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Review

# Salt-induced immobilization of affinity ligands onto epoxide-activated supports

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**Abstract**

The immobilization of affinity ligands onto epoxy-activated stationary phases is enhanced at high concentrations of certain salts, such as ammonium sulfate and potassium phosphate. This enhancement is thought to occur because of a salt-induced hydrophobic interaction between the affinity ligand and the surface of the stationary phase. The increase in concentration of the affinity ligand near the reactive epoxy groups leads to an increase in the rate of reaction between the nucleophilic groups on the affinity ligand and the epoxide. The salt-induced enhancement is applicable to proteins and nucleotides at neutral pH and to small affinity ligands at elevated pH. In most cases, the hydrolysis of the epoxy groups does not limit the amount of affinity ligand immobilized. This review discusses the use of high salt concentrations to immobilize proteins, oligonucleotides and peptides to epoxy-activated silica and polymer supports. These modified supports can be used in affinity applications such as affinity chromatography or immunoassays. 

*Keywords:* Reviews; Stationary phases, LC; Epoxy-activated stationary phases; Salt effects; Affinity adsorbents; Proteins; Oligonucleotides; Peptides

## Contents



## 1. Introduction

Epoxy-activated affinity supports have been widely used for many applications in affinity chromatog-

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raphy and other affinity applications such as immunoassays. Attachment of very diverse ligands, such as proteins [1,2], nucleic acids [3] and various small molecules [4,5], to the support via nucleophilic addition to the epoxide ring have been applied successfully. The mode of attachment of the affinity ligand to the epoxide phase is shown in Fig. 1A. The reactive groups on the affinity ligand are a nucleophilic primary or secondary amine, a sulfhydryl group or, less commonly, a hydroxyl group [4]. When both the support and affinity ligand are stable to high pH, the addition of affinity ligands is conducted under basic conditions. When the nucleophilic group is an amino group, the pH of the reaction is usually above nine, so that the amine is unprotonated, while for hydroxyl-containing ligands, the pH is usually above 11 so that the reactive nucleophile is the oxy anion [4]. Reactions with affinity ligands containing sulfhydryl groups are conducted at neutral pH [4] or at slightly alkaline pH where the sulfhydryl group becomes ionized [6].

For supports such as silica or glass and for ligands such as proteins or nucleic acids that are unstable under high pH conditions, epoxy-activated matrices have not been widely used. Because of the low reactivity of amino and hydroxyl groups at neutral pH, the epoxy-activated phases have been criticized as being unreactive compared to other activated phases [7]. Recently, a technique has been described for overcoming this problem of poor reactivity at neutral pH [1–3]. In the presence of high concentrations of certain salts, efficient immobilization of proteins [1,2] and nucleic acids [3] at neutral pH has been reported. The increased coupling reactivity

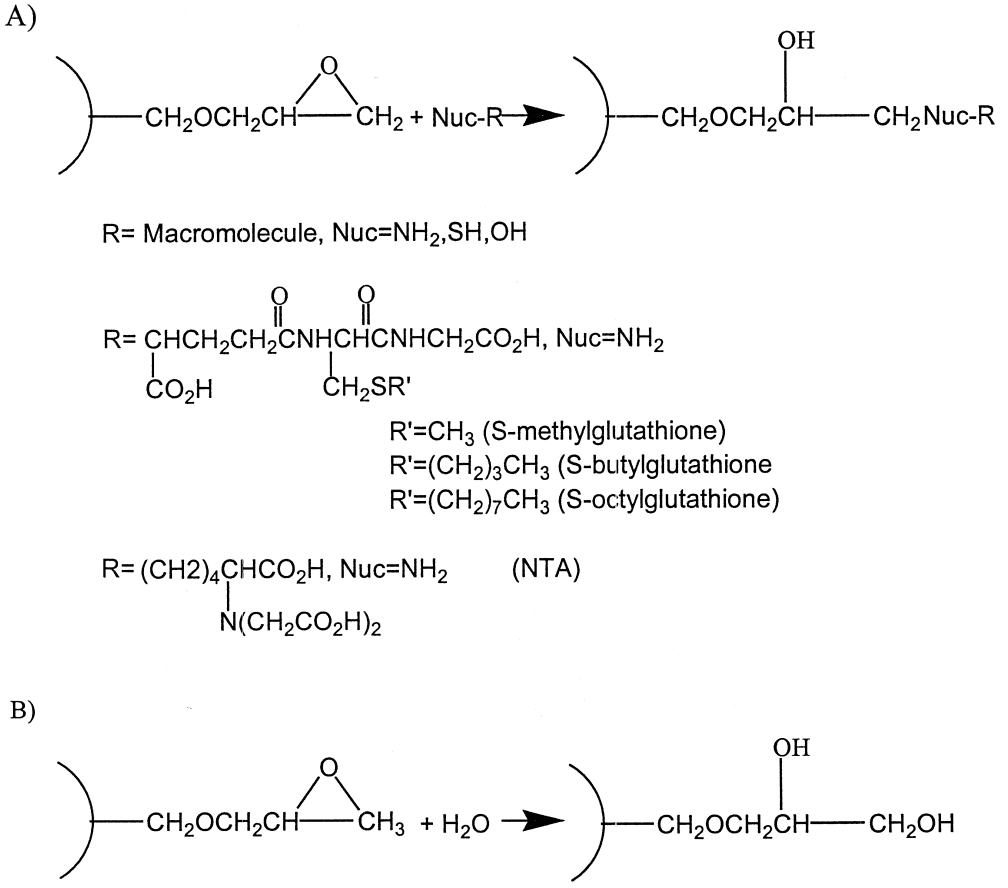


Fig. 1. Reaction of epoxy-activated affinity phase. (A) Nucleophilic affinity ligand and (B) hydrolysis.

was explained as resulting from a salt-induced association between the macromolecule and the surface of the affinity support, thereby increasing the effective concentration of the nucleophilic groups on the macromolecules near the epoxide reactive sites. The use of high salt concentrations to promote the reaction of small affinity ligands at high pH has also been shown to be beneficial [5]. This review summarizes these findings.

