2 ml) and triethylamine (200 μ l) was added fluorescein isothiocyanate isomer 1 (Aldrich Chemical Co.) (3.9 mg, 0.01 mmol) and the reaction was stirred at room temperature for 1 h. Volatile material was removed *in vacuo* and the residue was dried by forming an azeotrope with methanol (5 $\times$ 5 ml). The product was isolated by t.l.c. [developing solvent methanol/methylene dichloride/acetic acid (75:25:1)] to give the purified label (XVII) as yellow solid (R_f 0.75 in the above solvent system).

Characterization of synthesized compounds

High-field n.m.r. spectra and fast-atom-bombardment mass spectra were provided by the University of London Intercollegiate Research Service at Queen Mary and Westfield College with the use of a Bruker WH 400 MHz spectrometer and at the London School of Pharmacy with the use of a VG ZAB-IF mass spectrometer respectively.

Production of antibodies

Three mature multigeneration cross-bred ewes (numbered 270, 271 and 272) were each immunized subcutaneously and intramuscularly with 4 mg of the above immunogen (XI) in a water-(1 ml)-in-oil emulsion of Freund's complete adjuvant (3 ml). This was repeated at ten 4-weekly intervals except that 2 mg of immunogen and Freund's incomplete adjuvant were then used. The animals were bled from the external jugular vein 2 weeks after each re-immunization, and the blood was allowed to clot overnight and then centrifuged, and the serum was decanted and stored at -20°C .

Fluorescence-polarization antibody dilution curves

The fluorescent label (XVII) was dissolved in methanol and its concentration was determined by spectral analysis after dilution with 50 mM-sodium bicarbonate buffer, pH 9.0, and by using $\epsilon_{492} = 8.78 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Dandliker & Levison, 1967). A stock solution of the label (30 nM) was then prepared in 50 mM-sodium phosphate buffer, pH 7.5, containing 0.1% NaN_3 and 0.1% bovine γ -globulin. Antiserum (500 μ l) was dissolved in the

phosphate buffer (4.5 ml) to give initial dilution of 1:10. Doubling dilutions were then prepared from this, giving antibody dilutions ranging from 1:10 to 1:7680.

Each antibody dilution (1 ml) was incubated with the label (500 μ l of the stock solution) for 1 h at room temperature, and the fluorescence polarization of each tube was measured with an SLM4000 polarization fluorimeter (SLM Instruments, Urbana, IL, U.S.A.). Background values were obtained for each dilution by incubating the antibody dilution (1 ml) with the phosphate buffer (500 μ l), and these readings were subtracted from the values obtained with the label. Antibody dilution curves were constructed by plotting the resulting fluorescence polarization against antibody dilution for antiserum obtained from each sheep (270, 271 and 272) 22 weeks into the immunization schedule.

Purification of IgG from sheep 270 and from a non-immunized sheep

Anhydrous Na_2SO_4 (180 mg) was added, with vortex-mixing, to serum (1 ml) in one case from sheep 270 and in another case to provide a control from a sheep that had not been immunized. In each case the resulting suspension was gently mixed for 30 min, then centrifuged (3000 *g* for 10 min at 25°C), and the supernatant was aspirated to waste. The precipitated IgG was then washed [2×2 ml of 18% (w/v) Na_2SO_4] and redissolved in 50 mM-sodium phosphate buffer, pH 7.5 (2 ml). The resulting solution (2 ml) was subjected to chromatography on a Protein G–Sepharose 4 Fast Flow column (5 ml) with spectrophotometric (280 nm) monitoring of the eluate. The adsorbed IgG was eluted with 0.1 M-glycine/HCl buffer, pH 2.7, and 2 ml fractions of the eluate were collected in tubes containing aq. sodium 1 M- NaHCO_3 (0.1 ml). The fractions containing IgG were pooled and the buffer was exchanged by chromatography on a Sephadex G-25 column (32 cm $\times$ 3.2 cm) with 50 mM-sodium phosphate buffer, pH 8.0.

