0273-2289/93/4201—0019$04.40

Isonitrile Derivatives of Polyacrylamide as Supports for the Immobilization of Biomolecules

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Received December 15, 1992; Accepted January 17, 1993

ABSTRACT

Isonitrile derivatives of crosslinked polyacrylamide beads (Biogell P-100) were prepared by a two-step procedure: a. *N*-hydroxymethylation (methylolation) of amide groups on the polymer by treatment with formaldehyde; and b. Attachment of side chains, containing isonitrile functional groups by a displacement reaction involving 1-tosyloxy-3-isocyanopropane (p-CH_3-C_6H_4·SO_2·O·$(CH_2)_3$ NC) and alkoxide ions generated on methylolated polyacrylamide by treatment with a strong base in a polar aprotic solvent.

The modified polyacrylamide beads were tested as support for the immobilization of proteins, and low mol wt ligands by four component condensation (4CC) reactions.

Trypsin-polyacrylamide acting on *N*-benzoyl-L-arginine ethylester exhibited nonlinear Michaelis Menten kinetics and distorted pH activity profiles. The kinetic anomalies could be reduced by increasing the concentration of buffer. The data were consistent with a model assuming "buffer facilitated proton transport" in a diffusionally constrained system.

Index Entries: Polyacrylamide; polyacrylamide, isonitrile derivatives of; polyacrylamide-enzyme conjugates; polyacrylamide-trypsin, immobilized enzymes; enzyme immobilization; buffer-facilitated proton transport.

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INTRODUCTION

Polymeric matrices bearing isonitrile functional groups have been shown to serve as versatile supports for the immobilization of proteins and low mol wt ligands by four component condensation reactions (4CC) carried out in aqueous buffers at neutral pH (*1–4*).

This communication describes a method for the preparation of isonitrile derivatives of polyacrylamide beads (Biogel P-100). The modified polyacrylamide beads were used as supports for immobilization of proteins and low mol wt ligands. A cursory survey of the kinetic behavior of polyacrylamide-enzyme conjugates was carried out using a polyacrylamide-trypsin preparation.

MATERIALS AND METHODS

Chemical Reagent

Polyacrylamide beads (Biogell P-100) 50-100 mesh (wet hydrated diameter 150–300 microns) were a product of Bio-Rad (Richmond, CA). Trypsin (EC 3.4.21.4) Type II, 2 x crystallized was obtained from Sigma (St. Louis, MO). Potassium *tert*-butoxide was a product of Fluka (Buchs, Switzerland). Dimethyl sulfoxide, analytical grade, anhydrous (Merck) was stored over a molecular sieve. All other reagents and buffer salts were of the best grade available commercially.

Preparation of Reagents

Potassium Tert-Butoxide Stock Solution

Potassium *tert*-butoxide (1.8 g; 16 mmol) was suspended in 50 mL anhydrous dimethylsulfoxide and stirred vigorously at 40°C in a tightly stoppered flask. Undissolved material was separated by centrifugation or decantation. The solution was stored in polyethylene vials at −20°C. The overall base concentration as determined titrimetrically was about 0.3*M*.

1-Tosyloxy-3-isocyanopropane

The reagent was prepared from 3-aminopropanol via the *N*-formylaminopropanol derivative (*2,4*).

N-Formylaminopropanol

An equivalent amount of ethyl formate (26.4 mL, 0.44 mol) was added dropwise to strongly stirred 3-aminopropanol (25 mL, 0.33 mol). Stirring was continued for 1 h at room temperature. The ethanol formed in the reaction was removed in a rotatory evaporator and the residue distilled in vacuo. The yield of *N*-formylaminopropanol was about 26 g (77%), bp 115–118°C (10^{-2} mm Hg).

1-Tosyloxy-3-isocyanopropane

A pyridine solution (50 mL) of *p*-toluenesulfonyl chloride (38.2 g, 0.2 mol) was added dropwise over 30 min to a vigorously stirred, ice-cooled solution of *N*-formylaminopropanol (10.3 g. 0.1 mol) in pyridine (50 mL). The reaction mixture was stirred over ice for 1 h. Cold water (100 mL) was then added and the mixture was extracted with three 50 mL portions of diethylether-hexane (5:1, v/v). The combined extract was washed with cold water and dried over sodium sulfate. The solvent was removed by evaporation and the residue dissolved in diethylether-hexane (3:5:1, v/v). The solution was left at −18°C and the white crystalline solid that formed was collected on a filter, washed with 10 mL of ice-cold hexane, and air dried. The yield was 7 g (25%), mp 37–38°C.

Isonitrile Derivatives of Polyacrylamide Beads

The isonitrile derivatives of polyacrylamide were prepared by a two-step procedure: a. Partial hydroxymethylation of carrier amide groups by treatment with aqueous formaldehyde; and b. Displacement reaction involving 1-tosyloxy-3-isocyanopropane and alkoxide groups generated on the polymer by a strong base in a polar aprotic solvent (potassium *tert*-butoxide in dimethyl sulfoxide, *see* Eqs. 1 and 2).

