origin, but neither phytane nor olefinic isoprenoids have been isolated in detectable quantities (Blumer and Snyder, 1965). We do not, however, exclude the possibility that phytadienes and C_{19} olefins, formed *in situ* by catalytic dehydration of phytol play a role as intermediates in the formation of fossil phytane and, *via* degradation at greater depth and temperature, of its lower homologs.

Acknowledgment

We thank Mr. M. L. Rosenthal (Robeco Chemicals, New York) for samples and information.

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The Derivatization of Cross-Linked Polyacrylamide Beads. Controlled Introduction of Functional Groups for the Preparation of Special-Purpose, Biochemical Adsorbents*

John K. Inman and Howard M. Dintzis

ABSTRACT: Methods are described for the chemical modification of preformed, cross-linked polyacrylamide beads for the purpose of introducing any of a wide variety of functional groups to a predetermined level. Examples are given of the conversion of commercially available polyacrylamide beads into a variety of ion-exchange media, of differing titration range and capacity, capable of binding protein molecules. The use of chemically reactive bead derivatives for the covalent binding of proteins is seen in the examples of solid-phase coupling of trypsin and bovine serum albumin to beads. The reversible reaction of rabbit antibody with bovine serum albumin antigen covalently coupled to acrylamide beads is described.

Modified hydrophilic polymers of natural origin such as cellulose and cross-linked dextran have been used extensively as carriers for biochemical separations based on ion exchange (Sober *et al.*, 1965) or the covalent bonding of biochemically active molecules, either large (Campbell *et al.*, 1951) or small (Cuatrecasas *et al.*, 1968). These natural polymers are polysaccharide in nature and their chemical stability and reactivity is limited by the sugar groups of which they are composed. In the case of cellulose derivatives, furthermore, the complex physical structure of the starting material may be responsible for nonuniform substitution in the product.

Polyacrylamide beads as support medium offer the advantage of enhanced chemical stability by virtue of their polyethylene backbone structure, coupled with a statistically uniform physical state and porosity resulting from their formation as cross-linked synthetic polymers. The beads are readily available commercially in spherical form, in pregraded sizes and porosities, permitting the penetration of macromolecules up to molecular weights of approximately one-half million. It seems probable that wide use would be made of preformed polyacrylamide beads as carrier matrix for biochemical separations if methods were available for the controlled introduction of chemical groups suitable for ion-exchange or covalent formation. Methods fulfilling these requirements are described below.

Polyacrylamide beads contain a hydrocarbon skeleton to which is attached carboxamide side groups. These groups are chemically stable and resistant to hydrolysis in the pH range between 1 and 10. The amide ammonia nitrogen, however, is readily replaced by certain other nitrogen compounds per-

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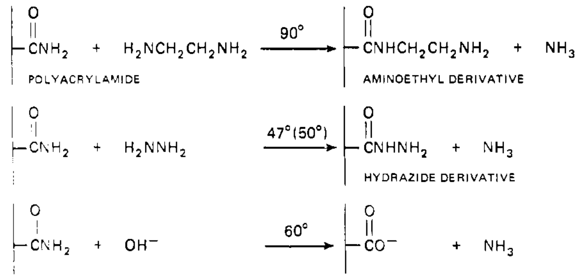

FIGURE 1: Reactions employed in the initial derivatizations of cross-linked polyacrylamide. In the first two reactions a large excess of the reagent was used. In the third reaction a sodium carbonate-bicarbonate buffer furnished OH^-. The symbol $-\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NH}_2$ represents a residue within a linear segment of polyacrylamide

$$-CH_2-\underset{CONH_2}{CH}-(CH_2-\underset{CONH_2}{CH})CH_2-\underset{CONH_2}{CH}-$$

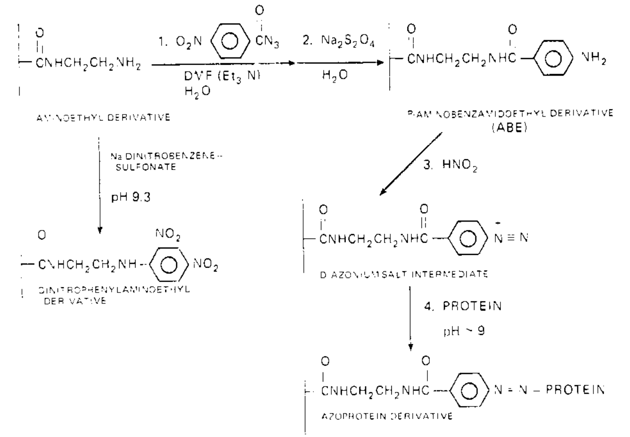

FIGURE 2: Reaction scheme used in preparing derivatives from aminoethylated polyacrylamide. DMF is *N,N*-dimethylformamide, Et_3N is triethylamine, $Na_2S_2O_4$ is sodium hydrosulfite (dithionite), and ABE is a symbol for the derivative so indicated.

mitting the formation of a series of derivatives with useful properties. Conversion of stable amide groups into more reactive nitrogen derivatives is illustrated in Figure 1 for reactions with anhydrous ethylenediamine and aqueous hydrazine. The aminoethyl and hydrazide derivatives of polyacrylamide beads thus formed serve as parent compounds for a further sequence of stable derivatives as illustrated in Figures 2 and 3. Not only is it possible to thus attach a wide variety of substituents to preformed neutral acrylamide beads, but as is discussed below, the degree of substitution may readily be varied over a wide range.

Experimental Procedures

Materials. The polyacrylamide beads used in this study were manufactured by Bio-Rad Laboratories, Richmond, Calif., and sold under the trade name Bio-Gel P. These beads, made of cross-linked copolymers of acrylamide and *N,N'*-methylenebisacrylamide, are spherical in shape and are available in several size ranges. When contacted by water or aqueous solutions, the beads swell to form spherical gel particles having pores of molecular dimensions. Pore size is controlled by the degree of cross-linkage. Various porosities are available which exclude solute molecules having molecular weights ranging from about 2000 (with type P2) to over 300,000 (with type P300). Chemicals used were of reagent grade quality if their sources are not designated.

