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CONTROLLED PRIMARY FUNCTIONALIZATION OF AGAROSE AFFINITY SUPPORTS BY CARBOXYMETHYLATION AND SUBSEQUENT ADDITION OF SPACER UNITS

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SUMMARY

Experimental details are given for (1) the O-carboxymethylation of cross-linked agarose beads with chloroacetate in aqueous sodium hydroxide, (2) formation of a tandem 2-aminoethylamide derivative, and (3) coupling of β-alanine residues to the aminoethyl groups using a new reagent, N-phthalimidyl N′-ethylsulfonylethoxycarbonyl-β-alaninate. An ideal support for attaching affinity ligands results. Extent of derivatization was controlled by the time and temperature of the first reaction. Functional group densities were estimated by using hydrogen ion binding measurements for carboxyls and a new reagent, N-succinimidyl 3-[2-(4-nitrophenylamino)ethyldithio]propionate, for assaying reactive amino groups on solid supports. Synthesis and use of the new reagents are described.

INTRODUCTION

Agarose gels in spherical bead form have been widely employed as a support matrix for the preparation of affinity chromatography adsorbents [1–3]. Agarose, an isolated natural polysaccharide, consists principally of chains of alternating 1,3-linked β-D-galactopyranose and 2,4-linked 3,6-anhydro-α-L-galactopyranose residues extensively bound together by hydrogen bonds into criss-crossing bundles of double helices [4]. Large open pores permit easy access of protein molecules to the interior structure of the beads. Agarose provides a matrix with a very low background of interaction with macromolecules and an ample number of potentially reactive hydroxyl groups for covalent anchoring of ligands.

A number of methods for functionalizing agarose supports have been employed [1, 5–9]. Not all of these methods, however, provide the following important features characteristic of a good general functionalizing approach:

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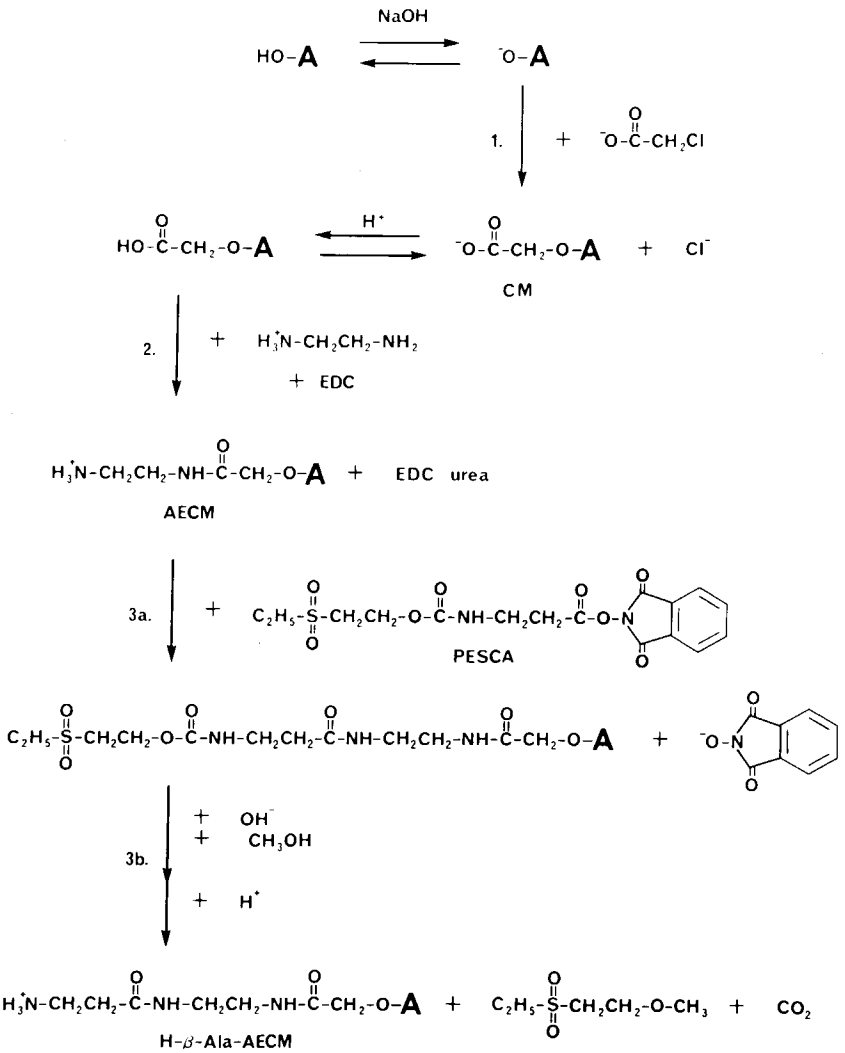

Fig. 1. Functionalization of agarose (A) and addition of two spacer units bearing primary amino groups. Reaction 1: carboxymethylation with chloroacetate to produce the O-carboxymethyl (CM) derivative. Reaction 2: aminoethylamide formation from excess ethylenediamine; coupling with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to give the N-(2-aminoethyl)carbamylmethyl (AECM) derivative. Reaction 3a: coupling of a protected β-alanine residue using phthalimidyl ethylsulfonylethoxycarbonyl-β-alaninate (PESCA). Reaction 3b: removal of the ethylsulfonylethoxycarbonyl (Esc) protecting group from the terminal primary amine.

