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PREPARATION AND MAGNETIC FILTRATION OF POLYACRYLAMIDE GELS CONTAINING COVALENTLY IMMOBILIZED PROTEINS AND A FERROFLUID

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Summary

Adenylate kinase, acetate kinase, and horseradish peroxidase have been immobilized covalently in good yields (20 - 90%) by free radical copolymerization of acrylamide, *N*,*N*'-methylenebisacrylamide, and *N*-acryloxysuccinimide, in a solution containing enzyme and enzyme-protecting reagents (substrates, DTT). To establish the sequence of events responsible for the formation of these enzyme-containing gels, rates for the reactions of *N*-acryloxysuccinimide and *N*-acetoxysuccinimide with oxygen-, nitrogen-, and sulfur-containing nucleophiles were determined. These data suggest that the rate of acylation of lysine ε-amino groups by *N*-hydroxysuccinimide-active esters is such that acylation and polyacrylamide gel formation are concurrent in the experiment procedures used. The yield of active, covalently-immobilized enzyme obtained by this procedure depends critically on active ester concentration and pH. To facilitate separation of these enzyme-containing gels from suspension, a ferrofluid — a surfactant-stabilized magnetite colloid — has been entrapped in them. The resulting magnetic gels can be manipulated using either conventional magnetic filtration and containment techniques, or more effective high-gradient procedures. Suspension polymerization can be used to produce spherical magnetic beads. The choice of suspending medium (a thixotropic carbon tetrachloride–n-heptane–silanized fumed silica mixture) and surface active agent (lecithin) are crucial to the success of this polymerization procedure.

Introduction

Cross-linked polyacrylamide gels are useful as immobilizing matrices for proteins and biochemicals for several reasons: they can be formed easily and economically, they are hydrophilic and electrically neutral, and they resist biological degradation. The simplest procedure for immobilization of proteins

in polyacrylamide gels — physical entrapment — has two limitations: first, included proteins leak from the gels at rates which are significant when long-term stability is required [1]; second, the low resistance of polyacrylamide gels to irreversible deformation on compression limits their usefulness in applications requiring high flow rates through packed columns. By preparing gels from vinyl monomers that are more hydrophobic than acrylamide, it is possible to increase both dimensional stability and long-term protein retention [2 - 4]. Gels of low hydrophilicity may, however, denature sensitive enzymes, and many vinylic monomers are unacceptably reactive toward protein thiol and amine groups. Further, decreased protein leakage usually required decreased gel pore size, with an accompanying increase in resistance of the gel to diffusion of substrates and products.

While developing procedures for immobilizing adenylate kinase (AdK)* and acetate kinase (AcK)*, to be used in regenerating ATP from AMP and ADP in cofactor-driven enzymatic synthesis [5 - 8], we examined polyacrylamide gels. The advantages of this polymer, combined with the ease with which its properties can be modified by copolymerization with other monomers, would make gels based on polyacrylamide very attractive for large-scale applications in applied biochemistry, provided that its deficiencies could be overcome. This paper describes studies to develop methods of eliminating protein leakage and compensating for dimensional instability. Copolymerization of *N*-acryloxysuccinimide (NAS) with acrylamide leads to a gel which is easily prepared as polyacrylamide and which is capable of immobilizing enzymes covalently. Inclusion of a ferrofluid — a surfactant-stabilized magnetite colloid — in the gel makes magnetic filtration possible, and permits magnetic recovery and containment of polyacrylamide gels under conditions that minimize the problems resulting from their poor mechanical properties.

Copolymers of acrylamide and NAS have been used previously for the covalent immobilization of affinity ligands [9] and for the prepatation of modified polyacrylamide resins [10, 11]. Related copolymers have also been used for enzyme immobilization [12 - 14]. Successful application of any procedure which involves carrying out the free-radical polymerization of a vinylic monomer in the presence of enzyme requires a detailed accounting of the factors contributing to enzyme deactivation during gel formation; the preceding paper provides this information for AdK and AcK in polyacrylamide [15].

Although the techniques described in this paper were developed specifically for immobilizing enzymes to be used in ATP regeneration, their simplicity and economy should make them valuable in a variety of problems

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*Abbreviations are: AdK, Adenylate kinase, AcK, acetate kinase; NAS, *N*-acryloxysuccinimide; PNP, *p*-nitrophenylacetate; NAcS, *N*-acetoxysuccinimide; Bis, *N,N'*-methylenebisacrylamide; DTT, dithiothreitol, Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPase, horseradish peroxidase; TMEDA, *N,N,N'*-tetramethylethylene diamine; Mops, morpholinopropane sulfonic acid.

requiring the immobilization, separation, or containment of biochemicals in polymeric gels.

Experimental

Materials were obtained and purified as described previously [15]. Horseradish peroxidase (Worthington) had a specific activity of 13 800 U/mg, and was used as purchased. AdK (Sigma) had a specific activity of 2 050 U/mg (1 U = 1 μmole/min) after reduction with DTT; AcK (Sigma) had a specific activity of 330 U/mg. The ferrofluid (a stable colloidal dispersion of single-domain magnetite particles) was obtained from Ferrofluidics Corp., Burlington, Mass.; it was a water-based material with magnetic saturation of 200 Gauss. Lecithin (Sigma), *o*-dianisidine (Sigma), and Tween 20 (Fisher) were used as obtained. Silanized fumed silica (Silanox 101) was obtained from the Cabot Corporation, Cambridge, Mass. Carbon tetrachloride and n-heptane used in suspension polymerizations were reagent grade solvents. Argon or nitrogen for inert atmospheres were welding grade.

Enzyme assays

AdK and AcK were assayed as described previously [15, 16]. HPase was assayed by following the oxidation of *o*-dianisidine (Worthington Enzyme Manual, 1972, p. 43).

Covalent immobilization of AdK and AcK

These immobilizations paralleled the experimental procedures outlined previously for non-covalent entrapment [15], except that NAS was included in the polymerization. NAS was prepared as a solution in dimethylsulfoxide (conveniently 1.0 - 2.5M). The desired quantity of this solution (typically, sufficient to make the final solution *ca.* 20mM) was added to the solution of acrylamide, *N,N'*-methylenebisacrylamide, riboflavin, and potassium persulfate, in aqueous buffer just before starting u.v. irradiation to initiate polymerization. As previously, the enzyme was added to the reaction mixture a few seconds before the gel point. All remaining steps have been described previously, and a more complete description can be found elsewhere [16]. An abbreviated procedure for HPase follows.

Seven stock solutions were required to immobilize HPase: S1: Hepes buffer (0.2M, pH 7.5) containing acrylamide (0.475 g/ml) and *N,N'*-methylenebisacrylamide (0.025 g/ml). S2: water containing a suspension of riboflavin (4 mg/ml). S3: Hepes buffer (0.05M, pH 7.5). S4: water (pH 7.6) containing potassium persulfate (50 mg/ml). S5: NAS (1M) in dimethylsulfoxide. S6: Hepes buffer (0.05M, pH 7.5), containing DTT (10mM) and ammonium sulfate (0.5M). S7: horseradish peroxidase, 13 800 U/ml. Degassed quantities of S3 (1.4 ml), S1 (0.5 ml), S2 (50 μl) and S4 (50 μl) were mixed in a capped, 5 ml beaker immersed in an ice bath under nitrogen. S5 (sufficient to give the desired concentration: 40 μl for a 20mM solution) was added to the

solution just before irradiation was started. A few seconds before the predetermined gel point, S7 (2.5 μl, 34.5 U of HPase) was added. Irradiation was continued for 25 s, and the resulting gel was kept at room temperature for 10 min. It was then transferred to a mortar pre-cooled to −15 °C, and broken up by brief, vigorous grinding. The gel particles were transferred to a 50 ml centrifuge tube using *ca.* 10 ml of S6. The particles were suspended in this wash solution, stirred briefly, and separated by centrifugation. The washing was repeated twice.

