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Oriented Immobilization of Periodate-Oxidized Monoclonal Antibodies on Amino and Hydrazide Derivatives of Eupergit C

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ABSTRACT

Amino and hydrazyno derivatives of Eupergit C were prepared by reaction of the beads with hexamethylene diamine (HMD) and adipic acid dihydrazide (ADH), respectively. Monoclonal antibodies (mAbs) against carboxypeptidase A (CPA) and horse radish peroxidase (HRP) were prepared, and those that did not inhibit the respective enzymatic activities were selected. The carbohydrate moieties of these antibodies were oxidized by reaction with sodium periodate and then coupled onto the modified beads. The oxidation and coupling reactions were optimized to achieve highly active matrix-conjugated antibodies. Thus, antibody-matrix conjugates that possessed antigen-binding activities close to the theoretical value of 2 mol antigen bound/mol immobilized antibody were obtained.

Index Entries: Monoclonal antibodies; Eupergit C; carbohydrates; periodate oxidation.

INTRODUCTION

Eupergit C is a synthetic polymeric matrix, based on polymethyl methacrylamide, bearing oxirane groups that are capable of binding proteins via their amino-, thio- and hydroxymoieties (*1*). An extensive study

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of the applicability of Eupergit C-immobilized antibodies and antigens to the immunopurification of the corresponding antigens and antibodies by conventional and HPLC-based affinity chromatography is being carried out in our laboratory. In our investigations, we use various enzymes as antigens and select for corresponding monoclonal antibodies (mAbs) that do not inhibit the enzymatic activity of these antigens. Binding of the antigen by the matrix-immobilized antibody, thus, may be monitored by direct measurement of the enzymatic activity of the matrix-bound immunocomplex (*2*). This approach enables determination of the antigen-binding activity of the conjugated antibodies at very high sensitivity.

An immunopurification system based on the Eupergit C-immobilized antibodies, which was developed by us as a result of these studies, was found to be highly selective, reproducible, and stable (*3*). However, the antigen-binding capacity obtained was relatively low (0.2–0.5 mol of bound Ag/mol mAb). This was attributed to: binding of the antibodies via amino acid residues located at, or close to, the antigen-binding site, which were essential for antigen binding activity, or improper orientation of the matrix-bound antibody molecules, which may impede their activity.

Immunoglobulins possess two carbohydrate chains located at the F_c region of the molecule, remote from the antigen-binding sites, which may be used for their oriented immobilization onto insoluble matrices. Periodate oxidation of the carbohydrate moieties, following their coupling to amino or hydrazino groups of a matrix by a Schiff base or hydrazone formation, respectively, should lead to antibody-matrix conjugates that possess higher antigen binding activity (*4–6*).

In the following communication, we describe the preparation and characterization of amino and hydrazyno derivatives of Eupergit C by treatment of Eupergit C beads with hexamethylene diamine (HMD) and adipic acid dihydrazide (ADH), respectively, and their application for the immobilization of periodate-oxidized antibodies at regions remote from the antigen-binding sites.

MATERIALS AND METHODS

Enzymes

Carboxypeptidase A (CPA) and horse radish peroxidase (HRP) were purchased from Sigma (St. Louis, MO).

Measurement of Enzymatic Activity

Carboxypeptidase A

CPA (1–50 ng in 10–50 μL of PBS containing 0.5*M* NaCl) was incubated with hippuryl L-phenylalanine (10 m*M* in the same buffer) at room temperature in wells of a microtiter ELISA plate for 10–60 min. At the end of the incubation period, phenylalanine liberated by the enzymatic reac-

tion was determined as follows. One hundred microliters of ninhydrin solution (3% in methyl cellosolve) and 50 μL of NaCN (0.2 m*M* in 3.8*M* acetate buffer, pH 5.3) were added to each well. The plate was covered and heated to 100°C for 20 min. The intensity of the color developed was measured by the absorbance at 550 nm (with no reference beam) in a SLT-210 ELISA reader (Grodig, Austria).

The activity of Eupergit C-bound CPA was determined by the same procedure except that after incubation of the immobilized enzyme with the substrate in Eppendorf tubes for 10–60 min, the beads were sedimented by centrifugation and aliquots of the supernatante were removed for reaction with ninhydrine, as described above.