(1) a highly stable linkage to the matrix; (2) easily controllable degree of functionalization; (3) no introduction of undesired ionic, hydrophobic or aromatic structures; (4) convenient means for adding uncharged, hydrophilic spacer arms using stable bonds; and (5) a generally useful anchoring (or pre-anchoring) group such as a primary amine. Illustrated in Fig. 1 is a series of reactions that have been used for some time with considerable success in the authors' laboratory in preparing affinity supports from commercially available,

chemically cross-linked agarose beads [10]. This approach to initial functionalization and addition of spacing structures meets the above criteria. Reaction 1 yields carboxyl groups linked by stable oxygen ether bonds through the agarose primary and secondary hydroxyl groups. Reaction 2 adds four atoms of spacing and a terminal primary amine, and reactions 3a and 3b do the same again, but in a manner that may be repeated if desired. These reactions have been described earlier by Inman [11–13]. In this paper we wish to give a full presentation of the methodology, semi-quantitative data on conditions for controlling the initial functionalization, and the first description of a new colorimetric reagent for assaying reactive primary (and secondary) amino groups on insoluble supports. The new reagent should be generally applicable to all matrices that can be chemically manipulated in solvent mixtures.

EXPERIMENTAL

Materials

Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Boehringer Mannheim Biochemicals (Mannheim, F.R.G.). Frinton Labs. (Vineland, NJ, U.S.A.) supplied 2-(ethylsulfonyl)ethanol (also available from K & K Labs., ICN). The following chemicals were purchased from Pierce (Rockford, IL, U.S.A.): N,N-dimethylformamide (Sequanal grade) and N-methylmorpholine. ICN Pharmaceuticals (NBC Division, Cleveland, OH, U.S.A.) was the source of 2,4,6-trinitrobenzenesulfonic acid. Aldrich (Milwaukee, WI, U.S.A.) supplied 2-mercaptopyridine and N,N-diethylethylenediamine. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride, dithiothreitol, dithioerythritol, N-hydroxysuccinimide (then recrystallized from ethyl acetate), isobutyl chloroformate, ethylenediamine dihydrochloride, *p*-nitrophenyl chloroformate, and N-hydroxyphthalimide were obtained from Sigma (St. Louis, MO, U.S.A.).

HEPES buffer containing EDTA (HE buffer) was prepared with the composition: 0.15 *M* HEPES, 0.07 *M* sodium hydroxide, 1.0 m*M* ethylenediaminetetraacetic acid, di- or trisodium salt (EDTA), pH 7.5. The solvent—HEPES—EDTA (SHE) buffer was prepared by mixing 3 vols. of HE buffer with 2 vols. of N,N-dimethylformamide (DMF, spectroscopic grade). These proportions were altered only where specifically indicated for modified SHE buffer.

Carboxymethylation at 25 or 37° C

Sepharose CL-4B beads (160 ml of settled bed volume) were suspended in 0.1 *M* sodium chloride to 900 ml, and fines were discarded after 60—70 min. This procedure was repeated three times, and the settled beads were transferred to a siliconized beaker; the volume was adjusted to 225 ml with 0.1 *M* sodium chloride. Next, 225 ml of 6.0 *M* sodium hydroxide were added and the temperature was adjusted to 10.0°C below the desired reaction temperature. Chloroacetic acid (monochloroacetic acid; 0.450 mol based on the equivalent weight determined by titration [13]) was added. Timing of the reaction was begun when the heat of neutralization reached 2° C below the control temperature, and the suspension was transferred to a siliconized, jacketed beaker

TABLE I

CARBOXYL AND AECM AMINO GROUP LEVELS VERSUS TIME AND TEMPERATURE OF CARBOXYMETHYLATION

Carboxymethylation		Functional group concentrations (μmol/ml of bed volume)				
Time (min)	Temperature (°C)	CM—agarose	Corresponding AECM—agarose			
		COOH as bound H^+	NH_2 via SNEP	Percentage of COOH	NH_2 via SPDP	Percentage of COOH
Untreated		0.68	0.35	51	N.D.*	
21	25.0	0.83	0.69	83	0.53	64
40	25.0	1.08	0.96	89	0.75	69
60	25.0	1.32	1.22	92	1.00	76
97	25.0	1.69	1.76	104	1.33	79
154	25.0	2.87	2.42	84	1.88	66
219	25.0	3.73	3.17	85	2.31	62
60	37	3.56	2.98	84	N.D.	
150	37	7.84	6.63	85	N.D.	
249	37	12.10	9.63	80	N.D.	

*N.D. = Not determined.

maintained at 25.0 ± 0.05°C or 37.0 ± 0.5°C from a circulating, constant temperature bath. The suspension was stirred with a motor-driven paddle. At designated times, aliquots were siphoned from the reaction mixture (see Table I) and transferred to a Buchner funnel. The reaction was terminated by washing the gel rapidly with 0.1 *M* sodium chloride. Washing was continued with 0.1 *M* sodium chloride, employing increasingly longer periods of time without applied suction. Each aliquot was allowed to stand overnight in 0.1 *M* sodium chloride and washed again slowly on a Buchner funnel, mostly without applying suction.

Carboxyl group concentrations

Carboxyl group concentrations (μmol/ml of settled bed) of agarose gels before and after carboxymethylation (Fig. 1, reaction 1) were estimated as hydrogen ion binding specific capacities (densities) between approx. pH 7 and 2 [14]. Thoroughly washed gel samples were transferred to siliconized graduated cylinders in 0.1 *M* sodium chloride suspensions and allowed to settle overnight. Following gentle tapping and further settling, settled bed volumes were read. Typically, a gel sample was then resuspended in 0.1 *M* sodium chloride to two or three times its bed volume, and the pH was adjusted to 7.0 ± 0.4 (with dilute sodium hydroxide or hydrochloric acid). A titration curve was obtained by adding increments of 0.05—0.25 *M* hydrochloric acid, stirring and recording pH after each addition. Values of pH were corrected to correspond to $p[H^+]$ by calibrating the meter (with Ross electrode) against a series of standard hydrochloric acid solutions in 0.1 *M* sodium chloride. Bound H^+ was calculated as total μmol of hydrochloric acid added minus μmol of free (unbound) H^+. The latter was determined as $1000 \times \text{antilog}(-p[H^+]) \times$ ml of suspension. Suspension volume was taken as initial net weight (g) × 1.0008 + ml of hydrochloric acid added.

