Imai, K. (1982) in *Allosteric Effects in Haemoglobin*, pp 189–193, Cambridge University Press, Cambridge.
Imai, K., & Yonetani, T. (1975) *J. Biol. Chem. 250*, 2227–2231.
Imai, K., & Yonetani, T. (1977) *Biochim. Biophys. Acta 490*, 164–170.
Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., & Kuroda, M. (1970) *Biochim. Biophys. Acta 200*, 189–196.
Imai, K., Ikeda-Saito, M., Yamamoto, H., & Yonetani, T. (1980) *J. Mol. Biol. 138*, 635–648.
Inubushi, T., Ikeda-Saito, M., & Yonetani, T. (1983) *Biochemistry 22*, 2904–2907.
Johnson, M. E., & Ho, C. (1974) *Biochemistry 13*, 3653–3661.
Lindstrom, T. R., & Ho, C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1707–1710.
Maeda, T., Imai, K., & Tyuma, I. (1972) *Biochemistry 11*, 3685–3689.
Miura, S., & Ho, C. (1982) *Biochemistry 21*, 6280–6287.
Ogawa, S., & Shulman, R. G. (1972) *J. Mol. Biol. 70*, 315–336.
Righetti, P., & Drysdale, J. W. (1971) *Biochim. Biophys. Acta 236*, 17–28.
Shaanan, B. (1983) *J. Mol. Biol. 171*, 31–59.
Smith, F. R., & Ackers, G. K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5347–5351.
Viggiano, G., & Ho, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3673–3677.
Viggiano, G., Ho, N. T., & Ho, C. (1979) *Biochemistry 18*, 5238–5247.
Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195–216.

Limitations of *N*-Hydroxysuccinimide Esters in Affinity Chromatography and Protein Immobilization†

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Received September 29, 1986; Revised Manuscript Received November 19, 1986

ABSTRACT: The carbodiimide-mediated reaction of *N*-hydroxysuccinimide with carboxyl groups immobilized to hydroxyl-containing polymers (such as Sepharose or Trisacryl) leads to an undesirable side reaction in high yields. The product of this reaction interferes with the application of such columns for further affinity-based purification. In addition to the desired *N*-hydroxysuccinimide ester, a bis(*N*-hydroxysuccinimide) derivative of β-alanine [namely, *N*-[(succinimidooxy)carbonyl]-β-alanine *N*-hydroxysuccinimide ester] is probably produced that reacts subsequently with the hydroxyl group of the polymer via ester and carbamate bonds. These β-alanine derivatives are formed upon interaction of dicyclohexylcarbodiimide with 3 equiv of *N*-hydroxysuccinimide followed by a Lossen rearrangement. The amount of β-alanine thus coupled is very high compared to the number of carboxyl groups present on the resin. The β-alanine bound through the ester bond comprises about 90% of the β-alanine bound. Alkaline treatment of the ester-bonded β-alanine-containing polymers (prior to coupling of amino-containing ligands) causes a rearrangement yielding β-alanine with a free carboxyl group coupled through a stable carbamate linkage. After coupling of amino-containing ligands, the above-described rearrangement cannot occur, and the β-alanine-linked ligand leaks from the polymer via hydrolysis of the ester bond. The newly formed carboxyl groups (derived from the rearrangement) can be used to prepare active esters. In view of the above, we developed methods for the preparation of nitrophenyl esters as well as *N*-hydroxysuccinimide esters free of unstable β-alanine derivatives on polymers containing hydroxyl groups. Upon coupling with amino-containing ligands, these esters yield resins bearing chemically stable bonds.

N-Hydroxysuccinimide (NHS)[1] esters were introduced by Anderson et al. (1964) and are widely used as coupling agents in peptide synthesis. These esters were later introduced by us for the modification of lysine residues on proteins (Becker et al., 1971) and cells (Becker & Wilchek, 1972). NHS esters are also widely used to activate carboxyl groups on spacer arms bound to agarose (Cuatrecasas & Parikh, 1972). Affinity gels containing NHS ester are commercially available and commonly used (Wilchek et al., 1984).

Recently, during an affinity study for the isolation of receptors and lymphokinases, we needed activated supports containing spacer arms that give stable products. We therefore applied both commercially available and "homemade" NHS derivatives of agarose and Trisacryl (Miron & Wilchek, 1985). To our surprise, upon reaction with ligands containing an amino group, all the gels yielded columns that were unstable to alkali. All of these columns were plagued by constant leakage during use. Furthermore, upon total hydrolysis of the activated gels or their coupled derivatives, a new amino acid appeared in large quantities. Leakage of ligands from affinity columns is a serious problem in affinity chromatography (Wilchek et al., 1975). This is particularly true when minute amounts of different biologically active factors are being pu-

† Supported in part by the Gerhardt M. J. Schmidt Fund for Preindustrial Research.

[1] Abbreviations: NHS, *N*-hydroxysuccinimide; ε-cap, ε-aminocaproic (or 6-aminocaproic) acid; DCC, dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenyl.

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rified and leakage can lead to misleading results (Pathy, 1985). Even more important, the leakage phenomenon can cause health hazards when immunoaffinity-purified compounds (e.g., prepared by genetic engineering) are complexed with mouse monoclonal antibodies. We therefore decided to study the reason(s) for the instability of the products of NHS esters on hydroxyl-containing polymers. We were also interested in defining the nature of the new amino acid and in determining whether there is a connection between the two phenomena.