N-Hydroxymethylation of Polyacrylamide Beads

Hydroxymethylation of polyacrylamide beads was routinely carried out as follows: Polyacrylamide beads (Bio-Gel, P-100; 50–100 mesh; 10 g) were suspended in water (200 mL) and allowed to swell overnight. Formaldehyde was added to a final concentration of 1.35*M* (23 mL of a 35% solution). The pH was brought to 9.5 and the reaction mixture incubated at 50°C with gentle shaking for 1 h. The resin was recovered by filtration on a sintered glass filter, washed exhaustively with water and lyophilized (*5*). The mean hydroxymethyl content of batches prepared by this procedure was 3.2 ± 0.4 mmol/g dry resin.

Isonitrile Derivatives of N-Hydroxymethylated Polyacrylamide

Dry *N*-hydroxymethylated polyacrylamide beads (1 g) were suspended in dry dimethylsulfoxide (25 mL) and preswollen overnight at room temperature, in a well stoppered flask. The swollen suspension was immersed in a 40°C bath. Solid potassium *tert*-butoxide (200 mg; 2.3 mmol) was added and allowed to dissolve with gentle manual shaking (alternatively an equivalent amount of an 0.3–0.4*M* $KBuO^t$ solution in dimethylsulfoxide was added). The suspension was incubated at 40°C for 5–10 min, 1-tosyloxy-3-isocyanopropane (560 mg; 2.5 mmol) in solid form was added in small portions and dissolved in the reaction mixture with shaking; the reaction was allowed to proceed for 4 h at 40°C in a shaker bath or with occasional manual shaking. The modified polyacrylamide beads were

separated by filtration on a sintered glass filter, washed on the filter with dimethylsulfoxide, cold aqueous acetone (1:1) and washed exhaustively with cold water. The wet, swollen polyacrylamide beads were used directly in coupling experiments or stored in dried form at 4°C.

The beads were dried either by lyophilization or by washing with graded concentrations of aqueous methanol and finally with pure methanol and then dried *in vacuo* over phosphorus pentoxide.

In view of the obnoxious smell of the 1-tosyloxy-3-isocyanopropane reagent all operations were performed in a well ventilated hood.

Binding of Trypsin and Other Proteins

a. 4CC Binding via ligand amino groups. Isonitrile derivative of polyacrylamide (30 mg) was suspended in cold 0.1*M* phosphate, 0.5*M* acetate pH 8 buffer (5 mL) and allowed to swell with gentle shaking for 1–2 h. Crystalline trypsine (10 mg), added in small portions, was dissolved in the reaction mixture and cold acetaldehyde added to a final concentration of about 0.14*M* (0.4 mL of a 10% aqueous solution, pH 8). The coupling reaction was allowed to proceed overnight at 4°C with gentle shaking. The enzyme-biogel conjugate was separated on a sintered glass filter, washed with water, 1*M* KCl and again with water, resuspended in water (5 mL), and stored at 4°C.

b. 4CC Binding via ligand carboxyl groups. Isonitrile derivative of polyacrylamide (30 mg) was suspended in cold 0.1*M* Tris pH 7 buffer (5 mL) and allowed to swell with gentle shaking for 1–2 h. All other operations were carried out as described in a. for binding via ligand amino groups.

Note: For the preparation of larger batches of protein-polyacrylamide conjugates it was more convenient, in order to avoid the carrier preswelling step, to use a freshly prepared isonitrile derivative of polyacrylamide (still wet and swollen).

Binding Capacity and Kinetics of Binding of Low Mol Wt Ligands to Isonitrile Derivatives of Polyacrylamide by 4CC Reactions

a. ^{35}S-Methionine coupling by 4CC, via ligand amino groups. Isonitrile derivative of polyacrylamide (5 mg) was suspended in 2.5 mL of ice-cold 2 m*M* ^{35}S-methionine (specific activity 10^6 cpm/μmol) in 0.1*M* potassium phosphate, 0.5*M* potassium acetate pH 8 in a stoppered vial. Cold acetaldehyde was added to a final concentration 0.45*M* (0.25 μL/mL reaction mixture) and the mixture shaken gently overnight at 4°C. Aliquots (1 mL) were removed, the ^{35}S-Met-polyacrylamide collected on glass-fiber filters (GF/C; Whatman; 2 cm diameter) and washed exhaustively with water (10×2 mL). The filters were placed in vials containing 5 mL scintillation liquid (Hydroluma) and the radioactivity assayed by liquid scintillation spectrometry.

b. Tyrosine coupling by 4CC via ligand amino groups. Isonitrile derivative of polyacrylamide (30 mg) was suspended in 1 m*M* tryosine in 0.1*M* phosphate 0.5*M* acetate pH 8 (10 mL) and allowed to swell for 2 h at 4°C in a stoppered vial. Cold acetaldehyde was added to a final concentration 0.14*M* (0.8 mL of a 10% aqueous pH 8 solution). The reaction was allowed to proceed overnight at 4°C with shaking. The tyrosine-polyacrylamide conjugate was separated by filtration, washed exhaustively with water, and resuspended in water (5 mL).