Aminoethylamide derivatives

Carboxymethylated (or untreated) agarose beads were transferred to a Buchner funnel and washed with four portions of 1.50 *M* (20%, w/v, in water) ethylenediamine (EDA) dihydrochloride (decolorized with activated charcoal and adjusted to pH 4.6); each portion was about one half the settled bed volume. Suction was applied briefly after each portion had run through. The suction-drained gel was transferred to a siliconized beaker, and the beads were suspended in 1.50 *M* EDA to a total volume of 1.8 ml/ml settled bed volume. After the pH was adjusted to 4.6 with 2 *M* hydrochloric acid or sodium hydroxide, EDC (9 mg/ml of suspension volume) was added in portions, with stirring, over a period of 30 min at room temperature. Stirring and pH control (4.6—4.8) were continued for an additional 30 min. Another similar portion of EDC was added as in the previous step. Stirring and pH control were continued for 4 h after the addition was completed. The suspension was transferred to a Buchner funnel and the beads were washed with 0.1 *M* sodium chloride, employing increasing periods without applied suction. Washing was continued with 0.4 *M* and finally with 0.1 *M* sodium chloride. The last drops of filtrate were checked for free EDA by adding 1 ml saturated sodium borate and 3 drops of 3% (w/v) 2,4,6-trinitrobenzenesulfonic acid (TNBS, in water) to 2 ml of filtrate [14] and looking for pronounced yellow color in 5 min.

SNEP synthesis

The synthesis and properties of N-succinimidyl 3-[2-(4-nitrophenylamino)-ethyldithio]propionate (SNEP) will be described in detail in a separate paper. Briefly, it was prepared as follows: N,N'-di-(4-nitrophenyl)cystamine was made by refluxing cystamine in methanol for six days with an excess of 4-nitro-1-fluorobenzene and diisopropylethylamine. The product, which crystallized from the reaction mixture, was recrystallized from 1-propanol, then 1,2-dichloroethane, and reduced with equimolar dithioerythritol in DMF (and the solution made basic with N,N-diethylethanolamine). After several hours, this solution was added to a large excess of 3,3'-dithiodipropionic acid in DMF. The mixed disulfide, 3-[2-(4-nitrophenylamino)ethyldithio]propionic acid, was isolated after removing DMF, by extracting into ethyl acetate over 0.5 *M* sodium phosphate buffer, pH 6.8, washing with the same and then with 0.2 *M* potassium hydrogen sulfate, and removing solvent. The crude product was crystallized three times from 1-chlorobutane plus acetonitrile.

SNEP was prepared by reacting essentially equimolar amounts of the above mixed disulfide (MW 302.4), N-hydroxysuccinimide and EDC in 2-propanol. The product (MW 399.45, m.p. 144—146°C) crystallized out from the initial solution (2 h) and was recrystallized twice from 1-propanol.

Molar absorptivities of pyridine-2-thione and NET

The molar absorptivity of pyridine-2-thione, the chromophore released in amino group assays via N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), was determined in several buffers. Pyridine-2-thione (predominant tautomer of 2-mercaptopyridine from Aldrich) was recrystallized five times from benzene. A sample in ethanol solution was diluted 100-fold in a chosen buffer; its absorbance was measured at the wavelength of peak value (near 343 nm).

The molar absorptivity of 2-(4-nitrophenylamino)ethanethiol (NET), the chromophore released in amino group assays via SNEP, was determined in several buffers. N,N′-Di-(4-nitrophenyl)cystamine (0.050 mmol) was dissolved in 10 ml of DMF. Dithiothreitol (DTT) (1.0 mmol) was added to produce 0.100 mmol of NET. After 2 h at room temperature, this reaction mixture was made up to 100.0 ml with water. Of this solution 2 ml were diluted to 50 ml with a chosen buffer. Absorbance was measured at the wavelength of peak value (near 404 nm).

Amino group determination with SPDP

A sample of N-(2-aminoethyl)carbamylmethyl (AECM)–agarose (3–4 ml bed volume) was washed on a Buchner funnel with 0.1 *M* sodium chloride, followed by SHE buffer (see *Materials*). It was then resuspended in 8 ml of SHE buffer. A five-fold molar excess of SPDP (synthesized according to Carlsson et al. [15]), freshly dissolved in 0.5 ml DMF, was added. The mixture was stirred 1–2 h and the gel was washed with SHE buffer (200–250 ml), 0.1 *M* sodium chloride (75 ml), and finally HE buffer (see *Materials*; 75 ml). For duplicate assays, 1.0–1.5 ml bed volume was placed in each of two, siliconized 10 × 75 mm test tubes using HE buffer to suspend the beads. The gels were packed by a 2-min centrifugation at approximately 1500 *g*. After the tubes were allowed to stand for at least 10 min, the position of the bed top was marked for subsequent volume measurement, and the supernatant buffer was removed. An approximately equal volume of freshly prepared 0.20 *M* DTT in HE buffer was added to each gel sample; the tubes were mixed intermittently (by inversion) for 45 min. Each suspension was quantitatively transferred to a 25-ml mixing cylinder and made up to the mark with HE buffer. The suspensions were mixed for 8–10 min, allowing time for the chromophore to diffuse out of the beads. Samples were centrifuged briefly to remove beads. Absorbances of the clear supernatants were measured at 343 nm versus a blank consisting of 0.01 *M* DTT in HE buffer. Amino group concentrations (μmol/ml of bed) were calculated from the expression $25 \times A_{343}/(8.21 \times \text{ml of gel bed})$, based on a molar absorptivity of 8210 for pyridine-2-thione in the above buffer.

