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Comparison of Antibody and Albumin Catalyzed Hydrolysis of Steroidal *p*-Nitrophenylcarbonates

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ABSTRACT

A monoclonal antibody (MAb) was produced against the *p*-nitrophenylphosphate derivative of 3α,5β-lithocholic acid, a transition-state analog for hydrolysis of a steroidal *p*-nitrophenylcarbonate. The indicated reaction was catalyzed by this Ab with kinetic constants k_{cat} = 4.0 × 10^{-2}/min and K_m = 3.3 μ*M* at pH 9.0 and 35°C. The Ab also hydrolyzed the isomeric *p*-nitrophenylcarbonate of 3β,5β-lithocholic acid with k_{cat} = 8.4 × 10^{-2}/min and K_m = 1.0 μ*M*. Bovine serum albumin (BSA) was found to catalyze the same reactions with similar turnover rates and Michaelis constants of 15 and 14 μ*M*, respectively. Although the BSA-catalyzed reaction was only weakly inhibited by the phosphate ester TSA (IC_{50} ca. 40 μ*M*), the Ab-catalyzed reaction was completely inhibited at less than 1 μ*M* of the TSA. The relative rates and efficiencies of the MAb-catalyzed and BSA-catalyzed reactions are discussed in the context of the hydrophobic sites and intrinsic reactivity of the protein surfaces, and the induction of groups on the Ab to enhance the enzymatic function.

Index Entries: Albumin; catalytic antibody; steroid; carbonate; phosphate hapten.

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INTRODUCTION

The development of catalytic antibodies (Abs) has generally focused on facile chemical processes with low activation energies *(1–4)*. Improvements in the designer approach can thus be assessed from incremental changes in catalytic efficiency of the Ab relative to an uncatalyzed reaction. Recently, it has been rediscovered that other nonspecialized protein molecules can exhibit catalytic activities similar to those of Abs elicited by mechanism-based antigen (Ag) design, including the transition-state analog (TSA) strategy. Bovine serum albumin (BSA), an off-the-shelf protein, has been shown to accelerate an elimination reaction with efficiency rivaling that of a tailor-made monoclonal antibody (MAb) *(5)*. Albumins are well known for their capacity to bind small molecules, including drugs and bile salts *(6–9)*. The esterase-like activity of human albumin has been studied previously *(10–12)*. The specific BSA-catalyzed hydrolysis of carbonate derivatives of diverse 3-hydroxy steroids described in this work is an example of such activity. Albumin-catalyzed hydrolysis could be regarded as another reference reaction used for evaluating a related Ab-catalyzed reaction.

Tailor-made Abs can display combining-site shape and chemical composition appropriate for a specific substrate and mechanism *(13)*. A combining site that makes extensive contact with the Ag is more likely to define a unique shape, which could distinguish a reaction intermediate or transition state from its substrate. An Ab with such a site could, in principle, make a significant contribution to catalytic efficiency through utilization of binding free energy. A larger haptenic molecule would allow Ab-affinity maturation to the flanking structure of a substrate, as well as against groups at the reaction center. In order to assess the potential to utilize this type of catalytic mechanism, the authors used a conventional phosphate ester hapten to generate an Ab specific for a substrate of moderate size. A steroid group appended on the phosphate was used to select Abs presenting a hydrophobic site similar to those found on albumins. Here are compared the catalytic properties of the Ab and BSA, in an effort to define the unique attributes of the immunological approach to catalyst design. A study of the kinetic features in relation to the tailored or adventitious ligand-binding functions of the respective proteins allows further insight into the potential of combining-site hypermutation for the refinement of Abs as artificial catalysts. Remarkably, both the Ab and albumin exhibited similar turnover rates and preference for a substrate containing a steroid with 3α-stereochemistry, different from that in the hapten used to generate the Ab.