The tyrosine content of the conjugate was determined by a modified Lowry procedure (*vid. infr.*).

c. Tyrosine coupling by 4CC via ligand carboxyl groups. To a 1 m*M* tryosine solution of 0.1*M* Tris buffer pH 7.0 (10 mL), 30 mg isonitrile derivative of polyacrylamide was added and allowed to swell at 4°C for 2 h. The reaction was started by the addition of cold acetaldehyde to a final concentration 0.14*M*. All other operations were as described for coupling tyrosine via its amino groups (*see* b. above).

d. Kinetic experiments. For the kinetic experiments, carried out 4°C in a shaker bath, isonitrile derivative of polyacrylamide (450–500 mg) was suspended in 25 mL of either 0.1*M* phosphate, 0.5*M* acetate pH 8, or 0.1*M* Tris pH 7.0 (for binding via ligand amino or carboxyl groups, respectively). The suspended particles were allowed to swell for 2 h with gentle shaking at 4°C. The reaction was started by the addition of cold acetaldehyde to a final concentration 1.15*M* (64 μL/mL reaction). At given time intervals, aliquots (2 mL) were removed into 15 mL conical graduated polyethylene test tubes containing 4 mL water. The tyrosine-polyacrylamide conjugate was spun down, washed exhaustively with water (5 mL×3) in the same test tube (to minimize loss of material), and resuspended in water to a final volume of 5 mL. The amount of bound tyrosine was determined by a modified Lowry procedure (*vid. infr.*).

Determination of N-Hydroxymethyl Groups

N-hydroxymethyl groups were estimated by a modification of the chromotropic acid (1,8-hydroxynaphtalene-3,6-disulfonic acid) method used for the determination of free formaldehyde (*5,6*). Under the strongly acidic conditions employed *N*-hydroxymethyl groups release equivalent amounts of formaldehyde that reacts with chromotropic acid (*5,6*).

Suspensions of *N*-hydroxymethyl derivatives of Biogel containing 0.5–3 mmol methylol groups in 0.5 mL were mixed with 0.5 mL of a 10% chromotropic acid solution. Concentrated sulfuric acid, 5 mL, was added slowly with vigorous shaking and the mixture kept in boiling water for 30 min. The mixture was then cooled, diluted to 50 mL, the solid was removed by centrifugation, and the absorbance determined at 570 nm. To ensure linearity, at least two determinations with different quantities of

polymeric hydroxymethyl derivative were carried out. Parent polyacrylamide served as blank. Under the conditions employed 1 μmol/assay of formaldehyde or low-mol-wt methylol derivative (*N*-hydroxymethacrylamide, BDH) corresponded to 0.31 OD at 570 nm.

Determination of Bound Tyrosine

Tyrosine bound to polyacrylamide was determined by a modification of the Lowry procedure (*7*): A suspension of polyacrylamide-tyrosine conjugate (containing 0.05–0.3 μmol tyrosine in 0.5 mL) was added to 5 mL 0.1*M* NaOH, 0.3*M* in Na_2CO_3 and the mixture immersed in boiling water for 20 min. The assay mixture was cooled to room temperature, 0.1 mL of copper tartrate solution (prepared by mixing equal volumes of 2% $CuSO_4$ and 4% sodium tartrate) were added and the mixture stirred magnetically for 10 min. Folin reagent (0.5 mL of ×3 diluted commercial reagent) was then added and stirring continued for another 20 min. The solid was separated by centrifugation or filtration and the absorbance of the supernatant determined at 660 nm.

Determination of Bound Protein

Bound protein was routinely determined by the modified Lowry method described for bound tyrosine; the results obtained by this method were confirmed by amino acid analysis of acid hydrolyzates of the appropriate polyacrylamide protein conjugates using the procedure reported by Amarant and Bohak (*5*).

Enzymic Activity

The enzyme activity of trypsin was determined at 25°C by the pH-stat method (*8*) using 0.1*N* NaOH as titrant. The substrate solution (5 mL) was 0.015*M* benzoyl-L-arginine ethyl ester, 0.05*M* in KCl. The assays were carried out at pH 8 for the native enzyme and at pH 9.0 for the polyacrylamide-trypsin conjugates. One unit of esterase activity was defined as that amount of enzyme that catalyzed the hydrolysis of 1 μmol of substrate/min under the specified assay condition. The specific activity of the batch of native trypsin used was 22 U/mg protein. The polyacrylamide-trypsin beads were ground in a hand homogenizer or alternatively broken by strong magnetic stirring for a few hours, prior to activity determination.

RESULTS AND DISCUSSION

Preparation and Characterization of Support

Isonitrile derivatives of crosslinked polyacrylamide beads were prepared by a modification of a procedure described earlier for the preparation of isonitrile derivative of polysaccharides and other polymers bearing hydroxyl groups (*2,4*).

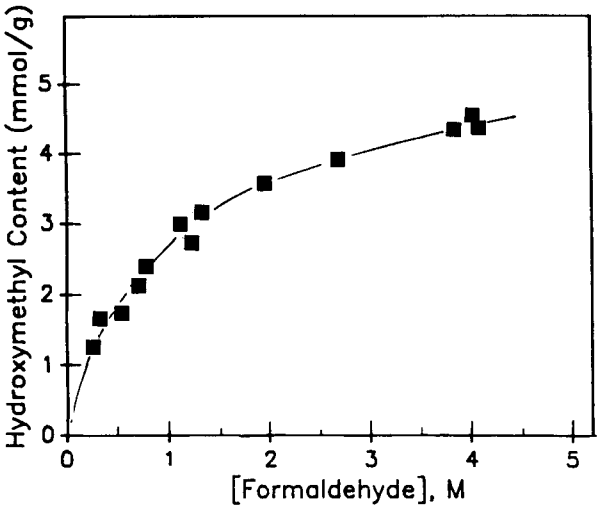


Fig. 1. Dependence of degree of hydroxymethylation of polyacrylamide beads on formaldehyde concentration (for details *see* Experimental Section).

