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Polyclonal Antibodies and Catalysis

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Abstract—Some recent results involving catalytic polyclonal antibodies are described. Polyclonal antibodies isolated directly from serum contain the complete distribution of different IgG antibodies elicited via immunization, so catalytic results obtained with polyclonal antibodies can be used to characterize the overall catalytic activity produced in an animal in response to a given hapten. This new window on catalytic antibodies should be especially useful for identifying general trends relating hapten structure to antibody catalytic activity, for monitoring the maturation of catalytic activity during immunization, and for studying the variability of catalytic activity elicited in different animals immunized with the same hapten. Furthermore, studying the catalytic activity of polyclonal antibodies in serum may aid in the development of novel immunization-based therapies.

Introduction

It is important to view a fine painting at close-range to examine the intricate details, but also from several feet back to appreciate how those details fit into the entire composition. Studying catalytic monoclonal antibodies has provided a wealth of details regarding the catalytic activity that can be produced in individual antibody binding pockets.[1–5] On the other hand, the discovery of substantial catalytic activity produced in polyclonal antibodies isolated directly from serum allows appreciation of the bigger picture involved with the entire catalytic antibody immune response.[6–10] Polyclonal antibodies contain the complete distribution of different IgG antibodies elicited via immunization, so catalytic results obtained with polyclonal antibodies provide a way to assess rapidly the overall catalytic activity produced in an animal in response to a given hapten. This new window on catalytic antibodies should be especially useful for identifying general trends relating hapten structure to antibody catalytic activity, for monitoring the maturation of catalytic activity during immunization, and for analyzing the variability of catalytic activity in different animals immunized with the same hapten. Furthermore, studying the catalytic activity of polyclonal antibodies in serum may aid in the development of novel immunization-based therapies.

An early attempt failed to identify catalytic activity in polyclonal antibodies elicited through immunization with a hapten intended to produce antibodies that catalyze Schiff base formation.[11] Later, ester hydrolysis activity was reported in rabbit polyclonal antibodies, but the activity was not reproducible.[12] In both of these cases, the polyclonal antibodies were isolated by hapten affinity chromatography. Enhancement of steroidal ester hydrolytic rates was found in a sample of polyclonal antibodies elicited by immunization with the steroid reaction product.[13] Catalytic activity attributed to antibodies has also been described in human patients not immunized with a designed hapten,[14,15] and a high level of catalytic activity has been reported in antiidiotypic polyclonal antibodies from rabbits.[16] The present paper is limited to a description of recent experiments involving catalytic polyclonal antibodies elicited in animals by immunization with transition state analog haptens.

Results

In 1990, Gallacher *et al.* reported that polyclonal antibodies isolated from the serum of a sheep immunized with the phosphate hapten **1** could catalyze the hydrolysis of the homologous carbonate substrate **2**.[6] In this case, the polyclonal IgG antibodies were not purified by hapten affinity chromatography, but were isolated using an initial Na_2SO_4 precipitation followed by purification on a Protein G-Sepharose 4 Fast Flow column (Pharmacia). Detailed kinetic analyses at pH 8.0 revealed that the catalytic polyclonal antibodies exhibited saturation behavior and plots of [S]/v versus [S] were linear, consistent with the Michaelis–Menten equation.[7] From these data, a value for K_m of 3.3 μM and V_{max} of 1.17×10^{-8} M^{-1} s^{-1} were obtained. No attempt was made to determine the percentage of hapten-specific antibody in the polyclonal sample, so k_{cat} values were derived from the observed V_{max} by estimating the concentration of catalytic antibody. For example, a k_{cat} of 0.029 s^{-1} was calculated assuming that every antibody in the sample was catalytic. This corresponds to a catalytic rate enhancement (as k_{cat}/k_{uncat}) of 150 over the background rate of hydrolysis for the same substrate in the absence of antibody. As pointed out by the authors, all of the polyclonal antibodies are not expected to be catalytic or even hapten-specific. Using a more reasonable estimate of 10 % active catalytic antibodies in the polyclonal sample generated a k_{cat} of 0.29 s^{-1} and a rate enhancement of 1500. This estimated rate enhancement agrees well with the rate enhancements reported for monoclonal catalytic antibodies that catalyze acyl transfer reactions.