Kinetics of the hydrolysis of the carbonate esters (I) and (II)

The release of 4-nitrophenolate from the carbonate ester (I) was monitored in 50 mM-sodium phosphate buffer, pH 8.0, containing 0.67% (v/v) acetonitrile at 25°C by recording the increase in A_{400} with the use of the 0–0.01, 0–0.02, and 0–0.05 *A* ranges of a Cary 16K spectrophotometer or the 0–0.005, 0–0.01, 0–0.02 and 0–0.05 *A* ranges of a Kontron Uvikon 810 spectrophotometer and quantified by using $\epsilon_{400} = 1.65 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Stock solutions of the substrate were pre-

pared in acetonitrile and their concentrations were adjusted so that, by addition of stock substrate solution and acetonitrile solvent to a total volume of 20 μl in a final reaction volume of 3 ml, final concentrations in the range approx. 0.2–16 μM were achieved. In some cases reaction mixtures contained also 0.2 μM -IgG prepared from sheep no. 270 or 0.2 μM -IgG prepared from the non-immunized sheep or pig liver carboxylesterase (0.33 $\mu\text{g}/\text{ml}$) or rabbit liver carboxylesterase (0.33 $\mu\text{g}/\text{ml}$) or 3000-fold-diluted whole serum from a non-immunized sheep or 3000-fold-diluted whole serum from a sheep immunized with a derivative of 3-*O*-methylnoradrenaline (3-*O*-methylnoradrenaline alkylated on the phenolic hydroxy group by reaction with methyl bromoacetate followed by hydrolysis to the carboxylic acid and coupling to keyhole-limpet haemocyanin via the hydroxysuccinimide ester; Mellor *et al.*, 1989). The concentration of IgG in the eluate from the Sephadex G-25 column was determined by using ϵ_{280} $2.0 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and was usually approx. 3 μM . Initial rates (v) were determined from slopes of linear progress curves. Those for the reactions carried out in the absence of IgG, carboxylesterase or whole sera or in the presence of IgG prepared from non-immunized sheep increased linearly with increase in $[S]$ and were closely similar at a given value of $[S]$. The initial rates obtained in the presence of IgG from non-immunized sheep were subtracted from those obtained at the same values of $[S]$ in the presence of the IgG prepared from sheep no. 270 to provide the rates of the polyclonal-catalytic-antibody-catalysed reaction. After demonstration of adherence of the corrected v -versus- $[S]$ data to the Michaelis–Menten equation by the linearity of an $[S]/v$ -versus- $[S]$ plot, values of the parameters V_{max} and K_m were determined by fitting the data to the Michaelis–Menten equation by weighted non-linear regression analysis performed by using the AR program from the BMDP statistical software package (1988 version for IBM PC/DOS) (Dixon *et al.*, 1988) and a Compaq Deskpro 386/20e computer equipped with a 20 MHz Jutel 387 co-processor and a 110 Mbyte fixed-disc drive. An error structure of constant relative error was assumed and weighting factors were inversely proportional to v^2 . The same procedure was used to determine values of the Michaelis–Menten parameters for the reactions catalysed by the two carboxylesterases and by catalysts (presumably esterases) present in the two types of sheep whole sera. For the former, apparent values of k_{cat} , based on protein concentration, were calculated by assuming one catalytic site per molecule and M_r values of 2×10^5 for the pig enzyme and 1×10^5 for the rabbit enzyme.

The kinetics of the release of 2-nitrophenolate from the carbonate ester (II) were studied in an analogous way except that reactions were monitored at 416 nm and initial rates were calculated by using ϵ_{416} $4103 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

RESULTS AND DISCUSSION

Design and synthesis of substrate and immunogen

The following considerations led to the design of the carbonate ester (I) as the substrate for the present investigation: (i) monoclonal antibody preparations had been shown to catalyse the hydrolysis of two other 4-nitrophenyl carbonates [(III) and (IV)]; the 4-nitrophenyl group provides for a chromogenic leaving group and also provides a strongly antigenic group in the corresponding immunogen; (ii) the additional phenyl ring and the amide group of compound (I), neither of which is present in compound (III) or compound (IV), were introduced to provide possible opportunities for recognition of the substrate by the antibody at hydrophobic and hydrogen-bonding sites; the amide group provides also the required link between the protein

(keyhole-limpet haemocyanin) and the hapten (X) in the immunogen [(XI) of Scheme 2(b)]. The isomeric carbonate ester (II) was designed to permit additional evaluation of the possibility of a contaminant esterase enzyme surviving the purification of IgG by chromatography on Protein G–Sepharose (see below).

Reaction of the carbonate substrate (I) with OH^- ion (Scheme 1) is assumed to proceed via the transient anionic tetrahedral intermediate (V), which should be similar in structure to the transition state. The stable phosphate analogue of this intermediate was used as the haptenic determinant in the immunogen (XI), synthesis of which is shown in Scheme 2(b).