The procedure consisted of:

1. *N*-hydroxymethylation (methylolation) of amide groups on the support by treatment with formaldehyde (*5*).

2. Attachment of sidechains containing isonitrile functional groups (*2,4*). This involved: a. The use of a strong base in a polar aprotic solvent (potassium *tert*-butoxide in dimethyl sulfoxide) to ionize methylol hydroxyl groups on the support; and b. Nucleophilic attack of the polymeric alkoxide ions thus generated on an isonitrile containing a good leaving group in the omega-position: 1-tosyloxyl-3-isocyanopropane (p-$CH_3 \cdot C_6H_4 \cdot SO_2 \cdot O \cdot (CH_2)_3$-NC).

The sequence of reactions employed is summarized in Eqs. 1 and 2.

$$\text{—CONH}_2 \xrightarrow[\text{pH 9.5}]{\text{HCHO}} \text{—CONHCH}_2\text{OH} \qquad (1)$$

$$\text{—CONHCH}_2\text{OH} \xrightarrow[\text{DMSO}]{\text{tert.BuO}^-} \text{—CONHCH}_2\text{O}^- \xrightarrow[\text{DMSO}]{\text{Tos}\cdot\text{O}\cdot(\text{CH}_2)_3\cdot\text{NC}} \text{—CONHCH}_2\text{O(CH}_2)_3\text{NC} \qquad (2)$$

Tos = p – $CH_3C_6H_4SO_2^-$

The degree of hydroxymethylation of polyacrylamide beads increased hyperbolically with increasing formaldehyde concentration (Fig. 1). Even at 4*M* CH_2O, however, only about 32% of the available amide groups were hydroxymethylated in 1 h at 50°C. For comparison, ground particles of polyacrylamide gel (5% crosslinking, highly swollen and permeable) and linear high mol wt polyacrylamide in solution underwent, under

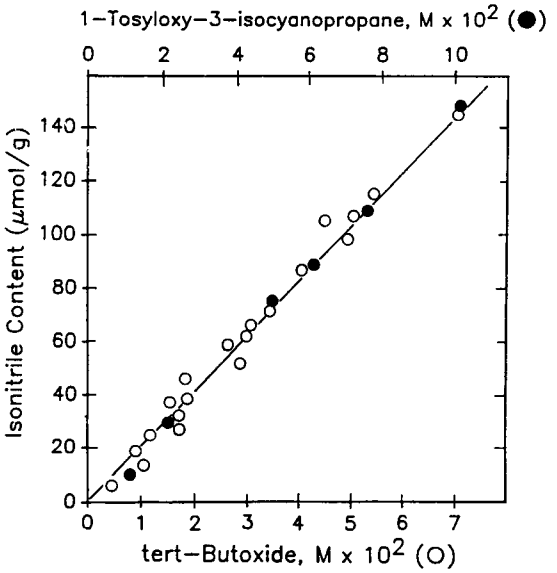


Fig. 2. Dependence of the isonitrile content of modified polyacrylamide beads on the concentration of reagents. Hydroxymethylated polyacrylamide beads (methylol content 3.5 mmol/g were treated at constant potassium *t*-butoxide concentration (0.07*M*) with increasing amounts of 1-tosyloxy-3-isocyanopropane—*open circles*—or alternatively at constant 1-tosyloxy-3-isocyanopropane concentration (0.1*M*) with increasing *t*-butoxide—*full circles*— as described in the Experimental section. The isonitrile content was estimated from the amount of ^{35}S-methionine bound.

similar conditions, 50 and 70% hydroxymethylation, respectively. This suggests that the extent of modification of amide groups in the crosslinked polymers is probably limited by parameters related to matrix density. Modified polyacrylamide beads of hydroxymethyl content 3–3.5 mmol/g (20–25% modification of amide groups) were routinely used.

The -NC content of the isonitrile derivatives of polyacrylamide, as estimated from the amount of [^{35}S]-methionine bound, varied linearly with the concentration of base or isonitrile reagent (at fixed coreagent concentration) (Fig. 2). This allowed considerable flexibility in the preparation of derivatized polyacrylamide beads of varying binding capacity, as indicated by the data of Fig. 2 and Table 1.

The isonitrile derivatives of polyacrylamide exhibited high storage stability when kept in dry form in the cold; no significant decrease in the binding capacity of samples refrigerated at 4°C over silica gel for up to one year could be detected.