MATERIALS AND METHODS

General Methods

Steroids and proteins for conjugation were obtained commercially and used as provided. ^{1}H-NMR spectra were recorded on a Bruker AC-300. Mass spectral analysis was obtained on a VG-70-70-EQ-HF instrument equipped with its own source, using xenon as carrier gas and glycerol as matrix. UV/Vis data were collected using a Perkin-Elmer lambda 6 spectrophotometer equipped with thermostated cell changer and data station, or Jasco UVIDEC 610. Molecular modeling, energy minimization, and dynamics were performed using the program Hyperchem (AUTODESK). Myeloma cell lines used in this work were obtained from the American Type Culture Collection (Rockville, MD). Immunoassay reagents were purchased from Promega (Madison, WS).

Preparation of Hapten and Substrates

3α-O-(4-nitrophenylphosphate)-lithocholic acid (**1,** Fig. 1) was prepared by adding a solution of 4-nitrophenylphosphorodichloridate (500 mg, 1.95 mmol) in 10 mL dioxane to methyllithocholate (400 mg, 1.02 mmol) in anhydrous dioxane:pyridine 19:1 (10 mL). The mixture was stirred for 1 h at room temperature. The reaction was diluted with water to give the phosphate diester. Solvent was removed under vacuum, and the crude residue was dissolved in 20 mL 0.1 *N* aqueous sodium hydroxide/pyridine (4:1) and left overnight at room temperature to obtain the carboxylic acid 1. The solvent was distilled from the reaction mixture, and the product was purified by flash silica gel chromatography (CH_2Cl_2:CH_3OH, 4:1) to give 451 mg (76% overall yield) of **1.** ^{1}H-NMR (DMSO) δ: 0.62 (3H, s, C-18 Me), 0.87 (3H, d, J = 6.3 Hz, C-21 Me), 0.88 (3H, s, C-19 Me), 4.02 (1H, m, H-3β), 7.40 (2H, d, J = 11 Hz) and 8.20 (2H, d, J = 11 Hz). Negative f.a.b. ms: m/z (relative intensity) 576 ([M-H] 100), 574 (21), 469 (14), 138 (70), 107 (98).

To obtain 3α-O-(4-nitrophenylcarbonate)-lithocholic acid (**2**), a solution of 4-nitrophenylchloroformate (250 mg, 1.24 mmol) in 10 mL dioxane was added to lithocholic acid (400 mg, 1.06 mmol) in 10 mL dioxane-pyridine (19:1), and the mixture was stirred for 5 h at room temperature. The solvent was evaporated and the residue purified by flash silica gel chromatography ($CHCl_3$:CH_3OH, 973) to give 130 mg (23%) of **2.** ^{1}H-NMR (DMSO) δ: 0.62 (3H, s, C-18 Me), 0.87 (3H, d, J = 6.3 Hz, C-21 Me), 0.88 (3H, s, C-19 Me), 4.71 (1H, m, H-3β), 7.40 (2H, d, J = 11 Hz) and 8.20 (2H, d, J = 11 Hz). Compounds 3 and 4 (Fig. 1) were prepared similarly by reacting 4-