The yields of immobilized enzyme from this and related procedures (obtained by comparing the activity of a washed, enzyme-containing gel suspension with the activity of a solution containing the quantity of enzyme used in preparing that gel) were not entirely reproducible. Although yields obtained by a particular operator showed adequate reproducibility, yields obtained by different operators might differ at the extreme by a factor of three. Yields reported in Figs. 5 and 6 were obtained by a single individual, and represent the high end of the observed range. Although atypical, they are used for illustration because they represent the best observed activity balances, and provide a useful basis for mechanistic discussion. Yields obtained in routine practice were usually approximately half of the maximum values reported in these Figures. The origin of this irreproducibility has not been established, but since a number of rapid reactions occur simultaneously during these immobilizations — polymerization and crosslinking of vinyl monomers, acylation of nucleophilic groups of the protein, consumption of active ester moieties by reaction with other nucleophiles in solution, free radical and redox processes involving the initiation system or the polymer radicals — uncontrolled variations in experimental technique might reasonably result in significant differences in the relative rates of protein deactivation and immobilization.

Covalent immobilization of adenylate kinase, acetate kinase, and horseradish peroxidase in magnetic polyacrylamide particles

These procedures paralleled those used in the absence of ferrofluid, except that the light/riboflavin initiation system was not used because solutions containing the black ferrofluid are opaque. Instead, since some component of the ferrofluid was capable of reacting with persulfate and initiating polymerization, the ferrofluid itself was used to start polymerization. A description of the procedure used to immobilize adenylate kinase follows: immobilization of other enzymes followed analogous protocols. Seven stock solutions were required: S1 Hepes buffer (0.2M, pH7.0) containing acrylamide (0.475 g/ml) and Bis (0.025 g/ml). S2, water containing potassium persulfate (12 mg/ml). S3, ferrofluid solution (used in the same form as supplied). S4, Hepes buffer (0.05M, pH 7.5) containing DTT (10mM), $MgCl_2$ (30 mM) and Na_2ADP (10mM). S5, Hepes buffer (0.05M, pH 7.5) containing DTT (10mM) and ammonium sulfate (0.5M). S6, NAS (2.5M) in dimethylsulfoxide. S7, adenylate kinase (*ca.* 2400 U/ml) in Hepes buffer (0.05M,

pH 7.5). Stock solutions S1 through S5 were degassed and stored as described previously [15, 16].

Degassed quantities of S4 (1.4 ml), S2 (50 μl) and S1 (0.5 ml) were mixed in a capped, 5 ml beaker immersed in an ice bath. The solution was stirred with a small magnetic stirring bar under argon. S2 (20 μl) was added to the solution just before S3 (50 μl) was added. The solution gelled 10 s after S3 was added. A 20 μl aliquot of S7 was added to the solution 5 s before the solution gelled. The gel was kept at room temperature for 10 min. It was then transferred to a mortar pre-cooled to −15 °C and broken up by brief, vigorous grinding. The gel particles were transferred to a 50 ml centrifuge tube using *ca.* 10 ml of S5. The particles were suspended in this wash solution, stirred briefly, and separated by brief centrifugation. The washing was repeated twice.

Preparation of polyacrylamide gel beads containing covalently immobilized enzymes by suspension polymerization

The thixotropic organic phase was prepared by adding lecithin (4% by weight), and Silanox 101 (5% by weight) to a solution of carbon tetrachloride and n-heptane (1.00:1.75 v:v). The resulting translucent mixture had approximately the same density as water. It behaved as a highly viscous liquid while being stirred, but rapidly set to a gel when stirring was stopped. The suspension polymerization was carried out by a procedure which followed that used to prepare gel blocks up to the point at which the enzyme was added (*ca.* 2 - 5 s before the gel point). Immediately after addition of the enzyme-containing solution to a reaction mixture to be used to prepare beads, the entire mixture was transferred to a rapidly stirred quantity of thixotropic organic phase, and irradiation was continued. As soon as the aqueous phase was dispersed in the organic phase, stirring was stopped. The organic phase set to a gel immediately, and irradiation was continued to complete the polymerization. The resulting beads were separated by addition of carbon tetrachloride (to increase the density of the organic phase and cause the polyacrylamide beads to float to the surface). The beads were collected by pipette and washed. A detailed procedure for preparation of polyacrylamide beads containing covalently immobilized adenylate kinase follows; procedures for other enzymes are analogous.

Eight stock solutions were prepared: S1, Hepes buffer (0.2M, pH 7.0) containing acrylamide (0.475 g/ml) and Bis (0.025 g/ml). S2, water containing a suspension of riboflavin (4 mg/ml). S3, water (pH 7.6) containing potassium persulfate (50 mg/ml). S4, Hepes buffer (0.05M, pH 7.5) containing DTT (10mM), $MgCl_2$ (30mM) and Na_2ADP (10mM). S5, Hepes buffer (0.05M, pH 7.5) containing DTT (10mM) and ammonium sulfate (0.5M). S6, NAS (2.5M) in dimethylsulfoxide. S7, adenylate kinase (*ca.* 2 400 U/ml) in Hepes buffer (0.05M, pH 7.5). S8, Hepes buffer (0.05M, pH 7.5) containing 1% (w:v) Tween 20. These solutions were degassed and stored as described previously [15, 16]. An excess of the thixotropic organic phase was prepared from carbon tetrachloride (100 ml), n-heptane (175 ml), lecithin

(11 g) and Silanox (13.8 g). The solvents were degassed by passing a slow stream of argon or nitrogen through them for 20 min. The components were mixed, and 30 ml of the mixture was transferred to a 50 ml, three-necked, round-bottomed flask equipped with a variable-speed overhead stirrer with a No. 1 (4.1 cm long) Teflon paddle. One of the free necks was fitted with a nitrogen inlet, and the other capped with a glass stopper. Nitrogen was passed briefly through the vessel to remove oxygen. The solution was kept at room temperature, and the stirring rate adjusted to the desired value (typically 1 000 - 2 000 r.p.m.).

Preparation of the aqueous part of the polymerization system was started by transferring 1.4 ml of S4 and 500 μl of S1 to a 5 ml beaker containing a small magnetic stirring bar and capped with a serum stopper, and by transferring S2 (100 μl) and S3 (50 μl) to separate, capped, 15 ml centrifuge tubes. Each solution was degassed. The mixture of S1 and S4 was cooled to 0 °C, and S2 and S3 were added. Just before the polymerization was initiated by irradiation, S6 (20 μl) was added to the solution. Five seconds before the previously determined gel point, a 22 μl aliquot of S7 was added by syringe. The entire reaction mixture was immediately transferred into the stirred aqueous phase by forced siphon through a stainless steel cannula under a positive nitrogen pressure. The u.v. light was moved to illuminate the resulting suspension. The aqueous phase dispersed in the organic phase after several seconds of stirring, and 3 s after completion of the transfer, the stirrer was turned off. Irradiation of the translucent mixture was continued for 30 s beyond the estimated gel time of the beads. The beads were kept for 2 min in the thixotropic gel. Carbon tetrachloride (20 ml) was then added. The polyacrylamide beads floated to the surface. The solution was centrifuged. The supernatant containing the polyacrylamide beads was transferred to a 50 ml centrifuge tube. The beads were collected by removal of the organic fluid by forced siphon through a cannula. The beads were then washed in S8 (30 ml), centrifuged, and washed again in S8 (30 ml). The beads were finally washed three times in S5 (10 ml each time) in the same manner as before.