The outcome of this study is that, upon reaction of carboxyl groups attached to hydroxyl-containing polymers (or the polyhydroxyl itself) with NHS and carbodiimide, a β-alanine derivative is formed and coupled to the hydroxyl groups of the resin through an ester bond. The instability of the ester bond between the β-alanine moiety and the resin causes the observed leakage. Alternative methods for activation of a carboxyl group on polysaccharides are described that yield stable carriers.

Experimental Procedures

Materials. Sepharose CL4B and activated CH-Sepharose 4B (lot IL 32483) were obtained from Pharmacia. Trisacryl GF2000 was a gift from Dr. E. Boschetti (IBF, Paris, France); Affi-Gel 10 (batch 20170) was obtained from Bio-Rad; 6-aminocaproic acid, dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine, *p*-nitrophenol, *o*-nitrophenol, *p*-nitrophenyl chloroformate, triethylamine, and lysine were obtained from Fluka. Bovine γ-globulin, *N*-hydroxysuccinimide (NHS), and trypsin were obtained from Sigma. N^{ϵ}-(Dinitrophenyl)-lysine was obtained from Bio-Yeda. *N*-Succinimidyl chloroformate was synthesized as described (Miron & Wilchek, 1985). Activation of Sepharose CL4B and Trisacryl GF2000 with chloroformates was performed as previously described (Wilchek & Miron, 1982; Miron & Wilchek, 1985) except that all activations were performed in the presence of 4-(dimethylamino)pyridine as catalyst.

Preparation of ε-cap Derivatives of Sepharose and Trisacryl and Their Active Esters. The chloroformate-activated gels were suspended in a 0.25 M 6-aminocaproic acid solution [in phosphate-buffered saline, pH 7.4 (PBS), for *N*-succinimidyl chloroformate activated gels or in 0.2 M $NaHCO_3$, pH 8.5, for *p*-nitrophenyl chloroformate activated resins], and the suspension was agitated for 24 h at 4 °C. After the usual washing procedure, gels containing between 15 and 20 μmol/g (wet weight) ε-cap were obtained. The NHS and *p*-nitrophenyl esters were prepared as described by Cuatrecasas and Parikh (1972) and Miron and Wilchek (1985), respectively.

Activation of Sepharose and Trisacryl with NHS and DCC. The gels, including the ε-cap derivatives, were dehydrated with increasing concentrations of dioxane in water (25%, 50%, 75%, 100%) and then washed with anhydrous dioxane. The dry gels (10 g of Sepharose or 5 g of Trisacryl GF2000) were suspended in dry dioxane (10 mL) containing NHS (5 mmol), and 10 mL of dioxane containing DCC (5 mmol) was added. The reaction mixture was shaken overnight at room temperature and washed with dioxane, methanol, and 2-propanol. Gels were stored at 4 °C in 2-propanol. Gels containing up to 180 μmol of NHS/g dry weight were obtained under these conditions, as determined by the previously described method (Miron & Wilchek, 1982). Higher substitutions could be obtained with larger amounts of reagents.

Conversion of β-Alanine Bound through Ester Bonds to Carbamate Bonds. The NHS/DCC-activated gels were suspended in 0.2 N NaOH for 20 min at room temperature with shaking. The gels were then washed with water, with 0.3 N HCl, and again with water.

Activation of β-Alanine-Containing Gels with DCC and Nitrophenols. Gels containing carbamate-bound β-alanine were dehydrated with dioxane as described above. The resins were suspended in dioxane containing either *p*-nitrophenol or *o*-nitrophenol (20-equiv excess over β-alanine) followed by an equivalent amount of solid DCC. Activation was allowed to proceed overnight at room temperature with shaking. The activated gels were washed with dioxane, methanol, and 2-propanol and kept in 2-propanol. The amount of *p*-nitrophenol or *o*-nitrophenol was determined (Wilchek & Miron, 1982).

Preparation of Chemically Stable NHS-Containing Resins. Gels (10 g of Sepharose) containing carboxyl groups (ε-cap, β-alanine, carboxymethyl) were dehydrated with dioxane as described above. The resins were suspended in dioxane (10 mL) containing DCC (5 mmol), and the suspension was shaken for 15–30 min. The resins were filtered immediately, washed with dioxane, and resuspended in a dioxane solution that contained NHS (5 mmol). The suspension was shaken for 3 h at room temperature, filtered, and washed successively with dioxane, methanol, and 2-propanol. Gels containing up to 135 μmol of NHS/g dry weight of resin were obtained. The gels were stored at 4 °C in 2-propanol.

Coupling of Ligands and Proteins to Activated Supports. Coupling was performed as previously described (Cuatrecasas & Parikh, 1972; Wilchek et al., 1984). To determine the amount of unstable β-alanine (coupled through the ester bond) and ligands thereof, the gels (after coupling of ligand) were treated with 0.2 N NaOH for 5 min, and the amounts of β-alanine and ligand coupled were determined before and after this treatment.

Preparation of N^{ϵ}-DNP-N^{α}-[(2-carboxyethyl)carbamoyl]-lysine (DNP-Lys-β-Ala-urea). Sepharose CL4B (10 g) was subjected to single-step activation with NHS and DCC as described above and reacted further with N^{ϵ}-DNP-lysine (20 mg/g of gel) for 24 h at room temperature in a solution of 30% dimethylformamide and 0.2 N $NaHCO_3$. The gel was washed with the same solvent followed by water until no yellow color could be detected and then treated with 0.3 N NaOH for 5 min and filtered. The yellow filtrate was acidified with 0.3 N HCl and extracted with ethyl acetate, and the organic layer was concentrated to dryness. The compound was pure on thin-layer chromatography with an R_f of 0.9 (butanol/acetic acid/water, 4:1:1). The structure of this compound was determined by NMR and elemental analysis.