Other investigators [8,9] have used conditions at high salt concentrations for the efficient coupling of proteins to silica-based epoxide affinity phases with the retention of biological activity, as evidenced by the affinities of the bound moieties for mobile phase components. Dependence of protein immobilization on salt concentration has also been described for the coupling to polymer-based supports [10–13].

## 2. Model for the reaction between affinity ligand and activated affinity phase

The reaction between affinity ligands and the epoxide group on the affinity support can be represented by the following reaction scheme

$$P + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} P \cdot L \xrightarrow{k_2} P\text{–}L$$

where P is affinity ligand in solution, L is the stationary phase containing the epoxy groups, P·L is the noncovalently bound ligand on the stationary phase and P–L is the ligand covalently bound to the surface through reaction with the epoxy groups. According to this model, any perturbation in the immobilization mixture that shifts the equilibrium to increase the ratio of [P·L]/[P] [L] will increase the concentration of ligand near the epoxide groups along the surface of the support. The increase in the concentration of P·L may result in faster rates and an increase in the amount of product, P–L.

When the reaction is quenched, the amount of ligand, $P_m$, measured in solution is the sum of P and P·L. The rate constant ($k_2$) for the attack of a ligand-associated nucleophile on an epoxy group is assumed to be small compared to both the rate constant of formation ($k_{+1}$) and the rate constant ($k_{-1}$) of breakdown of P·L. Based on this assumption, the concentration of P·L can be represented as

$$[P \cdot L] = [P][L]K$$

where $K = k_{+1}/k_{-1}$. Then,

$$d[P\text{–}L]/dt = k_2 [P \cdot L] = k_2 K[L][P]$$
$$= k_2 K[L_0][P] \quad (1)$$

where $[L_0]$ is the initial concentration of epoxide ligands on the stationary phase and is large compared to [P·L]+[P–L]. From the identities $[P\text{–}L] = [P_0] - [P_m]$ and $[P_m] = [P]\ (1 + K\ [L_0])$, Eq. (1) can be modified and integrated to yield Eq. (2)

$$\log f = -k_2 K[L_0]t/(2.3)(1 + K[L_0]) \quad (2)$$

where $f$ is the fraction of ligand that is not covalently bound to the stationary phase and is equal to $[P_m]/[P_0]$. To test this model, $f$ was determined after a constant time (20 h) for a number of proteins [2]. A plot of log $f_a$ ($f_a$ is the fraction of free protein remaining in solution after 20 h) as a function of ammonium sulfate concentration for ribonuclease A, lysozyme and α-chymotrypsin is shown in Fig. 2.

If $K[L_0]$ is less than one, Eq. (2) reduces to

$$\log f_a = -k_2 K[L_0]t_a/2.3 \quad (3)$$

where $t_a$ is the duration of the reaction (in this study, 20 h). According to Eq. (3), log $f_a$ is directly proportional to $K$. Partition of the protein along the epoxide phase, represented by the equilibrium constant $K$ in Eq. (3), is assumed to be a function of salt concentration, analogous to the salt-induced associations that occur between proteins and the stationary phase in hydrophobic interaction chromatography (HIC). Melander et al. [14,15] have shown for HIC that the distribution of the protein solute between the stationary and mobile phases parallels the dependence of protein solubility on salt concentration. Protein solubility at very low salt concentrations at first increases with increasing salt concentrations (the salting-in effect), and then decreases as the salt approaches high concentrations (the salting-out effect). The logarithmic dependence of protein solubility is non-linear with respect to salt at low concentrations and becomes linear at higher concentrations, a characteristic that is also found for log $k'$ versus salt concentration in HIC [14]. In this