Amino group determination with SNEP

A sample of amino-derivatized agarose (3–4 ml bed volume) was washed (on Buchner funnel) with 0.1 *M* sodium chloride, followed by modified SHE buffer [DMF–HE (65:35), see *Materials*], and then resuspended in 8 ml of the same buffer. A three- to five-fold molar excess of SNEP was dissolved in 0.5 ml DMF and added to the suspension. The reaction mixture (reaction 4, Fig. 2) was stirred at room temperature for a minimum of 2 h. The gel was washed with modified SHE, 0.1 *M* sodium chloride, then HE buffer, and set up for duplicate assays as in the preceding procedure. After marking the position of the bed top and removing the supernatant, an approximately equal volume of freshly prepared 0.2 *M* DTT in regular SHE [DMF–HE (40:60)] was added to each gel sample; the tubes were mixed intermittently (by inversion) for 45 min at room temperature (reaction 5, Fig. 2). Each suspension was quantitatively transferred to a 50-ml mixing cylinder and made up to the mark with HE

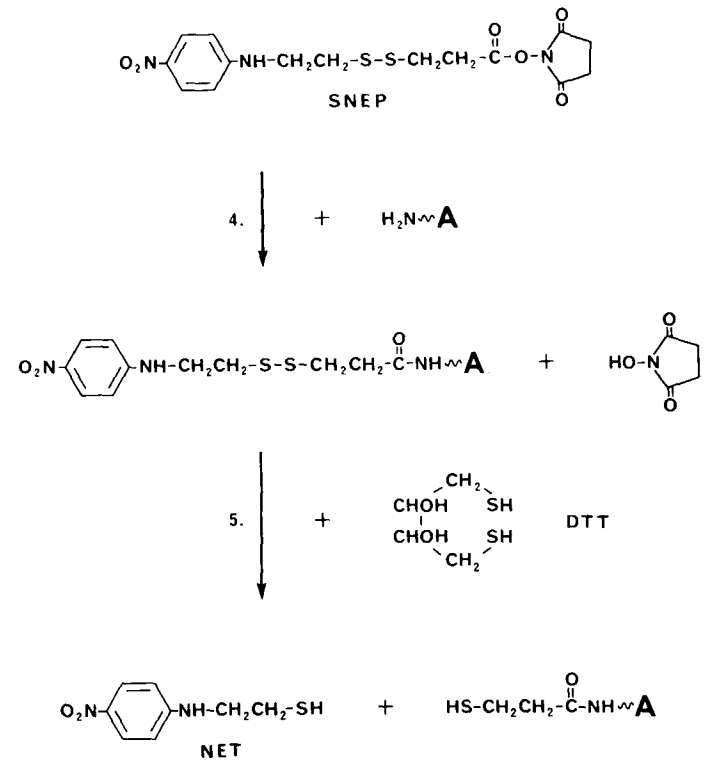

Fig. 2. Reactions employed in determining the level of amino groups attached to a solid support (A). Amino groups are reacted quantitatively with an excess of N-succinimidyl 3-[2-(4-nitrophenylamino)ethyldithio]propionate (SNEP) via reaction 4; excess reagent is removed by washing and the chromophore, 2-(4-nitrophenylamino)ethanethiol (NET), is released from the support by reducing the disulfide link with excess dithiothreitol (DTT) via reaction 5.

buffer containing 0.01 *M* DTT. The suspensions were mixed for 8—10 min. Samples were centrifuged briefly to remove beads. Absorbances of the clear supernatants were measured at 404 nm versus 0.01 *M* DTT in HE buffer. Amino group concentrations (μmol/ml of bed) were calculated from the expression $50 \times A_{404}/(18.78 \times$ ml of gel bed), based on a molar absorptivity of 18 780 for NET in the above buffer.

2-(Ethylsulfonyl)ethyl 4-nitrophenylcarbonate (Esc-ONp)

The synthesis of Esc-ONp was carried out as follows (reaction 6, Fig. 3: 16.60 g (120 mmol) 2-(ethylsulfonyl)ethanol (dried three days in vacuum over calcium chloride) were dissolved in 200 ml dichloromethane (DCM); 20.0 ml (248 mmol) pyridine were added and the mixture was cooled in an ice bath. To this solution was added slowly, with stirring, a solution of 16.13 g (80 mmol) *p*-nitrophenyl chloroformate in 120 ml DCM. The ice bath was removed, and stirring was continued at room temperature overnight. The mixture was shaken (separatory funnel) with 200 ml additional DCM plus 300 ml of 1 *M* hydrochloric acid. The lower phase was then shaken twice with 160-ml portions of 0.25 *M* hydrochloric acid and three times with 160-ml portions of

