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N-Hydroxysuccinimide-Activated Glycine-Sepharose

Hydrolysis of Activated Groups and Coupling of Amino Compounds

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ABSTRACT

Glycine-Sepharose CL 6B, activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), was used as a model compound to study the hydrolysis and aminolysis of immobilized NHS-activated groups. For comparison, the soluble analog *N*-*t*-BOC-glycine-NHS has been used.

Coupling of amino compounds, such as aminoethanol, aminohexane, amino acids, and esters of amino acids, is fast and efficient in both organic medium and buffered aqueous medium, e.g., coupling of aminoethanol is complete within 1 min. Hydrolysis of the activated groups in aqueous medium is general base catalyzed and is, particularly in borate buffer and at higher pH (>9.0), accelerated by addition of salt. The NHS-activated glycine-CL 6B is less sensitive toward hydrolysis as compared to *N*-*t*-BOC-glycine-NHS.

When amino compounds are coupled to NHS-activated Sepharose in aqueous medium, the use of a low buffer concentration and a pH of 8.5–9.0, without salt, is recommended. In combination with salt, phosphate buffer is preferred.

Index Entries: Sepharose; carbodiimide; *N*-hydroxysuccinimide; hydrolysis; immobilization.

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INTRODUCTION

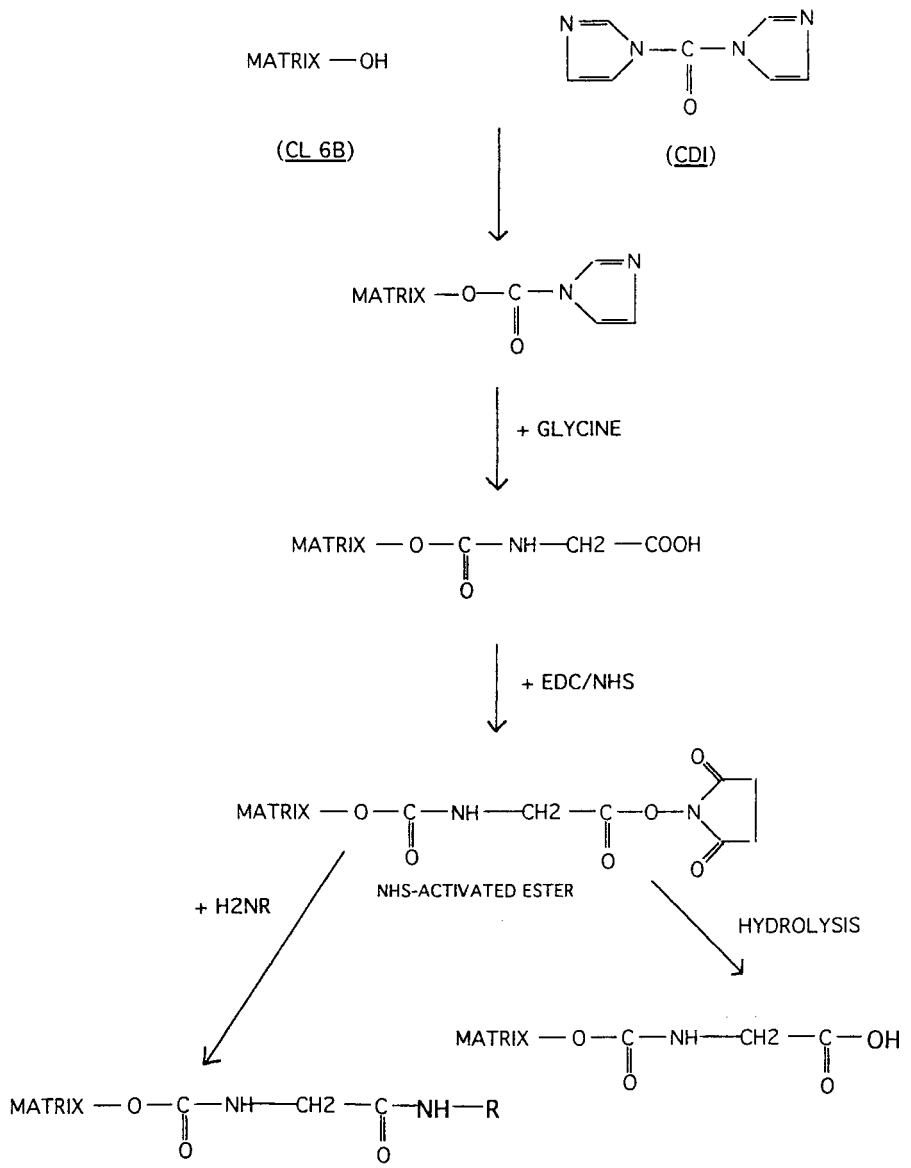
Affinity chromatography is a powerful method for isolation and purification of a wide range of biochemical substances, like enzymes, antibodies, antigens, nucleic acids, and hormones, as well as particles, like cells and viruses (*1*). The biospecific interaction between the immobilized ligand and its macromolecular counterpart may be hampered by steric hindrance, especially when no spacer is used (*2,3*).

Many different procedures have been described to activate hydroxylic groups on polymers, like Sepharose, dextran, and cellulose (*1*). To these activated polymers, bifunctional compounds can be coupled, yielding a spacer functionalized matrix. For the coupling of a ligand, the spacer must be activated. Depending on the type of functional group in the spacer (usually an amino or a carboxylate end group), a method of spacer activation must be choosen that has to be convenient in two respects: the corresponding coupling conditions do not result in deterioration of the ligand structure and the coupling efficiency as well as the ligand density must meet the requirements. Several methods for spacer activation can be used: conversion of immobilized amino groups into acyl azide groups (*4*), succinylation of carrier-bound amino groups with succinic anhydride followed by activation of the resulting carboxylate with carbodiimide (*5,6*), and reaction of amino groups with symmetrically reactive compounds, like imidoesters (*7*) or dialdehydes (*8*).

One well-known method is carbodiimide activation of gel-bound carboxylates, in the presence of *N*-hydroxysuccinimide (NHS) (*9*). NHS-activated Sepharose reacts with amino group containing ligands via the formation of a stable amide bond (Scheme 1). Gels activated this way are commercially available and are known under several trademarks. Many reports can be found in which the use of NHS-activated Sepharose derivatives is mentioned (*10,11*).

Wilchek and Miron (*12*) found substantial leakage from chromatographic media, obtained after coupling of antibodies to commercially available or homemade NHS-activated resins. It was proven that, as a result of the activation procedure with carbodiimide and NHS, β-alanine derivatives of the resins were formed (*12*). Part of the immobilized β-alanine is base-labile, and the application of the resin is less favorable for affinity purification because of the release of β-alanine.