Techniques. Polyacrylamide gel particles adhere strongly to clean glass surfaces. Therefore, in order to facilitate transfer and mixing operations all glassware was siliconized or, whenever possible, TPX™ (Nalgene) or polyethylene labware was used. Titrations were carried out in TPX beakers which allowed quantitative sample transfer to dry weight crucibles. Estimations of dry weight content of wet samples were made by measuring settled bed volumes in a TPX graduated cylinder and using the values for specific bed volume given by the manufacturer. Gels were washed free of reagents and by-products by suction filtration on Whatman No. 1 paper in porcelain Büchner funnels or by gravity sedimentation with removal of fines. Polyacrylamide derivatives were not dried, but were kept under refrigeration suspended in an approximately pH 7.3 buffer of the following composition: 0.20 M NaCl, 0.002 M Na_2EDTA, 0.10 M H_3BO_3, 0.005 M NaOH, and 5×10^{-6} M pentachlorophenol. Wet gel derivatives have been kept in this buffer for many months in the cold without apparent deterioration or microbial growth.

Direct Aminoethylation of Polyacrylamide. Ethylenediamine (anhydrous, Fisher reagent, assay 98%) was preheated in a glass vessel in an oil bath maintained at $90.0 \pm 0.1°$. The bath was set up in a fume hood. The reagent was stirred mechanically while dry polyacrylamide beads (as obtained from the manufacturer) were added. Each gram of polymer was added to 15 or 20 ml of ethylenediamine in the case of Bio-Gels P60 or P300, respectively. The addition was made gradually in order to minimize lumping; stirring was continued throughout the desired heating period (see Results). At the end of this time

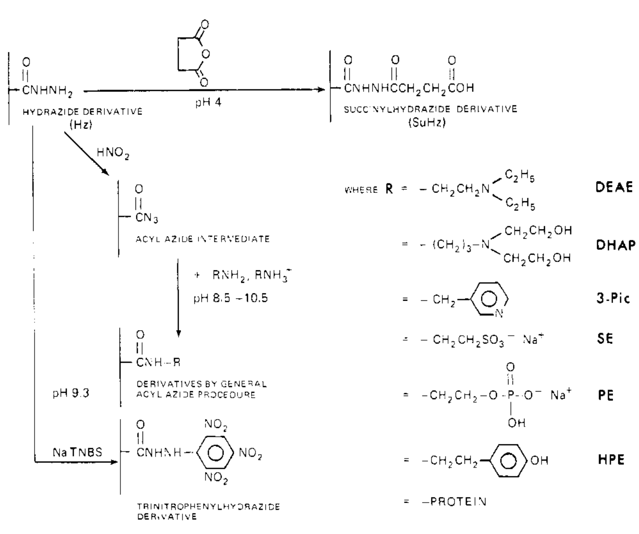

FIGURE 3: Reaction scheme used in preparing derivatives from the hydrazide derivative of polyacrylamide. NaTNBS stands for 2,4,6-trinitrobenzenesulfonate (picrylsulfonate); names corresponding with the symbol associated with each structure are listed in Table II. HPE symbolizes *p*-hydroxyphenethyl.

the reaction vessel was cooled for a short period in ice water, and the contents were mixed with an equal volume of crushed ice. The gel was washed with 0.1 M NaCl on a Büchner funnel and then by sedimentation until supernatants were free of ethylenediamine as indicated by a negative color test with sodium 2,4,6-trinitrobenzenesulfonate. A few drops of a 3% solution of the latter and 1 ml of saturated sodium tetraborate were mixed with 5 ml of the supernatant. After 5 min a positive test showed a stronger yellow color than a control tube with 5 ml of 0.1 M NaCl. The gel was given a filter wash with storage buffer, suspended in the latter, and kept under refrigeration. Samples for titrations were washed with 2 M KCl then 0.2 M KCl.

Preparation of the Hydrazide Derivative. Dry polyacrylamide beads were allowed to swell overnight in distilled water contained in a siliconized, conical, glass-stoppered flask. The amount of water was equal to 1.3 times the bed volume of the hydrated gel. The flask with the gel suspension and a glass-stoppered cylinder containing a calculated amount of hydrazine hydrate, or an aqueous dilution of hydrazine hydrate (Matheson, Coleman & Bell, assay 99–100%, 20.4 M), were immersed in a constant-temperature water bath. After about 45 min the hydrazine was added to the gel, the flask was stoppered, and the mixture was stirred by an immersible magnetic rotor. At the end of the reaction period the gel was washed with 0.1 M NaCl on a Büchner funnel (in fume hood) and further by sedimentation. The latter operation was repeated until supernatants were essentially free of hydrazine as indicated by a pale violet color when tested with sodium trinitrobenzenesulfonate in the manner described above for ethylenediamine derivatives. The gel was filter washed and suspended in storage buffer.

Trinitrophenyl Derivatives. Color tests for aminoethyl and hydrazide functional groups on gels were based on the formation of *N*-2,4,6-trinitrophenyl derivatives by alkylation with sodium 2,4,6-trinitrobenzenesulfonate. A small sample of the washed gel was placed on a spot test plate and suspended in saturated sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$). A few drops of freshly prepared solution of sodium 2,4,6-trinitrobenzenesulfonate were stirred in. Aliphatic primary amino groups yielded yellow-orange to orange products while unsubstituted hydrazides gave a rust color which changed to deep red (magenta).

Preparation of Carboxylated (Deamidated) Polyacrylamide. Carboxyl derivatives were obtained by partial alkaline hydrolysis of the polymer's amide side groups. The gel beads were hydrated overnight in water, washed with a solution of $NaHCO_3$ plus Na_2CO_3 (0.5 M total), suspended in the same buffer, and heated with stirring for a given period of time depending upon the capacity desired (see Results). The reaction products were washed with 0.1 M NaCl and storage buffer.