Hapten **1** inhibited the catalyzed reaction, and the purified polyclonal sample did not catalyze the hydrolysis of the isomeric carbonate substrate **3** with the nitro group in the

2 position. This hapten inhibition and isomeric discrimination is consistent with catalysis derived from antibodies. For comparison, experiments carried out at the same time with pig or rabbit liver carboxylesterases as well as whole serum from sheep showed no significant substrate discrimination of substrates **2** and **3**.

Polyclonal IgG antibodies isolated seven weeks later from the same sheep were shown to catalyze the hydrolysis of the *p*-nitroanilide substrate **4**.[8] Again, catalysis obeyed the Michaelis–Menten equation and a K_m of 5.4 μM was obtained. Using estimates of 100 % and 10 % active catalytic antibodies in the polyclonal sample yielded catalytic rate enhancements (k_{cat}/k_{uncat}) of 300 and 3000 respectively at pH 9.0. As expected, this polyclonal sample also catalyzed the hydrolysis of carbonate hapten **2**, displaying a similar K_m but 5-fold better V_{max} than was observed for the polyclonal sample isolated from the same animal seven weeks earlier. This was the first example of both carbonate and activated amide hydrolysis activity induced in antibodies by immunization with a phosphate hapten.

More recently, a total of thirteen purified polyclonal IgG antibody samples were prepared from three different sheep immunized with hapten **1**.[9] The samples were removed at various times during the immunizations, and *all* were found to catalyze the hydrolysis of the carbonate substrate **2**. The observed K_m values for these samples were spread over a 4-fold range from 1.2 μM to 4.75 μM. Without correcting for the amount of hapten specific antibody in each sample, the estimated k_{cat} values varied by over 31-fold. These results indicate that polyclonal antibody catalytic activity is a relatively general phenomenon, occurring in all three of the animals immunized with a phosphate hapten.

We have investigated catalysis of trityl ether hydrolysis in the polyclonal antibodies isolated from the serum of a rabbit immunized with the phosphonium hapten **5**.[10] The positive charge of the hapten should resemble the positive charge present in the protonated species formed during the acid catalyzed hydrolysis reaction. The antibodies are thus expected to bring about catalysis by stabilizing the positive charge of a protonated substrate by way of charge complementarity in the antibody binding pocket.

The phosphonium hapten and trityl ether hydrolysis reaction were chosen for two reasons. First, to the best of our knowledge, there is no natural enzyme that catalyzes trityl ether hydrolysis providing assurance that our assays will be free from any possible interference due to adventitious enzymatic activity. Second, we have previously carried out a *monoclonal* antibody study using this system, offering a basis for comparison.[17,18] A mere 270 was the best catalytic rate enhancement (as k_{cat}/k_{uncat}) observed with any of these trityl ether hydrolyzing monoclonal antibodies. This represents a relatively low rate enhancement relative to other catalytic antibody studies in which rate enhancements of 1,000 or more are common.[3–5] Therefore, the trityl hydrolysis reaction provides a stringent test of whether the polyclonal antibody method can be used to study even relatively modest catalytic activity.

A male New Zealand white rabbit was injected with the keyhole limpet hemocyanin conjugate of phosphonium hapten **5**. Periodically throughout the immunization regimen, IgG polyclonal antibodies were purified from the rabbit serum using a simple three step procedure consisting of caprylic acid treatment, saturated ammonium sulfate precipitation and chromatography with protein G sepharose. No catalytic activity or hapten specific titer was observed in a sample of purified IgG isolated from the serum removed prior to immunization. A small but measurable catalytic activity and titer were observed after the first injection, and the catalytic activity increased along with antibody titer throughout the rest of the immunization process.