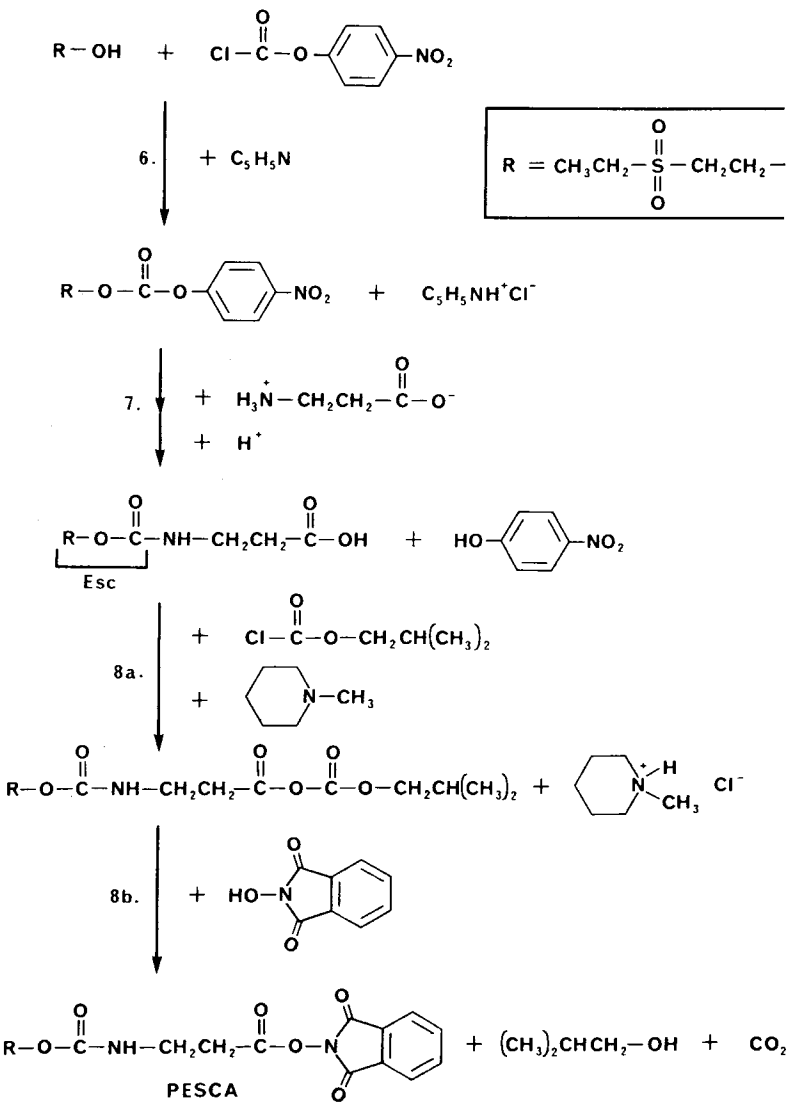

Fig. 3. Steps for synthesizing phthalimidyl ethylsulfonylethoxycarbonyl-β-alaninate (PESCA), the reagent used for adding β-alanine spacing units to amino groups on a solid support.

0.25 *M* $NaHCO_3$—0.25 *M* Na_2CO_3. All upper phases were discarded. To the lower layer were added 1.14 ml (8 mmol) N,N-diethylethylenediamine. After 2 h the mixture was shaken once with 160 ml of the above carbonate buffer and twice with 160 ml of 0.25 *M* hydrochloric acid (upper layers were discarded). The lower layer was filtered through Whatman No. 1PS paper, stirred with 50 g anhydrous sodium sulfate for 1 h and filtered again. Solvent was removed from the filtrate by rotary evaporation under reduced pressure. After standing in vacuum over calcium chloride, the crude product was dissolved in 110 ml warm ethyl acetate. The solution was cooled in an ice bath and 285 ml hexane were added dropwise with stirring, during which time

crystallization was induced by scratching or seeding. The mixture was allowed to stand overnight at 4°C; the product was collected on a Buchner funnel and washed with a mixture of 12 ml ethyl acetate and 30 ml hexane, then with hexane. The final product, dried in vacuum over calcium chloride, weighed 11.68 g (48% of theory) (MW 303.29; m.p. 63—65°C). Thin-layer chromatography (TLC) (silica, EM Labs.) in toluene—ethyl acetate (7:3, v/v) showed (UV) product of R_F 0.26 and a very minor component at R_F 0.01. Calculated for $C_{11}H_{13}NO_7S$: C, 43.56; H, 4.32; N, 4.62; S, 10.57. Found: C, 43.50; H, 4.33; N, 4.60; S, 10.51.

N-Phthalimidyl N'-ethylsulfonylethoxycarbonyl-β-alaninate (PESCA)

N-Ethylsulfonylethoxycarbonyl (Esc) β-alanine was made by adding a solution of 9.71 g (32 mmol) Esc-ONp in 175 ml DMF to a solution of 3.56 g (40 mmol) β-alanine in 88 ml water at room temperature (reaction 7, Fig. 3). The mixture was stirred overnight and solvent was removed by rotary evaporation (55°C) under vacuum. The residue was dissolved in 420 ml water; 5.65 g (19.2 mmol) of sodium citrate dihydrate were added, and the resulting solution was adjusted to pH 5.1 with 2 *M* hydrochloric acid, transferred to a separatory funnel and shaken with 120 ml diethyl ether. The lower phase was readjusted to pH 5.1 and reshaken with the same upper phase. The upper layer was discarded and the lower layer was extracted twice more with 120-ml portions of diethyl ether; the pH was checked and readjusted to 5.1 before each equilibration. Next, 112 g sodium chloride were dissolved in the lower phase and the pH was adjusted to 1.8 with 5 *M* hydrochloric acid. This solution was shaken three times with 180-ml volumes of ethyl acetate. The upper layers were pooled (final lower discarded), filtered (Whatman No. 1 paper) and rotary-evaporated. The residue was allowed to stand in vacuum over calcium chloride for several days. The crude product was dissolved in 40 ml ethyl acetate by heating to reflux, and the solution was allowed to cool to room temperature. Crystallization was induced, and 65 ml diethyl ether were added dropwise with stirring. The mixture was allowed to stand overnight at 4°C. The precipitate was collected by suction filtration, washed on the funnel with 3 ml ethyl acetate plus 6 ml diethyl ether and finally with diethyl ether alone. The vacuum-dried Esc-β-alanine weighed 3.58 g (44% of theory) (MW = 253.28).