2,4-Dinitrophenylaminoethyl Derivative. Approximately 1 g of Bio-Gel P60 aminoethyl derivative (2.0 mmoles of groups/g) was suspended in 30 ml of 0.09 M sodium tetraborate (pH 9.3). Three millimoles (0.81 g) of sodium 2,4-dinitrobenzenesulfonate (Eastman) was added, and the suspension was stirred 20 hr at room temperature. The deep yellow, dinitrophenylated polymer was washed with large volumes of 0.2 M NaCl–0.2 M Tris base and then 0.2 M NaCl. A small sample of the washed polymer with sodium 2,4,6-trinitrobenzenesulfonate showed no perceptible color change (toward orange) which indicated a fairly complete reaction with the amino groups.

Succinylhydrazide Derivative. A quantity of hydrazide gel corresponding to about 1 g dry weight was washed with 0.1 M NaCl, transferred to a TPX beaker, and resuspended in 30 ml of 0.1 M NaCl. The suspension was stirred efficiently while small portions of powdered succinic anhydride, totaling 0.6–0.9 g, were added during a 10-min period. Throughout this time the pH of the suspension was held near 4.0 with additions of 2 N NaOH. Completion of the reaction was checked by washing 1 ml of the suspension with saturated sodium tetraborate on a small Büchner funnel, followed by spot plate testing for unreacted hydrazide as described under trinitrophenyl derivatives. Additional succinic anhydride was added if a negative test was not obtained. The pH 4.0 suspension was stirred slowly for 2 hr, then washed free of succinate.

Coupling of Primary Amines via the General Acyl Azide Procedure. The procedure below describes how a number of primary aliphatic amines were coupled to polyacrylamide hydrazide *via* the acyl azide derivative (Figure 3) without intermediate washings or transfers. The quantities apply to 1 g (estimated dry weight basis) of the P60 hydrazide gel.

The hydrazide polymer was washed with 0.1 M NaCl and 0.25 N HCl (about 50 ml) and resuspended to 32-ml volume with 0.25 N HCl. The suspension was cooled to 0° and 8 g of crushed ice was added. The container was placed in an ice bath and 4.0 ml of 1.0 M sodium nitrite was added rapidly under efficient magnetic stirring; 90 sec later the coupling reagent containing the amine (see below for each derivative), which had been cooled to 0° was added rapidly. The mixture was stirred in the ice bath for a period of time specified below. Unreacted azide was reconverted into the hydrazide and then to the stable acetyl hydrazide in the following sequence of steps: 1.5 ml of hydrazine hydrate was added, followed by stirring for 0.5–1 hr longer. The derivative was then given the following sequence of washes on a Büchner funnel: (a) 0.1 M NaCl (100 ml), (b) 100 ml of 0.2 M sodium acetate in which 4 ml of acetic anhydride had been dissolved less than 1 min before, (c) 0.1 M NaCl (100 ml), (d) 2 M NaCl (50–100 ml), and (e) storage buffer or 0.2 M KCl (for titrations).

Diethylaminoethyl Derivative. The DEAE derivative was prepared by the general acyl azide procedure. The coupling reagent was 2.3 ml (16 mmoles) of *N,N*-diethylethylenediamine (Aldrich Chemical Co., redistilled). The coupling reaction was allowed to proceed 12 min before the addition of hydrazine hydrate.

Dihydroxyethylaminopropyl Derivative. The dihydroxyethylaminopropyl derivative was prepared by the general acyl azide method. The coupling reagent was a solution of 2.6 g (16 mmoles) of *N*-(3-aminopropyl)diethanolamine (Aldrich Chemical Co.) in 7.5 ml of H_2O. The coupling was carried out for 12 min before adding hydrazine hydrate.

3-Picolyl Derivative. The 3-picolyl derivative was prepared by the general acyl azide procedure. The coupling reagent was 2.5 ml (24 mmoles) of 3-picolylamine (3-aminomethylpyridine, Aldrich Chemical Co). Coupling proceeded 12 min before the addition of hydrazine hydrate.

Sulfoethyl Derivative. The sulfoethyl derivative was prepared by the general acyl azide method. The coupling reagent was a solution of 2.5 g (20 mmoles) of taurine (Eastman Organic Chemicals) in 16 ml of H_2O and 3.35 ml (24 mmoles) of triethylamine. The mixture was stirred at room temperature until a solution was obtained before cooling to 0°. The coupling was carried out for 70 min.

Phosphoethyl Derivative. The phosphoethyl derivative was prepared by the general acyl azide procedure. The coupling reagent consisted of two parts. A solution of 2.30 g (16 mmoles) of *O*-phosphorylethanolamine (Sigma Chemical Co) in 16 ml of H_2O and 3.35 ml of triethylamine (stirred until dissolved, then cooled) was added just after the 90-sec nitrous acid treatment. It was immediately followed by 0.8 ml of additional triethylamine (not precooled). The coupling proceeded for 30 min before addition of hydrazine hydrate.

p-Hydroxyphenethyl Derivative. The *p*-hydroxyphenethyl derivative was formed by the general acyl azide procedure. The coupling reagent consisted of a partial solution of 1.39 g (8 mmoles) of tyramine hydrochloride (Eastman Organic Chemicals) in 15 ml of dimethylformamide and 2.24 ml (16 mmoles) of triethylamine. The cooled suspension was added and the coupling was allowed to proceed for 2 hr before adding the hydrazine hydrate.

Coupling of Protein via the Acyl Azide. For the coupling of serum albumin, Bio-Gel P300 hydrazide derivative (1.3 mmoles of hydrazide/g) was washed with 0.3 N HCl. A 100-mg portion was suspended in 15 ml of 0.3 N HCl, cooled to 3°, and 1 ml of 1 M sodium nitrite was added. After 20 min in an ice bath, the suspension was poured onto a Büchner funnel and washed rapidly with 0.3 N HCl, 0.1 M sulfamic acid, and cold H_2O. The gel was immediately resuspended in 12 ml of 0.5% bovine serum albumin in 0.1 M sodium tetraborate (4°). The albumin had been lightly labeled with ^{131}I. The mixture was stirred in an ice bath for 1 hr. In order to convert unreacted azide groups back into amide, 2 ml of 1 M NH_4OH and 1 M NH_4Cl was added, and stirring was continued for 2 hr longer. The gel was washed with 0.2 M NaCl and then with H_2O; aliquots were resuspended in H_2O for dry weight determinations and scintillation counting of ^{131}I. The amount of bound protein was 67 mg/g of original polyacrylamide. Prolonged coupling under the same conditions increased the protein content to 78 mg/g.