Horseradish Peroxidase

HRP (0.1–10 ng in 10–100 μL of PBS) was incubated in microtiter ELISA plates with 100 μL of HRP substrate solution (2 mg/mL of O-phenylenediamine (Fluka, Buchs, Switzerland) and 0.008% H_2O_2 in 50 mM citrate buffer, pH 5.0) for 1 min at room temperature. Color development was stopped by the addition of 4*N* HCl (50 μL) into each tube. The intensity of the color developed was measured by an ELISA reader at 492 nm (with a reference beam at 405 nm).

Activity of HRP bound to Eupergit C-conjugated anti-HRP antibodies was determined by incubation of the beads (0.12 mg) with 1 mL of substrate solution for 1 min at room temperature. Color development was stopped by the addition of 4*N* HCl (0.5 mL) into each tube. The intensity of the color developed was measured by an ELISA reader, as described above.

Preparation and Purification of mAbs

Balb/C mice were immunized by two intrafootpad injections of CPA or HRP in complete Freund's adjuvant (50 μg/injection on d 0 and 10). Four days prior to fusion, 100 μg of CPA or HRP in PBS were injected into the peritoneum. The spleen cells of the immunized mouse were fused with the NSO/1 myeloma cell line (*7*). Fusion procedure and growth of antibody-secreting cell lines was essentially as previously described (*8*). Ascites were produced in Balb/C mice, primed by incomplete Freund's adjuvant, as previously described (*9*).

The antibodies were affinity-purified on antigen columns prepared as follows: Eupergit C beads (1.5 g) were thoroughly washed with PBS until no absorbance at 280 nm was detected in the supernatant, and then the beads were washed once with 1*M* potassium phosphate buffer, pH 7.4. CPA or HRP (15 mg in 1–2 mL of the same buffer) were added and incubated with the beads for 16 h at 4°C. The amount of bound enzyme was determined by a protein assay of the supernatant, as well as an activity assay of the bound enzyme. Excess oxirane groups on the matrix were blocked by incubation with 0.2*M* β-mercaptoethanol for 4 h at 4°C. The beads-conjugated enzymes were packed into HPLC stainless steel columns

(0.8×10 cm) and repeatedly washed with PBS and 0.2*M* ammonium acetate buffer, pH 10. The ascites fluids containing the various antibodies were fractionated by precipitation with 50% saturated ammonium sulphate solution at 4°C. The resulting precipitate was dissolved and extensively dialyzed against PBS. The dialysed protein (5–10 mg) was applied to the corresponding antigen column and washed with PBS at 1 mL/min. After elution on the nonbound protein, the column was washed with 0.2*M* ammonium acetate, pH 10, to elute the specific mAb.

Oxidation of Affinity-Purified Antibodies

MAbs (0.7–3.0 mg each) were oxidized by incubation with sodium periodate (0.1–100 m*M* in 1 mL of 0.1*M* acetate buffer, pH 5.5) for 10–120 min at 0°C in the dark. The oxidation reaction was stopped by the addition of three drops of ethylene glycol. Low molecular weight components of the oxidation mixture were removed by gel filtration on a PD-10 column (BioRad, Richmond, CA) preequilibrated with 0.1*M* acetate buffer, pH 5.5. The fraction containing the oxidized antibodies (3.5 mL) was collected and concentrated 10-fold in a 10 mL Amicon ultrafiltration cell equipped with a PM50 membrane. The amount of protein was determined by the Bradford method (*10*). The overall recovery of antibodies in this process was usually 50–70%.

Preparation of Hexamethylene Diamine-Modified Eupergit C

Eupergit C beads (1 g) were extensively washed with PBS, sedimented by centrifugation, and resuspended in 0.1*M* hexamethylene diamine in 0.2*M* carbonate buffer, pH 8.5. The mixture was incubated for 1 h at room temperature. The beads were then extensively washed with PBS and stored at 4°C (*11*).

Preparation of Adipic Dihydrazide-Modified Eupergit C

Eupergit C beads (1–5 g) were extensively washed with PBS and then reacted for 16 h at room temperature with 0.1–0.5*M* adipic dihydrazide (Sigma, St. Louis, MO) in 0.2*M* sodium carbonate buffer, pH 9.0 (10 mL/g dry weight of Eupergit C). The modified carrier was extensively washed with PBS and then incubated with 0.2*M* β-mercaptoethanol (10 mL/g of beads) for 16 h at room temperature in order to block residual, nonreacted oxirane groups on the Eupergit C beads. After extensive washing with 0.1*M* acetate buffer, pH 5.5, the beads were kept for further use at 4°C. The amount of ADH groups introduced onto the matrix was determined by reaction with trinitrobenzene sulfonic acid (TNBS) (*12*). Although a yellow color developed on the surface of the beads, this reaction could not be measured quantitatively, it enabled rough estimation of bound

ADH. Thus, the beads reacted with 0.5*M* of ADH and yielded a strong orange color, whereas those reacted with 0.1*M* yielded only a pale color. Beads not reacted with ADH remained colorless.