The synthesis of the carbonate substrate (I) is shown in Scheme 2(a). The *N*-hydroxysuccinimide ester of 4-hydroxyphenylacetic acid was generated *in situ* and allowed to react with *n*-butylamine to produce the amide (VII). The 4-hydroxy group was then acylated by reaction with 4-nitrophenyl chloroformate.

The synthesis of the immunogen (XI) is shown in Scheme 2(b). The methyl ester of 4-hydroxyphenylacetic acid (VIII) was allowed to react with 4-nitrophenyl phosphorodichloridate, and the intermediate diarylphosphoryl chloride was hydrolysed to produce the diaryl hydrogen phosphate (IX). More vigorous hydrolysis converted the methyl ester into the free acid (X), which was coupled to keyhole-limpet haemocyanin via the *N*-hydroxysuccinimide ester produced *in situ* to give the immunogen (XI).

Antibody production and initial evaluation

The kinetic data presented below were obtained for an IgG preparation obtained from the antiserum of a sheep (no. 270) that had been immunized with the immunogen (XI). Sheep were chosen as the source of IgG because of the large volumes of antiserum that they can provide and because those sheep from

Scheme 1. Reaction of 4-nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I) with OH^- ion

Reaction of compound (I) with OH^- is postulated to occur via the transient anionic tetrahedral intermediate (V). Loss of the preferred and chromophoric leaving group, 4-nitrophenolate, predicted on the basis of the relative $\text{p}K_a$ values of the conjugate acids of the two possible leaving groups, provides a monocarbonate ester, which decomposes to CO_2 and the higher- $\text{p}K_a$ phenol.

Scheme 2. Synthetic routes to (a) the isomeric carbonate substrates (I) and (II), (b) the phosphate immunogen (XI) and (c) the fluorescent label (XVII) used in the production and evaluation of polyclonal catalytic antibody preparations

KLH represents the carrier protein, keyhole-limpet haemocyanin; for further details of reaction strategy and of reagents, solvents and reaction conditions see the text.

Fig. 1. Demonstration of adherence to the Michaelis-Menten equation by, and determination of the characterizing parameters of, the hydrolysis of 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I) catalysed by the polyclonal catalytic antibody preparation from sheep no. 270 at pH 8.0 and 25 °C

(a) The linearity of the plot of $[S]/v$ against $[S]$ demonstrates the adherence of the catalysed reaction to the Michaelis-Menten equation. This type of plot is particularly effective in demonstrating any non-linearity that may exist. The lack of convincing evidence for non-linearity in the plot suggests that whatever structural heterogeneity exists in the IgG molecules that possess catalytic properties, it does not provide for significant variation in catalytic characteristics. Mixtures of catalysts with different characteristics provide for downward concavity in this type of plot (see Wharton *et al.*, 1974). (b) Best-fit values of $V_{\max}$ and K_m were obtained by fitting the v -versus- $[S]$ data to the Michaelis-Menten equation by weighted non-linear-regression analysis as described in the Materials and methods section. Constant relative error was assumed with weights inversely proportional to v^2 . The continuous line corresponds to $v = V_{\max} [S]/(K_m + [S])$ with $V_{\max} = 1.17 \text{ M}^{-1} \cdot \text{s}^{-1}$ (S.E. $\pm 0.04 \text{ M}^{-1} \cdot \text{s}^{-1}$) and $K_m = 3.34 \text{ } \mu\text{M}$ (S.E. $\pm 0.24 \text{ } \mu\text{M}$). By using the (unlikely, see the text) assumption that $[\text{active centre}] = 2 \times [\text{IgG}]$, $k_{\text{cat}} (= V_{\max}/[\text{active centre}]) = 0.029 \pm 0.001 \text{ s}^{-1}$ and $k_{\text{cat}}/K_m = 8766 \pm 359 \text{ M}^{-1} \cdot \text{s}^{-1}$. The standard error of the value of the ratio [S.E. (k_{cat}/K_m)] obtained from the best-estimate values of the individual parameters k_{cat} and K_m was calculated from S.E. (k_{cat}/K_m) $\approx \pm k_{\text{cat}}/K_m \sqrt{(c_{(k_{\text{cat}})}^2 + c_{(K_m)}^2 - 2\rho c_{(k_{\text{cat}})} c_{(K_m)})}$, where the best estimate of $k_{\text{cat}}/K_m = 8766 \text{ M}^{-1} \cdot \text{s}^{-1}$, $c_{(k_{\text{cat}})} = 0.03648174$, $c_{(K_m)} = 0.07036754$ (c for a given parameter being the coefficient of variation for that parameter, i.e. the S.E. expressed as a fraction of the mean) and $\rho = 0.8978$ (ρ being the correlation between k_{cat} and K_m , which is a normalized form of the co-variance). The convention suggested by Wilkinson (1961) is followed, whereby the square root of the variance is termed the standard error (S.E.) when the precision of a statistic, such as a regression coefficient, as an estimate of a parameter is being referred to. Standard error, which should not be confused with standard error of the mean (S.E.M.), is identical numerically with standard deviation, which is used to describe variability in experimental data. Values of $c_{(k_{\text{cat}})}$, $c_{(K_m)}$ and ρ are provided in the computer output from BMDP statistical package used to carry out the non-linear regression analysis (see the Materials and methods section).