Preparation of magnetic polyacrylamide beads containing covalently immobilized enzyme by suspension polymerization

This procedure modified that for the preparation of non-magnetic beads by initiating polymerization using the aqueous ferrofluid suspension. For a preparation incorporating AdK into the beads, stock solutions S1, S3, S4, S5, S6, S7, and S8 (described above for the suspension polymerization of AdK) were required. Solution S3 (riboflavin) was omitted and a u.v. light was not required. The function of initiator was served by ferrofluid and persulfate. The thixotropic organic phase was prepared as outlined previously, and S1, S3, S4, S5, and S6 were assembled in a 5 ml beaker as described. To initiate polymerization, 50 μl of aqueous ferrofluid solution were injected into the reaction mixture. The polymerizing aqueous phase was transferred into the stirred organic phase 3 - 4 s before its estimated gel point (*ca.* 10 s

after addition of the ferrofluid). The stirring was continued for 2 s, and then stopped. The suspension of beads was allowed to stand for 2 min, and then the process of separation begun by adding carbon tetrachloride. Further steps in separating and washing the beads followed in detail those described for the non-magnetic beads.

N-acroyloxysuccinimide

A 500 ml three-necked flask was equipped with a pressure equalizing dropping funnel, a drying tube and a Teflon-coated magnetic stirring bar and placed in an ice/water cooling bath. The flask was charged with *N*-hydroxysuccinimide (Aldrich, 23.0 g, 0.20 mol), chloroform (300 ml), anhydrous triethylamine (30 ml, 22 g, 0.22 mol), and 5 mg of 2,6-di-t-butyl-4-methylphenol (Aldrich), a polymerization inhibitor. Acryloyl chloride (Aldrich, 18 ml, 20 g, 0.22 mol) was added dropwise over a 20 min period and the reaction stirred at 0 °C for an additional 10 min. The solution was transferred to a 1 l separatory funnel, washed with 200 ml portions of water-saturated sodium chloride solution, then dried over anhydrous magnesium sulfate. Another 5 mg portion of 2,6-di-t-butyl-4-methylphenol was added to the filtered solution which was then evaporated *in vacuo* to *ca.* 40 ml. Dilution with a mixture of 30 ml of dry ethyl acetate and 70 ml of n-hexane, followed by quick warming with charcoal to near boiling, filtering, and setting aside overnight at 4 °C produced about 15 g (45%) of colorless crystals, m.p. 68.0 - 69.5 °C. This material is usually adequate for polymerizations. It can, however, be further purified by recrystallization from a mixture of ethyl acetate/n-hexane (1:2) with charcoal and a 5 mg portion of 2,6-di-t-butyl-4-methylphenol to inhibit polymerization. After standing overnight at 4 °C, the colorless crystalline product can be separated by filtration and dried *in vacuo*. This recrystallized product had a m.p. of 69.0 - 70.0 °C, n.m.r. ($CDCl_3$) 2.87 (s, 4H), 6.0 - 7.0 (m, 3H), p.p.m.; Ir (Nujol mull) 1800, 1775, 1735, 1260, 995, 870 cm^{-1}.

Kinetics of reactions of N-*acryloxysuccinimide by pH-stat titration*

Kinetics were followed by titration using a Radiometer pH-stat and GK 2321C glass electrode. Titration vessels were of polypropylene or glass, and solutions were degassed before use and covered with an argon atmosphere during kinetics run. Solutions were stirred magnetically. Carbonate-free sodium hydroxide (0.005M) was prepared by dilution of commercial 1M solution (Baker; Dilute-it), and standardized against potassium hydrogen phthalate. The ionic strength in solutions used for kinetics was maintained at $\mu = 1$ with KCl. NAS was added to solutions as 0.1M or 1.0M solutions in dioxane or dimethylsulfoxide. The solutions used for kinetics studies had a volume of 5 ml plus the volume of the added ester-containing solution. NAS was recrystallized before use. n-Butylamine was dried over potassium hydride and distilled under nitrogen (b.p. 78 °C). Determination of pK_a values of species of interest gave good agreement with literature values: *N*-hydroxysuccinimide, $pK_a = 6.0$ (literature = 6.0 [17]; acrylic acid, $pK_a = 4.25$ (liter-

ature = 4.25 [18]). Aminolysis reactions were carried out using an excess of amine, so that the rate equation (eqn. (6)), describing consumption of base would be pseudo zero order in amine. Reactions were run with the ratio of amine to NAS ranging from 1.5 to 10, with no significant differences in the derived rate constant, k_N.

Kinetics of aminolysis of NAS by g.l.c.

Aminolysis reactions were carried out as described for pH-stat studies, except that small aliquots were withdrawn periodically by syringe and immediately injected into a Perkin-Elmer Model 990 g.l.c., using a 4 ft glass column containing 10% Carbowax 20M-TPA on 40 - 60 mesh Chromosorb T. Areas of the peak corresponding to *N*-butylacrylamide were measured relative to $CH_3OCH_2CH_2OH$ internal standard using a Spectra Physics Autolab Minigrator electronic integrator.

Kinetics of reactions of N*-acryloxysuccinimide by u.v. spectroscopy*

Kinetics of the reactions of NAS were established by following the appearance of *N*-hydroxysuccinimide anion at 259 nm with a Gilford 240 Spectrophotometer. For these experiments, solutions were buffered with Hepes. NAS was added as a 10mM solution in dimethylsulfoxide. An extinction coefficient of $8\,600 M^{-1}\ cm^{-1}$ was used for the *N*-hydroxysuccinimide anion at pH 7.5 (Fig. 1). Two different experimental procedures were used. In the first, 3 ml of degassed Hepes buffer solution were transferred to a quartz cuvette, which was placed in the cuvette holder of the spectrophotometer. The sample compartment was thermostatted with circulating water or ethanol–water solution at constant temperature. The temperature of the solution in the cuvette was allowed to equilibrate (10 min), and a known volume of the NAS stock solution was added. The absorbance of the solution was monitored at 259 nm. The aminolysis reactions of NAS were studied similarly using solutions in which the total amine concentrations were initially 20 - 40 times larger than that of NAS. The pH of the solution was checked after the addition of the amine, and adjusted to the required pH, if necessary. The second procedure used to study the hydrolysis and aminolysis reactions of NAS involved circulating the reactor mixture to a flow-through cuvette in the thermostatted sample compartment from a 50 ml flask immersed in a constant temperature bath. An LKB 12000 Varioperpex pump was used to pump the solution rapidly (8.2 ml/min) through the cuvette. The circulating loop had a 5.2 ml volume and the flow-through cuvette a 0.5 ml volume. Before each run the solution was degassed by bubbling argon through it for 30 min. During each run the solution was flushed continuously with a stream of argon. The appearance of *N*-hydroxysuccinimide anion was monitored at 259 nm.