Analysis. The quantities of β-alanine, ε-cap, amino acids, and proteins coupled to the resins were determined by amino acid analysis after total hydrolysis according to Spackman et al. (1958).

Results

During a study involving the purification of different lymphokinases by immunoaffinity chromatography, we coupled several antibodies to commercially available, spacer-containing polymeric NHS esters (Affi-Gel 10, Affi-Gel 15, and activated CH-Sepharose) and found a substantial amount of leakage that characterized all of the above resins. We also coupled the same antibody preparation to a homemade NHS ester of ε-cap bound to cross-linked Sepharose or Trisacryl GF2000. These latter gels were prepared by coupling ε-cap to the respective chloroformate-activated (Wilchek & Miron, 1982) or cyanogen bromide activated (Kohn & Wilchek, 1982) matrices followed by coupling of NHS in the presence of DCC. These gels were also unstable. In order to determine the reason for the observed leakage and the precise amount of antibody coupled to the gels, the immunoaffinity matrices were hydrolyzed in 6 N HCl for 24 h and analyzed in an amino acid

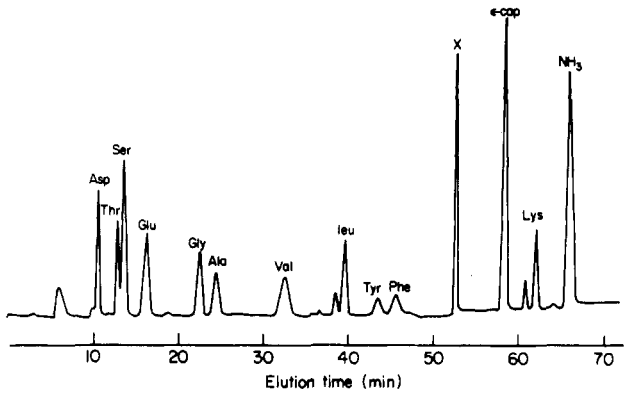

FIGURE 1: Amino acid analysis of immunoglobulin G coupled to Sepharose. X is an unidentified amino acid.

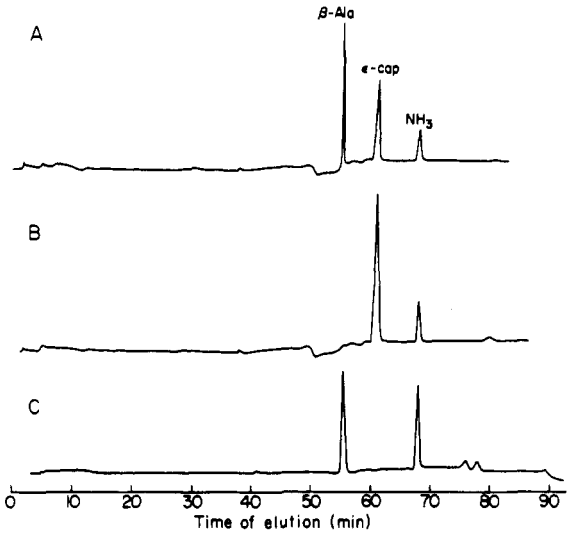

FIGURE 2: Amino acid analysis of modified Sepharose: (A) Sepharose–ε-cap NHS ester; (B) Sepharose–ε-cap; (C) β-alanine maker.

analyzer. In all the hydrolysates, large amounts of an unidentified amino acid were found (Figure 1). This amino acid was in large excess over the molar amount of protein coupled. Surprisingly, in many cases where ε-cap was used as the spacer, the amount of the new amino acid was even more than that of ε-cap.

In order to determine the source of this amino acid [i.e., whether or not it comes from the antibody (γ-globulin)], we coupled other proteins to the same matrices. In all cases, the new amino acid was present, suggesting that this amino acid is not a part of the ligand (protein) coupled but is derived from the resin. We therefore hydrolyzed the gels that contained ε-cap either as the free acid or as the corresponding NHS ester. Only the gel containing the NHS ester contained the new amino acid (Figure 2). Therefore, this new amino acid must result from the NHS ester or from its preparation.

In peptide chemistry, one of the side products for the preparation of NHS esters from blocked amino acids and DCC is *N*-[(succinimidooxy)carbonyl]-β-alanine *N*-hydroxysuccinimide ester (Gross & Bilk, 1968). The latter is formed by the reaction of 3 equiv of NHS and 1 equiv of DCC, the mechanism of which is shown in Figure 3 and includes a Lossen rearrangement. If this compound is also formed during the preparation of the polymeric NHS esters, it would be capable of reacting secondarily with the free hydroxyl groups on the polysaccharide or with the remaining hydroxyl group of the Trisacryl GF2000 gel (Figure 4, top), thus yielding matrices containing β-alanine. To check this possibility, we have compared, by amino acid analysis, the hydrolysates of the

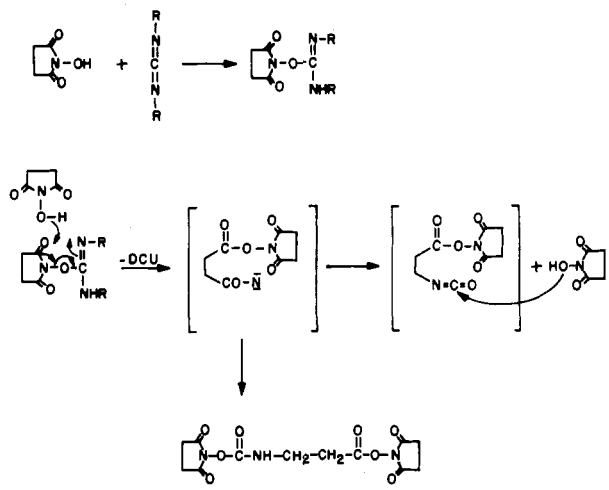

FIGURE 3: Mechanism of β-alanine formation from NHS and carbodiimide according to Gross and Bilk (1968). DCU, dicyclohexylurea.