For the coupling of trypsin, P100 hydrazide (0.5 g) was reacted in the cold nitrous acid solution for just 2 min and then washed with cold 0.1 M $CaCl_2$–0.001 N HCl. $CaCl_2$ was used to stabilize the trypsin. The gel azide was immediately transferred to 20 ml of 0.1 M $CaCl_2$–0.001 N HCl (0°) containing 100 mg of twice-crystallized trypsin (Worthington). The pH of the suspension was adjusted to and maintained at 9.0 with dilute NaOH. Stirring and ice-bath cooling were maintained for 60 min after which time 5.0 ml of 3.0 M NH_4Cl–1.0 M NH_4OH was added to couple with unreacted azide groups. Stirring was continued for 60 min at 0°, and the gel was washed with a large volume of 0.1 M $CaCl_2$–0.001 N HCl. When samples of the initial and final supernatants from the coupling reaction were examined and compared with control solutions, it was found that 84% of the protein had been bound to the gel, and 93% of the activity (against tosyl-L-arginine methyl ester) had been removed from the solution phase.

p-Aminobenzamidoethyl Derivative. Two grams (dry weight basis) of Bio-Gel P300 aminoethyl derivative with a specific capacity of 1 mmole/g was suspended in 140 ml of 0.2 M NaCl. A solution of 0.40 g (2.1 mmoles) of *p*-nitrobenzoyl azide (Eastman) in 140 ml of dimethylformamide was added with stirring followed by the addition of 0.20 ml (1.4 mmoles) of triethylamine. The mixture was stirred for 45 min at room temperature, then washed on a funnel with 50% v/v dimethylformamide (in 0.2 M NaCl), dimethylformamide, 50% dimethylformamide, 25% dimethylformamide, and finally 0.2 M NaCl. The product gave a pale yellow spot test with sodium 2,4,6-trinitrobenzenesulfonate indicating essentially complete conversion into the *p*-nitrobenzamidoethyl derivative. The latter was suspended in 120 ml of 0.2 M NaCl, warmed to 50° in a water bath, and 6.0 g of sodium hydrosulfite (Fisher Scientific Co., low iron grade; also termed sodium dithionite) was added. The mixture was stirred and warmed to 50–55° for 40 min, then washed on a funnel with 0.2 M NaCl. The resulting *p*-aminobenzamidoethyl derivative gave an orange color with sodium 2,4,6-trinitrobenzenesulfonate on a spot plate as expected for a primary amine (aryl amine in this case).

Coupling of Protein via the Diazonium Salt Intermediate. The P300 *p*-aminobenzamidoethyl derivative (about 200 mg) was taken up in 120 ml of 0.10 N HCl–0.2 M NaCl and cooled to near 0° in an ice bath. Under magnetic stirring, 2.5 ml of 1.0 M sodium nitrite was added, and stirring was continued for 10 min. Excess nitrous acid was destroyed by the addition of 2 ml of 1.0 M sulfamic acid. The gel was washed with cold 0.2 M NaCl and immediately suspended equally in two 50-ml portions of 2% bovine serum albumin solution in 0.05 M sodium tetraborate. In one portion the albumin had been labeled with ^{131}I. Both suspensions were stirred for 2 hr in an ice bath while the pH was maintained near 8.5 by additions of 1 N NaOH. The gel turned brown-orange as result of the gel–azoprotein linkages. The labeled gel was washed and counted. The uptake of albumin was about 300 mg/g of original polyacrylamide. The unlabeled preparation was able to bind reversibly 25–70 mg of [^{131}I]rabbit antibovine albumin antibody from nonspecifically purified immunoglobulin.

Hydrogen Ion Binding. Capacities (functional group contents) of many of the derivatives were determined by hydrogen ion binding measurements using the following general titration procedure. Approximately 1 g (dry weight basis) of gel derivative was washed with 0.2 M KCl and suspended in about 25 ml (for P60) of the same solution in a preweighed, 50-ml TPX beaker. A magnetic stirring bar and pH electrodes were inserted. Normal KOH was added to raise the pH to 2 units above the pK of the functional group being titrated. Standardized 1 or 0.2 N HCl was added slowly until the pH dropped to about 2 units below the pK value. When the final pH reading was steady, the stirring bar was removed, the beaker and contents were weighed, and the final fluid volume, V, was calculated as: V(ml) = 0.9935x (net weight of contents − w), where w is the estimated or subsequently determined dry weight in grams. The value 0.9935 is the specific volume of 0.2 M KCl at room temperature. The capacity c of the sample in millimoles was calculated by the expression: $c = (Nv - [H^+, OH^-]V - wB)xF \cong (Nv - [H^+, OH^-]V)xF - wB$, where N is the normality of the HCl, v is the volume of HCl required; $[H^+, OH^-]$ is the calculated final free hydrogen ion concentration if the titration ended below pH 4, *or* the calculated, initial, free hydroxyl ion concentration if the titration began above pH 10. In the latter case V is the final fluid volume as expressed above minus v. F is the factor correcting for the fraction of groups not titrated in the pH range employed. It was derived from the Henderson–Hasselbalch equation or taken from an actual extended hydrogen ion binding curve. Values for F are given in Table I. The background B represents

TABLE I: Values of F, the Factor Correcting for Functional Groups Not Titrated within pH Ranges with the Following Limit Values.