Not much is known about the aminolysis of the immobilized active ester when coupling is carried out with relatively small amounts of amino compounds. The level of coupling of alanine to a NHS derivative of agarose was significant, but seemed to be rather inefficient as deduced from the work of Cuatrecasas and Parikh (*9*). Coupling to NHS-activated gel is often performed in aqueous medium because of the poor solubility of most biomolecules in organic solvents. The rate of hydrolysis of NHS-



Scheme 1. Reaction scheme for the preparation, aminolysis, and hydrolysis of NHS-glycine-Sepharose.

activated carboxylated gel and the influence of various aqueous buffered media on hydrolysis may be very crucial for the coupling efficiency, especially when the ligand is not added in excess. In this study, Sepharose CL 6B, carboxylated with glycine and activated with carbodiimide and NHS (Scheme 1), is used as a model compound to investigate aminolysis and hydrolysis of the gel-bound NHS ester.

MATERIALS AND METHODS

Materials

Sepharose CL 6B was obtained from Pharmacia (Uppsala, Sweden). Aminoethanol, glycine, alanine, lysine, valine, arginine, glycine-*t*-butyl ester, glycine ethyl ester, arginine ethyl ester, poly(oxyethylene) bis-amine 3350, bovine IgG, acetic succinimide, and the NHS esters of *N*-*t*-BOC-glycine and *N*-t-BOC-leucine were all purchased from Sigma (St. Louis, MO). NHS and potassium tetraborate ($K_2B_4O_7$) were bought from Fluka AG (Buchs, Switzerland). Trinitrobenzene sulfonic acid was supplied by ICN (Costa Mesa, CA). All other chemicals were obtained from Merck (Darmstadt, Germany).

Methods

Preparation of NHS-Activated Glycine-Sepharose

Sepharose CL 6B was transferred from water to organic solvents, like acetone, *N,N*-dimethylformamide (DMF), or dimethylsulfoxide (DMSO), or from organic solvents to water by stepwise washing on a glass filter (Borosilikat, Robu Glas, G3) with threefold volumes of 30 and 70% solvent mixtures, followed by extensive washing with pure solvent (up to five times with fivefold volumes). Packed gel volumes were determined after centrifugation (10 min at 2600 rpm) in a Sorvall GLC-1.

Sepharose CL 6B was activated by adding 3 vol of 0.25*M* carbonyldiimidazole in acetone to 1 vol of packed gel in acetone. After 3–4 h of shaking, the activated gel was washed with acetone, transferred to water, and suspended in a 1*M* solution of glycine in water at pH 9.6–9.7. Reaction of CDI-activated gel with glycine was performed for at least 16 h. The amounts of glycine-derived carboxylates, coupled to the gels, were determined by conductivity titration. Titration was usually carried out with an amount of hydrogel corresponding with 100 μmol ligand or more. Before titration, the gel was washed extensively with deionized water, 2*M* NaCl, and again deionized water, after which the pH of the gel suspension was adjusted with 0.1*M* NaOH to a value of 10.0–11.0 (depending on the basicity or acidity of the ligand). For the assay, 0.100*M* HCl was added with an autoburette, whereas conductivity and pH were measured continuously.

Sepharose, carboxylated with glycine (three batches, glycine contents 100, 117, and 195 μmol/mL packed gel), was activated in DMF or DMSO by addition of a molar excess (calculated with regard to the amount of carboxylate groups) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and NHS (a fivefold molar excess in the case of Sepharose with a glycine content of 195 μmol/mL, and a tenfold molar excess for a glycine content of 100 and 117 μmol/mL). Activation time was at least 3 h, after which the gel was washed with organic solvent on a glass filter (G3). In most experiments, the gels with lower ligand densities (100 and 117 μmol/

mL) were used. The coupling of amino compounds in buffer was studied with the higher ligand density gel (195 μmol/mL).

Coupling Efficiency in DMF

The coupling yields of aminoethanol and aminohexane to NHS-activated gel were determined in DMF as coupling medium, at room temperature. The amino compounds were added in various amounts. All amounts were less than equimolar compared to the quantity of activated groups. After a coupling time of 2 h, the gels were transferred to buffer (PBS, pH 7.4) and incubated for 16 h or longer to hydrolyze activated ester, which was still present on the gel. Activated carboxylates that have undergone aminolysis with aminoethanol or aminohexane are not ionizable anymore. Conductivity titration of the gels was used to measure the amounts of carboxylates that had not reacted with aminoethanol or aminohexane.

Elemental analysis of washed and freeze-dried hydrogels was carried out with a computerized Elemental Analyzer 1106 (Carlo Erba Instruments, Milan, Italy) and resulted in values for the nitrogen content, after correction for hydration.

Trinitrobenzenesulfonic Acid (TNBS) Assay

The progress of the reaction between amino compounds and NHS-activated glycine-Sepharose was studied as a function of time. Reaction was performed in DMF (when using aminoethanol and aminohexane) and in DMSO (in the case of glycine-*t*-butyl ester, poly(oxyethylene) *bis*-amine 3350 and arginine ethyl ester), at room temperature. The coupling of amino compounds to activated gel was followed by taking samples of the supernatant and subsequent treatment of the samples with TNBS. Reaction between TNBS and amino compounds yields adducts that absorb light in the wavelength regions around 340 and 410 nm. The precise position of the absorption peak in both regions depends on the identity of the amino compound. In this study, the maximal extinction in the region around 350 nm was measured. Usually, 100–200 μL of supernatant were mixed with 1.00 mL 0.1*M* borate buffer ($K_2B_4O_7$) and 100 μL of 50 m*M* TNBS in deionized water. After a reaction time of at least 30 min, the TNBS sample solution was diluted with 0.1*M* borate buffer to give an extinction (at 350 nm) of < 2.0 OD.

Hydrolysis as a Function of Time

Hydrolysis rates were determined at 22°C unless indicated otherwise. NHS-activated gel, transferred from DMF or DMSO to water, was sampled at different times and added to a 25-fold molar excess of glycine ethyl ester (pH 7.5) to block the remaining NHS-activated carboxylate groups. Reaction time was 2 h or more followed by extensive washing of the gel with deionized water, 2*M* NaCl, and again deionized water. The ligand density

as measured with conductivity titration is a direct measure for the amount of hydrolyzed groups.

Hydrolysis of NHS-activated groups was also examined with a spectrophotometric method. NHS-activated Sepharose was transferred to dioxane, and most of the dioxane was then removed by means of a water aspirator. Application of low vacuum does not completely remove the dioxane. The partly dried NHS-glycine-Sepharose had a weight of yield per milliliter of packed gel that was more than the theoretical value for the dry content (105 mg vs 83 mg). The calculated content of residual dioxane, present after resuspending the "dry" gel in aqueous medium, is $< 2.5\%$. The dry gel was suspended in buffer (50 mL), present in a syringe (50 mL) equipped with a filter (Millipore, 0.45 μm). Samples were directly injected from the filled syringe into a sample cuvet (4.5 mL), and the extinction at 260 nm was measured. Thereafter, the sample was sucked into the syringe, and after mixing the content of the syringe, the next sample was injected just before the next measuring point of time (Uvikon 930, Auto Rate Assay program). From the extinction values as a function of time, initial slopes could be calculated and converted into values of hydrolysis half-life times.