Following the last immunization, the bulk of the rabbit serum was taken and the catalytic activity of the resulting purified polyclonal IgG antibodies was studied in detail. For the catalytic hydrolysis of substrate **6**, the polyclonal IgG preparation displayed classical saturation behavior consistent with the Michaelis–Menten equation. At pH 7.2, an apparent V_{max} of 8×10^{-2} μM min^{-1} and apparent K_m of 31 μM were determined. These values are termed apparent because they characterize the sample as a whole rather than any individual catalytic species. Hapten **5** was a potent inhibitor of the catalytic reaction, and complete inhibition was achieved when enough hapten was added to bind 12 % of the antibody binding sites in the polyclonal sample, suggesting that 12 % of the antibodies of the IgG preparation were hapten specific and/or catalytic. For these inhibition studies, we have assumed that the K_D for hapten is significantly lower than K_m for substrate and also lower than the concentration of hapten specific antibody being used. The validity of this assumption is strongly supported by the fact that we observed catalytic activity decreasing in a linear fashion with increasing hapten concentration.[10] If the hapten K_D were similar to the K_m for substrate, or higher than the concentration of hapten specific antibody concentration, then the inhibition data would have been markedly more curved.

Using 12 % as the estimated percentage of catalytic antibodies in the sample[19] yielded an apparent k_{cat} value of 0.02 min^{-1}, corresponding to an estimated rate enhancement as k_{cat}/k_{uncat} of 125. This estimate agrees well with the rate enhancement of 270 observed for the same reaction catalyzed by the previously reported murine monoclonal antibody 37C4.[17,18]

To the best of our knowledge there is no natural enzyme activity in sera, or anywhere else for that matter, that can catalyze the hydrolysis of trityl ether substrates. Nevertheless, several pieces of evidence verified that the observed catalytic activity resulted from the polyclonal antibodies and not an adventitious enzyme impurity or non-specific protein effect. First, the catalytic activity increased during the immunization regimen and was roughly correlated to antibody titer. Second, hapten **6** completely inhibited catalytic activity. Finally, there was no catalytic activity observed in the polyclonal IgG sample prepared from the serum removed from the same rabbit prior to

immunization, so IgG is not catalytic by way of some non-specific mechanism.

Five mice were also immunized with hapten **5**, and after five immunizations the serum from all five was removed and combined into one sample. The murine polyclonal antibodies prepared from the combined serum sample also showed significant catalytic activity. Inhibition studies revealed that the percentage of hapten specific and/or catalytic IgG was near 20 %, leading to an estimated apparent k_{cat} of 0.02 min^{-1}.

We have recently immunized a rabbit with the aryl phosphate hapten **7**. After five immunizations, the resulting polyclonal IgG antibodies were analyzed for their ability to hydrolyze the carbonate substrate **8**. We characterized the catalytic activity in the purified polyclonal sample, and some of the catalytic data are shown in Figure 1, panel B. Hapten **7** completely inhibited the catalytic activity, and quantitative studies indicated that 10 % of the polyclonal sample was hapten specific and/or catalytic. An apparent K_m value of 16 μM and estimated k_{cat} of 1.0 min^{-1} were determined, corresponding to an estimated rate enhancement as k_{cat}/k_{uncat} of 1800 over background. Thus, the findings of Gallacher *et al.*[6–9] using sheep have been qualitatively reproduced with a purified sample of polyclonal antibodies isolated from a rabbit immunized with a phosphate hapten. A sample of polyclonal antibodies isolated from the same rabbit prior to immunization with **7** failed to exhibit any catalytic activity.