Covalent Attachment of Proteins and Low Mol Wt Ligands

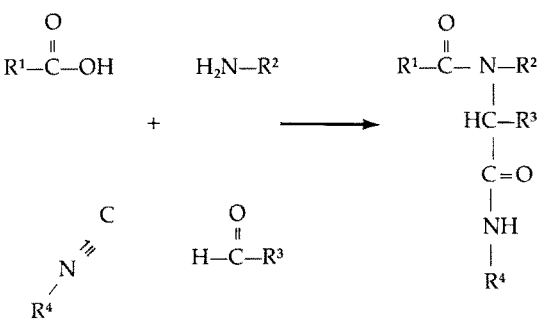
As shown in earlier publications from our laboratory, proteins and low mol wt substances such as peptides and amino acids can be attached covalently to polymeric supports containing isonitrile functional groups

Table 1
Isonitrile Derivatives of Hydroxymethylated Polyacrylamide Beads[a]

	Isonitrile derivative				Bound trypsin[c]	
	Preparation				Active	
No.	Potassium *t*-butoxide (*M*)	1-tosyloxy-3-isocyano-propane (m*M*)	Isonitrile content[b] μmole/g	Total mg/g	mg/g	% of total
1.	0.005	0.1	5.1	3.5	3.2	91
2.	0.018	0.1	32	27	23	85
3.	0.035	0.1	71	38	31	82
4.	0.070	0.05	74	40	32	80
5.	0.070	0.1	150	49	35	71

[a]Hydroxymethyl polyacrylamide beads (Biogel P-100) of methylol groups content 3.5 mmol/g were used.
[b]Estimated from the amount of ^{35}S-methionine or tyrosine bound.
[c]Amount of total bound trypsin was estimated by a modified Lowry procedure. Amount of active enzyme was determined by rate assays (*2,4,8*).

by four component condensation reactions (4CC), carried out in an aqueous buffer at neutral pH (*1–4*). 4CC reactions involve the simultaneous participation of amine, carboxyl, aldehyde, and isonitrile (Eq. 3) and lead to the formation of an *N*-substituted peptide bond between the carboxyl (R^1-COOH) and amine (R^2-NH_2) moieties; the aldehyde and isonitrile components (R^3-CHO and R^4-NC) appear as the sidechain attached to the amide nitrogen. The protein can serve as either amine or carboxyl donor in 4CC reactions, the actual mode of attachment to the support (R^4 in Eq. 3) being determined by the overall composition of the reaction mixture. In the presence of a water soluble aldehyde (acetaldehyde), coupling via ligand amino groups is obtained when the protein (or unprotected amino acid or peptide) is the sole amine donor, i.e., excess acetate is present in the reaction medium to provide carboxyl groups. Conversely, the presence of an amine (e.g., trishydroxymethyl aminomethane; Tris), in excess in the medium, allows the ligand to undergo covalent attachment through its carboxyl groups.



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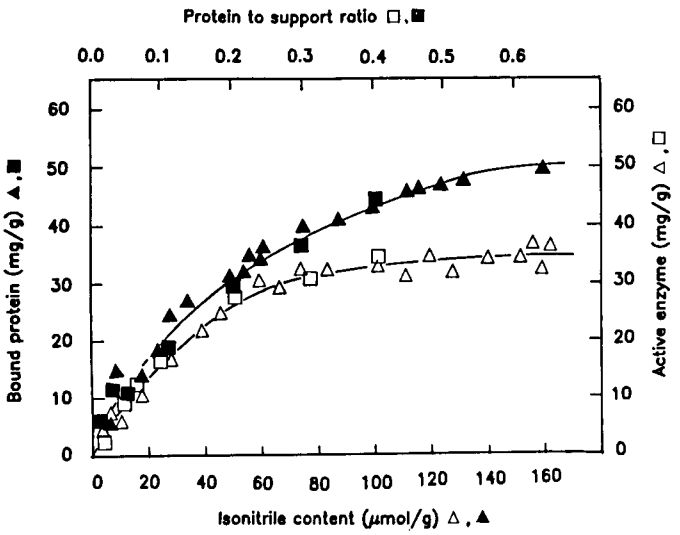

Fig. 3. Covalent binding of trypsin to isonitrile derivatives of polyacrylamide beads. Two types of 4CC saturation binding experiments were carried out: a. A constant amount of trypsin (20 mg) was reacted with polyacrylamide derivatives (50 mg) of varying isonitrile content (triangles); and b. A polyacrylamide derivative of high isonitrile content (99 μmol/g) was reacted with varying amounts of trypsin (squares). Full symbols—total bound protein; open symbols —enzymically active protein.

The protein binding capacity of the polyacrylamide supports was determined under two sets of conditions (Fig. 3): polyacrylamide derivatives of varying isonitrile content were contacted with excess trypsin under conditions of 4CC binding via protein amino groups (Fig. 3, circles). Alternatively, a derivatized polyacrylamide preparation of high isonitrile content (99 mmol/g) was contacted with increasing amounts of trypsin (Fig. 3, squares). The two sets of data points, which could be fitted on the same saturation curve, assymptoted toward 50 mg/g support and 35 mg/g support for total bound and enzymically active protein, respectively. These values imply the presence of diffusional constraints on the penetration of substrate in the case of polyacrylamide beads of high protein content.

Representative data on the saturative binding of a protein, trypsin, and of two amino acids, tyrosine and ^{35}S-methionine, to different batches of modified polyacrylamide beads are summarized in Table 1.

Stability of Bonds

In view of the fact that a number of bonds are generated in the course of the synthesis of polyacrylamide-ligand conjugates (*see* Eqs. 1-3), the susceptibility to hydrolysis of the bonds and/or groups generated at each step was investigated. To this end, residual capacities or contents were determined following exposure to buffers at different pH values.