In one case, the kinetics of hydrolysis of gel-bound and soluble NHS-ester was compared in a relative way. Hydrolysis times were converted into units of hydrolysis half-life time, and the relative amounts of released NHS were indicated as cumulative fractions E_t/E_m (extinction on time t divided by the maximal extinction). A comparison was made with a theoretical curve given by the equation $E_t/E_m = 1 - e^{-0.693^{t_r}}$ (t_r = relative time, expressed in units of hydrolysis half-life time).

Determination of Coupling Efficiencies

Coupling efficiencies of amino compounds to *N-t*-BOC-glycine-NHS in buffer were determined by spectrophotometry. The sample cuvet and reference cuvet contained the active ester with and without amino compound. Amino compounds were applied in an equimolar amount with regard to the active ester. The maximal extinction value at 260 nm (observed within a few minutes) and the extinction after 24 h, the last one measured with solely buffer in the reference cuvet, were used for calculation of coupling efficiencies.

The efficiency of the coupling of amino compounds was also determined with NHS-glycine-CL 6B. The content of activated groups present on the partly dried NHS-glycine-CL 6B used in this experiment was 1.40 mmol/g partly dried material (one gram was equivalent to 4.1 mL packed gel, after swelling in deionized water). The high dry substance content is, apart from the presence of dioxane, caused by crosslinking of the gel. This crosslinking occurred as a result of the drying procedure, which phenomenon was also reported by Cuatrecasas and Parikh (*9*). Amino compounds were added in half equimolar amounts as compared to the quantity of gel-bound NHS-ester. Glycine-Sepharose, obtained after suspending

NHS-glycine-CL 6B in 0.5*M* carbonate buffer (pH 9.0) for 2 h, was used as control and incubated with the amino compounds. After a coupling time of 16 h, the supernatants were sampled and the TNBS assay was applied.

RESULTS

Coupling Efficiency in DMF

The extent of coupling of aminoethanol and aminohexane to NHS-activated glycine-Sepharose in DMF was determined. The numbers of ionizable gel-bound groups present on the gel after reaction, as determined with conductivity titration, progressively decreased with increasing amounts of aminoethanol and aminohexane (Fig. 1A). For each measuring point in Fig. 1A, the decrease of the density of carboxylate groups on the gels (in μmol/mL) was equal to the amount of added amino compound (in μmol/mL gel).

Elemental analysis showed increasing values of the N content of the gels when larger amounts of amino compound were added for coupling (Fig. 1B). With 100 μmol amino compound added/mL gel, the nitrogen content of the gel increased about 100% as compared to the gel to which no amino compound was added (Fig. 1B).

Kinetics

The reaction between amino compounds and the NHS-activated hydrogel in organic solvent was followed in time by using the TNBS assay. The time needed for completion of the coupling reaction was <1 min for aminoethanol and aminohexane, 2–3 min for glycine-*t*-butyl ester and poly(oxyethylene) *bis*-amine 3350, and 10 min for arginine ethyl ester (not illustrated).

Hydrolysis

In mixtures of DMSO and water, hydrolysis of the NHS-activated Sepharose became only significant at a water content of 30% or more, with hydrolysis half-life times of about 10 h or less (data not shown). A study was made of the hydrolysis of the NHS-activated gel-bound groups in buffered aqueous media. Hydrolysis in phosphate buffer proved to be fast and dependent on pH (Fig. 2). Hydrolysis half-life times were found to be 15 min or less, and the rate of hydrolysis increased with higher pH. The calculated half-life times of hydrolysis of NHS-glycine-Sepharose seemed to correspond with the values obtained from measurements with the model compound *N-t*-BOC-glycine-NHS (Fig. 3). Hydrolysis of *N-t*-BOC-leucine-NHS was slower compared to the hydrolysis of NHS-activated glycine derivatives (Fig. 3).

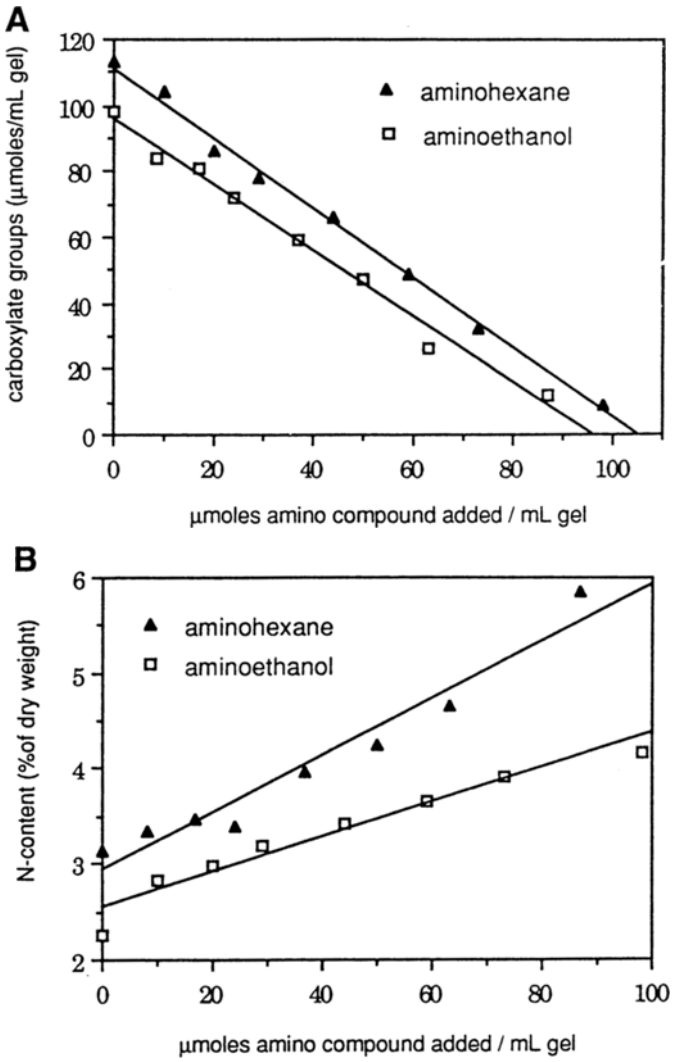


Fig. 1. Effect of addition of different amounts of aminoethanol and aminohexane, used for coupling to NHS-activated glycine-Sepharose CL 6B. **A**: The amount of carboxylate groups as determined with conductivity titration. **B**: The N content of the resulting gel. Coupling was performed in DMF for 2 h.