Coupling of Antibodies to Eupergit C Via Its Oxirane Groups

Monoclonal antibodies were immobilized onto Eupergit C beads by the same procedure described above (*see* "Preparation and Purification of mAbs").

Coupling of Oxidized Antibodies to Hydrazide and Amino Derivatives of Eupergit C

Oxidized mAbs (5–500 μg) were incubated for 0.5–16 h at 4°C with a suspension of hydrazide-modified Eupergit C beads (20–400 μL, corresponding to 5–100 μg dry weight) in 2.5 vol of 0.1*M* acetate buffer, pH 5.5. Unless otherwise stated, a ratio of 5 mg protein/g of beads was used. The amount of immobilized antibodies was determined from the difference between the initial and the residual amount of protein in the reaction mixture supernatant, determined by the Bradford method (*10*). In parallel experiments, periodate-oxidized ^{125}I-labeled anti-CPA ($mAb_{CPA}14$) or ^{125}I-labeled IgG were added as tracers in order to follow protein binding to the beads. These proteins were iodinated by the method described by Greenwood et al. (*13*). The specific activities obtained were 7.9×10^6 and 5.5×10^6 CPM/μg protein for the iodinated anti-CPA and IgG, respectively. Oxidized mAbs anti-CPA ($mAb_{CPA}3$ and $mAb_{CPA}5$) were also coupled onto ADH-agarose (Pharmacia, Uppsala, Sweden) using the same procedure.

Coupling of oxidized antibodies ($mAb_{CPA}100$) to the aminated beads was performed as follows: 0.5 mg of oxidized antibody in 1 mL PBS, pH 7.4, were incubated with 0.1 g of aminated Eupergit C beads for 1 h at 0°C in the dark. Sodium borohydride (0.01*M* final concn.) was added, and the reaction mixture was incubated for 2 h at 0°C. The amount of bound antibody was determined by a protein assay of the supernatant.

Determination of the Leakage of IgG from Eupergit C Derivatives

Anti-CPA antibodies ($mAb_{CPA}100$) were coupled to Eupergit C beads (10 mg Ab/1 g of beads) by the standard method (via oxirane groups) or the oxidation method (via ADH groups). The conjugates were extensively washed with PBS, 0.2*M* ammonium acetate buffer, pH 10, and 0.2*M* glycine–HCl buffer, pH 3.0, to remove all nonbound antibodies. Aliquots of the beads (100 mg each) were then incubated at 4°C with PBS for 4 d and with the elution buffers 0.2*M* glycine–HCl, pH 3.0, 0.2*M* ammonium acetate, pH 10.0, or 3*M* sodium thiocyanate for up to 2 h. The amount of IgG

Table 1
Binding of Antibodies to Standard Eupergit C

	anti CPA[a]			CPA[b]			
Ab #	Input, mg/g	Bound, mg/g	%	Input, mg/g	Bound, mg/g	%	CPA/Ig, mol/mol
mAb1[c]	0.7	0.7	100	0.5	0.10	20	0.60
	7.7	5.0	65	2.5	0.15	6	0.15
mAb3	1.2	1.1	92	0.5	0.20	40	0.76
	9.0	6.8	76	2.5	0.40	16	0.29
mAb4	1.5	1.3	87	0.5	0.14	28	0.45
	10.7	10.3	96	2.5	0.35	14	0.16
mAb8	1.5	1.5	100	0.5	0.21	42	0.59
	20.4	16.4	80	2.5	1.05	42	0.32
mAb9	1.5	1.4	93	0.5	0.23	46	0.69
	15.7	8.0	51	2.5	0.80	32	0.50
mAb14	1.4	1.4	100	0.5	0.25	50	0.75
	14.2	9.3	65	2.5	0.45	18	0.24

[a] Antibodies were immobilized onto 100 mg fractions of Eupergit C, as described in the "Materials and Methods" section. Input and bound protein were determined by the Bradford reaction.