Final p[H⁺][a]	Acetyl-hydrazide	Succinyl-hydrazide	Initial pH[b]	Aminoethyl
2.50	1.014	1.010	10.80	1.025
2.60	1.018	1.014	10.90	1.020
2.70	1.023	1.019	11.00	1.016
2.80	1.029	1.027	11.20	1.010
2.90	1.036	1.034		
3.00	1.046	1.043		

[a] The pH meter reading after the instrument was set to 2.00 for 1.000×10^{-2} N HCl in 0.20 M KCl at the temperature of the measurements. [b] The pH meter reading after the instrument was calibrated with commercial pH 10.00 standard buffer.

the milliequivalents per gram of dry derivative of H^+ bound, between the pH limits of the titration, due to groups present in the underivatized gel and to any extraneous groups introduced during the chemical procedures. Bio-Gels, as received, were found to bind only 0.02–0.04 mmole of hydrogen ion/g in a pH range of 4 units. The free hydrogen ion concentration was taken as $-\log$ of the pH meter reading after setting the instrument to 2.00 for a solution of 1.000×10^{-2} N HCl in 0.20 M KCl. Initial free hydroxyl concentration was assumed to be $-\log (14.00 - \text{pH})$ after setting the meter to 10.00 against commercial standard buffer. Titrations were carried out with a Radiometer Model 22 pH meter equipped with a type B, single probe, dual electrode.

Dry Weight Determinations. The titrated sample was quantitatively transferred to a previously weighed, 50-ml, glass, Gooch crucible (with coarse sintered disk) suitably mounted on a suction flask. In the case of amine-type derivatives the gel was converted into the uncharged form by a preliminary wash with 0.2 M sodium carbonate in order to minimize subsequent swelling. The gel was washed in the Gooch crucible successively with distilled water (to remove electrolyte), 60% v/v methanol in water, and absolute methanol. After the final rinse, air was drawn slowly through the crucible to evaporate most of the residual methanol. The crucible was allowed to stand overnight over anhydrous $CaCl_2$ in a vacuum desiccator that had been pumped to less than 1 mm through a Dry-Ice-cooled vapor trap. Air was slowly readmitted through a $CaCl_2$ tube, and the crucible was weighed immediately. The net weight obtained was used in calculating specific capacity.

Dried samples gradually gained weight (initially about 2%/hr) by absorbing moisture from room air and eventually, when left exposed, reached 107–110% of their dry weight *in vacuo*.

Specific Capacities. Specific capacity may be expressed as $C = c/w$, the measured capacity, c, of the sample divided by the dry weight, w. However, for calculating yields of reactions on gels it was necessary to correct for the weight increment of functional groups introduced. Corrected specific capacities, C', have been expressed as millimoles per dry weight gram of original polyacrylamide by use of the relationship

$$C' = \frac{C}{1 - \Sigma C_i \cdot \Delta r_i}$$

where Δr_i is 10^{-3} times the difference between the molecular weights of a residue of the ith type of functional group (with specific capacity ($C_i = c_i/w$) and a residue of linear polyacrylamide (see Table II for values). When specific capacities of by-product groups such as $-COO^-Na^+$ and acetylhydrazide were known or could be estimated, their $C \cdot \Delta r$ products were included in the indicated summation.

Hydrazine Concentrations. The concentrations of aqueous hydrazine solutions were checked by titration with standard HCl after diluting the sample with distilled water. The methyl red end point (in the vicinity of pH 5) was reached after the addition of 1 mole of acid/mole of hydrazine.

Specific Capacity of the Hydrazide Derivative. Hydrazide capacity can be estimated by direct titration, but because of the low group pK (2.6), the final free hydrogen ion term $[H^+] \cdot V$ becomes very large. Accuracy is low even when considerable care is exercised. It was found that the hydrazide gels could be readily and quantitatively converted into the succinylhydrazide form and the resulting terminal carboxyl function (with pK = 4.55) titrated accurately. Furthermore, background capacity (mainly due to carboxyl) could be measured after selectively blocking ionization of the hydrazide groups by acetylation using another sample. The resulting acetylhydrazide derivative was titrated in the same pH range to determine background.

The succinylhydrazide sample (about 1 g) was prepared as described previously. The acetylhydrazide derivative (from another 1-g sample) was prepared by suspending the hydrazide gel in 0.1 M NaCl and adding acetic anhydride (about 1 ml) dropwise with efficient stirring over a 5-min period. During this time the pH was kept near 4.0 with additions of 2 N NaOH. Completeness of the reaction was checked in the manner described for the succinylhydrazide derivative. Each preparation was washed thoroughly with 0.1 M NaCl, 2, and 0.2 M KCl, then resuspended in the latter and titrated from pH 6.7 to 2.7. Dry weights were determined on each sample. The general procedures for titrations and dry weights were followed as outlined above.

The corrected specific capacity of the hydrazide function C_{Hz}' was calculated in the following steps: (1) $C_{SuHz} = (Nv_1 - [H^+]_1V_1)F_1/w_1$ (from succinylhydrazide titration); (2) $C_{AcHz} = (Nv_2 - [H^+]_2V_2)F_2/w_2(1 + 0.058C_{SuHz})$ (from acetylhydrazide titration); (3) $C_{Hz} = C_{SuHz} - C_{AcHz}$ (mmoles/g of succinylhydrazide derivative); and (4) $C_{Hz}' = C_{Hz}/(1 - 0.115C_{Hz})$ (mmoles/g of original polyacrylamide). The term C_{AcHz} corresponds to the background specific capacity B in the previous general expression for capacity. The term $(1 + 0.058\ C_{SuHz})$ is an adequate correction for the different weight bases of the acetyl and succinyl derivatives.