Hydrolysis, Buffer Concentration, and Ionic Strength

The influence of buffer concentration and ionic strength on the hydrolysis of NHS-activated glycine derivatives was studied by measuring the extinction of filtrates of the aqueous gel suspensions at 260 nm. Hydrolysis half-life times of acetic succinimide and *N-t*-BOC-glycine-NHS, in 0.3*M* Hepes (pH 7.4, 25°C) were determined as references. Acetic succinimide

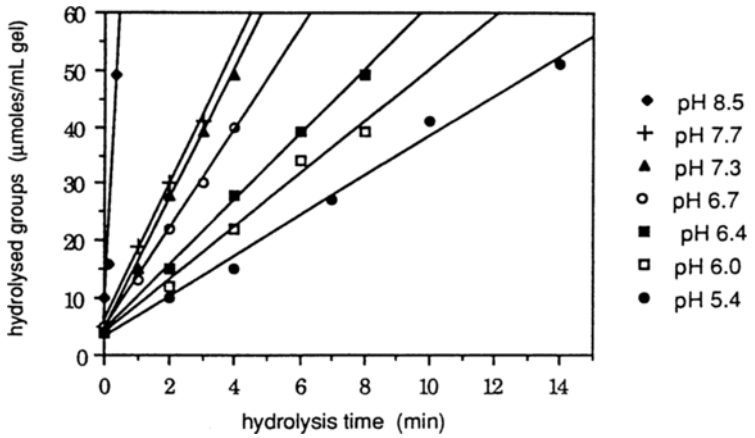


Fig. 2. Hydrolysis of NHS-activated glycine-CL 6B in phosphate buffer (PB) at different pH values as a function of time. Activated gel was transferred from DMF to water, immediately followed by the addition of phosphate buffer (t = 0) (2 mL 0.5*M* PB to 2 mL gel suspension [1 mL packed gel] in deionized water). After various times, the gel was treated with glycine ethyl ester (pH 7.5) (2 mL 1.3*M*/mL packed gel).

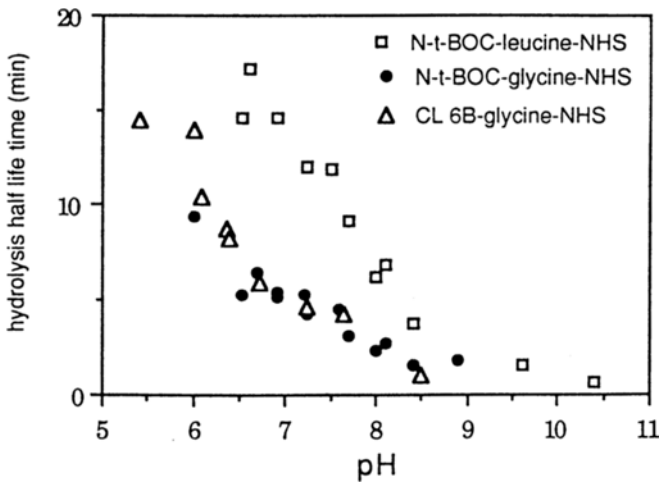


Fig. 3. Hydrolysis half-life times of NHS-glycine-CL 6B calculated from the data presented in Fig. 2, and hydrolysis half-life times of NHS-esters of *N-t*-BOC-glycine and *N-t*-BOC-leucine (addition of 100 μL of 16 m*M* *N-t*-BOC-amino acid-NHS in DMSO to 3.9 mL 5 m*M* phosphate buffer).

was more stable toward hydrolysis than *N-t*-BOC-glycine-NHS. The corresponding t50% values were 7.4 and 1.7 min, respectively (Table 1).

Using different concentrations of phosphate (pH 7.5) and borate (pH 9.3), it was found that the hydrolysis rate increased with increasing buffer concentration (Fig. 4A,B). Addition of NaCl (up to 1*M*) to phosphate buffer did not have any effect on the hydrolysis rates at pH 7.5 and 8.5; only at

Table 1
Hydrolysis Half-Life Times of Several NHS-Esters in Buffer, with or without NaCl (0.5*M*)[a]

Ester Compound	*T*-50%, min		Conditions
	−Salt	+Salt	
N-t-BOC-glycine-NHS	0.26 ± 0.01 (4)	0.17 ± 0.01 (4)	10 m*M* borate, pH 9.3, 22°C
Acetic succinimide	1.09 ± 0.02 (4)	0.74 ± 0.02 (5)	25 m*M* borate, pH 9.3, 22°C
N-t-BOC-leucine-NHS	0.53 ± 0.02 (4)	0.33 ± 0.02 (4)	25 m*M* borate, pH 9.3, 22°C
N-t-BOC-glycine-NHS	1.71 ± 0.07 (4)		0.3*M* Hepes, pH 7.4, 25°C
Acetic succinimide	7.44 ± 0.09 (3)		0.3*M* Hepes, pH 7.4, 25°C

[a]Means and standard deviations are given in minutes. The number of experiments is indicated in parentheses.

pH 9.5 was hydrolysis somewhat faster. In the case of borate, however, the ionic strength had a significant effect on the kinetics of the hydrolysis of gel-bound NHS-ester. The same concentration range of borate with or without NaCl (compensating for the differences in ionic strength within one of the two series) resulted in different hydrolysis half-life times (Fig. 5). The extinction as a function of time at one borate concentration, using different concentrations of NaCl, showed that higher salt concentrations gave higher hydrolysis rates (Fig. 6).

The salt effect was also observed with soluble NHS-esters, like *N-t*-BOC-glycine-NHS, acetic succinimide, and *N-t*-BOC-leucine-NHS. Hydrolysis half-life times of these esters, measured at pH 9.3, were very short (1 min or less), and the rate of hydrolysis proved to be significantly higher in the presence of 0.5*M* NaCl (Table 1).

Kinetics of hydrolysis of the gel-bound and the soluble NHS-ester of glycine was compared in a relative way. The curves of standardized hydrolysis as a function of time for the gel-bound and soluble ester had a similar shape, and the experimentally determined curves were parallel with the theoretical curve, but their position was somewhat lower (Fig. 7).

Coupling in Aqueous Media

Several amino group containing compounds were dissolved in buffer and added to dry NHS-glycine-Sepharose in an amount equal to half of the quantity of activated groups. The depletion of a number of amino compounds, added to NHS-activated *N-t*-BOC-glycine and NHS-activated glycine-CL 6B at different pH, was maximal at pH values of 8.0 and 9.0, respectively (Fig. 8A,B). The pH optima were rather sharp except for aminoethanol and glycine ethyl ester. Coupling of aminoethanol and glycine ethyl ester to immobilized ester was maximal between pH 8–9 and between pH 6.5–9, respectively (Fig. 8A).