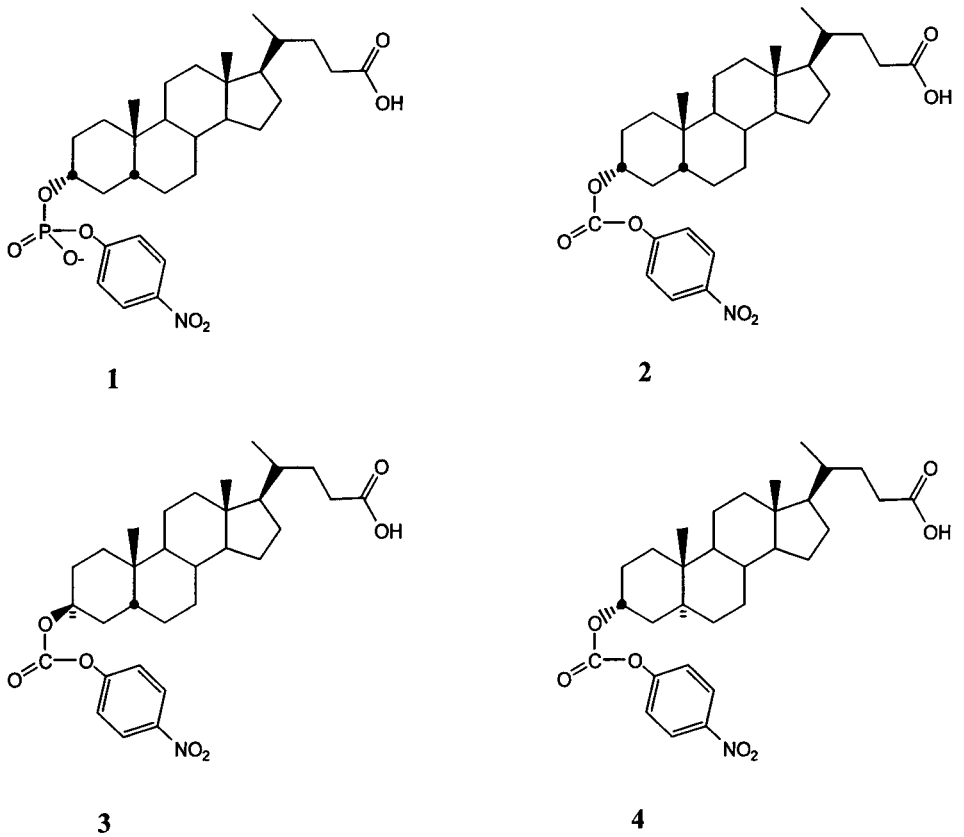


Fig. 1. Structures of steroid phosphate derivatives used as hapten for Ab production and corresponding carbonates used as substrates for Ab-catalyzed hydrolysis.

nitrophenylchloroformate with appropriate steroids obtained from commercial sources. Their ^{1}H-NMR and f.a.b. mass spectra were in accordance with the proposed structures.

Conjugation to Carrier Proteins

The hapten **1** (6 mg) was treated with dicyclohexylcarbodiimide (10 mg) and *N*-hydroxysuccinimide (8 mg) in 0.1 mL dimethylformamide. After 30 min at 23°C, the sample was added to a solution of 6 mg cationized BSA (cBSA, Pierce) or BSA in 1 mL 0.1 *M* aqueous sodium bicarbonate. The reactions were stirred gently at 4°C for 3 h. Protein conjugates were separated from unbound hapten and byproducts by filtration through G-25 Sephadex (PD-10 column, Pierce). Hapten-coupling density was determined by hydrolysis of an aliquot in 0.5 *N* sodium hydroxide and quantitation of nitrophenol release (A_{400} nm) relative to protein concentration determined by A_{280} nm.

Immunization, Hybridoma Production, and Antibody Purification

Female mice (Balb/c strain) were immunized by subcutaneous injection of the **1**-cBSA conjugate (1 mg/mL, 0.2 mL) in an equal volume of complete Freund's adjuvant. Two booster immunizations (0.2 mL of a 1:1 emulsion in incomplete Freund's adjuvant) were given intraperitoneally at 10–14 d intervals. Three to 4 d after the final boost, a serum sample was collected by retro-orbital eyebleed. The Ab response was determined by enzyme-linked immunosorbent assay (ELISA) using **1**–BSA conjugate and serum samples at 1:100–1:20,000 dilution. Hybridomas were prepared by standard protocol *(14)* by fusion of spleen cells with FO myeloma cell. Supernatants from cultures were assayed by ELISA on microtiter plates coated with **1**-BSA conjugate. Established positive clones were expanded and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) 5% fetal bovine serum. Supernatants (5 mL each) were harvested from confluent cultures, pooled into groups of 6, buffered by addition of *M* sodium phosphate, pH 8.0 (10% of volume), and up to 30 mL was loaded onto a protein G-Sepharose affinity column (0.2 mL gel volume). Antibody was eluted from the column with 0.1 *M* glycine buffer, pH 2.7, and fractions were buffered to pH 8.0 with 10% vol of 1 *M* Tris, pH 8.0. Protein concentration was estimated by absorbance at 280 nm, using $\epsilon_{1\%} = 14$.