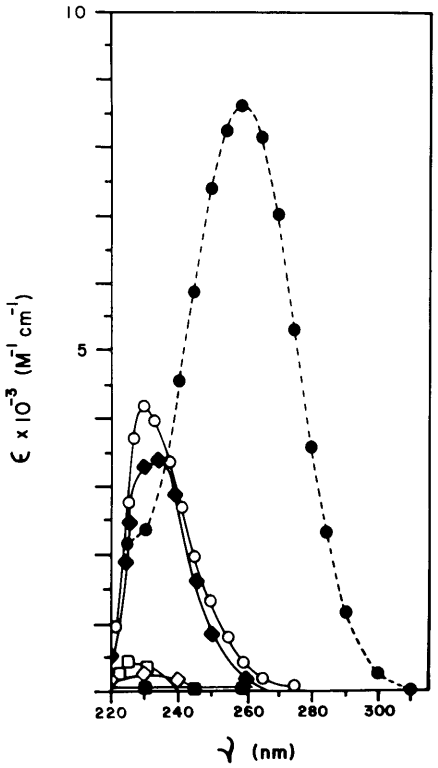


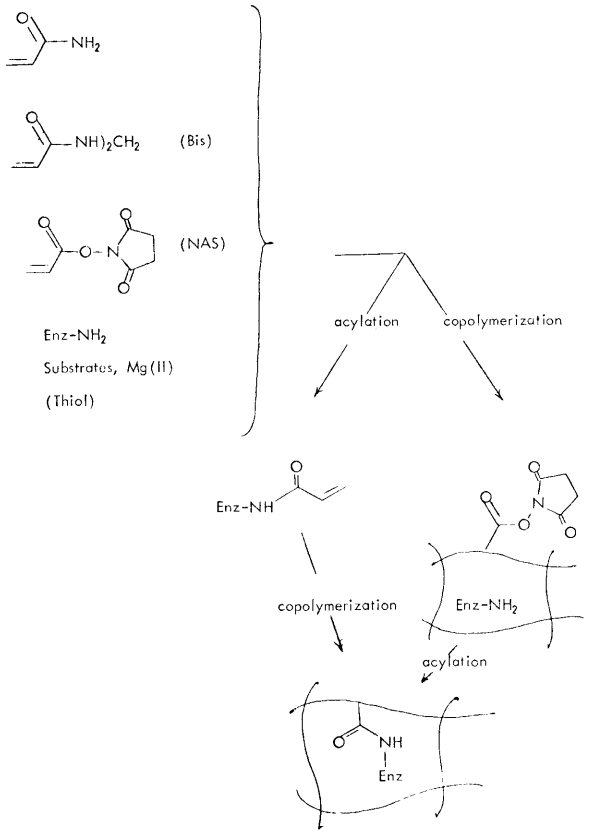
Fig. 1. Molar absorbances (M^{-1} cm^{-1}) of species present during hydrolysis or aminolysis of *N*-acryloxysuccinimide, or of models for these species (50mM Hepes buffer, pH 7.5, 25 °C): ●, *N*-hydroxysuccinimide; ○, *N*-butylacrylamide; ■, acetamide; ◇, DTT; □, acrylic acid; ♦, methyl thioacetate.

Results

Copolymerization of acrylamide and N-acryloxysuccinimide in the presence of enzymes produces gels containing good yields of covalently immobilized enzymes

The main limitation of physical entrapment in polyacrylamide gels as an immobilization method is leakage of enzyme from the gel. An alternative procedure — preparation of polyacrylamide gels functionalized to permit covalent linking, followed by treatment of the functionalized gel with a solution of the enzyme [9, 13, 19] — is also not entirely satisfactory; functionalization of polyacrylamide gels usually decreases pore sizes, and poor access to the functionalized sites may result in slow immobilization and poor yields of immobilized materials. This type of procedure is normally useful only when the enzyme can be used in excess and when the reactive group of the gel is stable in water.

One method which minimizes the disadvantages of the two types of procedure is to combine them in a way which permits the enzyme to be cova-



Scheme I. Covalent immobilization of enzymes in polyacrylamide gels.

lently coupled throughout the gel. The procedure used here is outlined in Scheme I. Polymerization of acrylamide, cross-linking agent (*N,N'*-methylenebisacrylamide, Bis), and NAS, in a solution containing enzyme and various components designed to protect the enzyme during the polymerization process, results in the formation of a polyacrylamide gel containing covalently immobilized enzyme. Comparison of the interval required to form the gel with estimated rates for reaction of the lysine ϵ-amino groups of the proteins with the *N*-hydroxysuccinimide active ester groups (see below) suggests that these processes probably proceed competitively. Since the polymerization takes place in a solution that includes the enzyme, the initially-formed gel includes all of the enzyme. The uniform distribution of the enzyme and active ester in the gel minimizes the time required for the enzyme to couple with the gel, and maximizes the yield of productive coupling relative to hydrolysis of the active esters. The major disadvantage of this procedure is that close attention to the details of experimental protocol is required to avoid deactivation of the enzyme by reactive vinyl monomers, radical initiators, and radicals. A more general procedure, which involves non-radical coupling of the enzyme with a preformed, soluble, copolymer of

acrylamide and NAS during conversion of this copolymer to cross-linked insoluble gel, is described elsewhere [20]. Although this latter procedure is clearly preferred for sensitive enzymes, the techniques described in this paper require fewer steps and should be useful with enzymes which are insensitive to deactivation by the polymer-forming conditions.

A number of parameters must be considered in carrying out these polymerizations and couplings: the physical properties (particularly mean pore size) required for the gel, protection of the enzyme against deactivation before and during the polymerization, the nature and concentration of the active ester included, the polymerization initiation system used. Each of these is considered in turn.

Gel physical properties

Two properties of polyacrylamide gels are important in designing matrices for enzyme immobilization: the effective mean pore size, and the gel hydrophilicity. Polyacrylamide gels can be prepared, having effective mean pore radii between 0.64 and 2.8 nm [21], by adjusting the total acrylamide concentration (denoted by $T = 100 \times$ g total monomer/ml solvent) and the concentration of cross-linking agent (expressed as a percentage of total monomer, and denoted by $C = 100 \times$ g cross-linking agent/g total monomer) [22 - 26]. In practice, low values of % T and % C lead either to weak gels, or to polymers that do not gel at all. For the gel to have significant mechanical strength, values of $T \geqslant 10$ and $C \geqslant 5$ are required. Throughout this work $T = 12$ and $C = 5$ were used, corresponding roughly to an effective mean pore radius of 1.2 nm.

The hydrophilicity of polymeric gels is difficult to define precisely. As an approximation, one can calculate Hansch Π values (normalized for differences in molecular weight) for the momomeric units along the polymer backbone using standard parameters: Π_{norm} = Π/monomer molecular weight [27 - 29]. For several matrices commonly used for immobilizing biochemicals, these values are: polymer, Π_{norm}; agarose, —1.7; polyacrylamide, —0.55; polyvinyl alcohol, +0.36; polyethylene oxide, +0.77; poly-2-hydroxymethylmethacrylate, +0.85; polyacrylonitrile, +0.91 [30]. These values neglect important details of hydrogen and hydrophobic bonding and are only crude guides to hydrophilicity. Nonetheless, they indicate that polyacrylamide is a very hydrophilic polymer. The inclusion of amide linkages connecting polymer and enzyme clearly should not markedly influence this hydrophilicity, because these linkages are present in low concentration and resemble the amide moieties normally present in polyacrylamide.

Enzyme protection during polymerization

This has been discussed in detail for AdK and AcK in the accompanying paper [15] and will only be summarized here. Polymerization was ordinarily initiated photochemically by riboflavin-sensitized decomposition of persulfate ion. The important contributors to enzyme deactivation are: autoxidation of cysteine moieties by oxygen present in solution, Michael addition of cysteine

sulfhydryl groups to vinylic monomers, oxidation of cysteine and other amino acids by singlet oxygen generated by energy transfer to oxygen from excited riboflavin, and attack on unidentified sites by (presumably) $SO_4^{\cdot}$ or buffer-derived free radicals. In addition, qualitative evidence accumulated during studies of covalent immobilization indicates that NAS itself deactivates enzymes rapidly, probably by acylation and by Michael addition: NAS is an α,β-unsaturated ester, and as such should be approximately 20 times more reactive than acrylamide in Michael addition [31 - 33]. Recent qualitative observations, to be described in detail in a separate paper, indicate that substitution of the *N*-hydroxysuccinimide active ester of methacrylic acid for NAS significantly decreases the deactivation of proteins during gel formation, and increases the immobilization yield*. Deactivation can be largely suppressed by manipulating the enzymes and carrying out the polymerization at 0 - 4 °C, with careful exclusion of oxygen, in the presence of substrates.