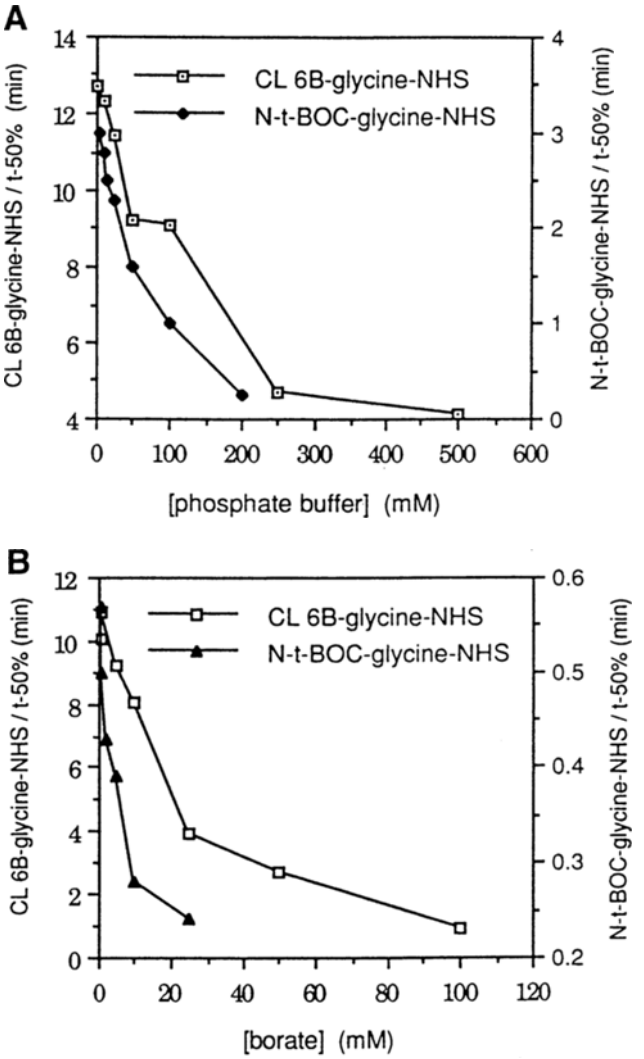


Fig. 4. The influence of buffer concentration on hydrolysis half-life times of NHS-activated glycine-CL 6B and *N-t*-BOC-glycine-NHS. **A**: Phosphate buffer (pH 7.5). **B**: Borate buffer (pH 9.3) (NHS-glycine-CL 6B: 10 mg dry gel suspended in 50 mL buffer; *N-t*-BOC-glycine-NHS: 100 μL of 16 m*M* in DMSO added to 3.9 mL buffer).

For the combination of aminoethanol and activated resin, not only depletion from supernatant, but also the content of ionizable groups of the gel was measured after coupling. The density of ionizable groups on the gel as a function of pH showed a minimum at pH 8–9 and increased at lower as well as higher pH values (Fig. 9).

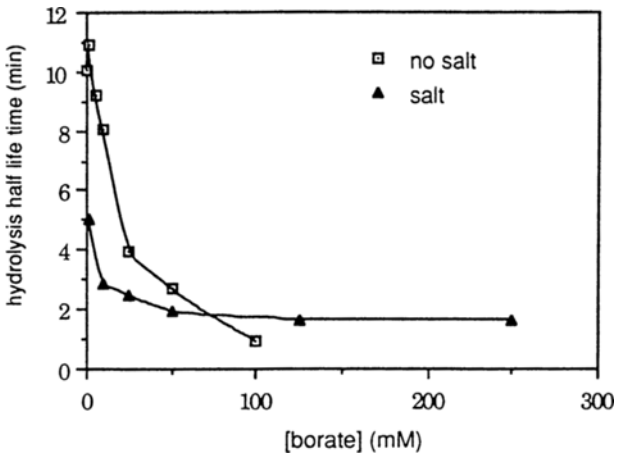


Fig. 5. Hydrolysis half-life times of NHS-glycine-CL 6B in borate buffer, pH 9.3, at different buffer concentrations with and without salt (NaCl). NaCl was added (up to 500 m*M*) in order to compensate for the differences in ionic strength caused by the use of different borate concentrations.

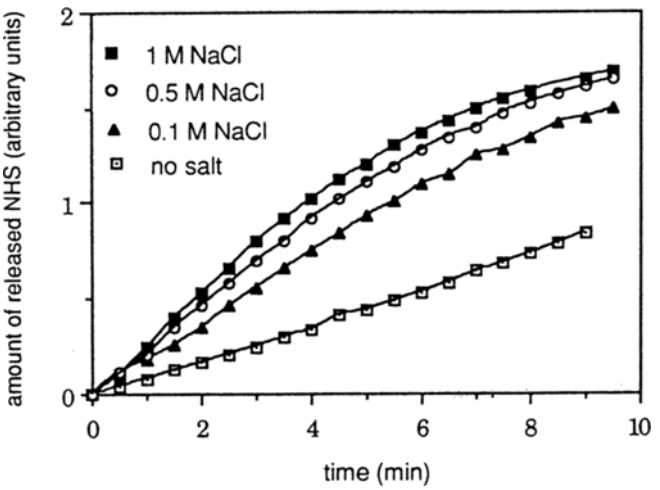


Fig. 6. Influence of different concentrations of NaCl, present in 10 m*M* borate buffer (pH 9.3), on the release of free NHS from NHS-glycine-CL 6B as a function of time. Free NHS was detected by measurement of the absorbance at 260 nm.

At pH 9.0, depletion of amino acids from supernatant ranged from 71% for lysine to 98% for glycine, with intermediate values for the other amino acids (Fig. 10). Depletion of aminoethanol, aminohexane, and glycine ethyl ester was complete (Fig. 10). Amino compounds added to the control gel (glycine-Sepharose containing no activated groups) also showed depletion from supernatant. This level of depletion was relatively low (16 ± 3%, summarized for all amino compounds) as compared with the depletion observed in the case of activated gel (Fig. 10).

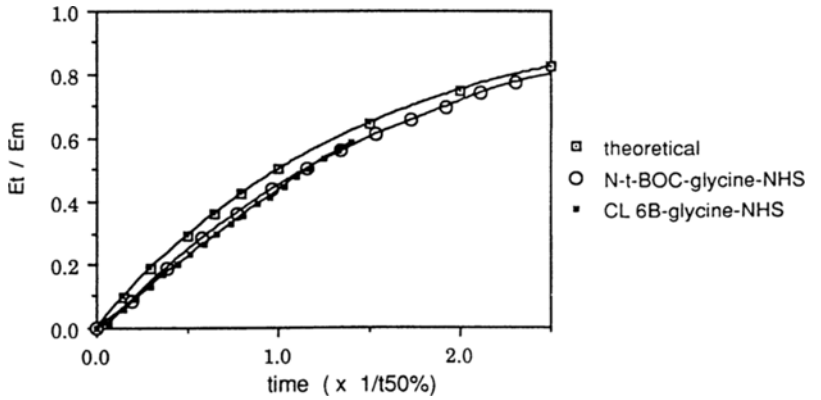


Fig. 7. Relative rate of hydrolysis of gel-bound and soluble NHS-ester as a function of time. The theoretical curve according to the equation $E_t/E_m = 1 - e^{-0.693 t_r}$ is also given (t_r = hydrolysis time expressed in $t50\%$ units) (hydrolysis in 10 m*M* borate, pH 9.3).