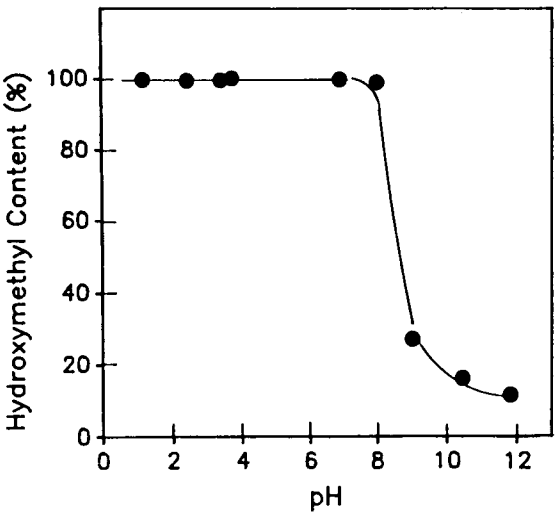

Fig. 4. Stability of methylol groups of hydroxymethylated polyacrylamide as function of pH. Aliquots (0.5 mL) of an aqueous suspension of hydroxymethylated polyacrylamide beads were added to 5 mL of an 0.1*M* buffer of the specified pH and immersed in boiling water for 15 min. The resin was separated by centrifugation and the methylol content determined as described by Amarant and Bohak (*5*).

The pH stability of the hydroxymethyl groups on methylolated polyacrylamide is shown in Fig. 4.

The stability to pH of a tyrosine conjugate of isonitrile polyacrylamide is shown in Fig. 5.

Comparison of the data of Figs. 4 and 5 establishes the increased stability of the —CONH·CH_2·O$(CH_2)_n$—R configuration relative to that of the hydroxymethylated amide groups (—CONH·CH_2OH). The susceptibility of the isonitrile functional group to spontaneous hydrolysis is presented in Fig. 6. The data show that hydrolysis of the —NC group is negligible around neutral pH values.

Binding Kinetics

The kinetics of binding of low mol wt substances to modified polyacrylamide beads by 4CC reactions was investigated using tyrosine as a model compound. The biphasic first order plots for binding of tyrosine via both amino or carboxyl groups shown in Fig. 7 conform with a two-sequential-reactions model ($k_1 = 7.7 \times 10^{-2}$ min^{-1}; $k_2 = 0.79 \times 10^{-2}$ min^{-1} and $k_1 = 4.8 \times 10^{-2}$ min^{-1}, $k_2 = 0.08 \times 10^{-2}$ min^{-1} for binding via the amino and carboxyl groups of tyrosine, respectively). The kinetics arguably imply the generation, with time, of diffusional constraints on ligand penetration, most probably related to changes in matrix density with the advance

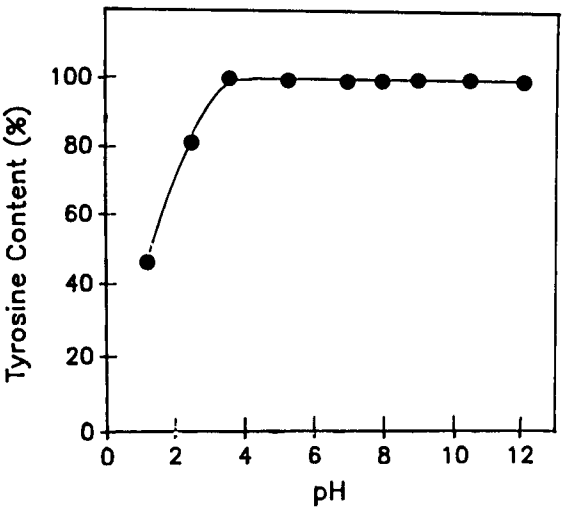

Fig. 5. Stability of tyrosine-polyacrylamide conjugates as function of pH. Aliquots (0.5 mL) of an aqueous suspension of tyrosine-polyacrylamide conjugate were added to 5 mL of 0.1*M* buffer of the specified pH. The reaction mixtures were immersed in boiling water for 15 min and the resin separated by centrifugation. The tyrosine content of the samples was determined by a modified Lowry procedure.

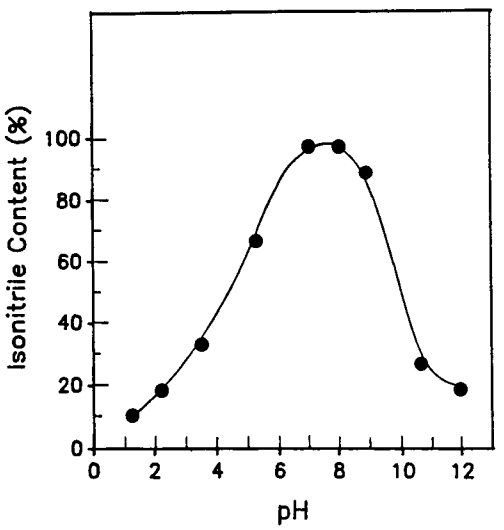

Fig. 6. Susceptibility of the isonitrile functional group to hydrolysis as a function of pH. Thirty mg of an isonitrile derivative of polyacrylamide were suspended in 5 mL of 0.1*M* buffer of the specified pH and incubated at 37°C for 48 h. The resin was removed by centrifugation and reacted with tyrosine under 4CC conditions. The tyrosine content of the *tyr*-polyacrylamide conjugate was determined by a modified Lowry procedure.