Assay for Hydrolytic Activity

Hydrolysis assays were performed by addition of a 0.4 m*M* stock solution of carbonate **2** in DMF to an aliquot of the Ab solution in 0.1 *M* Tris, pH 8.0, in quartz cuvets to obtain a final concentration of 20 μ*M* **2** and 5% DMF. Samples were incubated at 30°C, and absorbance changes at 400 nm were recorded over a 30-min period. Samples showing a rate increase over the background sample (carbonate **2** diluted into the protein fraction of identically purified culture media) were produced in larger quantity in ascites fluid for further characterization.

Kinetic Analysis

An aliquot of Ab solution (1.2 mg/mL in 50 m*M* Tris, pH 8.0) was mixed in a 0.5-mL cuvete containing **2** or other steroid carbonates, at concentrations ranging from 0.5 to 20 μ*M*, in 0.2 *M* Tris buffer and 5% DMF at pH 9.0. Cells were equilibrated for 5 min in the cell holder before absorbance changes at 400 nm were recorded over 60 min. Inhibitors were premixed with the Ab in Tris buffer prior to addition to the reaction. Initial rates were plotted as a function of substrate concentration, and kinetic constants were calculated by fitting the data to the Michaelis-Menten equation using nonlinear regression analysis.

RESULTS

Albumin-Catalyzed Hydrolysis of Steroidal 3-*p*-Nitrophenylcarbonates

Labile carbonates containing a steroid structure were examined as potential substrates for albumins. Among bile acids known to bind to serum albumins, lithocholic acid provides a convenient 3α-hydroxyl group for modification to the *p*-nitrophenylcarbonate **2.** Hydrolysis of **2** was observed in the presence of either bovine or human serum albumins. The reaction was characterized by an initial rapid phase, followed by a slower process. The reaction displayed modest rate dependence on alkalinity over a range from pH 8.0 to 10.0. At pH 9.0 and a concentration of 5 μ*M* BSA, the steady-state rate accounted for approx 11 μ*M*/h of product formation. Progress of the reaction over several hours was consistent, with production of more than 7 mol of substrate per mol of protein. Assuming a single hydrolytic site per BSA molecule, this rate suggests catalytic turnover by the protein.

Monoclonal Antibody Production and Screening

Lithocholic acid was elaborated to a phosphate-based TSA suitable for generating hydrolytic Abs. The p-nitrophenyl phosphate ester **1** corresponds to an analog for the hydrolysis of carbonate **2.** The carboxyl group in the D-ring side chain was employed for conjugation to a carrier protein in preparing the immunogen (Fig. 1). MAbs expressed in hybridoma culture supernatant were selected by ELISA for binding to **1**–BSA conjugate. These Abs displayed no crossreactivity in binding to unmodified BSA. Abs were purified from hybridoma culture supernatant by protein G-Sepharose adsorption prior to analysis for hydrolytic activity. The implied carbonate substrate **2** was used to screen for hydrolytic activity of the specific Abs by detecting absorbance changes caused by release of *p*-nitrophenol product. Two of 50 candidate Abs demonstrated significant activity in the assay, and one of these (2E10) was subcloned and expressed in ascites for further characterization.

Rates and pH Dependence of Antibody-Catalyzed Carbonate Hydrolysis

Deviation from first-order kinetics for spontaneous hydrolysis of *p*-nitrophenylcarbonate **2** was observed at concentrations above 100 μ*M*. This was attributed to limiting solubility of the steroidal derivative. A rate constant $k_{obs} = 1.1 \times 10^{-4}$/min for the spontaneous reaction at pH 9.0 was deduced from an Arrhenius plot over the range of 20–80°C. Kinetic analysis of the hydrolysis of carbonate by Ab 2E10 was done at 35°C under con-