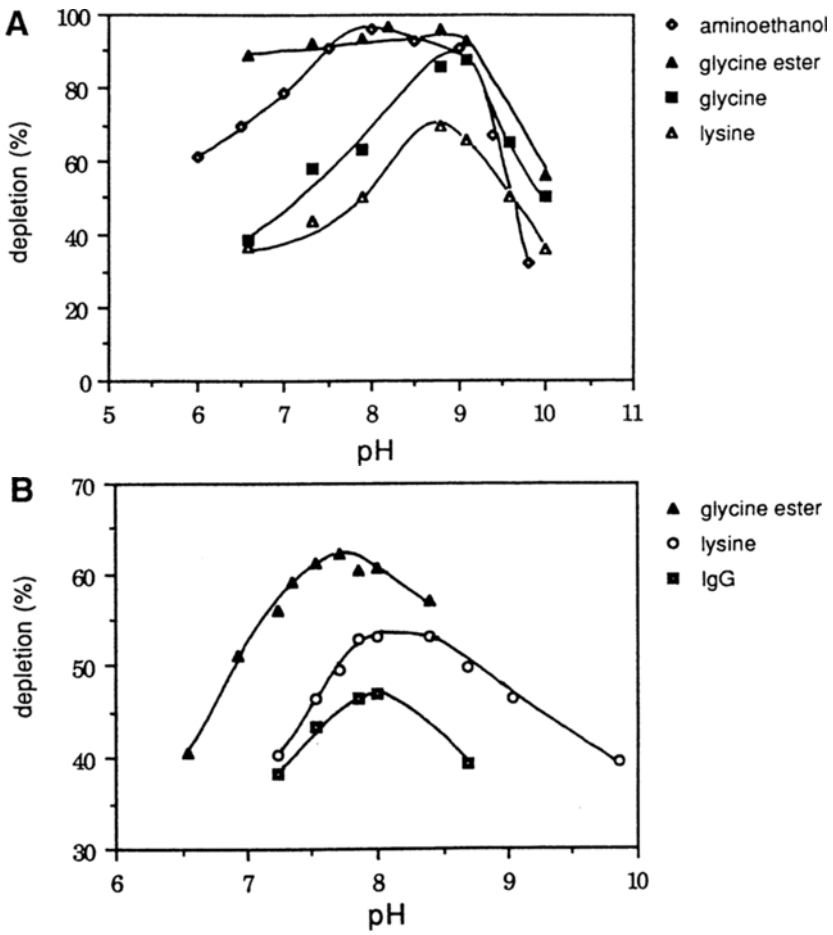


Fig. 8. Depletion levels of some amino compounds, added to NHS-glycine-CL 6B (**A**) and *N-t*-BOC-glycine-NHS (**B**) as a function of pH. **A:** 400 μL of 50 m*M* amino compound in 0.5*M* phosphate or carbonate buffer, added to 25 mg dry activated gel; coupling time 16 h. **B**: 50 μL of 16 m*M* ester (in DMSO) + 1.95 mL 5 m*M* buffer + 50 μL of 16 m*M* amino compound.

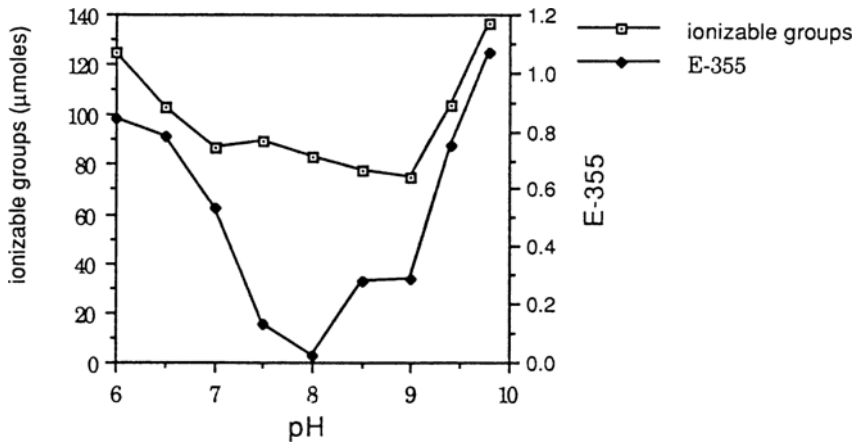


Fig. 9. Coupling of aminoethanol to NHS-glycine-CL 6B was studied both with the depletion method (E-355) and by measuring the amount of ionizable groups on the gel (µmol/150 mg dry gel) (for both methods: 150 mg dry gel were suspended in 3.0 mL 0.5*M* buffer and 60 µL of 2*M* aminoethanol in deionized water were added; coupling time 16 h).

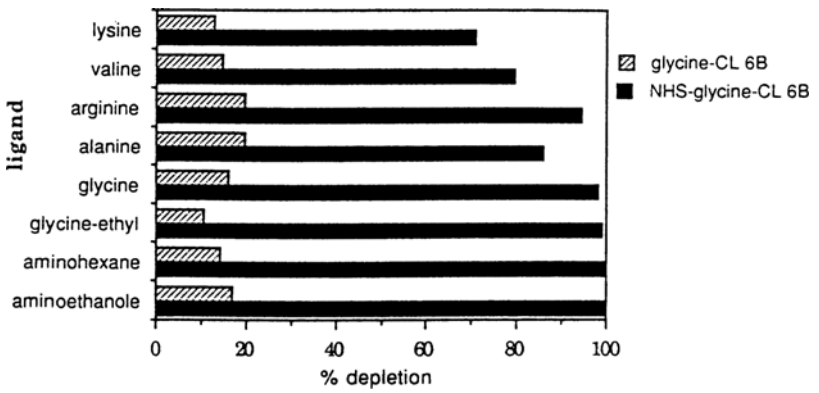


Fig. 10. Depletion levels of different amino compounds, added to (NHS-activated) glycine-Sepharose CL 6B in carbonate buffer (350 µL of 50 m*M* amino compound in 0.5*M* carbonate buffer, pH 9.0 and 25 mg dry gel; mixing time 16 h) (n = 3, sd < 3%).