multigeneration cross-bred flocks in particular have a good immune response.

Fluorescence-polarization antibody dilution curves for antisera obtained from sheep nos. 270–272, 22 weeks into the immunization schedule, were constructed as described in the Materials and methods section. The antibody titres were similar for all three sheep, i.e. 1:10000 for nos. 270 and 272 and 1:8000 for no. 271. The antiserum from sheep no. 270 was used for further study, and the IgG was isolated by Na_2SO_4 precipitation and chromatography on Protein G-Sepharose.

Evaluation of the kinetic characteristics of the IgG from sheep no. 270

The dependence of the initial rate (v) of antibody-catalysed hydrolysis of the carbonate substrate (I) on the substrate concentration for a typical set of data is shown in Fig. 1(a) as an $[S]/v$ -versus- $[S]$ plot and in Fig. 1(b) as a v -versus- $[S]$ plot. In each case the continuous line corresponds to the best fit. Values of the parameters $V_{\max}$ and K_m were obtained by weighted non-linear-regression analysis of the v -versus- $[S]$ data. The values of v were obtained by correcting the initial rates obtained in the presence of IgG from sheep no. 270 ($0.2 \text{ } \mu\text{M}$) for aqueous hydrolysis in the absence of IgG. The initial rates of hydrolysis in the absence of IgG vary linearly with $[S]$ (Fig. 2a), and closely similar results (values of v and their dependence on $[S]$) are obtained in the presence of $0.2 \text{ } \mu\text{M}$ -IgG from the antiserum of a non-immunized sheep (Fig. 2b).

In the data set shown in Fig. 1, 44 data points were collected in the range of $[S]$ approx. 0.2 – $16.5 \text{ } \mu\text{M}$ (with $K_m = 3.34 \pm 0.24 \text{ } \mu\text{M}$). Although in all cases the total concentration of IgG is $0.2 \text{ } \mu\text{M}$, Michaelis-Menten conditions should be met even

at the lowest values of $[S]$ because the concentration of catalytic antibody in the IgG preparation will be much less than $0.2 \text{ } \mu\text{M}$.

As in reactions catalysed by monoclonal antibodies, multiple turnovers were observed in the polyclonal-antibody-catalysed hydrolysis of compound (I). For example, with $[S] = 15 \text{ } \mu\text{M}$, at least 25 turnovers were demonstrated, even on the assumption that $[\text{active centre}] = 2 \times [\text{IgG}]$. The number of turnovers increases to 250 if only 10% of the IgG is catalytic antibody. Values reported for the number of turnovers observed in reactions catalysed by monoclonal catalytic antibodies are often in the range '> 10' to 'several hundred' (see, e.g., Tramontano *et al.*, 1986; Pollack *et al.*, 1989).

The results in Fig. 1 provide convincing evidence that a polyclonal antibody preparation capable of catalysing the hydrolysis of the carbonate substrate (I) has been produced by immunizing a sheep with the transition-state analogue immunogen (XI). A continuing anxiety in the catalytic antibody literature (see, e.g., Shokat & Schultz, 1990) is that catalytic activity in an antibody preparation might be due to the presence of a contaminant enzyme. Evidence that in the present work catalysis is antibody-mediated is found in the absence of any rate acceleration of hydrolysis of the isomeric 2-nitrophenyl aryl carbonate (II) in the presence of the IgG from sheep no. 270. Since antibody specificity is sensitive to structural changes, particularly at sites distant from the site of conjugation in the immunogen, the isomeric carbonate (II) would be predicted not to interact with the antibody. If the catalytic activity were the result of the presence of a contaminant enzyme, this marked discrimination between reactive ester substrates with identical aryloxy carbonyl groups and isomeric activated leaving groups would not be predicted.