[b] The amount of bound antigen was determined by enzymatic assay of the antigen bound to the matrix-conjugated antibody, as described in the "Materials and Methods" section.

[c] Name of antibody is abbreviated; mAb_1 is $mAb_{CPA}1$, and so on.

released to the solution was determined by ELISA using HRP-labeled rabbit anti-mouse IgG antibodies.

RESULTS AND DISCUSSION

Binding of the antibodies to Eupergit C may be achieved by reaction of their amino, thio, or hydroxy groups with oxirane moieties on the surface of the matrix. In many cases, highly active preparations were obtained (*2,3,11*). However, matrix immobilization may cause partial or complete inactivation of the antibodies. In most cases, this effect may be a result of modification of amino acid residues essential for activity, improper orientation of the immobilized antibody molecules, conformational changes induced immobilization, or restrictions induced by multipoint attachment of the protein molecules to the surface of the matrix.

This phenomenon was exemplified by the binding of Eupergit C of a series of anti-CPA mAbs. As shown in Table 1, although all the prepara-

tions of the immobilized mAbs were active in antigen binding, their specific activities were relatively low (below 0.5 mol antigen bound/mol immobilized mAb). This observation led us to seek an alternative approach for the binding of mAbs to Eupergit C, which would result in a higher antigen binding activities. Binding of mAbs via their carbohydrate chains, which are located at their Fc regions, remote from the antigen binding site, seemed to be appropriate especially for those mAbs that tend to lose their activity when coupled to the matrix via their amino groups.

Preparation of Amino and Hydrazyno Derivatives of Eupergit C

Coupling of periodate oxidized antibodies to Eupergit C, via their aldehyde groups, requires the conversion of oxirane groups on the surface of the matrix into amino or hydrazino groups. First, aminated Eupergit C beads were prepared by treatment of Eupergit C beads with 0.1*M* HMD. When 5 mg of $mAb_{CPA}100$, oxidized by treatment with 10 m*M* of sodium periodate for 1 h at 0°C as described in the Materials and Methods section, was allowed to react with the aminated beads, 20% of the protein were coupled within 1 h of incubation. The resulting antibody–matrix conjugate displayed high antigen binding activity, binding the antigen at 0.45 mol/mol immobilized antibody, compared to a value of 0.15 mol/mol obtained for the same antibodies when coupled directly to oxirane groups of Eupergit C. However, coupling of the oxidized antibodies to amino groups of the matrix, by the formation of a Schiff base at slightly basic pH values, may cause inter- and intramolecular cross-linking of the oxidized carbohydrates with amino groups on surface of the antibodies. Moreover, the Schiff base must be stabilized by reduction with borohydride, which may impede with antibody activity (*14*). In contrast, the hydrazone formation between the oxidized carbohydrates and hydrazyde-modified matrix should take place at slightly acidic pH values (pH 5–5.5), and cross-linking of aldehyde and amino groups of the antibodies is, thus, markedly reduced. This bond is stable and does not require further stabilization by reaction with borohydride.

An Eupergit C-hydrazide derivative was prepared by the reaction of Eupergit C beads with 0.1 or 0.5*M* ADH. As shown in Fig. 1, the preparations obtained bound oxidized antibodies efficiently when relatively low concentrations of antibodies were applied to the matrix (less than 5 mg antibodies/g of beads). At higher loads, binding was less efficient even when the amount of ADH groups on the matrix was increased. This observation led us to conclude that the amount of oxidized antibodies bound onto the modified matrix was limited by the amount of aldehyde groups on the surface of the oxidized antibodies rather than by the amount of ADH groups on the surface of the matrix. Therefore, in all successive experiments, Eupergit C–ADH was prepared by treatment of Eupergit C with 0.1*M* ADH.