Active ester

The most generally used procedures for coupling enzymes to insoluble supports depend on reaction of lysine ϵ-amino groups present in the hydrophilic exteriors of the proteins with alkylating agents, acylating agents, or related reactive groupings [20]. We based our coupling procedure on active esters of acrylic acid because these materials are readily synthesized and because they usually copolymerize smoothly with acrylamide. An enormous number of active esters have, of course, been developed for forming amide linkages in peptide chemistry; we utilized NAS as our starting material because it is an easily prepared, stable, crystalline solid with good water solubility and because *N*-hydroxysuccinimide is itself only a weak acid (pK_a = 6 [17]) and thus unlikely to produce large changes in solution pH on release from an active ester.

To control the copolymerization and coupling reactions of NAS, we required rate constants for reactions of *N*-hydroxysuccinimide esters with typical nucleophiles in aqueous solutions. Rate constants for reactions of these materials in non-aquous solutions have been collected [34], but only qualitative data are available for aqueous solutions [35]. In this work, we have determined both rates of hydrolysis of NAS in aqueous solution and rates of reaction with several representative nucleophiles. Rates were determined as a function of pH by following the uptake of base in a pH-stat at fixed pH and constant ionic strength (μ = 0.1 KCl), and in buffered solutions by analysis of the rate of appearance of *N*-butylacrylamide by gas–liquid chromatography or of *N*-hydroxysuccinimide by u.v. spectrophotometry.

The hydrolysis reaction represented by Scheme II can be described by rate equation (1).

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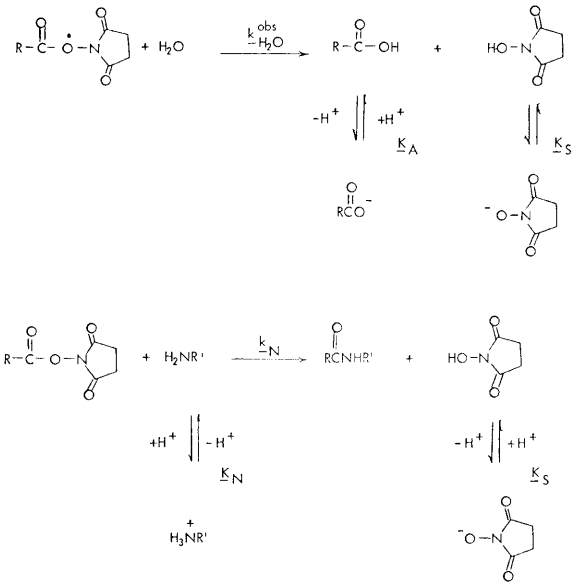
*These observations are drawn from unpublished work of R. Baughn, A. Pollak and J. R. Rasmussen.



Scheme II. Hydrolysis and aminolysis of an *N*-hydroxysuccinimide ester.

$$-\frac{d[\text{Ester}]}{dt} = k^{obs}_{H_2O}[\text{Ester}] = \left\{k_{H_2O}[H_2O] + k_{OH^-}[OH^-] + k_{H^+}[H^+] + \sum_Y k_Y[Y]\right\}[\text{Ester}] \quad (1)$$

In this equation k_{H_2O} is the rate constant for the uncatalyzed hydrolysis of the active ester, and k_{OH^-}, k_{H^+} and k_Y are rate constants describing its hydrolysis catalyzed by hydroxide ion, hydronium ion, and other species (Y), respectively.

Determination of the rates of hydrolysis by pH-stat titration are carried out in unbuffered solution under pseudo first-order conditions. Because the solution pH is maintained constant, the concentration of hydroxide ion remains constant. Although terms in k_Y might be important for the buffer or thiol components used during the immobilization of proteins, they are probably not important in the solutions used for these kinetics studies since no reactive species other than carboxylate ion are present. We therefore neglect these terms. The pH region of interest in immobilization lies between 6 and 9. We see no evidence of acid-catalyzed hydrolysis in this region (*vide infra*), and neglect k_{H^+}.

Kinetics were followed using initial-slope methods. Hydrolyses were allowed to proceed for a period of time long enough to establish the initial rate of consumption of hydroxide ion, but not long enough to change the concentration of ester significantly. Under these conditions, eqns. (2) and (3) describe the relation between the following variables: the starting ester concentration, $[\text{Ester}]_0$; the quantity of ester hydrolyzed, Δ [ester] (assumed

small relative to $[\text{Ester}]_0$); the time the hydrolysis is allowed to proceed, Δt; the quantity of hydroxide ion added over this interval to maintain constant pH, $\delta(OH^-)$, and $k^{obs}_{H_2O}$. The term C is a function of pH and accounts for the influence of the equilibria

$$k^{obs}_{H_2O}\,\Delta t\,[\text{Ester}]_0 = -\Delta[\text{Ester}] = C\delta(OH^-) \tag{2}$$

$$C = \left[\frac{[RCO_2^-]}{[RCO_2^-] + [RCO_2H]} + \frac{[NHS^-]}{[NHS^-] + [NHSH]}\right]^{-1}$$

$$= [(1 + 10^{pK_A - pH})^{-1} + (1 + 10^{pK_S - pH})^{-1}]^{-1} \tag{3}$$

between carboxylic acid and carboxylic ion, and between *N*-hydroxysuccinimide and its conjugate anion, on the number of equivalents of hydroxide ion consumed per equivalent of ester hydrolyzed. The individual terms up C are the fraction of carboxyl groups present as carboxylate ion and the fraction of *N*-hydroxysuccinimide groups present as anions, at the given pH (eqn. (3)).

Aminolysis represents a closely analogous problem (Scheme II). If these reactions are carried out at constant pH in the presence of an excess of amine, then neither amine nor ester concentration changes significantly during the time required to establish the initial rate of consumption of hydroxide, and eqn. (4) describes the appropriate initial slope approximation. Here, k^{obs}_N represents the contribution to the disappearance of active

$$-\frac{\Delta[\text{Ester}]}{\Delta t} = k_N[\text{Ester}]_0\,[R'NH_2]_0 + k^{obs}_{H_2O}\,[\text{Ester}]_0 \tag{4}$$

ester and release of protons due to amide formation. We neglect any catalysis of hydrolysis- or aminolysis reactions by $R'NH_2$ or $R'NH_3^+$. If the acid dissociation constant of alkylammonium ion is K_N, and the term $[\text{"}R'NH_2\text{"}] = [R'NH_2] + [R'NH_3^+]$ symbolizes the total concentrations of alkyl amine and alkylammonium ion, the relation between $\delta(OH^-)$ and $\Delta[\text{Ester}]$ is given by eqn. (5), and the rate constant k_N (Scheme II) for reaction of neutral amine with active ester by eqn. (6): $[\text{"}R'NH_2\text{"}]_0$ is the total concentration of amine-containing species at time = 0, and is assumed to be constant over a kinetics run.

$$\Delta[\text{Ester}]/\delta(OH^-) = D = [(1 + 10^{pH - pK_N})^{-1} + (1 + 10^{pK_S - pH})^{-1}]^{-1} \tag{5}$$

$$k_N[1 + 10^{pK_N - pH}]^{-1}[\text{"}R'NH_2\text{"}]_0 = \left\{\frac{D\delta(OH^-)}{\Delta t[\text{Ester}]_0} - k^{obs}_{H_2O}\right\} \tag{6}$$

Analysis of the rate of consumption of hydroxide ion by NAS in water at constant pH was carried out using eqn. (2), and used to generate the pH-rate profiles shown in Fig. 2. Acrylic acid and *N*-hydroxysuccinimide were established as the reaction products at pH 6. For convenience, Fig. 2 also gives half-lives (τ_{H_2O}) for the hydrolysis of NAS. Hydrolysis data were not collected at pH values sufficiently acidic to determine if the suggestion of a plateau at low pH is real. The accuracy of the rate data at low pH, particul-