Fig. 2. Demonstration of the linear dependence on substrate concentration of the initial rate of hydrolysis of 4-nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I) (a) in the absence and (b) in the presence of 0.2 μM -IgG from a non-immunized sheep at pH 8.0 and 25 °C

The points are experimental and the continuous lines are theoretical for $v = k[S]$ and best-fit values of k , determined by weighted non-linear-regression analysis as described in the Materials and methods section and in Fig. 1 legend with for (a) $k = 1.87 \times 10^{-4} \text{ s}^{-1}$ (S.E. $\pm 0.04 \times 10^{-4} \text{ s}^{-1}$) and for (b) $k = 1.99 \times 10^{-4} \text{ s}^{-1}$ (S.E. $\pm 0.04 \times 10^{-4} \text{ s}^{-1}$).

Table 1. Characteristics of the hydrolysis of 4-nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I) and of its 2-nitrophenyl isomer (II) catalysed by carboxylesterases and whole sheep sera in 50 mM-sodium phosphate buffer, pH 8.0, containing 0.67% (v/v) acetonitrile at 25 °C

Catalyst (and concentration or dilution)	Substrate (S)	Range of [S] (μM)	No. of data points	K_m (μM)	$10^8 \times V_{\text{max.}}$ ($\text{M} \cdot \text{s}^{-1}$)	$k_{\text{cat. (app.)}}$ (s^{-1})
Pig liver carboxylesterase (0.333 $\mu\text{g}/\text{ml}$, i.e. 1.67 nM assuming $M_r = 2 \times 10^5$)	(I)	approx. 2–60	20	< 2	3.8 ± 0.1	23
	(II)	approx. 2–70	20	3.4 ± 1.1	6.4 ± 0.4	38
Rabbit liver carboxylesterase (0.333 $\mu\text{g}/\text{ml}$, i.e. 3.34 nM assuming $M_r = 1 \times 10^5$)	(I)	approx. 2–25	20	4.5 ± 1.0	1.8 ± 0.1	5
	(II)	approx. 5–25	17	3.8 ± 1.1	5.5 ± 0.4	16
Whole serum from a non-immunized sheep (3000-fold diluted)	(I)	approx. 3–30	18	3.8 ± 1.7	0.21 ± 0.03	–
	(II)	approx. 2–20	17	10 ± 2	0.25 ± 0.03	–
Whole serum from a sheep immunized with the derivative of 3-O-methyl- noradrenaline given in the Materials and methods section (3000-fold diluted)	(I)	approx. 2–30	30	1.3 ± 0.4	0.17 ± 0.01	–
	(II)	approx. 2–20	20	3.0 ± 0.5	0.15 ± 0.01	–

Support for the view that hydrolytic enzymes would not be expected to display the marked (essentially absolute) specificity for substrate (I) as against substrate (II) found for the catalytically active IgG preparation is provided by the kinetic data collected in Table 1. Two carboxylesterases (from pig liver and from rabbit liver) and whole serum from non-immunized sheep catalyse the hydrolysis of both substrate (I) and substrate (II). Similar lack of discrimination between these substrates (Table 1) was observed for whole serum from a sheep immunized with a derivative of 3-O-methylnoradrenaline (see the Materials and methods section). This provides no evidence that immunization as such might produce enzymes capable of discriminating effectively between the isomeric substrates (I) and (II).

Moreover, when using the purification scheme described above, we have at no time found catalytic activity in IgG preparations

from non-immunized sheep. In addition, we have found that IgG preparations from sheep immunized with the sulphoxide (XVIII) or with the sulphone (XIX), structural variants of the phosphate immunogen (XI), are devoid of catalytic activity towards both the carbonate substrate (I) and their more hapten-congruent carbonate substrate (XX). Although not the primary objective of these sulphur-based studies, it is difficult to conceive of more appropriate control experiments to evaluate the possibility of contamination by enzymes.

The above kinetic data demonstrate that, whatever structural heterogeneity exists in the IgG, the catalytic characteristics of the active IgG species are all sufficiently similar that differences between them are not readily detected as deviations from a single-site saturation model. This type of antibody preparation therefore can be used to delineate catalytic characteristics of

Table 2. Comparison of the kinetic parameters for the hydrolysis of substrate (I) catalysed by the present polyclonal catalytic antibody preparation with those for the hydrolysis of substrates (III) and (IV) catalysed by monoclonal catalytic antibodies