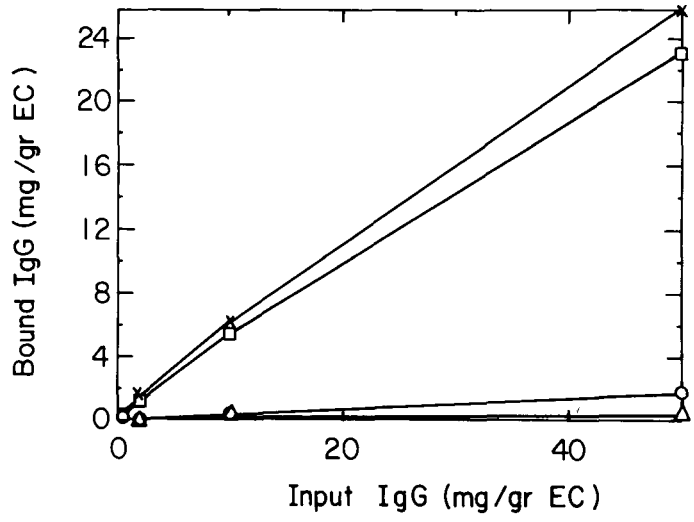

Fig. 1. Binding of radiolabeled oxidized ^{125}I IgG to Eupergit C-ADH beads. One hundred milligrams of Eupergit C beads were treated with 1 mL of 0.1*M* (□---□), or 0.5*M* (x---x) ADH in 0.2*M* sodium carbonate buffer, pH 9.0, for 16 h. The beads were then incubated for 4 h with β-mercaptoethanol (0.2*M* final concn.) and extensively washed with 0.1*M* sodium acetate buffer, pH 5.5. Oxidized IgG (containing 10,000 CPM/sample) were added to the reaction mixture (5 mg of beads/tube) and incubated for 4 h at room temperature. The amount of the bound IgG was determined by the radioactivity of the beads. Nonspecific absorption of the bound oxidized IgG was determined by extensively washing the beads with a solution containing 10% SDS in 8*M* urea and measuring the radioactivity in the beads supernatant (○---○). In control experiments, the binding of nonoxidized IgG to Eupergit C beads treated with 0.1*M* ADH (△---△) was also determined.

Treatment of Eupergit C beads with 0.1, or even 0.5*M* ADH, under the experimental conditions employed, does not convert all the oxirane groups into hydrazides. The amount of oxirane groups on the unmodified Eupergit C beads, determined by thiosulphate titration, is relatively high (0.8 mmol/g; 1 oxirane group/20Å^2). Titration of the residual active oxirane groups on Eupergit C beads after ADH modification showed that about 30% of the oxirane groups still remained active at the end of the reaction (Fig. 2). Although these groups may not participate in protein binding during antibody immobilization, which takes place at pH 5.5, they may react, at a later stage, with amino groups of the antigen to be purified by the immobilized antibodies at neutral pH. Therefore, blocking of the residual oxirane groups after ADH binding is essential.

Two reagents were tested as potential blockers, ethanolamine and β-mercaptoethanol. As shown in Table 2, ADH-modified beads reacted with 0.2*M* ethanolamine, pH 9.5, tended to adsorb proteins at pH 5.5, apparently owing to positive charges of the secondary amino groups formed by

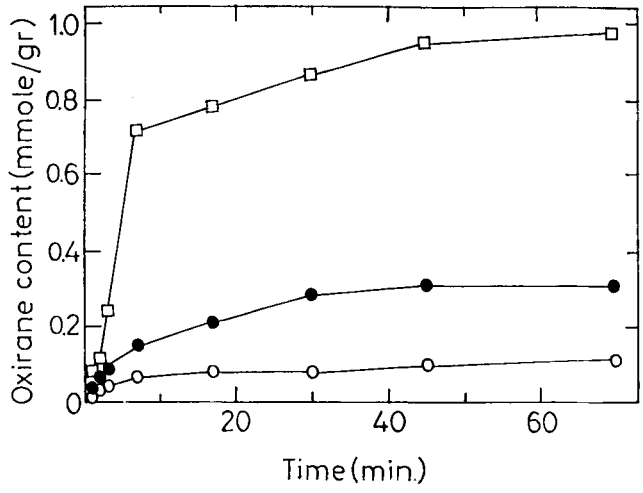

Fig. 2. Titration of oxirane groups on the surface of Eupergit C (□---□), Eupergit C-ADH (●---●), and Eupergit C-ADH beads blocked with β-mercaptoethanol (○---○). One hundred milligrams of beads were extensively washed with distilled water and then suspended in 0.5 mL of water. The pH was adjusted to 7.0 by 0.1*M* NaOH, using an Agla microsyringe, and then 5 mL of 0.3*M* sodium thiosulphate were added. At different time intervals, the pH was readjusted to 7.0 and the amount of 0.1*M* HCl added was recorded. The amount of oxirane groups were calculated accordingly.