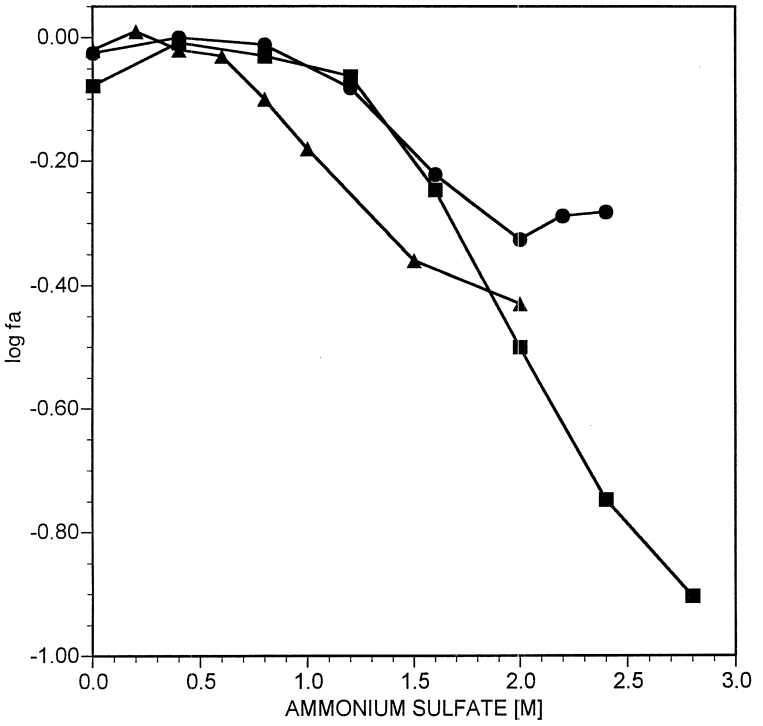


Fig. 2. Log $f_a$ vs. ammonium sulfate concentration. Protein was dissolved in solutions containing varying amounts of ammonium sulfate. The protein solution was reacted with the epoxy affinity phase and, after reaction for 20 h, the remaining protein was removed from the particles by washing. The amount of protein bound to the support was determined spectrophotometrically by the difference between the amount of protein added and the amount of protein in the washes [2]. ■ = Ribonuclease A; ● = lysozyme; ▲ = α-chymotrypsin.

study, trends similar to these [14] were found for the dependence of protein immobilization on the epoxide support. The dependence of log $f_a$ on ammonium sulfate concentration appears to be nonlinear at low salt concentrations and approaches a linear dependence at higher concentrations. This can best be seen for ribonuclease A in Fig. 2. Similarly for lysozyme and α-chymotrypsin, the fraction of protein immobilized is independent of salt concentration at low concentration and tends to become linearly dependent at higher concentrations (Fig. 2). Unlike ribonuclease, however, a region of salt concentrations is reached where the fraction of lysozyme and α-chymotrypsin immobilized becomes independent of concentration. In this region of salt concentrations, it is hypothesized that a very large fraction of the protein is already associated with the solid phase and any increase in salt concentration does not lead to an appreciable increase in P·L. The extent of immobilization at this region of salt concentrations is only a function of the rate constant $k_2$.

## 3. Immobilization of proteins

Figs. 3 and 4 show the dependence on ammonium sulfate concentration for the immobilization of 13 proteins onto an epoxy-activated silica-based HPLC support [2]. Table 1 lists the approximate concentration of ammonium sulfate needed for 90% immobilization in 20 h. All but three of the proteins exhibited 95–100% immobilization within 20 h, and the salt concentration at which this occurred depended on the individual protein examined. Of those that failed to react completely, ribonuclease A attained 85–90% immobilization while α-chymotrypsin reached 60 to 65% and lysozyme attained only about 50% immobilization. Some of the proteins in

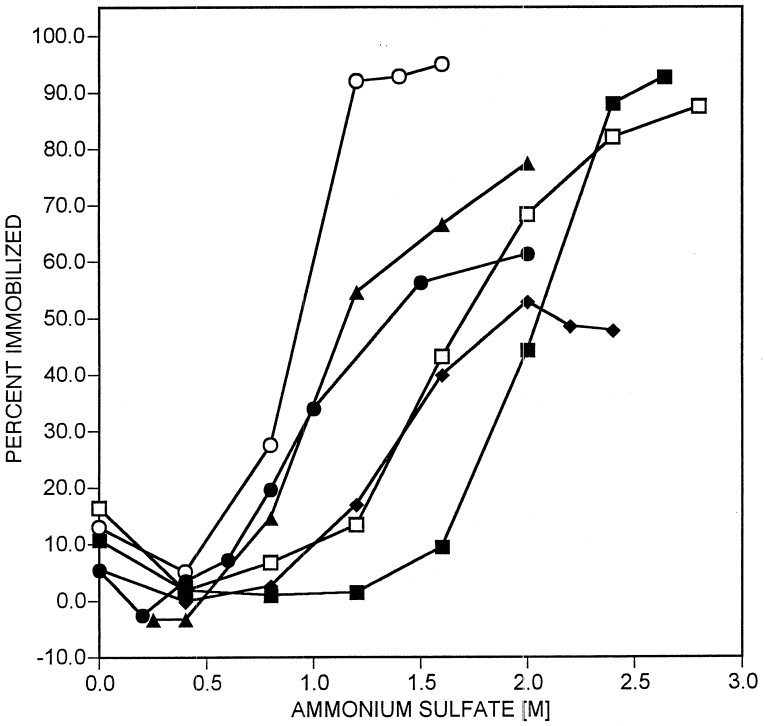


Fig. 3. Percent immobilization of proteins on an epoxy affinity support vs. ammonium sulfate concentration for proteins that exhibit elevated immobilization at zero salt concentration. See Fig. 2 for experimental details [2]. ○=Conalbumin; □=ribonuclease A; ▲=α-chymotrypsin; ■=avidin; ●=α-chymotrypsinogen A; ♦=lysozyme.

this study (Fig. 3) also exhibited a slight increase in immobilization as the concentration of ammonium sulfate approached zero. Such an increase in immobilization could occur as a result from an enhanced association between the protein and support in the absence of salt. Protein-stationary phase associations like these have been reported [16] for the isocratic retention of proteins on a silica-based HIC stationary phase (polyvinyl alcohol). In that study, the retention of some proteins first declined as ammonium sulfate concentration increased just above zero concentration. Increases in HIC protein retention in the absence of salt or the increases in immobilization seen in this study result presumably from enhanced associations between protein and support and are reminiscent of the salting-in effect discussed previously. For some proteins studied, no enhanced mobilization was noted at zero ammonium sulfate concentration (Fig. 4).