PESCA was next synthesized. A solution of 2.66 g (10.5 mmol) Esc-β-alanine in 33 ml DCM plus 4 ml DMF was cooled to −20°C using an alcohol bath and limited amounts of dry ice; 1.18 ml (10.5 mmol) N-methylmorpholine and 1.31 ml (10.0 mmol) isobutyl chloroformate were added, and the mixture was stirred for 20 min at −20°C. The resulting crude, mixed anhydride (reaction 8a, Fig. 3) was added to a solution of 1.75 g (10.7 mmol) N-hydroxyphthalimide in 20 ml DCM plus 4 ml DMF, precooled to −5°C. The mixture was stirred for 30 min at −5°C and for 3 h at 0°C (reaction 8b, Fig. 3). Solvent was removed by rotary evaporation, and the residue was equilibrated with 170 ml of DCM plus 50 ml of 0.5 *M* $NaHCO_3$. The lower layer was sequentially washed with 40 ml of 0.5 *M* $NaHCO_3$. 40 ml of 0.25 *M* hydrochloric acid and 40 ml of water; filtered through Whatman No. 1PS paper; stirred with 12 g sodium sulphate (1 h); filtered again (rinsing with DCM), and rotary-

evaporated. The residue was vacuum-treated over calcium chloride overnight, then dissolved in 40 ml absolute ethanol by heating to reflux with stirring. Crystallization was induced (scratching/seeding) during cooling to room temperature. Crystals of PESCA were collected after standing overnight at 4°C, washed twice with 4 ml cold ethanol and dried in vacuum over calcium chloride. Yield was 3.06 g (77% of theory) (MW = 398.39; m.p. 128—130°C). Calculated for $C_{16}H_{18}N_2O_8S$: C, 48.24; H, 4.55; N, 7.03; S, 8.05. Found: C, 48.00; H, 4.61; N, 6.97; S, 8.13.

Preparation of β-alanyl—AECM—agarose

AECM—agarose (15 ml) was washed on a Buchner funnel with 0.1 *M* sodium chloride, followed by a mixture of 0.15 *M* HEPES—sodium hydroxide, pH 7.6 buffer (3 vols.) and DMF (2 vols.) (designated, HS buffer). The gel was suspended in HS buffer to a 25-ml volume. A five-fold molar excess of PESCA (based on amino group content of the AECM derivative, but never less than the amount calculated for 1.5 μmol/ml) was dissolved in DMF (to make about 0.12 *M*). This solution was added to the gel, and the suspension was stirred at room temperature for 2 h. The resulting Esc-β-alanyl—AECM—agarose (reaction 3a, Fig. 1) was washed (Buchner funnel) with HS buffer (150—200 ml) and 0.1 *M* sodium chloride (100 ml). Completion of the reaction was checked by adding a few drops of 3% aqueous 2,4,6-trinitrobenzenesulfonic acid (TNBS) to a suspension of gel in 1 ml of 0.15 *M* HEPES—sodium hydroxide buffer, pH 7.6 (without DMF; Esc groups begin to dissociate in borate buffer at pH 9, giving falsely positive reactions). An essentially negative reaction on the gel should be seen after several minutes.

Esc groups were removed (reaction 3b, Fig. 1) by allowing the gel to remain on the Buchner funnel and washing successively and slowly with 0.1 *M* sodium chloride—methanol (3:1, then 1:1) (125 ml each); next, the composition 0.05 *M* sodium hydroxide—0.1 *M* sodium chloride—50% (v/v) methanol (250 ml) for 30 min; then 0.1 *M* sodium chloride—methanol (3:1), 0.002 *M* hydrochloric acid in 0.1 *M* sodium chloride, and finally 0.1 *M* sodium chloride.

RESULTS AND DISCUSSION

Carboxymethyl (CM) derivatives

Initial functionalization of cross-linked agarose beads (Sepharose CL-4B) was carried out by O-carboxymethylation with aqueous 1.0 *M* chloroacetate in 2.0 *M* excess sodium hydroxide (reaction 1, Fig. 1) in the manner reported earlier [11—13]. This same reaction was used by Peterson and Sober [16] in preparing carboxymethyl cellulose adsorbents. A large excess of chloroacetate was employed in order to minimize exposure time of the agarose to the strong alkali necessary for promoting the reaction. It can be seen from the data in Table I, as plotted in Fig. 4, that it is quite feasible to reach any desired concentration of stable carboxymethyl ether groups; the reaction is conducted at a controlled temperature and is terminated at a preset time by rapid funnel washing with a saline solution.

The determination of carboxyl group content of gel samples was based on the assumption that binding of hydrogen ions between pH 7 and 2 was due

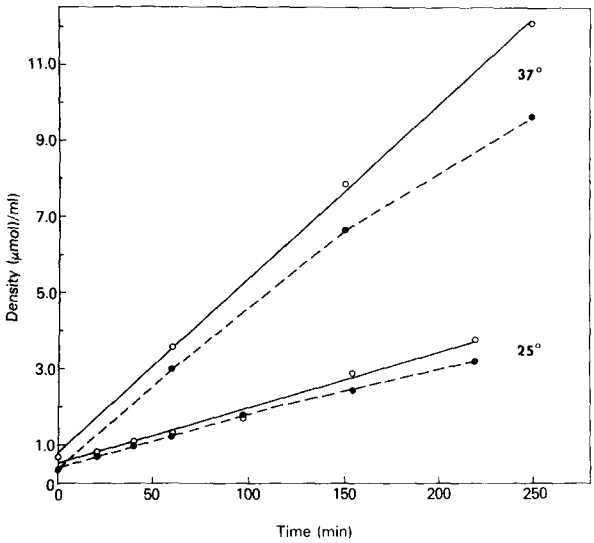

Fig. 4. Time course of temperature-controlled carboxymethylation of agarose (Sepharose CL-4B) at 25 and 37° C (solid lines) and amino group levels of the AECM—agarose prepared from the corresponding CM derivatives (dashed lines). Functional group densities (i.e., concentrations) are expressed in μmol/ml of settled gel bed. Open circles are estimations of carboxyl densities from hydrogen ion binding measurements and filled circles are results of amino group assays using SNEP (see Experimental and Table I). Solid lines are linear regressions; dashed lines are drawn curves.