Table 2
The Effect of Blocking of Eupergit C-ADH
on the Adsorption of Nonoxidized Antibodies[a]

Beads	Blocking	Adsorbed mAb, OD_{410}	
		pH 5.5	pH 7.5
EC[e]-ADH	-	0.07	0.55[b]
EC-ADH	ethanolamine	0.53[c]	0.07
EC-ADH	β-mercaptoethanol	0.02	0.04
EC	-[d]	0.1	1.75[b]

[a]Eupergit C-ADH beads were prepared with 0.1*M* ADH as described in the "Materials and Methods" section and then residual oxirane groups were blocked by incubation for 4 h with 0.2*M* β-mercaptoethanol, pH 8.0, or 0.2*M* ethanolamine, pH 9.5. These beads were then washed extensively with 0.2*M* sodium acetate buffer, pH 5.5, or 1*M* potassium phosphate buffer, pH 7.5. To samples containing 5 mg of beads, 50 μg of oxidized anti-HRP antibodies $mAb_{HRP}2$ were added for 1 h. The amount of adsorbed antibody was determined after incubation of the beads with HRP for 30 min and measuring the activity of the bound enzyme.

[b]These figures represent covalent binding to oxirane groups.

[c]This figure represents nonspecific ionic adsorption to the beads.

[d]The beads were blocked with β-mercaptoethanol after the antibodies binding step.

[e]EC = Eupergit C.

the reaction. In contrast, when β-mercaptoethanol was used as a blocker, protein adsorption at pH 5.5, as well as covalent protein binding to residual oxirane groups at pH 7.5, were markedly reduced. Consequently, the ADH-modified beads were routinely treated with 0.2*M* β-mercaptoethanol, pH 8.0, for 4 h at room temperature before protein binding. As shown in Fig. 2, the blocking step eliminated most of the residual oxirane groups. The 10% of the original oxirane groups that still seem to remain active even after the blocking step should represent groups that are remote from the outer surface of the beads, apparently not available for protein binding.

Periodate Oxidation of Antibodies

Like many other glycoproteins, the carbohydrate moieties of mAbs may be oxidized to form aldehyde groups that, in turn, may react with hydrazide groups on the surface of the matrix (*15*). However, when antibodies are being oxidized by sodium periodate, certain amino acid residues, particularly *N*-terminal serine, threonine, and methionine, may be oxidized as well. Whenever these residues are essential for the antigen-binding activity of an antibody, their oxidation may harm its activity. The oxidation period recommended in the literature varies from 30 min (*16*) to 24 h (*17*). Therefore, before attempting optimization of the binding of a certain oxidized antibody onto the amino- or hydrazyde-modified matrix, it is necessary to examine the sensitivity of this antibody to periodate oxidation in order to minimize the damage to the antibody activity.

The sensitivity to periodate oxidation of a series of anti-CPA antibodies, as well as an antiperioxidase antibody, was examined by incubation of these antibodies with various concentrations of sodium periodate for different periods of time. As shown in Table 3, all the antibodies tested were relatively stable to periodate oxidation at low concentrations (up to 10 m*M*), short oxidation periods (1 h), and low temperature (0°C). Increased periodate concentration (100 m*M*) or a longer oxidation period (16 h) had a marked effect on the activity of some antibodies (e.g., $mAb_{CPA}1$), but only a minor effect on others (e.g., $mAb_{CPA}14$). Similarly, increasing the temperature during the oxidation step to 4°C or room temperature resulted in a rapid inactivation of the more sensitive antibodies, whereas the more stable antibodies were less affected (data not shown).

As shown in Fig. 3, the concentration of periodate in the oxidation mixture had a marked effect on the antigen binding activity of the matrix-bound antibodies. Assuming that at low concentrations of periodate the activity of the immobilized antibodies is directly related to the amount of bound antibodies, the above results indicate that the amount of aldehyde groups on the oxidized antibody molecules were a limiting factor in the rate of the binding. Loss of activity, which was observed with the 100 m*M* periodate-oxidized antibody, was attributed to irreversible damage to the antibodies caused by the high periodate concentration.

Table 3
Effect of Sodium Periodate on the Activity of mAbs[a,b]

mAb	IO_4, m*M*	Incubation time 1 h	Incubation time 16 h
mAb1	2.5	90	65
	5.0	80	25
	10	74	17
	25	68	15
	50	52	12
	100	4	0
mAb3	2.5	85	70
	5.0	78	62
	10	74	62
	25	72	58
	50	81	52
	100	3	0
mAb9	2.5	100	97
	5.0	100	93
	10	88	91
	25	86	89
	50	80	87
	100	70	ND
mAb14	2.5	93	90
	5.0	85	71
	10	81	67
	25	77	66
	50	77	58

[a]Antibodies were incubated with different concentrations of sodium periodate for 1 or 16 h in the dark. *See* "Materials and Methods" section for details.
[b]Figures represent residual activity, as measured by reverse ELISA. *See* "Materials and Methods" section for details.