The concentration of salt needed to immobilize a protein at pH 7 is highly protein-specific. For the commonly used IgG, we have found that concentrations of ammonium sulfate or potassium phosphate of 0.5 *M* are appropriate for efficient immobilization during a time course of 16 to 20 h. For avidin, higher concentrations (2.5 *M*) of these salts are required for immobilization in this time period. For the immobilization of proteins that have not been studied previously, a preliminary study such as depicted in Figs. 3 and 4 is required to optimize the salt concentration for immobilization in a fixed time period. During immobilization, salt-induced hydrophobic interactions of proteins can lead to either protein precipitation or to interaction with the stationary phase. In our experience, protein–stationary phase interactions occur at lower salt concentrations than those necessary for protein precipitation. This probably occurs because of the greater surface area and more hydrophobic character of the stationary phase compared to the protein.

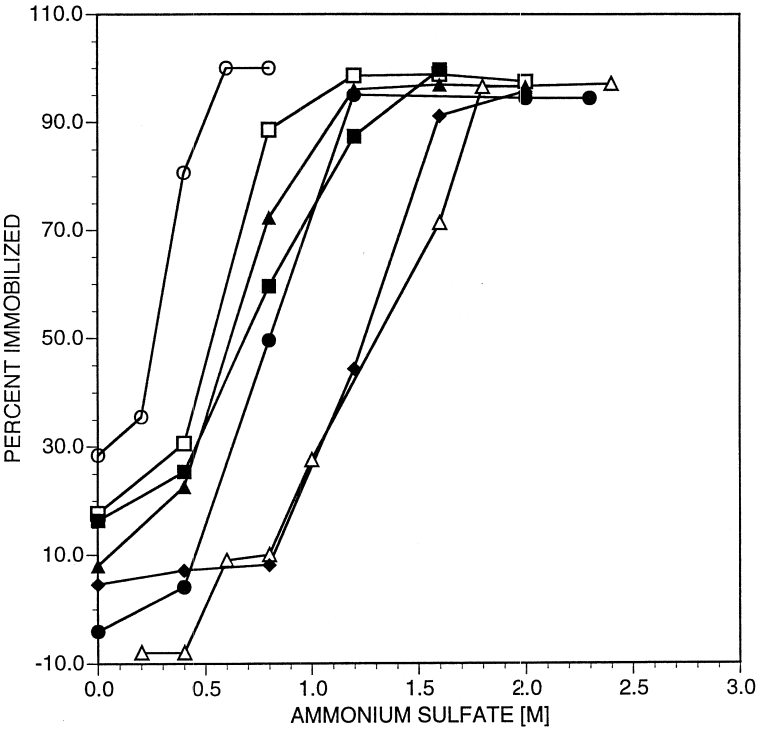


Fig. 4. Percent immobilization of proteins on an epoxy affinity support vs. ammonium sulfate concentration for proteins that did not exhibit elevated immobilization at zero salt concentration. See Fig. 2 for details [2]. ○=IgG1 k; □=transferrin; ▲=α-lactalbumin; ■=myoglobin; ●=bovine serum albumin; △=carbonic anhydrase; ♦=β-lactoglobulin B.

Table 1
Ammonium sulfate concentration required for 90% immobilization [2]

| Protein | Molecular mass | p*I* | Ammonium sulfate concentration [*M*] |
|---|---|---|---|
| Lysozyme[a] | 14 000 | 11.0 | – |
| Avidin | 68 000 | 10–10.5 | 2.4 |
| α-Chymotrypsinogen A[a] | 25 000 | 9.5 | – |
| Carbonic anhydrase | 29 000 | 8.4,9.5 | 0.8 |
| Ribonuclease A[b] | 13 700 | 9.4 | 2.8(87%) |
| Chymotrypsin[b] | 21 600 | 8.8 | 2.0(77%) |
| Myoglobin | 17 500 | 7.1 | 1.2 |
| IgG1 k monoclonal | 155 000 | N.A. | 0.4 |
| Conalbumin | 77 000 | 6.5 | 1.4 |
| Transferrin | 77 000 | 5–6 | 0.8 |
| β-Lactoglobulin B | 35 000 | 5.1 | 1.1 |
| Bovine serum albumin | 68 000 | 4.4–4.8 | 0.9 |
| α-Lactalbumin | 14 200 | 4.5–4.7 | 1.0 |

[a] These proteins were not 90% immobilized. See text.
[b] This was the highest concentration examined.