Discussion

The experiments described above have demonstrated that significant catalytic activity can be produced and studied in the polyclonal antibodies isolated from the sera of immunized animals. In addition, the polyclonal method has several features ideally suited to the study of antibody catalysis. For example, polyclonal antibody samples can be analyzed within three months from the first immunization, they are technically easy to prepare, they can be analyzed without sacrificing the animal, and the procedures involved are relatively inexpensive. Thus, compared to using monoclonal antibodies, polyclonal antibodies provide a more efficient way to screen new hapten designs for their ability to elicit catalytic antibodies. In other words, high levels of catalytic activity in polyclonal samples elicited by a new hapten would indicate the presence of highly efficient and/or large concentrations of catalysts, either way a system worthy of further investigation. Lack of significant polyclonal activity would indicate an ineffective hapten design.

Catalytic antibodies are not constructed by researchers, they are created by animal immune systems in response to immunization. As a result, it is important to study all aspects of the catalytic immune response, not just the chemistry of catalytic antibodies. Along these lines, polyclonal antibodies could be used to study the variability of the overall catalytic immune response in several different animals of the same or different species immunized with the same hapten. The restrictions imposed by hybridoma methods limits the number of different animals and animal species that can be analyzed using monoclonal antibodies, and virtually all previously reported monoclonal catalytic antibodies have been derived from mice only. Furthermore, these murine monoclonal catalytic antibodies were derived from inbred mouse strains, providing an even more myopic view of the catalytic immune response. Genetic diversity may play an important role in optimizing antibody catalysis, and studying polyclonal antibodies from genetically diverse animals will provide a practical means of screening different animals for a favorable combination of hapten design and immune system genetics.

Furthermore, as opposed to hybridoma methods, preparing polyclonal antibody samples does not require sacrificing the animal. Thus, polyclonal antibodies can be used to study the development of catalytic activity during and after immunization of the same animal. In this way, immunization procedures and schedules could be systematically studied and optimized. In addition, the

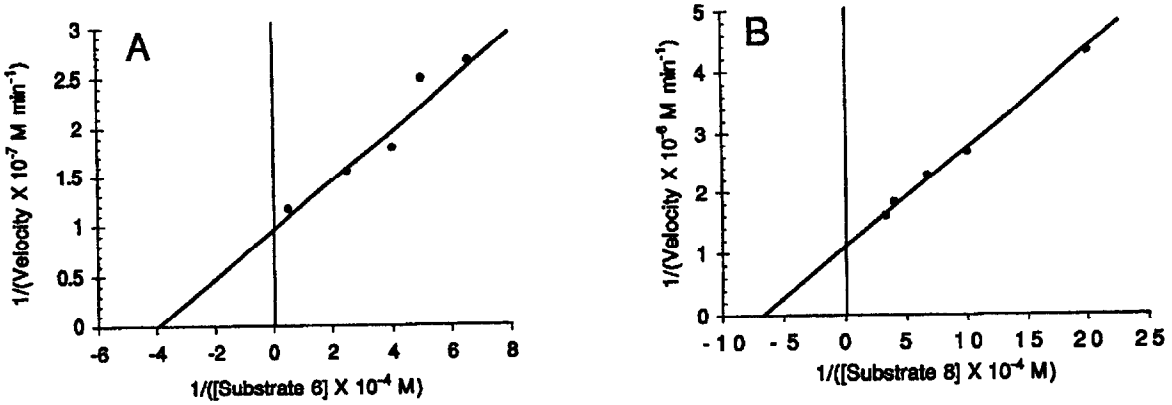


Figure 1. *Panel A*, A Lineweaver–Burk plot for the hydrolysis of trityl ether substrate **6** catalyzed by the purified polyclonal antibodies raised against phosphonium hapten **5**. 30 μM total IgG was used in phosphate buffered saline (10 mM phosphate, 100 mM NaCl, pH 7.2) and the concentrations of substrate were varied as indicated. Each data point represents an average of two independent measurements. Errors are estimated as ±10 %. *Panel B*, A Lineweaver–Burk plot for the hydrolysis of carbonate substrate **8** catalyzed by the purified polyclonal antibodies raised against phosphate hapten **7**. Everything is the same as in panel A except 4.7 μM total IgG was used in 10 mM Tris–HCl, pH 8.0.