Activity of the Immobilized Antibodies

As a result of the above studies, we adopted an oxidation and binding procedure as follows: antibodies (0.7–3.0 mg) are oxidized by 10 m*M* periodate in 1.0 mL of 0.1*M* sodium acetate buffer, pH 5.5, for 1 h at 0°C. Three drops (approx. 100 μL) of ethylene glycol are then added to destroy excess periodate; the sample is applied to a gel filtration column, and the fraction containing the antibodies is collected. Eupergit C-ADH is prepared by treatment of Eupergit C with 0.1*M* ADH and blocking of residual oxirane groups by reaction with β-mercaptoethanol under the conditions

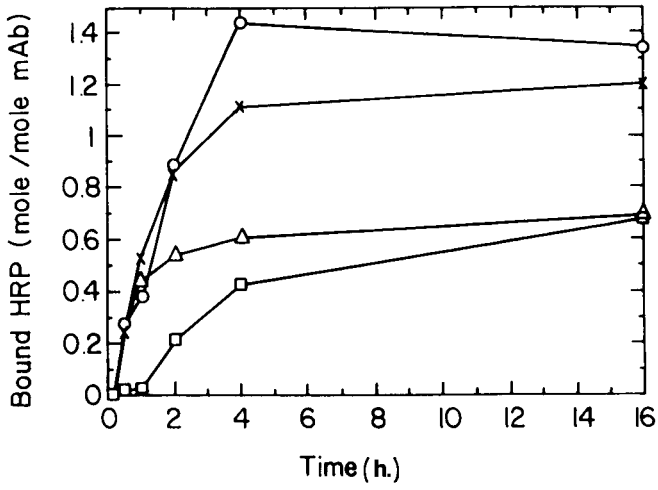

Fig. 3. Antigen binding activity of oxidized antibodies coupled onto Eupergit C-ADH beads. Samples of anti-HRP antibodies $mAb_{HRP}2$ (25 μg), which were oxidized in the presence of 0.3 m*M* (□---□), 3.0 m*M* (x---x), 10 m*M* (○---○), and 100 m*M* (△---△) of sodium periodate, were incubated with portions of 5 mg of Eupergit C-ADH beads (prepared with 0.1*M* ADH) for various periods of time. The amount of HRP bound by the conjugated antibodies which was, in turn, determined by an HRP assay.

described in the Materials and Methods section. Oxidized antibodies are coupled to the modified matrix by reaction at 0°C in the dark for 1 h.

The superiority of the new binding method over the direct binding procedure (via oxirane groups of the matrix) was exemplified by coupling, after periodate oxidation, of the same series of anti-CPA antibodies described above in Table 1 to Eupergit C-ADH. As shown in Table 4, the antigen-binding activity of the anti-CPA antibodies was considerably enhanced when coupled by the new procedure. With some antibodies, the antigen binding activity increased to a level of close to 2 mol antigen bound/mol of antibody, which is the theoretically expected value. It is pertinent to note that, although with most antibodies binding of periodate-oxidized antibodies to Eupergit C-ADH apparently yields more active antibody preparations, exceptions may be anticipated. For example, anti-HRP antibodies ($mAb_{HRP}2$) showed very high antigen binding activity (approx. 1.8 mol Ag bound/mol immobilized antibody) when coupled to Eupergit C via oxirane groups. When the same antibody was oxidized and coupled to Eupergit C-ADH, its activity decreased to 1.5 mol Ag bound/mol immobilized antibody.