DISCUSSION

The NHS active ester of glycine, immobilized on Sepharose, is very reactive toward compounds containing amino groups. In organic medium, there is no need to use excessive amounts of reactant to yield significant amounts of products. Each small quantity is coupled completely (Fig. 1A,B). Any desirable ligand density, equal or less compared to the density of activated groups, can be attained controllably just by adding the appro-

priate amount of amino compound. The results presented in Fig. 1A and B also indicate that, in contrast to the results of Wilchek and Miron (*12*), no substantial amount of β-alanine is immobilized on Sepharose, which could be the result of activation of the gel with carbodiimide and NHS. If significant amounts of β-alanine were formed, this should lead to a higher content of ionizable groups on the gel as compared to the glycine-Sepharose before activation. Conductivity titration of the gel before and after activation yielded nearly the same content of ionizable groups. This was concluded after comparison of the points of intersection with the *y*-axis in Fig. 1A and the carboxylate densities on the gel before activation, with an average of 100 (aminoethanol series) and 117 μmol/mL gel (aminohexane series), respectively. The values for the two parameters in Fig. 1A and B reveal that the total amount of reactive groups on the activated gel is equal to the amount of immobilized glycine on the gel before activation. Complete aminolysis of activated groups is achieved by addition of about 100 μmol amino compound/mL activated gel (Fig. 1A), which is an equimolar amount compared to the original glycine content of the gel. From Fig. 1B, it can be deduced that the N content of the gel is doubled after reaction of all activated groups with amino compound. These results strongly suggest that immobilized NHS-activated β-alanine does not significantly contribute to the total amount of activated groups after activation. The glycine density on the glycine-CL 6B used in this study (100–200 μmol/mL packed gel, roughly equivalent to 1.25–2.5 mmol/g dry Sepharose CL 6B) is high compared with the density of β-alanine on Sepharose CL 4B as analyzed by Wilchek and Miron (about 100 μmol/g dry gel or less) (*12*).

Organic solvents used as suspending medium do not have to be completely dry to avoid hydrolysis of the *N*-hydroxysuccinimide ester of Sepharose. Hydrolysis becomes apparent in DMSO at a water content of 30% with a hydrolysis half-life time of about 10 h. Polar ligands that poorly dissolve in pure organic solvents are usually more soluble in mixtures of organic solvent and water. Such mixtures of solvents may be used to improve the coupling yield.

The structure of *N-t*-BOC-glycine-NHS is very similar to that of the NHS-activated immobilized glycine, including the urethane moiety present in both substances. Therefore, *N-t*-BOC-glycine-NHS was used as a soluble analog of the immobilized NHS-ester. The hydrolysis of NHS-activated *N-t*-BOC-glycine is faster compared to NHS-activated *N-t*-BOC-leucine (Fig. 3), very likely because of steric hindrance of the reaction site by the side chain of leucine. Hydrolysis half-life times of acetic succinimide and *N-t*-BOC-glycine-NHS in 0.3*M* Hepes, pH 7.4 (25°C), were found to be 7.8 and 1.7 min, respectively (Table 1). The hydrolysis half-lives as found for *N-t*-BOC-glycine-NHS and *N-t*-BOC-leucine-NHS are short compared with data reported for other NHS-activated compounds. Pollak et al. (*13*) measured hydrolysis rates of acetic succinimide (*t* 50% = 11 min), *N*-acryloxysuccinimide (*t* 50% = 4.7 min), and methacryloxysuccinimide (*t* 50% = 35 min) dissolved in 0.3*M* Hepes, pH 7.4 (25°C). The

hydrolysis half-life time of 7.8 min for acetic succinimide derived from our experiments is smaller than the value found by Pollak et al. (11 min). However, Pollak et al. did not give a standard deviation for their result, so not much can be said about the significance of the difference between the two values.

Hydrolysis half-life times of the immobilized and soluble NHS-esters of glycine are about the same (Fig. 3). The buffer concentrations used, however, were very different (5 and 500 m*M*). The hydrolysis of NHS-activated immobilized glycine and *N-t*-BOC-glycine-NHS is catalyzed by buffer solutes (Fig. 4A and B). In the presence of phosphate buffer, the hydrolysis rate is not influenced by the addition of salt (*see* Results: Hydrolysis, Buffer Concentration, and Ionic Strength). In the case of borate, ionic strength is only partially responsible for the accelerated hydrolysis found with the higher buffer concentrations (Fig. 5). This indicates that the hydrolysis of the NHS-esters is general base catalyzed.

Activated glycine-CL6B is more stable toward hydrolysis as compared with the soluble analog *N-t*-BOC-glycine-NHS, differing roughly by a factor 3 at pH 7.5 (phosphate buffer) and a factor 20 at pH 9.3 (borate buffer) (Fig. 4A and B). Pollak et al. (*13*) found that *N*-acryloxysuccinimide, copolymerized with acrylamide, was more stable toward hydrolysis than the monomeric *N*-acryloxysuccinimide. At pH 7.4, the hydrolysis half-life times of the polymeric and the monomeric forms of the NHS-ester were reported to be 18 and 4.7 min, respectively.

A possible explanation for the relatively high stability of the gel-bound activated groups is a local acidification of the matrix microenvironment. The immobilized NHS-ester of glycine has a high concentration in the matrix, and hydrolysis gives rise to a high local concentration of H^+, which cannot be immediately neutralized by base present in the medium. The low pH will result in a decrease of the hydrolysis rate.

Steric hindrance of the reaction site toward hydroxylic ions exerted by the polymer backbone is very unlikely. As reported in this study, the reaction of activated glycine-Sepharose with poly(oxyethylene) *bis*-amine 3350 is fast (completion within 3 min), so steric constraints seem to be negligible for small molecules like OH^-.

Another mechanism underlying the stability of the immobilized ester could be the so-called autoretardation. Hydrolysis of the immobilized ester gives rise to polyelectrolyte molecules. Examples of autoretardation of polymer reactions can be found in the literature, e.g., the quaternization of poly(4-vinylpyridine) (*14*). Plate (*15*) describes autoretardation for the KOH saponification of poly(methyl methacrylate), which he explains by repulsion between carboxylate anions and hydroxylic ions.

However, in our study, initial hydrolysis rates were determined, and in this early stage of reaction, no considerable amounts of carboxylate

groups have yet been formed. Therefore, it is unlikely that differences in initial hydrolysis rates are caused by autoretardation.

Moreover, autoretardation would result in a progressive decrease of hydrolysis rates in later stages of reaction. Standardized kinetics of hydrolysis for NHS-glycine-CL 6B is similar to that for *N-t*-BOC-glycine-NHS (Fig. 7). The steepness of the curves is nearly comparable to that of the theoretical curve throughout the reaction (Fig. 7). These results indicate that autoretardation effects can be excluded.