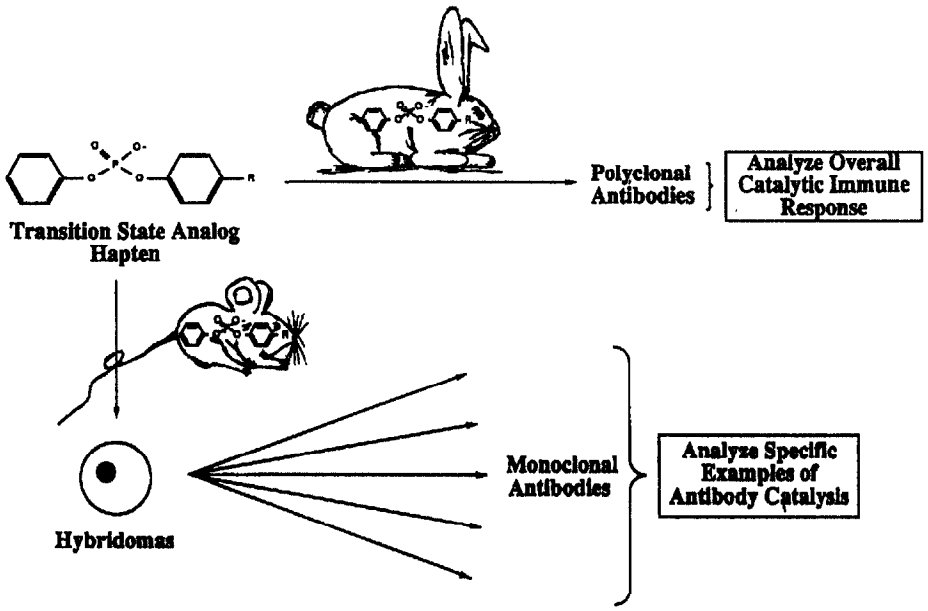
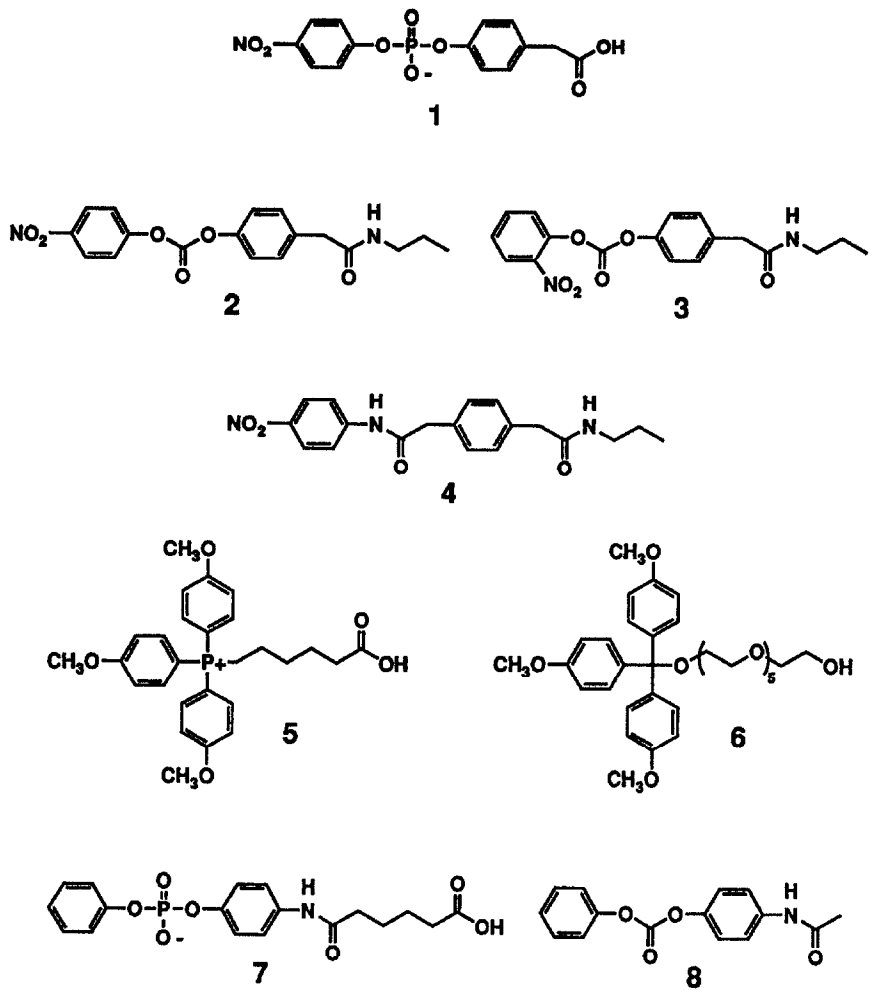