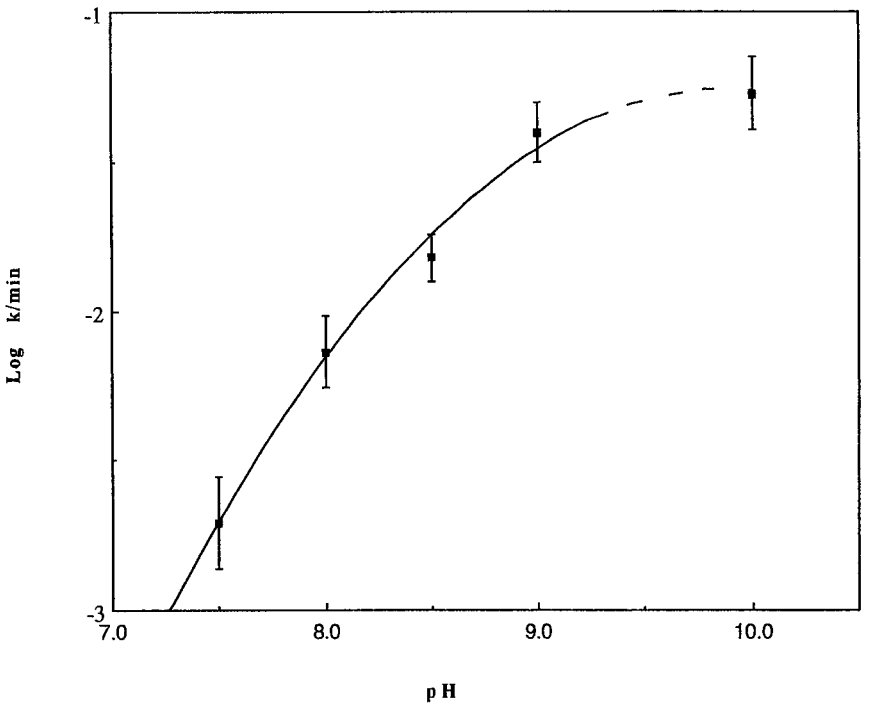


Fig. 2. Dependence of the rate of Ab 2E10-catalyzed hydrolysis of **2** as a function of pH in 0.1 *M* Tris, 5% DMF. The Ab concentration was 200 μg/mL and [**2**] = 100 μ*M*, which represents 30 × K_M at pH 9.0. The value for *k* is obtained from replicate measurements, and is an approximation of k_{cat}.

ditions in which background hydrolysis was negligible. The reaction obeyed saturation kinetics over a range of substrate concentrations from 0.5 to 100 μ*M*. Rate constants of K_M = 3.3 μ*M* and k_{cat} = 4.0 × 10^{-2}/min were derived from a fit to the Michaelis-Menten equation by nonlinear regression. The Ab-catalyzed reaction showed increasing activity with pH, as shown in Fig. 2. The reaction rate appeared to be independent of pH at values above pH 9.0 Ab activity was stable to incubation at pH 10.0 for several hours, suggesting that the deviation was not caused by inactivation of the protein by alkaline denaturation.

Alternative Steroidal *p*-Nitrophenylcarbonate Substrates

Additional steroidal 3-nitrophenylcarbonate (Fig. 1) were prepared and examined as potential substrates for Ab hydrolysis. These studies were undertaken in order to probe the consequence of destabilizing interactions with remote substrate features on the catalytic efficiency of the Ab. Isomeric lithocholate derivatives containing the charged 17α-side chain and altered stereochemistry in the A-ring were prepared by procedures similar to those for **2.** Carbonate **3,** derived from 3β-lithocholate, is

Table 1
Comparison of Antibody 2E10 and BSA as Catalysts for Hydrolysis of Carbonates

Substrate/Catalyst		k_{cat} (10^2/min)	K_M (μM)	k_{cat}/K_M (min/M)
2	Ab	4	3.3	12,120
	BSA	3.8	15	2533
3	Ab	8.4	1.0	84,000
	BSA	7.8	14	5570

epimeric at the carbonate linkage to the steroid. Its solubility and hydrolytic stability were analogous to those of carbonate **1.** In the standard hydrolysis assays, compound **3** proved to be a better substrate than **1** for either Ab or BSA. The kinetic constants are compared in Table 1. The steroid derivative **4,** with 5α (A/B trans) stereochemistry, also had similar stability and solubility as carbonates **2** and **3.** However, no rate acceleration was observed when a 20-μM solution of **4** was treated with Ab 2E10. The lack of a reaction is evidence for the specificity of the Ab for the steroid framework features.