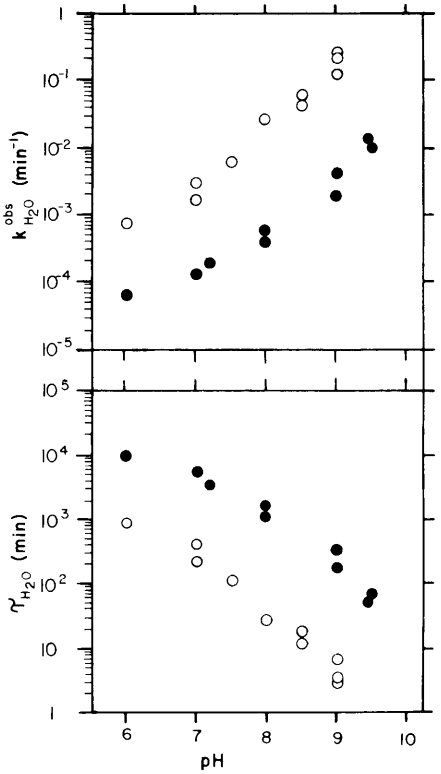


Fig. 2. (Upper) Observed rate constants ($k^{obs}_{H_2O}$, eqn. (2)) for hydrolysis of *N*-acryloxysuccinimide in unbuffered water (μ = 1, KCl), obtained by pH-stat titration. (Lower) Half-lives (τ_{H_2O}) for hydrolysis derived from these rate constants. For both plots, ○ indicates data obtained at 25 °C, and ● indicates data obtained at 0.2 °C.

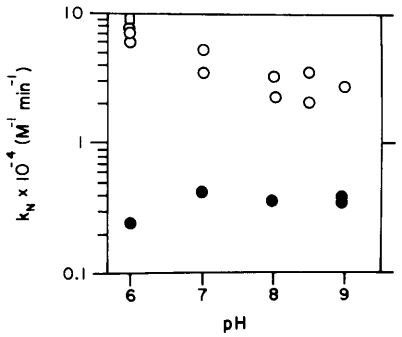


Fig. 3. Rate constants (k_N, eqn. (6)) for reaction of n-butylamine with *N*-acryloxysuccinimide in water (μ = 1.0, KCl): ○, pH-stat titrimetric data, 25 °C; ●, titrimetric data, 0.2 °C; □, rate constant derived by g.l.c. analysis of the rate of formation of *N*-butylacrylamide.

arly at 0.2 °C, is lower than that at higher values of pH and temperature because reactions at low pH are very slow.

Figure 3 summarizes rate constants, k_N, obtained for reaction of NAS with n-butylamine. Most of these constants are based on titrimetric data

(eqn. (6)), but direct analysis by g.l.c. of the *N*-butylacrylamide produced was also included. Rate constants at 0.2 °C are in reasonable agreement over the pH range examined, and give an average value of k_N (0.2 °C) ≅ 3.6 × $10^3 M^{-1}$ min^{-1}. Rate constants at 25 °C show an apparent decrease with increasing pH, for reasons that were not established. The good agreement between values of k_N derived from analysis of independent pH-stat and g.l.c. data at pH 6 provides a check of the accuracy of these data. The yield of *N*-butylacrylamide formed by reaction of 0.004M NAS with 0.03M n-butylamine was 99 - 100% (by g.l.c.). This high yield confirms that neither hydrolysis of NAS nor Michael addition of n-butylamine to NAS competes significantly with amide formation.

The rate studies based on pH-stat titration and g.l.c. analysis summarized in Figs. 2 and 3 were carried out in unbuffered aqueous solution. Enzyme immobilizations were always conducted in buffered solutions. It was necessary to use yet another analytical technique — u.v. spectrophotometry — to establish the influence of a typical buffer system on aminolysis and hydrolysis rates: pH-stat titration is unreliable in highly buffered systems, and the buffer components (or their decomposition products) interfered with the g.l.c. analysis. A survey of pertinent u.v. spectra (Fig. 1) established that it was possible to follow the appearance of the anion of *N*-hydroxysuccinimide at 259 nm without interference from other species. Kinetics data were analyzed by procedures analogous to those described previously. Rates of hydrolysis of NAS ($k_{H_2O}^{obs}$) and rate constants for coupling of NAS with n-butylamine (k_N) as a function of the concentration of Hepes buffer (pH 7.6, 25 °C) are summarized in Fig. 4. Addition of 1M KCl to these solutions produced no significant change in the observed rates. Extrapolation of $k_{H_2O}^{obs}$ to zero buffer concentration gave a rate of 0.006 min^{-1}, in satisfactory agreement with the value of 0.005 min^{-1}, estimated from Fig. 2. Extrapolation of k_N to zero buffer concentration yielded a value of 5 × $10^4 M^{-1}$ min^{-1} (pH 7.6, 25 °C), again in satisfactory agreement with the value of 3.2 × $10^4 M^{-1}$ min^{-1} obtained by pH-stat titration (Fig. 3).

The data of Fig. 4 establish that buffer catalysis may increase the rates of attack of nucleophiles on NAS by relatively small factors (2 - 4) at buffer concentrations representative of those used for enzyme immobilizations. Two other characteristics of the solutions used in these immobilizations differ from those used in studying the kinetics of the model coupling reactions: the former contain high concentrations of acrylamide (originally 1.7M) and its polymers, and, occasionally, appreciable quantities of DTT or 2-mercaptoethanol used as protein antioxidants. The rate constant for reaction of NAS with n-butylamine in Hepes buffer (pH 7.5, 25 °C) containing 1.7M acetamide was k_N = 12 × $10^4 M^{-1}$ min^{-1}. This value is close to that observed in the absence of acetamide, and suggests that even high concentrations of polar amides do not markedly influence the rate of reaction of NAS with amines. Table 1 summarizes these rate constants, together with those for two other nucleophiles that might be included in solutions containing *N*-hydroxysuccinimide active esters: DTT, and tetramethylethylenediamine (TMEDA,

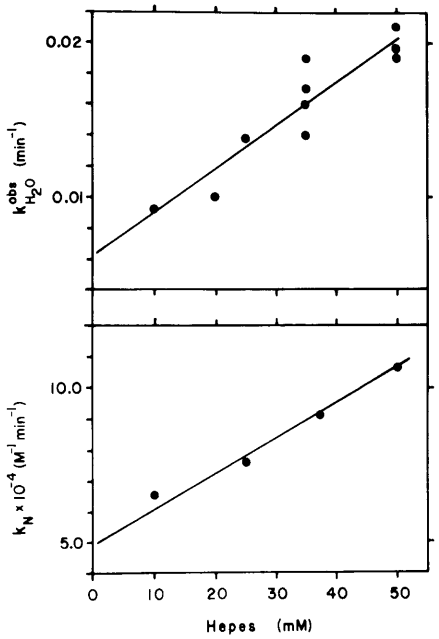


Fig. 4. Observed rates of hydrolysis ($k^{obs}_{H_2O}$, 25 °C, upper), and aminolysis (k_N, 25 °C, n-butylamine, lower) of *N*-acryloxysuccinimide as a function of Hepes buffer concentration at pH 7.62. All experiments were carried out in aqueous solutions containing Hepes, n-butylamine and *N*-acryloxysuccinimide. The initial concentrations of *N*-acryloxysuccinimide were between 33 and 100μM, and the initial n-butylamine concentrations between 0.5 and 5mM.

a tertiary amine commonly used as one component of redox systems for initiating acrylamide polymerization). One rate of aminolysis of a saturated *N*-hydroxysuccinimide active ester (*N*-acetoxysuccinimide, NAcS) is also included to establish that the rates of reaction of saturated and α,β-unsaturated active esters do not differ markedly. Since alkyl substitution on *p*-nitrophenyl acetate has relatively little influence on its reactivity toward nucleophiles, *N*-hydroxysuccinimide active esters, by analogy, should have similar reactivity, whether present as monomeric NAS or as part of a copolymer with acrylamide [36].