Figure 2.



information gained from these studies should aid the development of any future therapeutic strategies involving the introduction of beneficial catalytic antibody activity into patients through immunization.[20]

To summarize, catalytic activity has been characterized in polyclonal antibody samples derived from a variety of immunized animals. This new window on catalytic antibodies should provide important information regarding

the bigger picture involved with the catalytic immune response.

Experimental

For immunizations, 5 pound male New Zealand white rabbits (R & R Rabbitry, Pipe Creek, Texas) or Balb/cJ mice (Jackson Laboratories, Bar Harbor, Maine) were used, as appropriate. All chemicals were purchased from Aldrich unless indicated otherwise, and were used without further purification. Analytical HPLC was performed using a Hewlett–Packard 1090M liquid chromatograph with diode array detection and a Hewlett–Packard 79994A analytical workstation. For the work involving haptens **5** and **7**, all hapten conjugations to protein, injections, antibody purifications and antibody analyses were carried out as described earlier.[10,16,17] The synthesis of compounds **5** and **6** as well as a description of the trityl hydrolysis catalytic reaction analyses have been reported previously.[16,17] Complete synthetic details for new compounds follow.

N-(4-Hydroxyphenyl)-5-methoxycarbonyl pentanoate

Adipic acid monomethyl ester (Aldrich, 2.3 mL, 16 mmol) was dissolved in CH_2Cl_2 (50 mL) and oxalyl chloride (Lancaster, 2.0 mL, 23 mmol) was added at 25 °C. After the mixture had been stirred for one hour at room temperature, CH_2Cl_2 and excess oxalyl chloride were removed *in vacuo* to give the crude acid chloride. In a separate flask, 4-aminophenol (1.8 g, 16 mmol) was dissolved in DMF (50 mL) and the acid chloride was added dropwise as a DMF solution (10 mL). After the mixture had been stirred for 15 h, the solvent was removed *in vacuo*. The crude product was redissolved in water and extracted with ethyl acetate (3 × 50 mL). The combined ethyl acetate solution was washed with saturated NaCl, dried over $MgSO_4$ and concentrated to yield the product (2.5 g, 10 mmol, 63 %): ^{1}H NMR (250 MHz, acetone-d_6) δ 1.68 (m, 4H), 2.05 (t, J = 9.8, 4H), 3.61 (s, 3H), 6.75 (d, J = 6.5, 2H), 7.43 (d, J = 5.9, 2H), 8.12 (s, 1H), 9.90 (s, 1H).