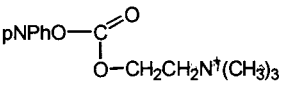
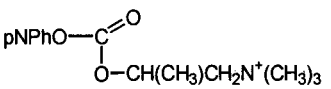
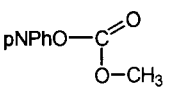
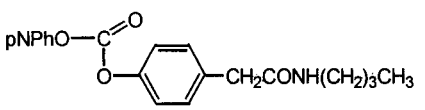
Inhibition of Carbonate Hydrolysis by Haptenic Phosphate Ester

Strong inhibition an Ab-catalyzed reaction by the analog used for immunization is regarded as evidence of a catalytic site elaborated through affinity maturation. The Ab 2E10 was incubated in the presence of phosphate ester **1** at concentrations ranging from 0.5 to 25 μM prior to initiation of the reaction with carbonate **2.** The reaction was completely inhibited, even at the lowest concentration of **1.** Thus, it can be assumed that the inhibition constant is in the nanomolar range. Lithocholic acid also inhibited Ab 2E10, although concentrations greater than 25 μM were required for 50% inhibition. By contrast, the BSA-catalyzed hydrolysis of **1** was only 50% inhibited in the presence of 40 μM of **1.** An apparent difference of about two orders of magnitude could be deduced for the inhibitory potency of the TSA **1** acting on the specific Ab site vs the albumin site.

DISCUSSION

Features of an enzyme-active site are also found on diverse nonenzymatic proteins. Albumins are known to present binding sites for fatty acids and other drug-like substances *(6–9).* The hydrolytic activity of albumin toward labile esters has been attributed to the presence of chemically reactive residues in these binding sites *(10).* This activity provides a meaningful reference for the catalytic efficiency of Abs which act on identical or

Table 2
Substrates and Rate Factors for Antibody-Catalyzed Carbonate Hydrolysis

Ref.	Substrate	k_{cat} (10^2/min)	K_M (μM)	k_{cat}/k_{uncat}
This work	2	4	3.3	364
2		40	200	770
2		20	2000	380
4		140	660	810
17		7200	980	5500

analogous substrates. Such a reference reaction reflects the intrinsic chemical reactivity of a protein surface not adapted by immunological affinity maturation. Differences in the kinetic properties of albumins and Abs could therefore assist in determining the value of the design approach for access to improved catalysis.

Transition state theory predicts a direct correlation between TSA binding affinity and Ab catalytic efficiency *(15)*. However, catalytic Abs from diverse studies suggest significant deviations from this model *(13,16)*. The authors reasoned that a TSA hapten containing a steroid could engage a number of combining-site residues in the Ab, which might be used to constrain or distort a bound substrate in favor of hydrolysis. The Ab described in this study expressed only modest catalytic activity relative to other hydrolytic Abs generated against phosphate or phosphonate TSA haptens. A rate enhancement of about 400-fold above background for the 2E10-catalyzed reaction is at the low end of the range for Abs with carbonate hydrolase activity (Table 2). This result is consistent with the reported trend toward a reduced level of catalytic activity in polyclonal Abs elicited with haptens of increasing size and hydrophobicity *(18)*. Identification of the Ab by screening with a large hapten such as **1** could also bias against efficient catalysis, because of the dominant interactions with the steroid group. In this regard, short TSA derivatives may be advantageous in the screening procedure to select Abs that use chemically reactive residues in the binding interaction *(19,20)*.