Table 1 also gives a rate constant for hydrolysis of *p*-nitrophenyl acetate measured using the same techniques as those employed in the studies of NAS, and a rate constant estimated from a careful spectrophotometric study of the hydrolysis of this ester [37]. The reasonable agreement between these two sets of measurements lends support to the reliability of our data and makes it possible to compare, qualitatively, the hydrolysis and aminolysis rates for the *N*-hydroxysuccinimide esters with the enormous body of research that has been carried out using *p*-nitrophenyl acetate as substrate [38].

Several features of the data in Table 1 and Figs. 2 - 4 are pertinent to protein immobilization by the techniques described in this paper. First, the

TABLE 1

Rate constants for reaction of nucleophiles with active esters, and estimated relative reactivities of these nucleophiles at pH 7.6 and 25 °C

Ester	Nucleophile (R)	pK_a	Soln[a]	Method	$k_R(M^{-1}\,min^{-1})$ ($\times 10^{-4}$)	$k^{obs}_{H_2O}$ (min^{-1})
NAS	$OH^-(H_2O)$	15.7	μ = 1.0 (KCl)	pH	1.2[b]	0.005
				u.v.[c]	1.5	
			Hepes, 50mM	u.v.	3.3	0.013
	$C_4H_9NH_2$	10.8	μ = 1.0 (KCl)	pH	3.2	
				u.v.[c]	5.0	
			Hepes, 50mM	u.v.	11	
			d	u.v.	12	
	TMEDA	5.7, 9.1[e]		u.v.	(0.003 0)[f]	
	DTT^-	9.2[g]		u.v.	1.5[g]	
NAcS[h]	$C_4H_9NH_2$	10.8		u.v.	9.1	
PNPA[i]	$OH^-(H_2O)$	15.7		u.v.		0.003
			Tris, 100mM	u.v.		0.002[j]

[a] Buffers or other materials present in solution. Buffer, salts used to adjust ionic strength, and other organic additives are listed explicitly: if no buffer is mentioned, none was present. Solutions contain 0.3 - 0.5% v:v DMSO used as a solvent for the active ester, unless indicated otherwise.

[b] This value is based on the assumption that all of the observed hydrolysis at pH 7.6 involves attack of OH^-; see the text for a discussion.

[c] Extrapolation of u.v. data in buffered solution to zero buffer concentration.

[d] This solution contained 1.7M acetamide.

[e] Refs. 39, 40.

[f] Calculation based on the assumption that the TMEDA is entirely in the monocation form. In fact, at the pH used for these kinetics runs (7.95) approximately 6% of the TMEDA was present as unprotonated material. No effort was made to separate contributions from neutral and cationic species.

[g] DTT^- is the monothiolate anion of DTT. We assume that only monoanion is present in solution. The rate constant has been corrected statistically for the presence of two thiol groups.

[h] NAcS is *N*-acetoxysuccinimide.

[i] PNPA is *p*-nitrophenylacetate.

[j] Ref. 37.

hydrolysis and aminolysis rates define qualitatively the rates of the various processes required to form a polyacrylamide gel containing covalently immobilized enzyme. The half-life for hydrolysis of NAS in water at ~0.2 °C and pH 7.6 is 3600 min (Fig. 2); assuming the same buffer dependence as is observed at 25 °C (Fig. 4), the half-life for hydrolysis in 50mM Hepes buffer (pH 7.6, ~0 °C) should be approximately 900 min. Since the entire immobilization process is complete in *ca.* 10 min, loss of NAS by hydrolysis is not significant. The rate constant, k_N, for reaction of NAS with n-butylamine is $3.6 \times 10^3 M^{-1}\,min^{-1}$ at 0.2 °C, and implies the half-life for coupling of this amine (pK_a = 10.76) with 20mM NAS in water (no buffer) at pH 7.6 is *ca.* 15 min. Buffer catalysis by 50mM Hepes should decrease this half-life by

approximately a factor of three to *ca.* 5 min. This estimate is in accord with qualitative observations in related systems [35]. The exposed lysine ϵ-ammonium groups of proteins have a range of pK_a values ($pK_a \sim 9.4 - 10.6$ [41]) but the average is lower than n-butylammonium ion. The Bronsted coefficient which should be used to adjust k_N for the difference in basicity of these amines has not been determined, but is probably small ($\beta \cong 0.2$ [42]). Thus, the half-life for coupling a single lysine ϵ-amino group having pK_a = 10 of a protein to NAS present in 20mM concentration in solution (pH 7.5, 0 °C, 50mM Hepes) can be estimated to be *ca.* 3 min, and that for coupling at least one NAS molecule with a protein having 10 exposed lysine groups should be *ca.* 0.3 min. This estimate probably overestimates the lifetime for coupling under the conditions encountered in the polymerization reactions, since these reactions are significantly exothermic (the temperature increase on polymerization in gel volumes larger than 10 ml can be greater than 20 °C). Thus, although the time the enzyme is in contact with the NAS-containing solution before gel formation is less than 10 s, acylation is fast, and gel formation and acylation appear to require roughly comparable intervals to occur.

This analysis is clearly an approximate one, but it, and the rate data on which it is based, permit several useful conclusions concerning the relative rates of important processes. First, spontaneous hydrolysis of the active ester groups is negligibly slow. At pH 7.6 and 25 °C, the half-lives for disappearance of NAS by hydrolysis, reaction with 0.01M amine (pK_a = 10), and reaction with 0.01M thiol (pK_a = 9) would be 50 min, 0.1 min, and 0.1 min, respectively; at 0 °C, these half-lives would be greater by approximately a factor of 10. Second, acylation of protein lysine ϵ-amino groups is very fast. Since multiple acylation is probably a significant contributor to deactivation of enzymes in this procedure, gel formation should be carried out at low temperature and low pH, if possible, and contact of the enzyme with the active ester-containing solution before gel formation minimized. Third, thiols react with these active esters at approximately the same rates as do amines. High concentrations of DTT or other thiol reducing agents are thus undesirable. Fourth, the rate constants for these reactions are significantly larger then the rate constants for Michael additions of amines and thiols to acrylic ester. For example, the rate constants for Michael additions of glycine to methyl acrylate is approximately $60M^{-1}\ min^{-1}$ at 30 °C, and that to acrylamide is $2.4M^{-1}\ min^{-1}$; corresponding rate constants for thiolate anions are greater by $10^2 - 10^3$ [31, 32]. Thus, modification of protein amino functions by acylation is probably a more important contributor to protein deactivation than is modification by Michael addition; modification of thiol functionalities by Michael addition, may, however, occur at rates comparable with or faster than acylation.

Polymerization initiation system

The possible choices for the radical initiator system to be used in forming the polyacrylamide gel are limited by the requirement that the compo-

nents of this system must be compatible with both proteins and NAS. The polymerization initiator must work efficiently at temperatures close to 0 °C in aqueous solution. Ideally, it should not contribute transition metal ions to the system, since these catalyze protein autoxidation, and it should not contain a strongly nucleophilic component, since this component would be reactive toward NAS. These considerations made the common redox initiators based on a peroxide and copper, iron, or organic sulfinates, appear less attractive than photochemical initiation at the start of this work, and we have used riboflavin-sensitized homolysis of persulfate ion during most of it. The particular hazards to AdK and AcK posed by this initiation method have been considered and largely circumvented [15]. Reaction of persulfate ion with other nucleophiles, especially tert-amines, does, however, provide an effective method for initiating polymerization. This type of initiation is probably responsible for the initiation observed in systems containing only persulfate, Hepes, and ferrofluid.