O-(4-(5-Carboxypentanamide)phenyl), O-phenyl phosphate, triethylammonium salt

Triazole (1.3 g, 19 mmol) and triethylamine (2.2 mL, 16 mmol) were dissolved in THF (30 mL). The mixture was cooled to 0 °C and phosphoryl chloride (Aldrich, 300 μL, 3.2 mmol) was added. After the mixture was stirred for 0.5 h, phenol (250 mg, 2.7 mmol) was added dropwise as a solution in DMF (2 mL). The mixture was stirred for 1 h at 0 °C, then allowed to slowly reach room temperature over an additional 1.5 h, after which time *N*-(4-hydroxyphenyl)-6-(methoxycarbonyl)hexanamide (670 mg, 2.6 mmol) was added slowly as a solution in DMF (3 mL). After stirring at room temperature overnight, excess water was added and the pH was neutralized. Solvents were removed *in vacuo* and the aqueous solution was brought to pH 8 with $NaHCO_3$. The aqueous fraction was washed with ether (2 × 30 mL), then the pH brought to 5 with 1.0 M HCl and finally extracted with ethyl acetate (3 × 50 mL). The combined ethyl acetate fractions were dried over $MgSO_4$ and concentrated.

The crude phosphate was dissolved in THF (6 mL) and H_2O (4 mL) and 1.0 M NaOH was added (1.8 mL). The reaction mixture was stirred at room temperature for 1 h, then the pH was brought to 5 with 1.0 M HCl before the mixture was extracted with ethyl acetate (3 × 30 mL). Drying and concentration, followed by low pressure chromatography purification yielded 46 mg of the pure product (0.12 mmol, 4.6 %): ^{1}H NMR (250 MHz, D_2O) δ 1.08 (t, J = 15, 11H), 1.49 (m, 4H), 2.22 (m, 4H), 2.95 (m, 4H), 7.04 (m, 5H), 7.23 (m, 4H); ^{13}C NMR (250 MHz, D_2O) δ 8.95, 24.7, 25.5, 34.7, 36.7, 47.3, 120.9, 121.3, 121.4, 123.9, 125.2, 130.5, 133.9, 152.4, 175.8, 180; MS m/z calcd for $C_{18}H_{19}NO_7P$: 392.35, found 392.09.

4-Acetamidylphenyl, phenyl carbonate

Triethylamine (300 μL, 3.7 mmol) and phenylchloroformate (330 μL, 2.6 mmol) were added to *N*-(4-hydroxyphenyl) acetamide (200 mg, 1.3 mmol) dissolved in CH_3CN (6 mL). The reaction was stirred for 3 h before solvent was removed *in vacuo*. The crude compound was dissolved in CH_2Cl_2 and washed first with a dilute aqueous solution of $NaHCO_3$, then with a saturated solution of NaCl. The organic fraction was dried over $MgSO_4$ and concentrated *in vacuo:* ^{1}H NMR (250 MHz, acetone-d_6) δ 2.07 (s, 3H), 7.26 (d, J = 12, 2H), 7.32 (d, J = 8.8, 3H), 7.46 (m, 2H), 7.71 (d, J = 9.1, 2H), 9.27 (s, 1H); ^{13}C NMR (250 MHz, acetone-d_6) δ 120.697, 121.949, 122.061, 127.072, 130.402; MS m/z calcd for $C_{15}H_{13}NO_4$: 271.29, found 272.09.

Catalytic hydrolysis of 4-acetamidylphenyl, phenyl carbonate

The catalytic reactions contained 10 mM Tris buffer, pH 8, along with the antibodies elicited against *O*-(4-(6-carboxyhexanamide)phenyl), *O*-phenyl phosphate (4.7 μM total IgG concentration) and varying concentrations of the carbonate substrate. These reactions were incubated at room temperature for 15 min, then analyzed quantitatively via HPLC. The identity of the product was confirmed by comparison of retention time and UV-visible spectrum with an authentic sample. The inhibition experiments using hapten were carried out as described above, except the polyclonal antibodies were incubated with a given concentration of hapten for 30 min prior to reaction with substrate.

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19. Of course 12 % is probably an overestimate for the actual amount of catalytic antibody, since this value can include antibodies than can bind hapten but are not catalytic.

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