Hydrolysis of immobilized ester at higher pH (>8.5) is accelerated by addition of salt (Figs. 5, and 6). The increase of the hydrolysis rate is nearly 100% in the presence of 0.1*M* NaCl (Fig. 6). This phenomenon may have an adverse effect in biochemical practice, because standard buffered saline solutions often have relatively high salt concentrations.

Not only gel-bound, but also soluble NHS-esters show accelerated hydrolysis rates in the presence of salt (Table 1). In contrast with this finding, Adalsteinsson et al. (*16*) found no differences in the hydrolysis rate of *N*-acryloxysuccinimide with or without 1*M* KCl. It must be noted, however, that the salt effect as described in this article was only observed at higher pH (>8.5), whereas Adalsteinsson et al. used a pH of 7.6.

The influence of salt on the hydrolysis rate has been described for a number of simple esters (*17*). Salt can accelerate or slow down the hydrolysis reaction depending on the salt species and the type of ester. Increase of the hydrolysis rate has been related with an increase of the activity coefficient of the ester or with the solvation of the ester (*17*).

Efficiencies of the coupling of amino compounds to activated gel were calculated from the relative depletion of amino compounds from the supernatant of the gel suspensions. Despite its indirect approach, depletion of amino compounds is considered to be an accurate system to estimate coupling levels. Suitable controls must be used to ascertain that depletion is not caused by noncovalent binding, i.e., by physicochemical interaction. In the case of the coupling of aminoethanol to activated gel, results of conductivity titration agree with data obtained with the depletion assay (Fig. 9). This means that depletion of aminoethanol from the supernatant and coupling of aminoethanol to the gel are correlated.

The pH optima observed for coupling of amino compounds to immobilized active ester are significantly higher than those found for the coupling to activated *N-t*-BOC-glycine (>9.0 and 8.0, respectively) (Fig. 8A,B). This difference in location of pH optimum can be explained by the higher stability of the activated glycine-CL6B toward hydrolysis as compared to the solute analog.

Despite of the significant hydrolysis of the NHS-ester of glycine-Sepharose in buffer, nearly all amino compounds indicated in this article seemed to have rather high coupling efficiencies. For glycine, a depletion of 98.2%

was found (Fig. 10). Coupling of amino acids with more bulky side chains, like for example arginine or valine, is less favorable. Nevertheless, depletions of 71% or more can still be obtained (Fig. 10). Aminoethanol, aminohexane, and the ethyl ester of glycine seem to couple completely (depletion >99.2%) (Fig. 10).

It appears that depletion of amino compounds from the supernatant cannot be directly used for calculating coupling efficiencies. In combination with the control gel, i.e., glycine-Sepharose lacking activated groups, all amino compounds showed depletion from the supernatant (±16%; Fig. 10). Therefore, some part of the depletion found with activated gel may be caused by noncovalent interaction. The differences between the activated and nonactivated Sepharose with respect to the decrease in the amount of amino compound in the supernatant are rather large. The minimal coupling efficiencies to be expected are proportional to these differences, and the actual coupling efficiencies are likely to be even higher.

A relation is given by Cline and Hanna (*18*) between the rate constant for aminolysis (k) of anisoyl-NHS and the pK_a of the amino group of (sterically unhindered) nucleophiles: $\log k = pK_a - 8.8$. The rate constant was obtained from calculations in which only the concentration of nonprotonated amine (i.e., free amine) was considered. Coupling rate is determined not only by nucleophilicity of the amine, but is also dependent on the concentration of free amine ($[\text{amine}]_{\text{free}} = K_a\,[\text{amine}]_{\text{total}}/([H^+] + K_a)$). Higher basicity is correlated with a higher nucleophilicity of the amine, but also with a lower proportion of free amine. For the experiments summarized in Fig. 10, experimental values of depletion are compared with the theoretical calculated product of the rate constant k and the relative fraction of free amine (Table 2). The fraction, $D/(F \times k)$, has a mean value of 67 (±9) for lysine, valine, alanine, aminohexane, glycine, and aminoethanol (Table 2). The value for glycine ($D/(F \times k) = 72.5$) is higher compared to lysine, valine, and alanine, probably because of the absence of steric hindrance in the case of glycine. Arginine ($D/(F \times k) = 120$) and glycine ethyl ester ($D/(F \times k) = 1176$) show much higher values for the calculated fraction, as compared to the other amino compounds, which difference is difficult to explain. From Table 2, it can be deduced that the extent of coupling of six of the eight amino compounds, used in this study, seems to be primarily determined by the nucleophilicity of the amine and, to some extent, also by steric hindrance of the amino group.

From this study, it can be concluded that NHS-activated glycine-Sepharose CL 6B is a very efficient system for coupling of amino compounds, both in organic as well in aqueous media. For maximal coupling yields, especially in the case of dilute ligand solutions or with ligands having bulky substituents, one should take care to use the lowest possible buffer concentration, with a pH of 8.5–9.0. If salt has to be present, for example, to stabilize the ligand, the use of phosphate-buffered coupling medium is preferred. The EDC/NHS activation method is promising for the coupling

Table 2
Comparison of Depletion Results with Theoretical Predicted Relative Coupling Level

Amino Compound		PK_a	Fraction free amine, F	Rate constant, k	F × k	Depletion, %, D	D / (F × k)
Lysine	α	9.20	0.39	2.51	0.98	71.1	72.6[b]
	γ	10.80	0.016	100	1.60		44.4[b]
Valine		9.72	0.16	8.32	1.33	79.4	59.7
Arginine		8.99	0.51	1.55	0.79	94.3	119.4
Alanine		9.87	0.12	11.75	1.41	86.3	61.2
Glycine		9.78	0.14	9.55	1.34	98.3	73.4
Glycine ethyl ester		7.75	0.95	0.089	0.085	99.3	1168
Aminohexane		10.60	0.025	63.1	1.58	99.4	62.9
Aminoethanol		9.5	0.24	5.01	1.20	99.4	82.8

[a]For each amino compound the fraction free amine ($F = [amine]_{free} = K_a\,[amine]_{total}/([H^+] + K_a)$, at pH 9.0), the rate constant for aminolysis (k; $\log k = pK_a - 8.8$ [*18*]), and the product F × k, were calculated. Levels of depletion (D), obtained from the experiments corresponding with Fig. 10, were converted into values D / (F × k) (last column).
[b]Mean = 58.5.

to Sepharose of enzyme cofactors and enzyme inhibitors as well as substrate analogs, ligands commonly used in the purification of enzymes. The mentioned ligands are small and preferentially need a spacer. In addition, many of these ligands contain one or more amino groups, so they can be coupled easily and efficiently to NHS-activated carboxylated Sepharose, resulting in high-capacity gels for the binding of enzymes or other proteins.

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