Catalytic antibody (CA)	Substrate	Conditions	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$\frac{k_{cat}}{K_m [OH^-]} = \tilde{k}_{cat}$ ($M^{-2} \cdot s^{-1}$)	$\frac{k_{cat}}{K_m [OH^-]} = \tilde{k}_{cat}^*$ [reaction of (I), (II) or (III) with OH^-] ($M^{-2} \cdot s^{-1}$)	$\tilde{k}_{cat}/\tilde{k}_{non-cat.}$	Reference
				$\left\{ \begin{array}{l} 0.029 \\ 0.29 \\ 2.9 \end{array} \right\}$ (if $[CA] = 2 \times [IgG]$) (if $[CA] = 0.2 \times [IgG]$) (if $[CA] = 0.02 \times [IgG]$)	$\left\{ \begin{array}{l} 8.8 \times 10^4 \\ 8.8 \times 10^4 \\ 8.8 \times 10^5 \end{array} \right\}$	$\left\{ \begin{array}{l} 2.9 \times 10^3 \\ 2.9 \times 10^5 \\ 2.9 \times 10^6 \end{array} \right\}$	$\left\{ \begin{array}{l} 8.8 \times 10^9 \\ 8.8 \times 10^{10} \\ 8.8 \times 10^{11} \end{array} \right\}$	$\left\{ \begin{array}{l} 1.5 \times 10^2 \\ 1.5 \times 10^3 \\ 1.5 \times 10^4 \end{array} \right\}$	Present work
Polyclonal	(I)	pH 8.0, 0.67% (v/v) acetonitrile, 25 °C	3.34				199		
Monoclonal	(III)	pH 7.0, 2% (v/v) acetonitrile, 30 °C	208				87		Pollack <i>et al.</i> (1986)
Monoclonal	(IV)	pH 8.5, 2% (v/v) acetonitrile, 30 °C	660				9		Jacobs <i>et al.</i> (1987)

* $\tilde{k}_{cat}/\tilde{K}_m$ is the pH-corrected value of k_{cat}/K_m for catalyses in which $K_m (= \tilde{K}_m)$ is pH-independent and k_{cat} varies linearly with $[OH^-]$.

catalytic antibodies towards a variety of substrates and their responses to various types of reversible-binding inhibitor and chemical-modification reagents.

Some preliminary inhibition experiments have been carried out with polyclonal IgG preparations (Jackson, 1991). The catalytic activity of anti-phosphate IgG is inhibited by the phosphate (IX). With $[IX] = [I]$, the catalytic activity at pH 8.0 was abolished. Studies with the more immunogen-congruent phosphate (XXI) established that it acts as a competitive inhibitor of the catalysed hydrolysis of compound (I) with $K_i = 9$ nM at pH 8.0 determined by the method of Dixon (1953).

Parameters for the comparison of kinetics characteristic of catalytic antibodies for hydrolytic reactions

Reactions catalysed by catalytic antibody preparations that display single-site saturation phenomena, as in the present work, are reasonably characterized operationally by consideration of K_m , k_{cat} , and k_{cat}/K_m as in analogous Michaelian enzyme-catalysed reactions. Comparisons of interest include characterization of the reaction of a given antibody preparation with a range of structurally different substrates, those of reactions of different antibody preparations with a given substrate or type of substrate, and those of the reactions of a given antibody towards a particular substrate and the corresponding non-catalysed reaction. In the evaluation of enzyme catalysis k_{cat}/K_m , the specificity constant (Fersht, 1977), is a particularly valuable parameter. It provides a value of the apparent second-order rate constant for product formation from reaction of enzyme and substrate. It is not affected by non-productive binding (Bender & Kézdy, 1965; Brocklehurst *et al.*, 1968) and is a valuable parameter with which to evaluate enzyme effectiveness (see, e.g., Brocklehurst & Cornish-Bowden, 1976; Brocklehurst, 1977). Values of this and the other kinetic parameters can be compared at particular pH values or, better, if the pH-dependence character-

istics are known, the pH-independent or pH-corrected parameters (e.g. $\tilde{k}_{\text{cat.}}/\tilde{K}_m$; see, e.g., Brocklehurst & Brocklehurst, 1988) are compared. One difference between most enzymes and at least some catalytic antibodies for hydrolytic reactions is noteworthy. In the case of some hydrolytic reactions catalysed by catalytic antibodies [such as the catalysed hydrolyses of compounds (III) and (IV) (Pollack *et al.*, 1986; Jacobs *et al.*, 1987)], OH^- ion appears to be a reactant (although the requirement for the base form of a high-pKa general acid with water as reactant cannot be excluded). For catalyses in which $k_{\text{cat.}}$ is a linear function of $[\text{OH}^-]$ and K_m is pH-independent [as for the catalysed hydrolyses of compounds (III) and (IV)] and a mechanism involving HO^- ion as a reactant is postulated, it may be convenient to use the apparent third-order rate constant $k_{\text{cat.}}/K_m[\text{OH}^-] = \tilde{k}_{\text{cat.}}/\tilde{K}_m$ for product formation from reaction of antibody, substrate and OH^- ion, as an operational parameter for evaluating specificity and for comparing the effectiveness of different antibody preparations. This may be particularly useful in comparisons with results in the literature where the kinetic evaluations have been carried out at different (alkaline) pH values.