Under the conditions of high salt concentrations at pH 7, a number of immobilized proteins have retained activity. Human IgG, albumin and $\alpha_1$-acid glycoprotein were immobilized on a silica-based high-performance liquid chromatography (HPLC) epoxy-activated support using ammonium sulfate and the resulting affinity phase was then used to purify the corresponding protein antibodies from whole goat antiserum [1]. Likewise, the antibodies to these three proteins were immobilized at different densities on this support and used to study the binding capacity for the corresponding proteins. Using potassium phosphate, proteins have been immobilized onto epoxy-activated 6 mm glass beads [17] for use in immunoassays. These beads were found to be convenient supports for radioimmunoassays and for enzyme linked assays. Avidin was exposed for 16 h at increasing concentrations of potassium phosphate. The avidin-derivatized beads were treated with horseradish peroxidase coupled with biotin and the amount of avidin immobilized on the bead was measured by a coupled enzyme assay (Fig. 5). The amount of avidin bound to the beads as a function of the potassium phosphate concentration shows the same salt concentration dependence as for many of the proteins shown in Figs. 3 and 4. At low potassium phosphate concentrations, there is little or no immobilization of avidin, while at higher concentrations, the amount immobilized is proportional to the salt concentration. Other protein molecules that have been successfully immobilized onto epoxy-activated affinity supports with retention of activity are the $\alpha$ chain of the interleukin-2 receptor, a human IgM monoclonal antibody and a human/mouse chimeric $IgG_1$ [17].

## 4. Immobilization of nucleotides

Cyanogen-bromide-activated supports, in particu-

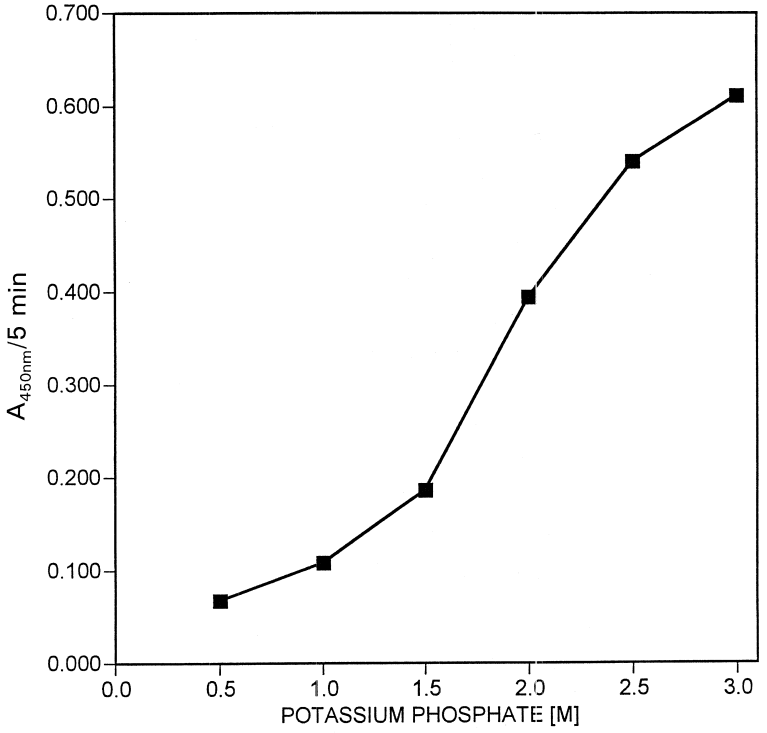


Fig. 5. $\triangle A/5$ min vs. potassium phosphate concentration. Epoxy-activated glass beads were incubated with avidin at various concentrations of potassium phosphate. A single avidin-derivatized bead was incubated with a solution of biotin–horseradish peroxidase. After washing, the resulting bead was incubated with a horseradish peroxidase substrate and the absorbance was determined after 5 min [17].

lar, have been used extensively for the immobilization of DNA strands, and the resulting supports were subsequently used to isolate transcription factors [18]. With this very reactive activation chemistry, the DNA strands can become covalently attached at several points along the strand. This multisite attachment may interfere with DNA binding specificity. This possibility has led to an interest in the site-specific immobilization of DNA. Immobilization of oligonucleotides modified at the 5′-phosphate terminus with a mercaptoalkyl group provides two possible advantages. First, it introduces a nucleophilic sulfhydryl group that has a greater reactivity than the nucleotide bases. Thus, other less reactive activation chemistries for affinity supports could be explored for the immobilization of DNA at sites other than the nucleotide bases. Second, the alkyl chain may act as a spacer arm to displace the DNA affinity ligand away from the surface of the support. Using this approach, 5′-mercaptopropyl derivatives of double-stranded eicosomeric and dodecameric oligonucleotides were immobilized on an epoxide-activated Sepharose phase [19]. In this study, about 15–20% of the DNA was immobilized in 0.50 *M* carbonate buffer at pH 10.

We have used a thiol-modified DNA strand to explore the immobilization of nucleotides onto an epoxide-activated HEMA polymeric phase [3]. A 24-base oligomer modified at the 5′-terminus with a mercaptohexyl group (HS-d24) and a complementary 22-base oligomer (d22) were synthesized. The annealed double-stranded DNA (dsDNA) included a recognition site for the transcription factor NF-κB. The extent of immobilization at pH 7 of the dsDNA as a function of potassium phosphate concentration was determined after a reaction time of 120 h (Fig. 6). Similar results were obtained at pH 8. At salt concentrations approaching 3 *M*, about 95% of the dsDNA was immobilized on the stationary phase, as estimated by the amount of DNA in the wash filtrate.