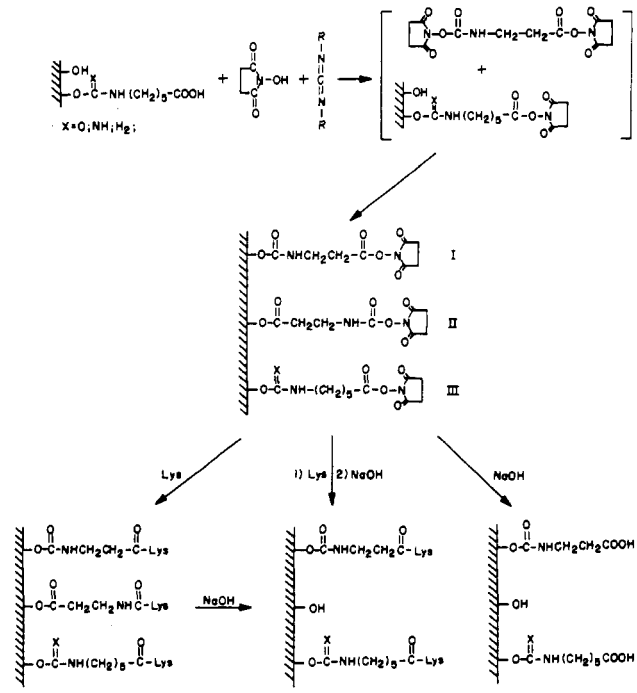

FIGURE 4: Mechanism of binding and structure of β-alanine derivatives coupled to hydroxyl-containing polymers (top and middle). Mode of lysine binding to the NHS-activated resins (bottom left) and structure of NaOH-treated resin before and after lysine coupling (bottom middle and right).

NHS esters or their reaction products with authentic β-alanine and found them to be identical. Furthermore, underivatized agarose or Trisacryl GF2000, subjected to reaction with NHS and DCC, gave β-alanine as the only amino acid upon total hydrolysis, thus establishing β-alanine as the new amino acid.

As can be seen in Figure 4, β-alanine can be coupled to the hydroxyl groups of the carriers in two different modes: (I) through the amino side [the (succinimidooxy)carbonyl group], which is an activated carbamate possesing a good leaving group, and/or (II) via the carboxyl side of the β-alanine (the NHS ester). The β-alanine coupled through the carboxyl group (via an ester bond) should be labile and unstable to alkaline conditions. On the other hand, the β-alanine coupled through the oxycarbonyl group should be completely stable since it yields a carbamate linkage which is known to have high stability (Wilchek & Miron, 1982). Therefore, some of the amino-containing ligands coupled to these activated gels would

Table I: Quantitative Amino Acid Analysis of Ligands Coupled to NHS Ester of Sepharose–ε-cap[a]

amino acid	before coupling	after coupling	NaOH treatment: before coupling	NaOH treatment: after coupling
β-alanine	132	105.6	100	15.8
ε-cap	42.5	42.0	40	43.0
lysine	0	102	0	35.5
β-Ala-Lys (urea)	0	~66.7[b]	0	0

[a] Results are expressed as micromoles of amino acid per gram (dry weight) of resin. [b] Taking into consideration the same factor as for lysine.

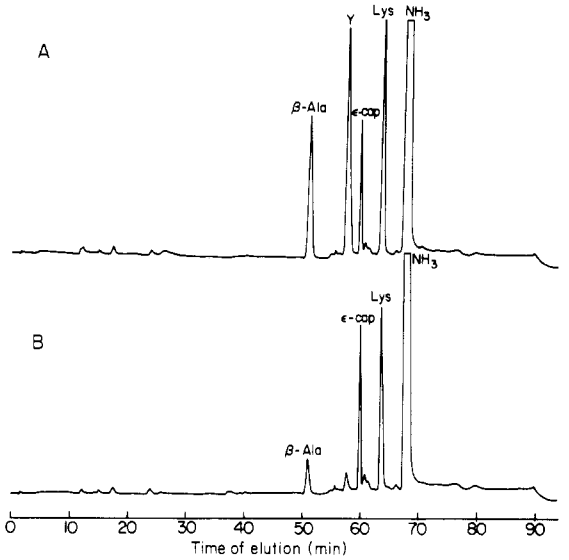

FIGURE 5: Amino acid analysis of lysine coupled to NHS-activated Sepharose–ε-cap: (A) before NaOH treatment; (B) after NaOH treatment. Y is a new amino acid.