Capacity of the Aminoethyl Derivative. A sample of washed aminoethyl derivative was resuspended in 0.2 M KCl and titrated from pH 11.0 to 6.2. The hydrogen ion bound was taken as a measure of the aminoethyl capacity. The background term B was usually of the order of 0.02 mequiv/g in this range. The titration was continued from pH 6.2 to

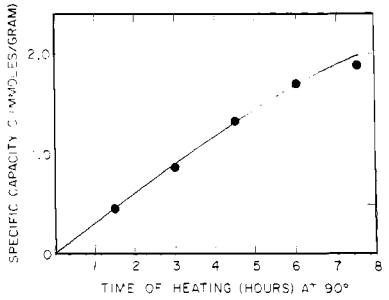

FIGURE 4: Time course of the reaction between polyacrylamide (Bio-Gel P60, 100–200 mesh) and anhydrous (98%) ethylenediamine at 90°. Specific capacity, C', refers to mmoles of aminoethyl groups found on the amount of derivative produced from 1 dry g of original polyacrylamide.

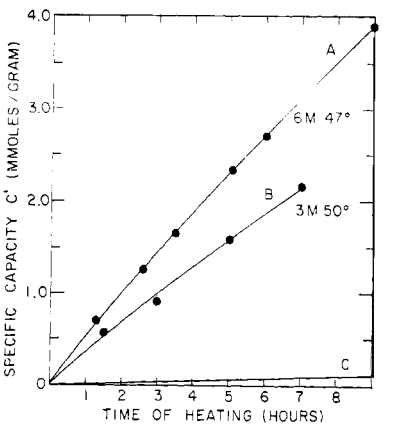

FIGURE 5: Time course of the reaction between polyacrylamide (Bio-Gel P60, 100–200 mesh) and aqueous hydrazine. Molar concentrations of hydrazine and temperature are indicated alongside curves A and B. Curve C is the average time course of carboxyl group formation during the runs for curves A and B. Specific capacity, C', is defined in the legend to Figure 4.

2.7 for the determination of carboxyl function in the preparation.

Capacity of the Sulfoethyl Derivative. The sulfoethyl derivative could not be titrated directly because of its low pK. Treatment with strong acid followed by subsequent water wash and titration of the sulfonic acid group was feasible as a method for determining capacity except for the danger of generating some carboxyl groups by acid hydrolysis of background amide. Preparation of the pyridinium salt and titration of the pyridinium cation to pyridine with standard base proved to be a satisfactory method of analysis. Specific capacity calculated from sulfur analysis agreed within 2% of the value obtained in this way.

A 1-g sample of the sulfoethyl derivative was titrated from pH 8.2 to 2.8 in 0.2 M NaCl to determine binding capacity wB of background groups (mainly carboxyl). The gel was transferred to a no. 2 (Coors) funnel and washed with small portions, totaling 200 ml, of 1.0 M pyridinium chloride (pyridine + HCl to pH 2.8). The gel was washed next with small portions of distilled water, totaling 200–300 ml, and the swollen gel was carefully transferred to a 100-ml TPX beaker. A suspension of the gel, made with 8 ml of 2 M NaCl and water, was titrated to pH 8.0–8.5 with standard 1.0 N NaOH. The capacity of sulfoethyl groups was taken as the milliequivalents of NaOH minus the milliequivalents of background groups determined in the first titration. The dry weight of the sample corresponded to the sodium salt of the sulfoethylated polymer.

Results

Preparation of Primary Derivatives. The course of the reaction of polyacrylamide with anhydrous ethylenediamine at 90° is shown in Figure 4. Similar reaction curves for the polymer in aqueous hydrazine solutions are plotted in Figure 5. In the case of alkaline hydrolysis of polymer amide groups, the extent of formation of carboxylate as a function of pH is illustrated in Figure 6.

Aminoethylation was routinely carried out in anhydrous (98%) ethylenediamine. When this reagent was diluted with water, the reaction rate increased somewhat, but at the same time a much higher proportion of carboxyl groups was formed. Reactions in buffered aqueous solutions (ethylenediamine plus its hydrochloride) showed greatly decreased reaction rates over unbuffered solutions and still yielded a higher proportion of carboxyl to aminoethyl groups than was obtained with the 98% (anhydrous) diamine. Bio-Gels P100 and P300 followed the same reaction curve (within ±6%). Carboxyl groups formed during the aminoethylation varied irregularly with time of heating from 0.02 to 0.18 mmole per g.

The proportion of carboxyl groups formed during hydrazinolysis was smaller than in the case of aminoethylation (2–3% *vs.* about 8%). The average course of carboxyl formation, shown by curve C (Figure 5), was 0.008 mmole/g per hr for 6 M hydrazine at 47° and 0.010 mmole/g per hr for 3 M hydrazine at 50°. A lower ratio of carboxyl formed to hydrazide (1.7%) was obtained with 6 M reactions as compared with 3 M ones (3.1%). Buffering of 3 M hydrazine with 0.06 and 0.24 M HCl decreased the reaction rates to 25 and 11%, respectively, of that obtained in unbuffered systems, and lowered the yield of carboxyl groups formed per hydrazide residue to 2%.

Ion-Exchange Derivatives. The hydrazide derivative which can be formed to a predetermined substitution level (Figure 5) can be converted with high efficiency into other derivatives

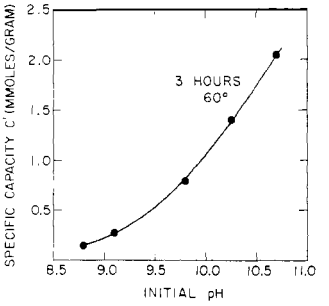

FIGURE 6: Formation of carboxyl groups by alkaline hydrolysis of amide groups on polyacrylamide (Bio-Gel P60, 100–200 mesh) as a function of pH. The medium was aqueous 0.50 M carbonate ($NaHCO_3$ plus Na_2CO_3). Gel beads were heated for 3 hr at 60°. Specific capacity, C', is defined in the legend to Figure 4 and was not corrected for initial content of ionizing groups.

TABLE II: Apparent pK Values and Yields of Derivatives Prepared from Polyacrylamide Hydrazide Derivative.