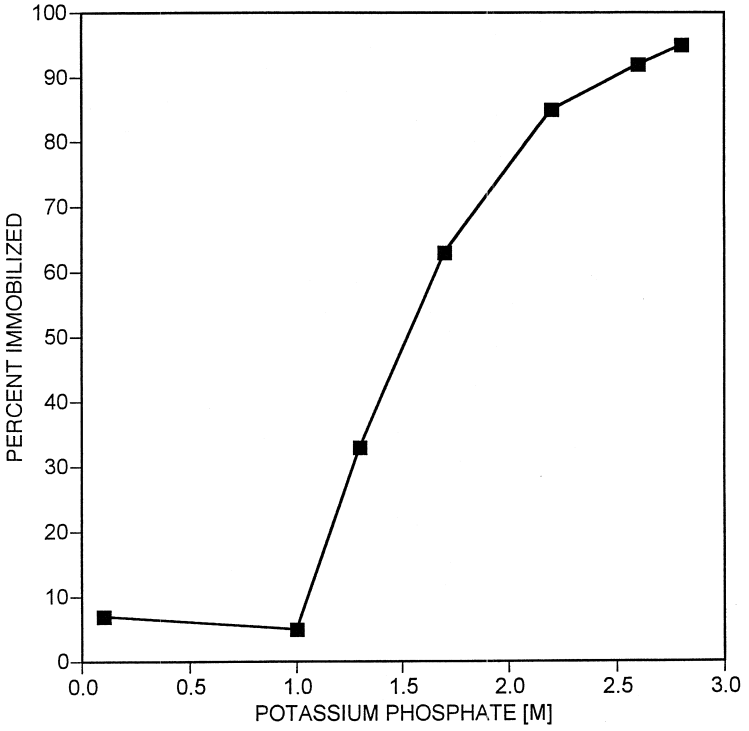


Fig. 6. Percent immobilized double-stranded DNA vs. potassium phosphate concentration. Double-stranded DNA was incubated with the epoxy-activated stationary phase for 65 h with various concentrations of potassium phosphate. After washing the product, the unreacted DNA was determined from the $A_{280}$ of the product wash [3].

The shape of the curve for the fraction of dsDNA immobilized at increasing concentrations of salt is similar to that observed for proteins. At low salt concentrations (0–1 *M*), the amount of dsDNA immobilized is insensitive to salt concentration. Above 1 *M* potassium phosphate, the fraction immobilized is quite dependent upon salt concentration. In a time course study, at 2.7 *M* potassium phosphate and pH 7, it was found that 90% of the dsDNA was associated with the stationary phase after 70 h. For the dsDNA, there seemed to be little reaction of the nucleotide bases with epoxide groups. The dsDNA affinity phase was subjected to conditions (water at 80–90° for 10 min) that should lead to the dissociation of the d22 strand from the immobilized HS-d24 strand. The filtrate of this treatment was subjected to analysis by reversed-phase HPLC, and it was found that 85% of the d22 associated with the phase was in the supernatant. If there had been immobilization of the DNA strands through reaction of the epoxides with the bases, a much lower fraction of d22 would have been expected in the supernatant. The immobilized dsDNA was packed into an HPLC column and was successfully used to purify p50 protein, the DNA binding element of NF-κB. Also, p50 protein from the nuclear extracts of phorbol-stimulated HeLa and Jurkat cells was retained on the affinity column and eluted with a salt gradient.

## 5. Immobilization of small affinity ligands

The immobilization of small molecules on epoxy-activated phases (Fig. 1 A) is generally carried out at higher pH values, from 9 to 11, so that the reactivity of unprotonated amino groups can be utilized [4]. These pH conditions can be used since small molecules are often not as susceptible to degradation at high pH as are macromolecules. Attachment of the small molecule affinity ligand via nucleophilic addition of an amino group to the epoxy moiety has a potential advantage for affinity chromatography at physiological pH, because the resulting amino group in the coupled product is able to retain its positive charge. Since the binding of a small molecule to a protein site often has an electrostatic component, retention of this positive charge is often important. For many affinity activation chemistries, the amino group on the affinity ligand becomes attached to the stationary phase via the formation of an amide bond. At physiological pH, this nitrogen in the amide bond is uncharged and may result in decreased binding between the affinity ligand and the protein site.

Development of more efficient ways to immobilize small affinity ligands is of value since, often, the affinity ligands are not available in large quantities. We have investigated the effect of high salt concentration on the immobilization of three tripeptides of increasing hydrophobicity and a highly polar ligand, nitrilotriacetic acid (NTA) (see Fig. lA). The three tripeptides were *S*-alkyl derivatives of glutathione with increasing hydrophobicities in the order *S*-methyl, *S*-butyl and *S*-octyl. The immobilization of these ligands was investigated on a HEMA-based, epoxy-activated HPLC phase [5]. The extent of reaction of small molecules with an epoxy-activated stationary phase was generally in accord with previous findings for the extent of reaction of this type of surface with macromolecules. As the hydrophobicity of the small molecule increases, the amount of peptide immobilized increases for a defined reaction time. For the *S*-alkylglutathiones, the hydrophobicity increases as the length of the alkyl chain increases. Thus, one expects that *S*-octylglutathione would react to a greater extent in 90 h than would *S*-butylglutathione, and that *S*-butylglutathione would be expected to react to a greater extent than *S*-methylglutathione. In carbonate buffer, this was found to be the case (Table 2). NTA, which is a much smaller molecule than the *S*-alkylglutathione tripeptides and which has three negative charges at pH 10.5, is more polar than the *S*-alkylglutathiones and, therefore, would be expected to be immobilized to a lesser extent in a given time period. As seen in Table 1, the percentage of NTA immobilized from solution was less than that for any of the glutathiones. The concentration of NTA in the initial solution was approximately threefold greater than for any of the glutathiones, yet, under these more severe conditions, only 2% of the available ligand could be immobilized in 90 h. Except for *S*-methylglutathione, the concentration of carbonate buffer had little effect on the amount of small ligand immobilized. For *S*-methylglutathione, as the salt concentration decreased, the amount of ligand immobilized increased. It is unclear to us why *S*-methylglutathione should