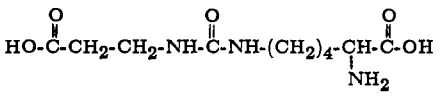

FIGURE 6: Structure of Y (Figure 5), N^{ϵ}-[(2-carboxyethyl)carbamoyl]lysine (β-Ala-N^{ϵ}-Lys-urea).

be labile to alkali and would be removed together with β-alanine upon exposure to alkaline conditions, a phenomenon that could explain the observed leakage. Such an instability toward alkali had already been noticed in the original paper on the use of the NHS esters of agarose (Cuatrecasas & Parikh, 1972). In that work, the authors suggested the use of 0.1 M NHS and DCC in order to minimize this problem. Throughout our studies we used this concentration or less, but columns activated under these conditions were still unstable.

In order to determine the relative amounts of I and II, we performed the experiments illustrated in the bottom portion of Figure 4. Lysine was coupled to the NHS-activated Sepharose–ε-cap, and the respective amounts of ε-cap, β-alanine, and lysine were determined before and after treatment with NaOH for 15 min. As can be seen in Table I, after NaOH treatment of the lysine-containing resin, the amount of ε-cap did not change while the amount of lysine and β-alanine were reduced by about 65% and 85%, respectively. After the coupling of lysine, an unidentified amino acid appeared upon 24-h hydrolysis (Figure 5A). The latter amino acid could not be detected following treatment with NaOH (Table I and Figure 5). The structure of this amino acid was found to be N^{ϵ}-[(2-carboxyethyl)carbamoyl]lysine (Figure 6). This amino acid (which represents lysine coupled via a urea linkage to β-alanine) had been bound to agarose through an ester bond.

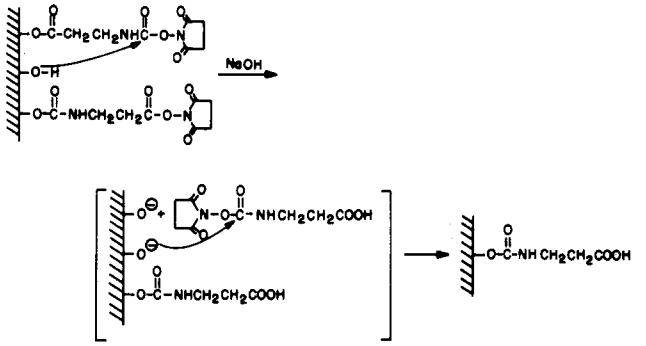

FIGURE 7: Mechanism of rearrangement of β-alanine from the unstable ester bond to a stable carbamate linkage in the presence of strong alkali (NaOH).

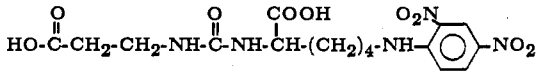

FIGURE 8: Structure of N^{ϵ}-DNP-N^{α}-[(2-carboxyethyl)carbamoyl]-lysine (N^{ϵ}-DNP-Lys-β-Ala-urea) formed upon coupling N^{ϵ}-DNP-lysine to NHS- and DCC-activated Sepharose.

Table II: Reaction of Agarose with Different Ratios of NHS and DCC

ratio of NHS:DCC	NHS[a] (μmol/g dry weight)	β-alanine[b] (μmol/g dry weight)
0.5:1	109	162
1:1	126	178
2:1	86	91
3:1	87	94
1:1[c]	291	295

[a] NHS was determined after treatment with NH_4OH. [b] β-Alanine was determined by amino acid analysis after total hydrolysis. [c] In the presence of triethylamine.

The appearance of this amino acid reflects the stability of the urea bond to hydrolysis under standard conditions of peptide hydrolysis (Spackman et al., 1958). Upon prolonged hydrolysis (72 h) of this amino acid, additional amounts of both β-alanine and lysine were obtained. The same amino acid was found in the solution after NaOH treatment.

A more surprising result was that the amount of β-alanine decreased by only 20–30% when samples of the NHS-activated Sepharose– or Trisacryl GF2000–ε-cap were treated with NaOH *before* coupling of lysine (Figure 4, bottom right). This finding can best be explained by a rearrangement of the β-alanine molecule from the ester linkage to form a carbamate bond, as illustrated in Figure 7.

To determine whether this reaction does indeed take place, we reacted underivatized agarose with NHS and DCC for 12 h, using different ratios of the reactants (Table II). The amount of β-alanine was determined by amino acid analysis. In all cases, the amount of β-alanine was consistently higher than that of the active ester due to partial hydrolysis of the latter (Table II). The elevated amounts of β-alanine (particularly in cases where lower ratios of NHS:DCC are used) can also be explained by the fact that the NHS ester of β-alanine is formed (Figure 3), but there is not enough NHS to react after the Lossen rearrangement to form the carbamate bond. This intermediate is coupled to the agarose. The amount of active ester and β-alanine could be further increased when the reaction was performed in the presence of triethylamine (Table II).

Part of the NHS/DCC-activated agarose was then reacted with N^{ϵ}-DNP-lysine. The DNP-containing gel was treated with 0.3 N NaOH for 5 min. This treatment removed 80%

Table III: Analysis of NHS- and DCC-Activated Hydoxyl-Containing Resins before and after NaOH Treatment[a]

resin	treatment (min)	NHS before NaOH	NHS after NaOH	β-alanine before NaOH	β-alanine after NaOH
Sepharose CL4B	2	135	0	189	110
	20	135	0	189	138
	2	291	0	295	181
	20	291	0	295	260
Trisacryl GF2000	2	103	0	95	58
	20	103	0	95	82

[a] Results expressed in micromoles per gram dry weight.

of the DNP coupled. The resultant product was acidified and extracted with ethyl acetate. The product was chromatographically pure on thin-layer chromatography. NMR and elemental analysis revealed the structure to be N^{ϵ}-DNP-N^{α}-[(2-carboxyethyl)carbamoyl]lysine as shown in Figure 8. This structure can only result from the binding of N^{ϵ}-DNP-lysine via a urea linkage to the β-alanine moiety bound to the resin through the ester bond. Analysis of the agarose after removal of DNP showed that only 20% of the β-alanine remained, indicating that about 80% of β-alanine was bound through the ester bond.