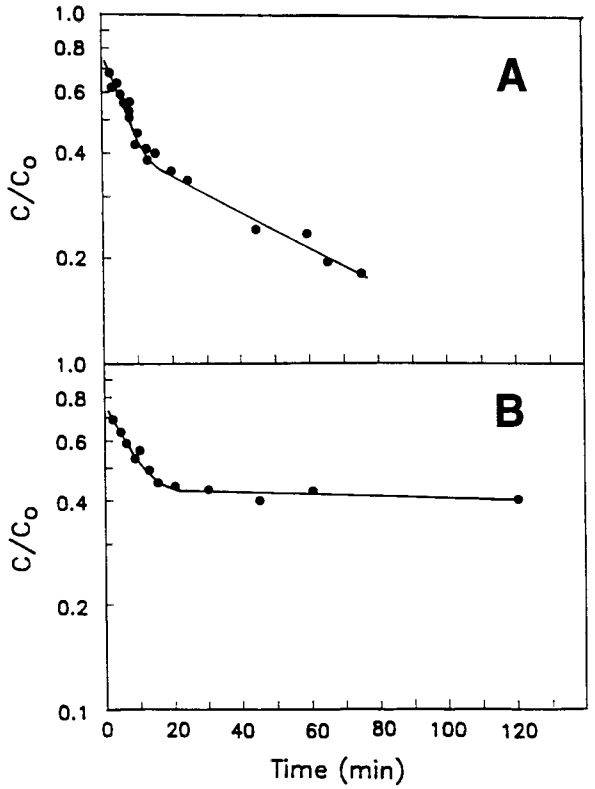

Fig. 7. Kinetics of 4CC binding of tyrosine to an isonitrile derivative of polyacrylamide beads. A—4CC binding via tyrosyl amino groups. B—4CC binding via tyrosyl carboxyl groups. A polyacrylamide derivative of high isonitrile content (80 mole/g) was reacted with a 1 m*M* solution of tyrosine 1.15*M* in acetaldehyde and 0.1*M* in phosphate 0.5*M* in acetate for binding via tyrosol amino groups (A), or 0.1*M* Tris 1.15*M* in acetylaldehyde for binding via tyrosyl carboxyl groups (B).

of the coupling reaction. The lower rate constant for 4CC binding of tyrosine via its —COOH group, estimated from the data of Fig. 7, go together with lower limiting amounts of tyrosine bound to polyacrylamide under the same conditions (80 μmole/g vs 42 μmol/g for binding of tyrosine via $-NH_2$ and —COOH, respectively). This suggests the presence of competing reactions, most probably with the solvent.

Kinetic Behavior of Polyacrylamide Trypsin Conjugates

A precursory survey of the kinetic behavior of polyacrylamide-enzyme conjugates was carried out using polyacrylamide-trypsin preparations of high enzyme content acting on an ester substrate, *N*-benzoyl-L-arginine ethylester, as a model system.

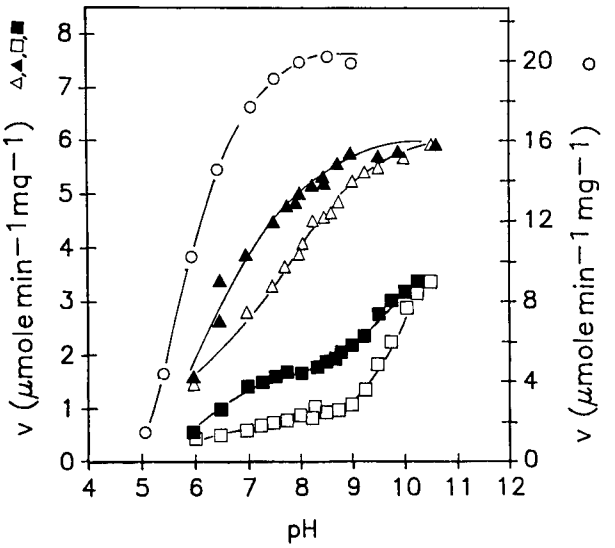

Fig. 8. pH dependence of the specific activity of trypsin-polyacrylamide conjugates. The enzyme activity of the trypsin-polyacrylamide conjugate was determined pH-statistically (*4,8*) using 0.015*M* *N*-benzoyl-L-arginine ethylester (0.01*M* in KCl). Specific activities were calculated/mg immobilized protein. Open squares: intact trypsin-polyacrylamide beads, 0.01*M* KCl (mean diameter 270 ± 80 μm); full squares: intact beads, 0.01*M* KCl, 1 m*M* borate. Open triangles: ground trypsin-polyacrylamide beads (mean diameter 35 ± 10 μm), 0.01*M* KCl. Full triangles: ground trypsin-polyacrylamide beads, 0.01*M* KCl, 1 m*M* borate. Open circles: native trypsin, 0.01*M* KCl.