Table 2
Immobilization of NTA and *S*-alkylglutathiones as a function of salt type and salt concentration[a]

| Buffer | NTA[b] | | *S*-Methylglutathione[b] | | *S*-Butylglutathione[b] | | *S*-Octylglutathione[b] | |
|---|---|---|---|---|---|---|---|---|
| | μmol/g | % Immobilization | μmol/g | % Immobilization | μmol/g | % Immobilization | μmol/g | % Immobilization |
| 2.0 *M* Carbonate | 29.0±1.0 | 2 | 16.1±0.6 | 3 | 40.9±0.5 | 9 | 68.5±1.4 | 15 |
| 1.0 *M* Carbonate | 24.9±0.1 | 2 | 21.3±2.0 | 4 | 35.4±2.1 | 8 | 67.6±1.3 | 14 |
| 0.5 *M* Carbonate | 25.8±0.5 | 2 | 29.0±0.7 | 6 | 40.3±0.4 | 9 | 67.7±0.6 | 14 |
| 2.0 *M* Phosphate | 32.9±3.5 | 2 | 83. 8±2.8 | 18 | 111.1±4.0 | 24 | 92.9±2.4 | 20 |
| 1.0 *M* Phosphate | 34.4±2.8 | 2 | 56.2±4.1 | 12 | 91.8±14.6 | 20 | 84.3±2.2 | 18 |
| 0.5 *M* Phosphate | 35.9±1.2 | 2 | 32.7±0.6 | 7 | 48.5±9.1 | 10 | 57.0±2.2 | 12 |

[a] The amount of immobilized alkyl glutathione was determined by acid hydrolysis of the stationary phase and analysis of the resulting amino acids by HPLC. The amount of NTA was determined by the amount of nickel bound by the derivatized stationary phase [5].
[b] Per gram of epoxy-activated packing, the μmoles of ligand initially in solution were 1530 for NTA, 470 for methylglutathione, 470 for butylglutathione, and 470 for octylglutathione.

act differently from the other ligands. Immobilized *S*-octylglutathione on a HEMA epoxide stationary phase was used for the successful affinity chromatography of a number of glutathione *S*-transferase enzymes [20–24].

The effect of potassium phosphate concentration on the extent of immobilization is dramatically different from the effect of potassium carbonate concentration (Table 2). For all three of the *S*-alkylglutathiones, as phosphate concentration increases, the amount of ligand immobilized also increases. Increasing phosphate concentration is known to increase hydrophobic interactions [6] and is presumably responsible for causing salt-induced hydrophobic association between the small affinity ligand and the stationary phase. This association then leads to an increase in reaction rate, resulting in more efficient immobilization. Phosphate at 0.5 *M*, however, does not appear to be any more effective than carbonate at the same concentration. For the highly polar ligand NTA, the differences in the salt type and phosphate concentration seem to have little effect. The increase in the extent of immobilization of NTA when comparing phosphate and carbonate is at best minimal, with perhaps a slight advantage in using phosphate. Increasing the phosphate concentration had very little effect. Apparently, for very hydrophilic ligands such as NTA, the phosphate concentrations examined in this study are not sufficient to induce a hydrophobic interaction with the stationary phase. The resulting NTA phases were successfully used to produce a nickel affinity column to purify a recombinant hexa-histidine-tagged recombinant protein [5].

## 6. Hydrolysis of epoxy groups

At elevated pH or for long reaction times, the loss of epoxy groups due to hydrolysis might limit the amount of affinity ligand immobilized. The hydrolysis rate (Fig. 1B) at 23°C and pH 10.5 was measured by taking aliquots of the epoxy-activated HEMA [5] suspended in potassium carbonate buffer (pH 10.5) at predetermined time points. Using the method of Scoble and Scopes [25], the hydrolysis reaction at 23°C and pH 10.5 was followed by determining the number of epoxy groups remaining. The results are shown in Fig. 7 and the fitted curve is derived from the first order equation:

$$\mu\text{mol epoxy} = 135\, e^{-at}$$

where the first-order rate constant, $a = 0.005\ 2\ h^{-1}$. The second-order rate constant is $2.6 \cdot 10^{-8}$ mol·s based on the assumption that the rate of hydrolysis at pH 10.5 is a pH-independent reaction. Previous investigation of the solution kinetics for the addition of water to epoxy groups indicates a pH-independent region from 7 to 11 [26]. The rate of water addition to the epoxy groups of activated HEMA resin is very similar to the rate of water addition in solution to the hindered isobutylene epoxide. For the hindered epoxide, the second-order rate constant is $3.0 \cdot 10^{-8}$ l/mol·s [26].