Another sample of the NHS/DCC-activated agarose was treated with 0.2 N NaOH at room temperature for either 2 or 20 min (Table III). In all cases, the amount of β-alanine remaining on the respective matrices was about 75–85%, again indicating a rearrangement of the unstable ester bond to the stable carbamate bond. This rearrangement can occur in two possible ways, as shown schematically in Figure 7: the activated *N*-succinimidyl carbamate is attacked by hydroxylate groups either before or after the ester is hydrolyzed by the base.

In order to differentiate between these two possibilities, time-dependent measurements of the reaction products were taken. When the activated gels were treated with NaOH for only 0.5 min, only about 30% of the original β-alanine remained attached to the matrix, indicating that the reaction occurs after hydrolysis of the ester. The results also suggest that the *N*-succinimidyl carbamate is more stable than the ester bond between the carboxyl group of β-alanine and the hydroxyl group of the agarose; i.e., the *N*-succinimidyl carbamate can thus react with the hydroxylate ions of agarose. Maximum rearrangement was achieved in 15–20 min, reaching 70–80% of the original β-alanine on the matrix, after which no further increase in β-alanine was observed (Table III). The higher stability of the *N*-succinimidyl carbamate was explains why most of the β-alanine is coupled through an ester bond to polyhydroxyl carriers. The hydroxyl groups of the resin are nucleophilic enough to react with the NHS ester of the β-alanine, but they react much less so with the *N*-succinimidyl carbamate, which requires strong basic conditions for reaction.

The above-described process can be used as a mild method for the introduction of carboxyl groups with a short β-alanine chain onto these matrices (Figure 9). These carboxyl groups can then be used to couple amino-containing ligands by different means, either by direct use of carbodiimides or by preparation of active esters.

As a result of this study, it is obvious that NHS esters, if prepared by the described standard procedures (Cuatrecasas & Parikh, 1972), are not suitable for use as affinity matrices due to leakage. Instead, other esters that do not give this side product should be applied. Alternatively, one can use the two-step procedure described under Experimental Procedures for the preparation of NHS esters; lower amounts of active ester and coupling are obtained. In our laboratory we have

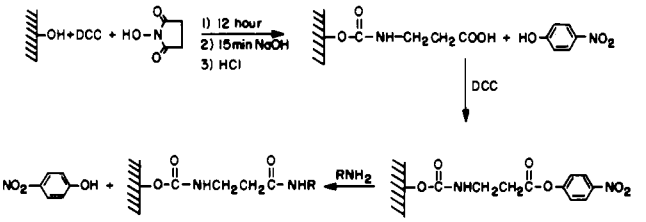

FIGURE 9: Activation conditions of polymers containing hydroxyl groups (Sepharose, Trisacryl), with DCC and NHS introducing β-alanine via stable carbamate linkages (with a free carboxyl group), and formation of *p*-nitrophenyl active ester followed by coupling of ligands (peptides, proteins) that contain an amino group.

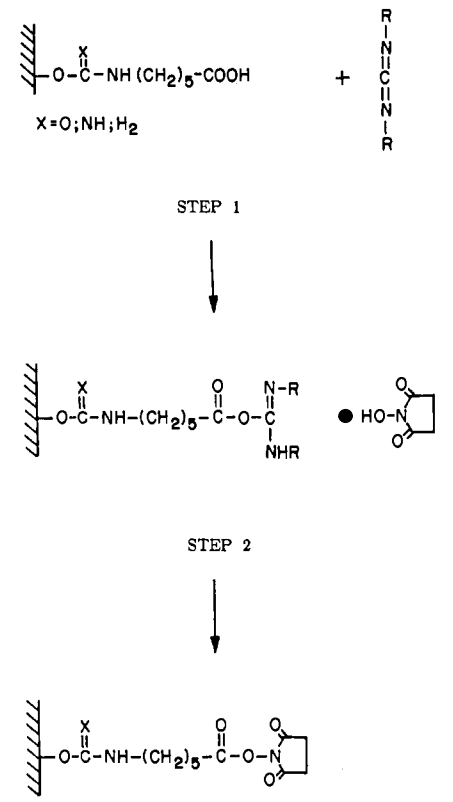

FIGURE 10: Two-step reaction for formation of NHS esters: step 1, activation with carbodiimide; step 2, reaction with NHS.

always used *p*-nitrophenyl esters (originally for economic reasons). These esters are easy to prepare (Bodanszky & Funk, 1973; Miron & Wilchek, 1985) and react with amino-containing ligands (Figure 9), albeit with slightly less efficiency than the corresponding NHS ester.[2] Several ligands and proteins were coupled to these *p*-nitrophenyl-β-alanine-activated matrices, yielding very stable conjugates (Table IV). The reaction of NHS and DCC was also used by us to supplement gels with carboxyl groups in cases where the amount of ε-cap coupled was low. After the rearrangement with NaOH, *p*-nitrophenyl or *o*-nitrophenyl esters were prepared (Table IV) and used for coupling.

Due to the release of the visible yellow color during the coupling of proteins to nitrophenyl esters, it is difficult to estimate the amount of protein coupled directly by UV, and therefore, researchers have been reluctant to use this approach.