If a mechanism is assumed whereby OH^- ion reacts with the antibody-substrate complex, a rational comparison between catalysed and non-catalysed reactions may be made by comparing the second-order rate constant $k_{\text{cat.}}/[\text{OH}^-] = \tilde{k}_{\text{cat.}}$ with the second-order rate constant for the reaction of the substrate with OH^- ion.

Kinetic characteristics of the reaction of IgG from sheep no. 270 with compound (I) and comparison with the parameters for the monoclonal-catalytic-antibody-catalysed hydrolyses of compounds (III) and (IV)

The kinetic parameters for these three reactions are presented in Table 2. It is a striking result that, even if the concentration of polyclonal catalytic antibody is assumed to be equal to twice the total IgG concentration, the value of $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ ($8.88 \times 10^9 \text{ M}^{-2} \text{ s}^{-1}$) for the catalysis of the hydrolysis of compound (I) is considerably larger (27 times and 800 times respectively) than the analogous values for the hydrolysis of compounds (III) and (IV) catalysed by monoclonal antibodies. The value of $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ for the polyclonal-antibody-catalysed reaction becomes even larger of course if, as seems probable, the concentration of active catalytic antibody is only a small fraction of the total concentration of IgG in the preparation (see Table 2). On this assumption, even the value of $\tilde{k}_{\text{cat.}}$ (i.e. $k_{\text{cat.}}/[\text{OH}^-]$) for the polyclonal-antibody-catalysed reaction is considerably larger than the value of $\tilde{k}_{\text{cat.}}$ for the monoclonal-antibody-catalysed reactions even when differences in the intrinsic reactivities of the substrates (I), (III) and (IV) towards OH^- ion are taken into account, i.e. when values of $\tilde{k}_{\text{cat.}}/\tilde{k}_{\text{non-cat.}}$ are compared.

Concluding comment

This first successful production of polyclonal catalytic antibodies is considered to result from the appropriate design of immunogen and substrate. In particular, the provision of phenyl rings on either side of the central carbonate group is predicted to provide for a strong hydrophobic effect. The deformations involved in the progression from ground state to transition state might be facilitated by the presence of these phenyl rings.

The demonstration that it is possible to produce polyclonal catalytic antibodies with simple Michaelian characteristics should facilitate the kinetic investigation of a wide range of catalytic antibody systems. The simplicity of production of polyclonal antibodies should aid the screening of immunogens for their ability to generate catalytic antibodies with particular kinetic

characteristics. It remains to be discovered, for example, whether by making changes to the structure of the immunogen it is possible to change the values of the kinetic parameters in desired directions, e.g. to produce increases in both K_m and $k_{\text{cat.}}$.