Name of Derivative	Symbol	pK[a]	Δr[b]	% Yield from the Hydrazide[c]		
					By-Products	
				Derivative[c]	COOH[c]	AcHz[d]
Hydrazide	Hz	2.6	0.015			
Aminoethyl	AE	9.2	0.043	81	15	4
Succinylhydrazide	SuHz	$4.5\bar{5}$	0.115	100		
Diethylaminoethyl	DEAE	9.1	0.099	80	13	7
Dihydroxyethylaminopropyl	DHAP	8.1	0.145	80	13	7
Phosphoethyl	PE (1)	6.8	0.124	60	9	31
	(2)	<2.0				
3-Picolyl	3-Pic	$4.6\bar{5}$	0.091	88		12
Sulfoethyl	SE	≪2.0	0.130	70	12	18

[a] Midpoints of the hydrogen ion binding curves (Figure 7) were taken (as "apparent" pK). [b] The difference in molecular weight $\times 10^{-3}$ between a derivatized and original residue of polyacrylamide (*cf.* Experimental Procedures, Specific Capacities). [c] Corrected specific capacity of the group times 100 divided by corrected specific capacity of the hydrazide. [d] Nonionizing groups: acetylhydrazide (AcHz) in all cases except with AE where azide was reconverted into amide. Values were remainders after summing other two.

by reaction with aliphatic amines (Figure 3) giving a variety of products of predetermined charge (positive or negative), titration range, and charge density. Data summarizing the pK values and yields of several such derivatives are given in Table II and titration curves for the same derivatives are shown in Figure 7.

Ion-Exchange Binding of Proteins. The ion-exchange derivatives described above are capable of reversibly binding large quantities of protein of high molecular weight. As is illustrated in Table III, in acid pH solutions where the dihydroxyethylaminopropyl derivative bears a positive charge, it will bind more than its weight of bovine serum albumin (mol wt 67,000) at a pH where the protein bears a negative charge of several units (pH 6.3). However, below the isoelectric pH of the serum albumin (approximately pH 5) very little binding occurs since the protein and the dihydroxyethylaminopropyl derivative both carry positive charges. In a like manner, the very basic protein lysozyme, which is positively charged at pH values below its isoelectric pH of 10, binds very tightly to the negatively charged succinyl hydrazide derivative near neutral pH (Table III), but binds very poorly as the negative charge on the succinylhydrazide derivative is lowered by reducing the pH to values below the pK of the gel derivative

Similarly, human hemoglobin (mol wt 68,000, isoelectric point near neutrality) binds well to highly substituted aminoethyl P100 gel derivative in 0.01 M Tris-chloride buffer (pH 8.5), where the gel is positive and the hemoglobin slightly negative; and may be removed as a chromatographic peak by linearly increasing the NaCl concentration to 0.05 M.

Solid-Phase Enzymes and Immunoadsorbents. Two separate methods were used to attach protein covalently to gel beads. One method consisted of using the acyl azide bead inter-

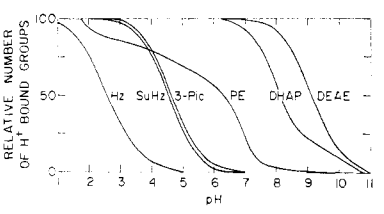

FIGURE 7: Hydrogen ion binding curves for various polyacrylamide derivatives. They are acid titration curves in 0.20 M KCl (25 ± 1°) corrected for free H^+ and OH^- ions. The ordinate is an expression of total milliequivalents of bound $H^+ = Nv - [H^+, OH^-] \cdot V$ (*cf.* Experimental Procedures) normalized to 100 for total H^+ bound over the pH range of the titration.

TABLE III: Ion-Exchange Adsorption and Elution of Proteins with P100 Gel Derivatives.

Derivative	Protein	pH	% of Protein Bound[b]	g of Protein Bound/g of Derivative
DHAP[a] (1.2 mmoles/g)	Bovine serum albumin	6.3	85	1.06
		4.7	5	0.06
		3.5	5	0.06
SuHz[a] (1.5 mmoles/g)	Lysozyme (chicken egg)	6.9	99+	1.24
		3.3	4	0.05
		2.6	3	0.04

[a] Symbols are defined in Table II. [b] The gel derivative (0.8 g) was equilibrated at room temperature with 1.0 g of the protein in 100 ml of 0.02 M sodium formate near neutrality. The pH was subsequently adjusted downward with 5 N HCl. Samples of supernatants were diluted and adsorbance at 280 nm was measured to determine unbound protein.

mediate formed from the hydrazide to react with amino groups on the protein molecule (Figure 3), linking the protein to the acrylamide by an amide bond. The second method involved the formation of a diazonium salt derivative of the acrylamide beads (Figure 2) which couples to the protein by reacting with tyrosine, histidine, and lysine side chains to form azoprotein derivatives.

When 100 mg of crystalline trypsin was coupled to 0.5 g of P100 gel hydrazide through formation of the azide intermediate, 84% of the protein was bound to the gel. The resulting covalent enzyme–gel complex catalyzed the hydrolysis of *N*-benzoyl-L-arginine ethyl ester at a rate 35% of that measured for a comparable amount of trypsin in free solution. In a similar manner a protein as large as bovine serum albumin could be coupled to P300 hydrazide derivative to a level of over 70 mg/g.

By use of the diazonium salt intermediate formed from P300 gel, bovine serum albumin could be coupled covalently to the extent of 300 mg/g of original acrylamide. The solid-phase bovine albumin complex thus formed was able to bind reversibly 25–70 mg of [^{131}I]rabbit antibovine albumin/g of acrylamide fom nonspecifically purified immunoglobulin. The resulting antibody–antigen complex was washed with 1% NaCl in sodium borate buffer (pH 7.8) and the antibody was then eluted with 0.05 M glycine-HCl (pH 2.4), 93% of the antibody was eluted when bound to the extent of 70 mg/g and 76% was eluted when bound to the extent of 25 mg/g of adsorbent.