[2] It is clear from this study that the higher yields usually claimed with NHS esters are due to the coupling of the amino-containing ligands to the *N*-succinimidyl carbamate of the β-alanine that is bound via an ester bond to the carrier. If the column is carefully washed and treated with ethanolamine, low yields of coupling (10–20%) are obtained (Yu et al., 1985) due to hydrolysis.

Table IV: Formation of *p*-Nitrophenyl Esters and Coupling of Ligands and Proteins

carrier	designation[a]	activated resins (μmol/g dry weight)			ligand coupled		
		β-Ala	ε-cap	PNP[b]	lysine (μmol/g)	γ-globulin (mg/g)	trypsin (mg/g)
Sepharose CL4B	A	138		65	65	18.5	NT[c]
	B		712	400	416	NT	82
	B		240	223		86	53
	C	177	241	307		154	112
Trisacryl GF2000	A	50		42	25	2.3	NT
	B		28	28		5.7	9.3
	C	154	31	68	66	10.5	NT
	C	44	13	34		12.6	12.1

[a] (A) Prepared by direct activation with NHS and DCC followed by NaOH treatment; (B) ε-cap coupled to chloroformate-activated resin; (C) ε-cap coupled to chloroformate-activated resin activated by NHS and DCC followed by NaOH treatment. [b] PNP, *p*-nitrophenyl. [c] NT, not tested.

Table V: Comparison of Extent of Activation and Coupling Capacities Obtained with Carbodiimide and NHS under Various Reaction Conditions

	standard reaction[a]	two-step reaction[b] (μmol/g dry weight)	low-temperature reaction[c]
NHS	356	115	58
β-Ala	100	0	13
Ala	260	85	55
ε-cap	258	259	260

[a] Sepharose–ε-cap was activated with NHS and DCC and reacted with alanine. [b] Treatment with 30 min with DCC followed by NHS for 3 h. [c] Reaction performed in tetrahydrofuran according to the standard conditions but at −10 °C.

We have therefore developed a two-step method for the preparation of NHS esters based, first, on the reaction of the carboxyl-containing resins with DCC; after filtration of excess DCC, NHS is added to yield NHS esters free of β-alanine (Figure 10). NHS esters with only small amounts of β-alanine were also prepared by the standard procedure if the reaction was performed at −10 °C. When the two-step method is used, three possibilities may account for reduced yields observed: (a) hydrolysis or incomplete reaction in one of the steps shown in Figure 10, (b) production of any anhydride (formed during activation between two carboxyls on the polymer and a carbodiimide molecule which would result in 50% yield, and (c) rearrangement of the carbodiimide intermediate to a stable ureido compound (Bauminger & Wilchek, 1980). These two methods obviously reduce both the amount of NHS ester formed and the amount of ligand coupled (Table V). For very high capacity columns, alternative methods should be developed.

Discussion

The problem of leakage has previously been considered to be mainly of an academic nature, since it had been thought that its major consequence would be an erroneous result (Wilchek et al., 1975). In recent years, the leakage phenomenon has posed a much more serious problem, since many proteins produced by genetic engineering for pharmacological use are purified by immunoaffinity chromatography on columns containing monoclonal antibodies. Leakage of even low levels of mouse antibodies which contaminate the final product would then be coinjected into patients (Wilchek et al., 1984).

In many laboratories, the NHS ester of agarose has been the support of choice for immobilization of proteins and low-molecular-weight amino-containing ligands in cases where a spacer is required (Twining & Brecher, 1975; Caldwell & Kuo, 1977; Tsao & Kim, 1978; Kanellopoulos et al., 1979; Conn et al., 1981). The widespread use of NHS agarose stems from the efficiency of the coupling reaction, the mild reaction conditions (physiological pH; also pH 3–10), and the previous claim that such reactions provide stable bonds (La Porte et al., 1977; Conn et al., 1981). However, the instability to alkali of alanine coupled to an NHS-containing support had already been noted in the first publication (Cuatrecasas & Parikh, 1972) on the use of NHS ester for affinity chromatography; the authors worked out conditions that reportedly minimized or eliminated this lability.

Many commercial companies are presently supplying these supports with claims of their complete stability; it had been assumed that the ligands are coupled to the NHS-activated terminal carboxyl group of the spacer arm to yield amide bonds. This would indeed have been the case, barring the latent side reaction described in this paper. This side reaction also occurs under previously described conditions assumed to give "leakage-free matrices".

Among the various supports examined in this work (checked for labile β-alanine after coupling of ligands), the least amount of leakage was observed with CH-activated Sepharose. It seems that β-alanine is coupled to this resin mostly through the stable carbamate linkage. A possible explanation for this is that cyanogen bromide activation modifies most of the primary hydroxyl groups on the Sepharose. Secondary hydroxyl groups are then available, which react better with the NHS-activated carbamates.

Since NHS esters are very convenient to use and have many advantages, we tried other known methods to produce NHS esters that do not use carbodiimides [e.g., using *N*,*N*′-disuccinimidyl carbonate (Wilchek & Miron, 1985)]. In all cases, other side reactions were observed (such as the production of activated carbonates), in addition to the desired ester (Miron & Wilchek, 1985).