The pH-dependence of the specific activity of intact and ground polyacrylamide-trypsin beads in the absence and in the presence of buffer (1 m*M* borate) is shown in Fig. 8 for the hydrolysis of *N*-benzoyl-L-arginine ethylester. The pH activity profile of intact beads (mean diameter 270 ± 80 μm) at low salt concentration (0.01*M* KCl) and in the absence of buffer, initially S-shaped, levels off as the pH increases and then rises again at high pH values. The presence of 1 m*M* borate raises the whole pH activity curve but does not significantly change its shape. Grinding of the beads (to a mean diameter 35 ± 10 μm) results in additional increase in activity as well as in the abrogation of the anomalous shape of the curves. The pH activity profiles of ground beads are, however, broader than the corresponding curves of native trypsin, even in the presence of 1 m*M* borate buffer.

This type of behavior is consistent with a "buffer-facilitated proton transport" model proposed by Engasser and Horvath (*9–11*) to explain the effects of buffers in proton generating enzyme reactions controlled by diffusional constraints, viz. buffers effect protogenic catalytic events by functioning as carriers that clear the proton from the site of the reaction (*9,12*).

Table 2
Kinetic Constants for the Hydrolysis of *N*-Benzoyl-L-Arginine Ethylester Catalyzed by Trypsin-Polyacrylamide Beads at pH 9[a]

	Borate buffer concentration, m*M*	V_{max}(app) μmol-min^{-1}-mg^{-1}	K_M(app), m*M*
Intact beads[b]	0.25	1.25±0.3	0.94 ±0.3
	0.5	1.8 ±0.3	1.0 ±0.2
	1	2.4 ±0.2	1.1 ±0.2
Ground beads[c]	—	4.7 ±0.3	0.064±0.08
Native enzymes	—	21.9 ±0.2	<0.001

[a]Rates of hydrolysis determined by the pH state method (*8*) (*see text*).
[b]mean diameter 270±80 μm.
[c]mean diameter 35±10 μm.

The substrate concentration dependence of the rate of hydrolysis of *N*-benzoyl-L-arginine ethylester catalyzed by intact and ground polyacrylamide-trypsin beads at pH 9 in the absence and in the presence of borate (pK_a of boric acid 9.2) is shown in Fig. 7.

In the absence of buffer, intact beads exhibit large deviations from Michaelis-Menten behavior, reflected in concave rather than linear Hofstee plots (*see 13,14*). Borate even at relatively low concentration ($2.5\times10^{-4}M$) leads to the linearization of the Hofstee plots and to increased values of V_{max} (app). Increasing the buffer concentration leads to additional increase in V_{max}(app) without, however, significantly affecting the values of K_M(app) (Table 2). Grinding the beads causes further increase in V_{max}(app) in parallel with a drastic decrease in the values of K_M (app) (Table 2).

The data of Fig. 9 is in agreement with the expected behavior for a diffusionally restricted enzyme catalyzed reaction inhibited by product (viz. protons), the latter acting as a noncompetitive inhibitor (*9,12–16*). In such systems, diffusional constraints would be reduced by either decreasing particle size or by enhancing the diffusional clearance of protons by facilitated transport away from the site of the reaction through the addition of buffer.

Reducing particle size, with the concomitant reduction of mass-transfer effects, would be expected to lower diffusion limitations on both substrate and product and hence affect K_m(app) as well as V_{max} (app). Clearance of the proton product by facilitated diffusion would on the other hand be expected to effect only V_{max} (app). These effects should be investigated further.

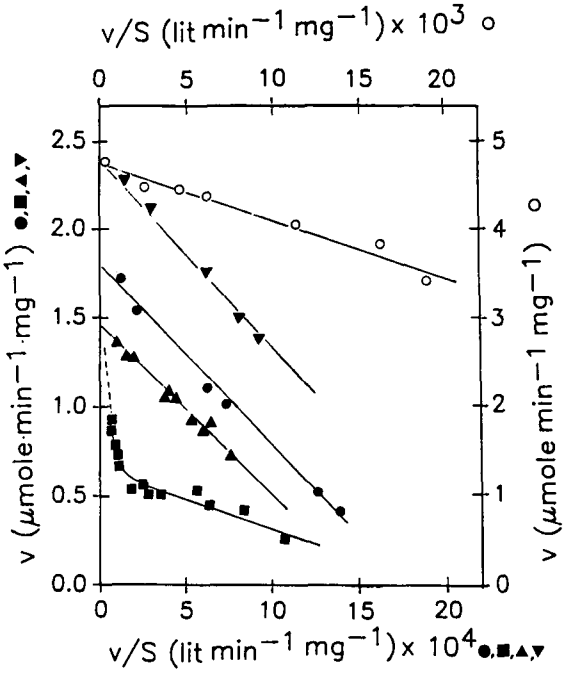

Fig. 9. Hofstee plots for the hydrolysis of *N*-benzoyl-L-arginine ethyl-ester by trypsin-polyacrylamide beads at pH 9.0. Rates of substrate hydrolysis catalyzed by trypsin-polyacrylamide (batch 4, Table 1) were calculated/mg immobilized protein. Full symbols: intact beads (mean diameter 270±80 μm). Full squares: 0.01*M* KCl; full triangles: 0.01*M* KCl, 0.25 m*M* borate; full circles: 0.01*M* KCl, 0.5 m*M* borate; Full inverted triangles: 0.01*M* KCl, 1 m*M* borate. Open circles: ground beads (mean diameter 35±10 μm), 0.01*M* KCl.

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