In a parallel experiment, two of the anti-CPA antibodies, $mAb_{CPA}3$ and $mAb_{CPA}5$, were also coupled to a commercial preparation of ADH-agarose (*18*). As shown in Table 4, the degree of binding of the oxidized antibodies to the agarose was similar to that obtained for Eupergit C. The

Table 4
Binding of Oxidized Antibodies to Eupergit C-ADH[a]

	anti CPA			CPA			CPA/Ig,	
Ab #	Input, mg/g	Bound, mg/g	%	Input, mg/g	Bound, mg/g	%	mol/mol	Enhancement factor[b]
mAb1[c]	0.7	0.7	100	0.5	0.22	44	1.3	2.16
mAb3	1.5	1.0	66	0.5	0.36	72	1.5	1.97
mAb4	1.2	1.0	83	0.5	0.40	80	1.7	3.77
mAb5	0.6	0.5	87	0.5	0.20	40	1.8	-
mAb8	1.5	1.2	80	0.5	0.40	80	1.4	2.37
mAb9	0.5	0.5	100	0.5	0.20	40	1.6	2.31
mAb14	0.8	0.8	100	0.5	0.30	60	1.6	2.13
mAb3[d]	0.95	0.5	55	0.5	0.12	24	1.0	
mAb5[d]	0.5	0.4	80	0.5	0.14	28	1.5	

[a]For experimental details, *see* legend to Table 1.
[b]Figures obtained by division of the mol/mol values of this table with those of Table 1.
[c]Name of antibody is abbreviated; mAb1 is $mAb_{CPA}1$, and so on.
[d]Binding of antibodies to ADH-agarose.

amount of CPA bound to the antibodies coupled to ADH-agarose was, however, lower than that bound to Eupergit C-ADH under similar experimental conditions.

In Fig. 4, the antigen-binding activity and the specific binding activity of the $mAb_{CPA}14$ antibody, coupled to the matrix by the two methods, are presented as a function of antibody load to the matrix. The values obtained for the oxidized antibodies were always higher than those obtained by the standard binding procedure. As might be expected, in both cases, the specific activities tended to decrease with increasing antibody loads on the beads, apparently owing to steric hindrance effects and diffusion limitations.

Leakage of Immobilized Antibodies

A prerequisite for the application of any method for immobilization of antibodies on solid carriers for use in immunoaffinity purification of antigens is the stability of the bond between the antibody and matrix. In order to test the leakage of antibodies from the oxirane- and ADH-modified matrices, intact or oxidized IgG, respectively, were coupled to the matrices, which were in turn incubated with PBS and several elution buffers. No leakage was detected when the ADH-modified beads were stored in PBS for over 4 d or when incubated for 2 h with 0.2*M* glycine-HCl buffer, pH 2.0, or 3*M* Na-thiocyanate, pH 7.5. In contrast, incubation of the oxirane beads under the same conditions with 0.2*M* glycine-HCl buffer, pH 3.0, or 3*M* sodium thiocyanate, resulted in the release of 15 and 4 ng of

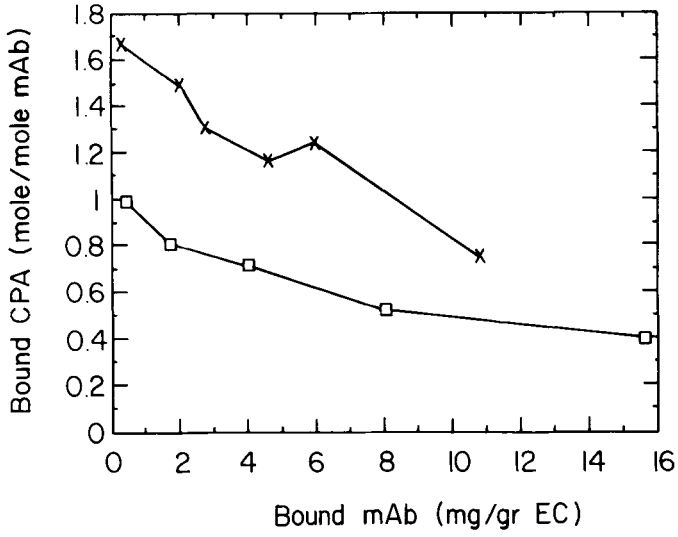

Fig. 4. Specific antigen-binding activity of immobilized $mAb_{CPA}14$. Antibody was immobilized onto Eupergit C beads, as described in the legend to Fig. 1. Specific-binding activity was calculated as the antigen-binding activity, determined by the enzymatic activity of CPA bound to the conjugated antibodies, divided by the amount of bound antibody. (x---x) Oxidized antibodies coupled to Eupergit C-ADH beads, (□---□) nonoxidized antibodies coupled to Eupergit C beads.

IgG, respectively, out of the 5 mg that were immobilized (corresponding to 1.5 and 0.5×10^{-6} of the immobilized antibodies released per hour, respectively).

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