In view of the findings in this paper (with all the limitations we describe here, i.e., the observed levels of leakage and modification of the bound ligands), the question is whether we should continue to use currently available forms of NHS esters, prepared by standard methods, or we should search for active esters or other coupling methods that would not yield side reactions during their preparation. If one persists in using NHS esters, one has to take into consideration that if a ligand is coupled through a single amino group, a large portion of the ligand will be lost (up to 80%) during the blockage of the active group with ethanolamine or NaOH after coupling of the ligand. If one couples a protein to the NHS ester of agarose or other hydroxyl-containing matrices, the chances of losing so much protein are much smaller, since the protein binds to multiple sites, including the stable one. On the other hand, when binding a protein to NHS-containing resins, one should take care to destroy all the excess active groups, including the β-alanine bound through the ester bonds. This can be accomplished by prolonged incubation with ethanolamine or hydroxylamine or (if the protein is stable) by short

treatment with 0.1 N NaOH. Even then one should remember that most of the lysine residues on the protein probably contain β-alanine linked via a urea bond and that the charge of the modified lysine is reversed (in some cases this may even be helpful). When such a protein leaks from the column, detection is difficult, since the protein is more negatively charged and tends to stain very poorly with Coomassie Blue or other stains (unpublished observations). More troublesome, however, is the possibility that one does not remove all the β-alanine bound through the ester bond and then uses the column for affinity chromatography. In this case the protein to be purified would bind to the *N*-succinimidyl carbamate of β-alanine, and the purified protein would contain some β-alanine (particularly when harsh conditions are used for evolution from the column). Otherwise, the protein would remain bound to the column, thereby reducing its capacity. We have observed all these problems during our recent studies with NHS ester.

From the present study it becomes clear that new activation methods for carboxyl groups on polymers containing hydroxyl groups are required. In this study we described the use of *p*-nitrophenyl or *o*-nitrophenyl esters and an improved modified method to prepare NHS esters that are free from side reactions but are less advantageous than the original NHS regarding final coupling yields. We are presently investigating alternative methods for activation of carboxyl groups on insoluble supports. We have started to check other activation methods of carboxyl groups such as Woodward's reagent K (Woodward et al., 1961) or active esters formed with 1-hydroxy-2-nitrobenzene-4-sulfonic acid (Klausner et al., 1977) for basic proteins or 2-hydroxypyridine esters for acidic proteins (Dutta & Morley, 1971).

Registry No. NaOH, 1310-73-2; DNP-Lys-β-Ala-urea, 107037-31-0; β-Ala-N^{ϵ}-Lys-urea, 107037-32-1; *N*-[(succinimidooxy)-carbonyl]-β-alanine *N*-hydroxysuccinimide ester, 21994-89-8; dicyclohexylcarbodiimide, 538-75-0; *N*-hydroxysuccinimide, 6066-82-6; *p*-nitrophenol, 100-02-7; *o*-nitrophenol, 88-75-5; Affi-Gel 10, 60454-66-2; Affi-Gel 15, 79920-18-6.

REFERENCES

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839.

Bauminger, S., & Wilchek, M. (1980) *Methods Enzymol. 70*, 151.

Becker, J. M., & Wilchek, M. (1972) *Biochim. Biophys. Acta 264*, 165.

Becker, J. M., Wilchek, M., & Katchalski, E. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2604.

Bodanszky, M., & Funk, K. W. (1973) *J. Org. Chem. 38*, 1296.

Caldwell, H. D., & Kuo, C. C. (1977) *J. Immunol. 118*, 437.

Conn, D. M., Smith, R. G., & Rogers, D. C. (1981) *J. Biol. Chem. 256*, 1098.

Cuatrecasas, P., & Parikh, I. (1972) *Biochemistry 11*, 2291.

Dutta, A. S., & Morley, J. S. (1971) *J. Chem. Soc. C*, 2896.

Gross, H., & Bilk, L. (1968) *Tetrahedron 24*, 6939.

Kanellopoulos, J., Rossi, G., & Metzger, H. (1979) *J. Biol. Chem. 254*, 7691.

Klausner, Y. S., Meiri, T. H., & Schneider, E. (1977) in *Peptides, Proceedings of the Fifth American Peptide Symposium* (Goodman, M., & Meienhofer, J., Eds.) pp 536–538, Wiley, New York.

Kohn, J., & Wilchek, M. (1982) *Biochem. Biophys. Res. Commun. 107*, 878.

La Porte, D. C., Rosenthal, K. S., & Storm, D. R. (1977) *Biochemistry 16*, 1642.

Miron, T., & Wilchek, M. (1982) *Anal. Biochem. 126*, 433.

Miron, T., & Wilchek, M. (1985) *Appl. Biochem. Biotechnol. 11*, 445.

Pathy, L. (1985) *Eur. J. Biochem. 151*, 197.

Spackman, D. H., Moore, S., & Stein, W. H. (1958) *Anal. Chem. 30*, 1190.

Tsao, D., & Kim, Y. S. (1978) *J. Biol. Chem. 253*, 2271.

Twining, S. S., & Brecher, A. S. (1975) *Proc. Soc. Exp. Biol. Med. 150*, 98.

Wilchek, M., & Miron, T. (1982) *Biochem. Int. 4*, 629.

Wilchek, M., & Miron, T. (1985) *Appl. Biochem. Biotechnol. 11*, 191.

Wilchek, M., Oka, T., & Topper, Y. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1055.

Wilchek, M., Miron, T., & Kohn, J. (1984) *Methods Enzymol. 104*, 3.

Woodward, R. B., Olofson, R. A., & Meyer, H. (1961) *J. Am. Chem. Soc. 83*, 1010.

Yu, K.-T., Pessin, J. E., & Czech, M. P. (1985) *Biochimie 67*, 1081.