Comparison of the kinetic constants for Ab 2E10-catalyzed hydrolysis of **2** with those of BSA shows that the Ab binding site provides only a small

advantage over the albumin site for the hydrolysis of a steroidal carbonate (Table 1). This advantage is associated principally with improved substrate affinity, and could be considered a stochastic property of the ligand-binding function, which is mediated primarily by hydrophobic interactions. A previous report of esterase-like activity of antisteroid Abs also appears to reflect such reactivity *(21)*. In that case, only the stoichiometric reaction of Ab and steroid ester is accelerated. Despite the apparent specificity for lithocholate carbonates with the 5β (A/B *cis*) stereochemistry, Ab 2E10 was found to accept substrates with either 3β- or 3α-carbonate configuration in the steroid. This result suggests that a precise alignment of the steroid group relative to the reactive residue(s) is not necessary for productive binding. It has been noted that catalytic Abs exhibit improved turnover of substrates that are structurally varied at positions remote from the reaction center *(3,22)*. Alternative substrates may undergo more efficient catalysis because of weakened interactions with the Ab in the ground state.

Solvent interactions could also account for different kinetic activities of the two substrates. Examination of the carbonates **2** and **3** by molecular modeling, subject to energy minimization and dynamics, suggests that the former structure could adopt a conformation in which a strong internal hydrophobic interaction exists between the phenyl ring and the α-surface of the steroid (Fig. 3). Association with the protein in this state could also hinder access of water or hydroxide ion into the active site, resulting in a reduced rate of hydrolysis. This model provides a general explanation for the analogous substrate preference by BSA. Evidence for combining site flexibility and crossreactivity of antisteroid Abs has been reported in X-ray crystallographic structure analysis *(23)*. The cleft-like combining site in this structure presents additional possibilities for an active site analogy.

CONCLUSION

This study supports the idea that a component of the catalytic potential of Abs is derived from the natural properties of hydrophobic sites on a protein surface. Basic or nucleophilic residues on the periphery of these sites could have enhanced chemical reactivity because of proximity or medium effects. The precise orientation of reactive groups arising through affinity maturation is likely to contribute to the catalytic efficiency of a designer Ab. However, increasing size and hydrophobicity of the Ag was shown to lead to Abs of attenuated catalytic activity. Similar rates of Ab-catalyzed and albumin-catalyzed reactions suggests that immunological complexation need not recruit precise alignment of reactive groups. The function of peripheral groups in such a complex should be to exclude water from the hydrophobic interface. Solvation of these residues could

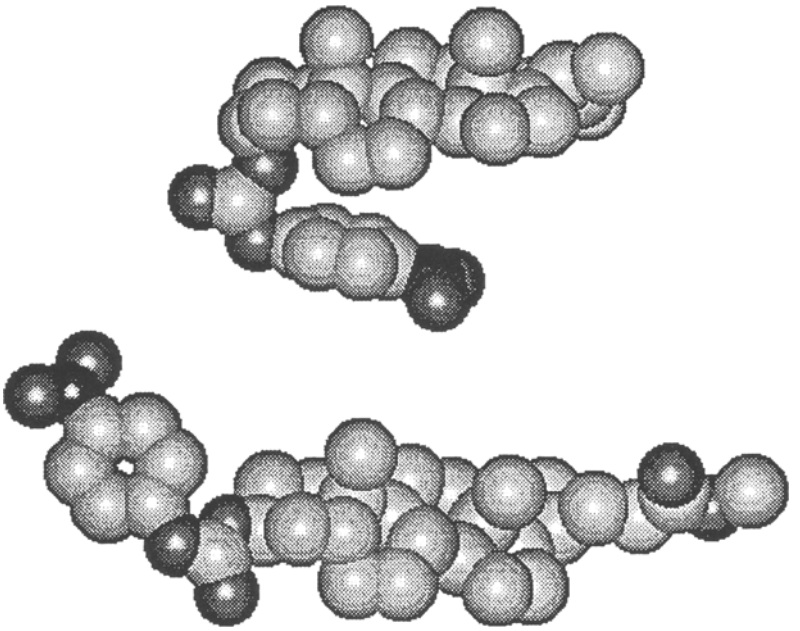


Fig. 3. Energy minimized structures of steroidal carbonates **2** (upper) and **3** (lower) indicating the intramolecular hydrophobic interaction between the nitrophenyl group and the steroid skeleton in substrate **2.** Although the minimization was done in a vacuum, it is reasonable to presume that the interaction shown would be even stronger in aqueous solution.

preclude alignment through ion-pair of hydrogen-bonding interactions that are more favorable at buried residues.

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