mainly to carboxylic acid groups of the glycolic ether type. Indeed, plots of bound H^+ versus pH (not shown) give smooth, sigmoid titration curves with pK_a values in the expected range of 3.7—3.8. This low pK_a, coupled with the low capacity of many of the gels, made free hydrogen ion corrections large and uncertain below about pH 3, so that most full-range hydrogen ion binding values (pH 7—2) were estimated by doubling the amounts bound from pH 7 to pH = pK_a = 3.72, determined with the highest capacity gel (249 min at 37° C). However, the pK_a may actually vary slightly with COOH concentration, introducing some error. All functional group densities (specific capacities) in this study were expressed as millimolar concentrations, that is, μmol/ml of settled bed volume. Because of possible variation in degree of bed packing, bed volume is not as precise a quantity for normalizing capacity as, say, a dry weight, but concentrations are a direct and practical measure for both theory and applications of the adsorbent density parameter. Therefore, all results were obtained in this form, in order to serve as a practical guide in derivatizing agaroses.

The washed, untreated Sepharose CL-4B employed in this study had a low level of groups ionizing in the carboxyl range. The average value of the intercepts of the 25 and 37° C curves (excluding use of the zero-time value), as well as the concentration determined by titration of the untreated Sepharose, was 0.68 μmol/ml. From the linear regression curves (solid lines) of Fig. 4, a Q_{10} (ratio of reaction rates over a 10° C interval) of 2.6 was obtained, which is in the range found for many chemical reactions. The two temperatures employed allow convenient reaction periods for low and high ranges of functional group

concentrations (C) normally used for preparing affinity adsorbents. Results from using other temperatures can be predicted from $\log C_2/C_1 = (t_2 - t_1/10) \log Q_{10}$.

AECM derivatives

In reaction 2 (Fig. 1) the AECM derivative is formed by amide coupling of ethylenediamine to the CM carboxyl group in aqueous solution at pH 4.6 by means of the water-soluble condensing agent, EDC. Mainly one-ended coupling, leaving a free amino terminus, is ensured by employing a large excess of ethylenediamine (1.5 M). This type of reaction is further promoted by the fact that the first pK_a of ethylenediamine is about 7.1, much nearer to the reaction pH than $pK_a = 9.2$ of an attached amino group [11, 14]. Thus, an unreacted diamine is intrinsically more reactive than an attached molecule since a higher proportion of its amino groups are in the reactive, unprotonated form at pH 4.6. The AECM derivative provides a primary amino group with six atoms of spacing.

The density of AECM groups will be determined by reaction 1 since reaction 2 is allowed to go as fully as possible to completion. An advantage of this general approach is that carboxyl groups originally present in the agarose will also participate in reaction 2; therefore, less residual charge will remain when the amino groups are all subsequently reacted with desired moieties (ligands and terminating or capping groups, such as acetyl). Further practical details for preparing AECM—agarose are given by Inman [13].

It was of interest to measure AECM amino group concentrations to check on the efficiency of reaction 2. However, few really satisfactory methods were known for analyses involving insoluble matrices. A good approach would be to derivatize the amino groups as completely as possible with a dissociable chromophoric reagent which would permit subsequent spectrophotometric determination of the chromophore in free solution. The use of SPDP in such a manner has been proposed [15]. In the authors' laboratory it was found that, upon reduction of the bound pyridyldithiopropionyl groups with dithiothreitol, some hydrogen sulfide was released; its presence in vapors over the reaction mixture was verified by a positive reaction on moist lead nitrate paper. The nature of this side-reaction has not yet been investigated, but it probably involves the sulfur atom of the expected, released chromophore, pyridine-2-thione. The result would be a lower reported value for the derivatized amine. This side-reaction appears to occur to a greater or lesser degree with all thiol reductions of disulfides on solid phases or in solution [17]. We accordingly designed and synthesized a reagent, SNEP, in which the disulfide sulfur atoms are not an essential part of the chromophore. Its use is illustrated in Fig. 2 (also, see Experimental). The dissociated chromophore, NET, has additional advantages over pyridine-2-thione in having a higher maximum absorbance wavelength (easily visible, brilliant yellow) so that UV-absorbing substances will not interfere; it allows higher sensitivity by having over two-fold higher molar absorptivity, and its absorptivitiy is less sensitive to pH changes.

The results of SNEP assays for amino groups of AECM—agaroses, prepared from the corresponding CM derivatives, are given in Table I; these results are plotted as the dashed curves in Fig. 4. Although there are considerable

combined margins of error in comparing carboxyl and amino values, the latter levels appear to be consistently slightly lower (average of 89.5% of carboxyl values in the 25°C series and 83% in the 37°C series). The causes of this difference, of course, could be incomplete conversion of CM to AECM due to incomplete reaction and side-reactions such as cross-linking (mentioned above) or some N-acylurea formation between carboxyls and the carbodiimide. In a study on the formation of AECM—Ficoll from CM—Ficoll [11], 12% of the carboxyls remained unchanged; both amino and carboxyl groups had been assayed by titration. In the present study, 18 mg/ml rather than 12 mg/ml EDC was used, so a slightly higher conversion would be expected. Data from one experiment in which varying amounts of SNEP were used (more and less than in the regular procedure) suggest that perhaps not all amino groups were combined with SNEP (see Table II), but that probably greater than 90% were combined. Taking into account the above questions, it can be seen that the SNEP reagent and assay can work in a reliable manner to give an estimate of reactive amino group concentrations. Washed, dissociated gels were completely colorless and tested strongly positive for bound sulfhydryl in a nitroprusside color reaction.