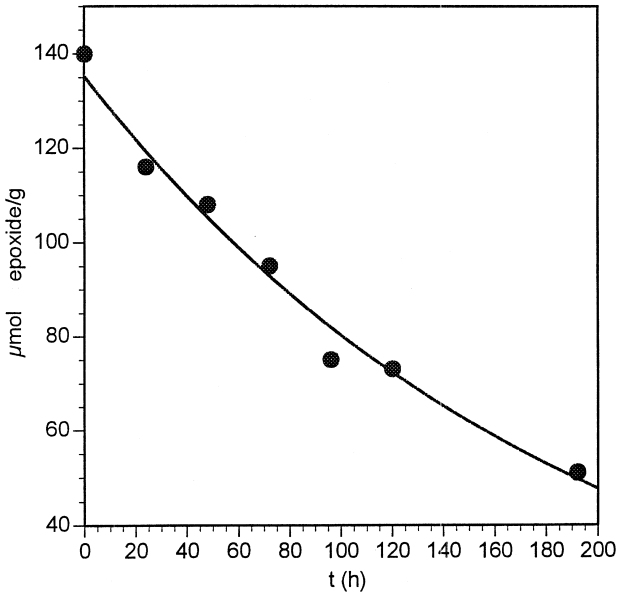


Fig. 7. Hydrolysis of epoxy-activated HEMA at pH 10.5. Epoxy-activated HEMA was suspended in potassium carbonate (pH 10.5) and, at selected time points, an aliquot was removed. The aliquot was washed and reacted with mercaptoacetic acid. The amount of unreacted epoxy groups was determined by titration of the immobilized mercaptoacetic acid [5,25].

At 2 *M* potassium phosphate for the three *S*-alkylglutathiones and at 1 *M* potassium phosphate for *S*-butylglutathione and *S*-octylglutathione, a maximum of 80 to 100 μmol/g of immobilized ligand is approached (Table 2).The limiting factor seems not to be the total amount of epoxy groups on the HEMA, since, from the titration data, this value was found to be 140 μmol/g. A more likely source of the limitation in the amount immobilized is due to the competing hydrolysis of the epoxy groups during the immobilization reaction. In the absence of any ligand, 40% of the epoxy groups hydrolyze, leaving a total of 83 μmol/g of epoxy groups after 90 h. The amount of ligand immobilized at 90 h would be a function of the rate of immobilization balanced against the rate of hydrolysis. For NTA at any phosphate concentration, the limiting factor would be the rate of immobilization and not the rate of hydrolysis. For the *S*-alkylglutathiones at higher phosphate concentrations, the limit of reaction is probably a function of the rate of epoxide hydrolysis. For immobilization in carbonate buffer, the extent of immobilization for any of the ligands investigated here is a function of reaction rate only.

For the immobilization of macromolecules at pH 7–8, the amount of either protein or oligonucleotide immobilized will not be limited by epoxide hydrolysis, even though the rate of epoxide hydrolysis at these pH values is expected to be similar to that measured at pH 10.5 [26]. Because of the greater steric bulk of the macromolecules, the number of the macromolecules on the surface will be far less than for small affinity ligands. Since both macromolecules and small affinity ligands need to be attached to the surface by only one covalent linkage, far fewer reactive epoxide groups are required for the maximum number of macromolecules than for the maximum number of small ligands.

## 7. Conclusion

High salt concentrations of potassium phosphate or ammonium sulfate are clearly beneficial in the coupling of affinity ligands to epoxy-activated stationary phases. This has been demonstrated for proteins on epoxy-activated silica HPLC particles, for proteins on epoxy-activated glass beads, as well

as for oligonucleotides and small affinity ligands on an epoxy-activated HEMA-based HPLC particle. For proteins and oligonucleotides, high coupling efficiencies have been demonstrated at neutral pH, thereby helping to preserve the integrity of the immobilized ligand in the coupling process. The coupling of proteins and oligonucleotides is described by a model where the high salt concentrations cause a hydrophobic-induced association between the macromolecule and the epoxy-activated surface. This association results in an increased effective concentration of the macromolecule, which, in turn, leads to an increase in the immobilization rate. For small, hydrophobic molecules, the high salt concentrations also increase the efficiency of immobilization. For a highly polar ligand such as NTA, no effect was seen by using high salt.

In the coupling of single-stranded oligonucleotides, it appears as if the chains are bound at multiple sites to the surface of the stationary phase [3]. In the case of the dsDNA, this multiple binding is minimized. The lack of multiple binding to the surface of the dsDNA probably results from a steric protection of the reactive guanine and adenine bases in the helical structure such that they are unable to react with the epoxy groups. It is probable that the immobilization of proteins also occurs at multiple sites, but this possibility was not investigated.

As a general rule, for the immobilization of small molecules through reaction of their amino groups with epoxy-activated supports, a greater efficiency of reaction will be seen using increased concentrations of potassium phosphate. Increasing concentrations of carbonate buffer are not effective in increasing the immobilization of small ligands. For very polar ligands such as NTA, increasing the concentration of phosphate or carbonate is not effective in increasing the immobilization efficiency. For cases where the efficiency of immobilization is low, such as in the case of NTA, it may be necessary to extend the time of reaction. Extending the time of reaction does have limits, however. Although the rate of epoxide hydrolysis is slow, it can in some cases be limiting as to the final amount of ligand immobilized. Another alternative is to increase the amount of ligand in solution so as to increase the rate of the immobilization reaction.

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