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REFERENCES

- Balan, A., Doctor, B. P., Green, B. S., Torten, M. & Ziffer, H. (1988) *J. Chem. Soc. Chem. Commun.* 106–108
- Bauminger, S., Kohen, F. & Linder, H. R. (1974) *J. Steroid Biochem.* **5**, 739–747
- Bender, M. L. (1987) in *Enzyme Mechanisms* (Page, M. I. & Williams, A., eds.), pp. 56–66, Royal Society of Chemistry, London
- Bender, M. L. & Kézdy, F. J. (1965) *Annu. Rev. Biochem.* **34**, 49–76
- Blackburn, G. M., Kang, A. S., Kingsbury, G. A. & Burton, D. R. (1989) *Biochem. J.* **262**, 381–390
- Brocklehurst, K. (1977) *Biochem. J.* **163**, 111–116
- Brocklehurst, K. & Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 165–166
- Brocklehurst, K., Crook, E. M. & Wharton, C. W. (1968) *FEBS Lett.* **2**, 69–73
- Brocklehurst, K., Kowlessur, D., Patel, G., Templeton, W., Quigley, K., Thomas, E. W., Wharton, C. W., Willenbrock, F. & Szawelski, R. J. (1988a) *Biochem. J.* **250**, 761–772
- Brocklehurst, K., Brocklehurst, S. M., Kowlessur, D., O'Driscoll, M., Patel, G., Salih, E., Templeton, W., Thomas, E., Topham, C. M. & Willenbrock, F. (1988b) *Biochem. J.* **256**, 543–555
- Brocklehurst, S. M. & Brocklehurst, K. (1988) *Biochem. J.* **256**, 556–558
- Dandliker, W. B. & Levison, S. A. (1967) *Immunochemistry* **5**, 171–183
- Dixon, M. (1953) *Biochem. J.* **55**, 170–171
- Dixon, W. J. (chief ed.), Brown, M. B., Engelman, L., Hill, M. A. & Jennrich, R. I. (1988) *BMDP Statistical Software*, pp. 389–417, University of California Press, Guildford
- Fersht, A. R. (1977) *Enzyme Structure and Mechanism*, pp. 96–97, W. H. Freeman, Reading
- Gallacher, G., Coxon, R., Landon, J., Rae, C. J. & Abukinesha, R. (1988) *Ann. Clin. Biochem.* **25**, 42–48
- Gallacher, G., Jackson, C. S., Topham, C. M., Searcey, M., Turner, B. C., Badman, G. T. & Brocklehurst, K. (1990) *Biochem. Soc. Trans.* **18**, 600–601
- Jackson, C. S. (1991) Ph.D. Thesis, University of London
- Jacobs, J. W., Schultz, P. G., Sugawara, R. & Pollack, S. J. (1987) *J. Am. Chem. Soc.* **109**, 2174–2176
- Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 282–320, McGraw-Hill, New York
- Kang, A. S., Kingsbury, G. A., Blackburn, G. M. & Burton, D. R. (1990) *Chem. Br.* **26**, 128–132
- Klotz, I. M. (1987) in *Enzyme Mechanisms* (Page, M. I. & Williams, A., eds.), pp. 14–34, Royal Society of Chemistry, London
- Knowles, J. R. (1987) *Science* **236**, 1252–1258
- Kowlessur, D., Thomas, E. W., Topham, C. M., Templeton, W. & Brocklehurst, K. (1990) *Biochem. J.* **266**, 653–660
- Landsteiner, K. (1947) *The Specificity of Serological Reactions*, pp. 156–210, Harvard University Press, Cambridge, MA
- Leatherbarrow, R. J. & Fersht, A. R. (1987) in *Enzyme Mechanisms* (Page, M. I. & Williams, A., eds.), pp. 78–96, Royal Society of Chemistry, London
- Mellor, G. W., Gallacher, G. & Landon, J. (1989) *Biog. Amines* **6**, 473–486
- Paul, S., Volle, D. J., Beach, C. M., Johnson, D. R., Powell, M. J. & Massey, R. J. (1989) *Science* **244**, 1158–1162
- Pauling, L. (1946) *Chem. Eng. News* **24**, 1375–1377
- Pauling, L. & Pressman, D. (1945) *J. Am. Chem. Soc.* **67**, 1003–1012
- Pike, V. W., Wharton, C. W., Brocklehurst, K. & Crook, E. M. (1978) *Biochem. Soc. Trans.* **6**, 269–271
- Pollack, S. J., Hsivn, P. & Schultz, P. G. (1989) *J. Am. Chem. Soc.* **111**, 5961–5962

- Pollack, S. J., Jacobs, J. W. & Schultz, P. G. (1986) *Science* **234**, 1570–1573
- Powell, M. J. & Hansen, D. E. (1989) *J. Protein Eng.* **3**, 69–75
- Raso, V. & Stollar, B. D. (1975) *Biochemistry* **14**, 591–599
- Schultz, P. G. (1988) *Science* **240**, 426–433
- Schultz, P. G. (1989) *Acc. Chem. Res.* **22**, 287–294
- Schultz, P. G. & Jacobs, W. (1988) in *Environmental Influences and Recognition in Enzyme Chemistry* (Liebman, J. F. & Greenberg, A., eds.), pp. 315–316, VCH Publishers, New York
- Shokat, K. M. & Schultz, P. G. (1990) *Annu. Rev. Immunol.* **8**, 335–363
- Stoddart, J. F. (1987) in *Enzyme Mechanisms* (Page, M. I. & Williams, A., eds.), pp. 35–55, Royal Society of Chemistry, London
- Tramontano, A., Janda, K. D. & Lerner, A. (1986) *Science* **234**, 1566–1570
- Wharton, C. W., Cornish-Bowden, A., Brocklehurst, K. & Crook, E. M. (1974) *Biochem. J.* **141**, 365–381
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332

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