Discussion

The reaction of polymer amide groups with ethylenediamine or hydrazine (Figure 1) may be interpreted as an acylation of an amine by an amide. In the reaction of polyamides with diamines some molecules of the latter may react at both amino groups forming a cross-link in the polymer. Statistically, this reaction should not be favored because of the large excess of diamine used. Throughout the course of the reactions, amino groups on unbound diamine molecules were usually more than 100 times as plentiful as the terminal amino groups on bound molecules. Shrinkage of beads and lower specific bed volumes of derivatized beads, which might have been expected from a significant increase in cross-linkage, were not observed. Symmetrical bisacylation of diamines by the polymer is apparently not favored by steric or chemical factors. This was further supported by the result of the reaction between ethylenediamine and polymer acyl azide intermediate where diamine amino groups were in 25-fold excess. Only 4% of the original hydrazide was unaccounted for as ionizing products (Table II) and probably much of this remainder was amide regenerated from the ammonia scavenger that was added following the coupling reaction (rather than nonionizing cross-links).

The hydrogen ion binding curve of the aminoethylated polymer so nearly coincided with that for DEAE-polyacrylamide that it was not drawn in Figure 7. The binding curve for the dihydroxyethylaminopropyl derivative suggested that the reagent, *N*-(3-aminopropyl)diethanolamine, contained impurities including, perhaps, structures with only one hydroxylethyl group. The alkaline-most ionization (charge 1− to 2−) of the dibasic phosphoethyl group occurs with an apparent p*K* of about 6.8, but only part of the acidic ionization (charge 0 to 1−) is seen on the binding curve below pH 3.5.

The method described for coupling amines to the hydrazide polymer through an acyl azide intermediate appears to have general applicability. The HCl present in the azide formation step functioned along with excess amine (primary amine reactant plus triethylamine where extra amine was required) to form a buffer for the coupling reaction. Excess nitrite did not appear to interfere at the prevailing alkaline pH and low temperature. Thus, the two-step reaction sequence was conveniently carried out without an intermediate separation of reactants. This strategy lessened decomposition of the unstable acyl azide making large-scale preparations feasible.

At the end of each coupling period hydrazine was added as a scavenger for unreacted azide groups since it is somewhat more reactive than primary amines or ammonia. The assumption was made that a minor proportion of azide groups may be less reactive because of steric factors and would eventually hydrolyze to increase the background of negatively charged carboxylate groups. The hydrazide produced was converted into the neutral acetylhydrazide form in the aqueous acetic anhydride wash. The per cent yields based on the hydrazide, for the desired derivative and carboxylate formed by the general acyl azide procedure were determined by hydrogen ion binding. The values are shown in Table II. The content of neutral by-product (usually acetylhydrazide) was taken as the remainder unaccounted for by hydrogen ion binding.

Diazotized arylamine derivatives of cellulose have been used to form covalent complexes with protein antigens (Campbell *et al.*, 1951; Gurvich, 1964; Behrens *et al.*, 1967). In each method cited nitrophenyl groups were introduced into the adsorbent and reduced with sodium hydrosulfite; the resulting amine was diazotized and coupled to protein. Nitrophenyl compounds with an acylating function have been linked to commercial aminoethylcellulose. Acylation was effected through an active ester (Behrens *et al.*, 1967) or more conveniently by an acyl azide (*p*-nitrobenzoyl azide) (J. K. Inman, 1967, unpublished data). The latter method, adapted from a histochemical procedure for fixing chromagens to protein (Geyer, 1965), was applied equally well to aminoethyl polyacrylamide (Figure 2, Experimental Procedure). Since the diazo linkage to a biologically active protein may involve sensitive residues of tyrosine or histidine, it may be preferable to form covalent bonds principally with free amino groups of the protein. Coupling of enzymes to acyl azide derivatives of cellulose has been reported (Micheel and Ewers, 1949; Mitz and Summaria, 1961; Hornby *et al.*, 1966) wherein specificity for protein amino groups would be expected. The hydrazide derivative of polyacrylamide can be converted directly into an acyl azide and coupled to proteins under similar conditions.

The polyacrylamide derivatives reported here allow a versatile approach to the preparation of immunoadsorbents for isolating specific antihapten antibodies and subdividing these further according to differences in binding affinity and cross-reactivity. The dinitrophenyl and trinitrophenyl derivatives are examples. A variety of azohapten adsorbents can be formed by coupling diazotized arylamines to the *p*-hydroxyphenethyl derivative in a manner similar to that reported for tyramine-cellulose (Vannier *et al.*, 1964).

The ion-exchange derivatives described in the present study may find applications similar to those of their counterparts

with cellulose and cross-linked dextran carriers, but there will be opportunity for one to choose from a much wider range of functional groups and capacities. The hydrazide derivative is stable, can be stored in a variety of specific capacities, and used to prepare special derivatives when required. As with the Sephadex derivatives, gel filtration can be designed to operate simultaneously with the adsorption chromatographic process. Polyacrylamide as a chromatographic carrier has special advantages over dextran and cellulose in terms of background adsorption, improved resolution, and chemical stability (Carrara and Bernardi, 1968; Sun and Sehon, 1965). Problems may arise in column chromatography employing charged polyacrylamide derivatives with elution gradients which span ionic strengths somewhat above and below 0.01, since large bed volume changes can occur in the lower cross-linked forms.

Perhaps the most useful applications of tailored polyacrylamide derivatives will involve separations depending upon specific chemical and biochemical interactions as, for example, antigens (or haptens) with antibodies, enzyme with substrate-like moieties (Cuatrecasas *et al.*, 1968; Dennis, 1968), mercurials with molecules having free sulfhydryl groups (Shainoff, 1968; Eldjarn and Jellum, 1963), and metal ions with chelating structures.

Peptide syntheses or degradations could be carried out on polyacrylamide supports if reactions can be adapted to solvents which cause the gels to swell and allow passage of reagents and by-products in and out of the pores. Solvents which meet this requirement include water, formamide, glacial acetic acid, formic acid, and some water–solvent mixtures.

Acknowledgment

We thank Dr. William C. Alford of the National Institute of Arthritis and Metabolic Diseases for performing the elemental analysis for sulfur.

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