TABLE II

AMINO GROUP ASSAYS USING VARIOUS LEVELS OF SNEP

Applying the SNEP assay to AECM—agarose derived from 60 min at 37°C CM—agarose (COOH, 3.56 m*M*).

Concentration of SNEP* (m*M*)	Mol SNEP used per mol COOH**	Amino group concentration*** (μmol/ml bed volume)
2.1	1.76	2.90
2.7	2.28	3.07
4.2§	3.52	2.98
5.6	4.75	3.36
6.7	5.63	3.25

*Initial concentration of the reagent, SNEP, in reaction 4 (Fig. 2).
**Total mol of SNEP used per mol of COOH in the precursor, CM—agarose.
***Each value is an average result from two reductions via reaction 5 (Fig. 2) from a single sample carried through reaction 4.
§The level of SNEP used for the assay reported in Table I.

Substantially lower values for amino group concentrations were obtained with a corresponding SPDP assay (Table I, last two columns). AECM samples were shown to be saturated with pyridyldithiopropionyl groups by negative TNBS reactions. The molar absorptivitiy of pyridine-2-thione was carefully measured in the buffer employed (Experimental). The suggestion can be made that the H_2S-producing side-reaction could be primarily responsible for these lower values, but a definitive answer must await further investigation.

Addition of a β-alanine spacer unit

The AECM derivative allows a spacing of six atoms from the matrix. This marginal separation can be increased by four atoms ideally by adding a residue

of β-alanine. The advantages of this spacing unit are the stable bond, the hydrophilic character of the peptide bond with only two CH_2 links, no added charge (aside from the functional amine), and a flexible featureless structure. A convenient reagent for coupling β-alanine residues onto anchored amino groups was designed, synthesized and reported earlier [12]. N-Hydroxyphthalimide active esters [18] are highly selective in acylating amino groups rather than matrix hydroxyl groups [17]. In Experimental, full details are given for the synthesis of PESCA or, alternatively, Esc-β-Ala-ON<Pht.

The two-stage reaction employing PESCA (reactions 3a and 3b) is shown in Fig. 1. The quantitative acylation of AECM amino groups by the active ester function was carried out by using an excess of PESCA (preferably the stoichiometric amount plus an amount calculated to give 4—5 m*M* concentration in the reaction mixture). The resultant Esc-blocked β-alanyl gel derivative was treated with dilute sodium hydroxide to remove the Esc group by a base-catalyzed β-elimination reaction. The immediate cleavage product, reactive ethyl vinylsulfone, is destroyed by addition to water or methanol; the latter is perhaps the better scavenger, preventing Michael addition to the released amino group (while the carbamate intermediate provides some temporary protection of the amine). PESCA is a stable, nicely crystalline compound that can be stored for years over calcium chloride at 4°C. It should be usable with any matrix that can withstand 0.05 *M* sodium hydroxide for 30 min at room temperature.

The synthesis of PESCA is outlined in Fig. 3. Esc groups were introduced (reaction 7) by the reagent Esc-ONp, prepared (reaction 6) in a similar way to the methylsulfonyl analogue [19]. Esc was chosen because of the commercial availability of 2-(ethylsulfonyl)ethanol and the convenience of higher melting points of the intermediates and final product. PESCA was prepared by means of the mixed anhydride of Esc-β-alanine with isobutylcarbonic acid [20, 21] (Fig. 3, ractions 8a and 8b), giving a very clean product. Reactions 8a and 8b have been replaced successfully by a single-step condensation using either EDC in DMF or dicyclohexylcarbodiimide in dioxane.

The extent of conversion of AECM to β-alanyl-AECM was determined by assays employing SNEP for two separate preparations: AECM from CM (219 min at 25°C) and CM (249 min at 37°C) having amine concentrations of 3.17 and 9.63 μmol/ml, respectively, were carried through reactions 3a (with excess PESCA) and 3b (Fig. 1; and see Experimental). SNEP assays on the products gave 3.01 and 9.18 μmol/ml, 95% conversion in both cases. Once prepared, PESCA can be used in many different applications; its use is facile and rapid. Additional spacing units may be added by repeating reactions 3a and 3b. The above approach yields stable, well characterized agarose derivatives that provide bland spacer arms with terminal primary amino groups for subsequent attachment of specific affinity ligands. The new colorimetric reagent, SNEP, should be applicable for estimating the levels of functionally reactive amino groups on other affinity matrices that can be treated in solvent-containing media.

β-Alanyl—AECM—Sepharose CL-4B [13] was reacted with SPDP and treated with DTT to give a thiol derivative to which was attached an S-(2,4-dinitrophenylaminohexyl)carbamylmethyl ligand [12]. This ten-atom-spaced amino

agarose also was combined with N-(2,6-dinitro-4-carboxyphenyl)-β-alanylglycylglycyl groups via an acyl azide coupling reaction [22, 23]. The above derivatives and similar nitrophenyl adsorbents have been used successfully in quantitative affinity chromatography experiments for determining binding constants of anti-dinitrophenyl monoclonal antibodies with a wide variety of ligands, employing a competitive inhibition technique [22, 17].

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