NAS concentration, pH, and protein concentrations

The concentrations of the above used in these immobilizations were optimized empirically. Figure 5 summarizes several experiments carried out with AdK, AcK, and HPase, in which the concentration of NAS present in solution at the initiation of polymerization was varied from 0 to 60mM, and in which the influence of a typical change in pH was explored. These Figures are each composed of two curves, whose significance is best appreciated by reviewing briefly the experimental procedure followed in obtaining them. A solution of acrylamide, Bis, persulfate, riboflavin, DTT, ADP, and $MgCl_2$ was prepared at 0 °C in degassed Hepes buffer. NAS was added to this solution, and polymerization initiated by u.v. irradiation: just before the gel point was reached (*ca.* 45 s), enzyme was added. Irradiation was continued for 10 s. The resulting block was kept at room temperature for 10 min and then rapidly broken into 20 - 30 μm particles at −10 °C. These particles were suspended in ten times their volume of Hepes buffer containing ammonium sulfate and DTT and stirred for 15 min at room temperature. The gel particles were separated by centrifugation, and washed twice, again using the same procedure. The collected washings and the washed particles were assayed separately for activity. The curve for "immobilized activity" shown in Fig. 5 is based on the enzymatic activity of the particles alone. The curve for "total activity" is based on the sum of the activity of the particles and the washings. Both refer to the activity of the starting enzyme in free solution: that is, "90% immobilized activity" indicates that 90% of the activity present before polymerization was retained in the gel in immobilized form. We emphasize that these curves represent atypically high yields, and are shown because their mechanistic interpretation is simpler than that for curves with poor activity balances. *Typical* yields range from 25 to 50% for AcK and from 40 - 60% for AdK, depending, in a manner not fully understood, on the operator and the batch of enzyme used.

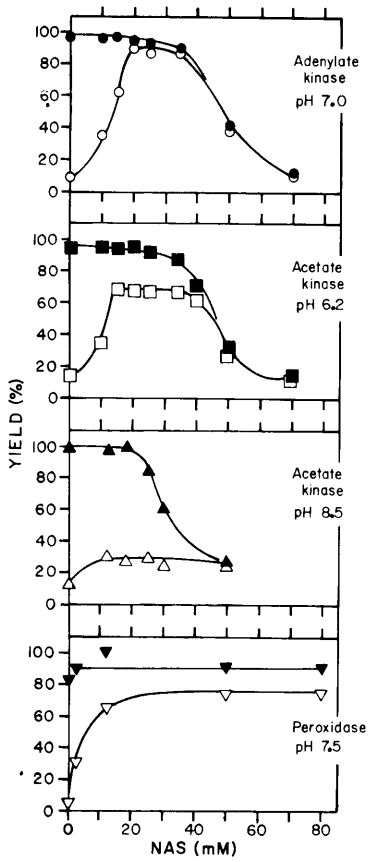


Fig. 5. Immobilized (○), and total recovered (●) yields of adenylate kinase in Hepes, acetate kinase in Mops at pH 6.2 and Hepes at pH 8.5, and peroxidase in Hepes as a function of the concentration of the *N*-acryloxysuccinimide included in the gel.

The important feature of Fig. 5 is that a high immobilization yield may depend on using a carefully chosen concentration of NAS: if too little active ester is present, the enzyme is not covalently immobilized and is washed from the gel; if too much is present, little or no active protein escapes on washing the gel, but the overall activity is low. The origin of this decrease in yield at high NAS concentration is not known in detail, but it presumably involves loss in activity when the enzyme is acylated several times or multiple covalent links are formed between gel and individual enzyme molecules.

Figure 5 also compares data for AcK, obtained following polymerization at two different pH values. The yield of immobilized enzyme depends strongly both on NAS concentration and on pH. This Figure demonstrates that immobilization of horseradish peroxidase is relatively insensitive to the concentration of NAS over a broad range. It does depend significantly on the concentration of enzyme in solution, since only low concentrations of enzyme are immobilized in high yields.

The enzyme-containing gels produced by copolymerization of NAS and protein with vinyl monomer showed good stability for the enzymatic activity. Less than 10% of the activity was lost from gels containing AdK, AcK, or HPase on storage for three months, provided that the enzymes were protected against autoxidation.

Inclusion of a ferrofluid in the polymerization mixture results in a gel that can be separated magnetically from suspension

Polyacrylamide gel has poor mechanical properties; it can be used in packed columns only if the pressure drop and the liquid flow rate are kept low, because the beads deform irreversibly during compression. Similarly, recovery of small polyacrylamide particles from suspension by filtration or centrifugation must be carried out cautiously if the particles are to be reused. As a result, filtration of small ($<10\ \mu m$) soft polyacrylamide particles can be very slow. We have developed a technique to avoid the problems associated with separating these particles when using conventional filtration or centrifugation techniques. This technique involves preparation of gels containing colloidal magnetite particles, and separation of these particles using magnetic filtration. Magnetic separation procedures are often more rapid than conventional techniques, particularly for small particles [43 - 48]. These procedures for preparing and separating small, magnetically responsive, polyacrylamide gel particles should be useful for manipulating a variety of types of immobilized biochemicals besides enzymes — for example, in radioimmunoassay procedures. Magnetic filtration of enzymes immobilized by, *e.g.*, glutaraldehyde crosslinking to magnetite has been described previously [49 - 51], and magnetic filtration of catalysts discussed [52, 53].

The enzyme-containing polyacrylamide gels were made magnetic by including a ferrofluid in the original polymerization. A ferrofluid is a magnetic (Fe_3O_4) colloid composed of *ca.* 5 nm dia. particles stabilized in aqueous suspension with a suitable surfactant coat (2 - 5 nm thick) [54 - 58]. Addition of 5% (v/v) of an aqueous ferrofluid to the mixture of vinyl monomers, substrates and stabilizing agents, and polymerization initiator, produces a magnetic gel. Since the diameter of the magnetite particles is larger than the pore diameter of the gel (~2.4 nm), the magnetite particles do not leak from the gel. Since this diameter is much smaller than the diameter of the individual gel particles, and a significant quantity of ferrofluid is added, each gel particle is uniformly magnetized (a 1 μm gel particle contains 10^2 - 10^3 magnetite particles).

Three points concerning the preparation of these gels deserve explicit mention. First, it seemed possible that the surfactant used to stabilize the ferrofluid might denature proteins. Control experiments established that at 0 °C for 30 min in the presence of air, DTT, and ferrofluid 5% (v:v), solutions of AdK lost 5% of their activity, AcK 10%, and HPase 0%; indistinguishable results were observed when the ferrofluid was omitted. Thus, the surfactant used to stabilize the ferrofluid has no apparent influence on the stability of the proteins. Second, solutions containing ferrofluids are either black or

deeply colored, and they might be expected to exclude the light used for photochemical initiation. We have found that an aqueous mixture of ferrofluid and persulfate ion provides an adequate polymerization initiator in the absence of light and riboflavin. Thus, mixing acrylamide, Bis, NAS, persulfate, DTT, $MgCl_2$, ADP, AdK, and ferrofluid in degassed Hepes buffer at 0 °C results in polymerization with a gel time of *ca.* 7 s. under the conditions that were used. Third, the presence of the ferrofluid did not alter greatly the yield of immobilized activity obtained following polymerization in the presence of NAS, or the response of this yield to changes in the NAS concentration. Figure 6 shows a representative curve obtained for immobilization of AdK in magnetic gel particles. Comparison of this Figure with Fig. 5 indicated that the optimum yield of immobilized enzyme obtained in the presence of ferrofluid is *ca.* 85%, compared with 90% in its absence, and that the forms of the curves in the two figures are very similar. Qualitatively similar results are obtained for AcK and HPase.

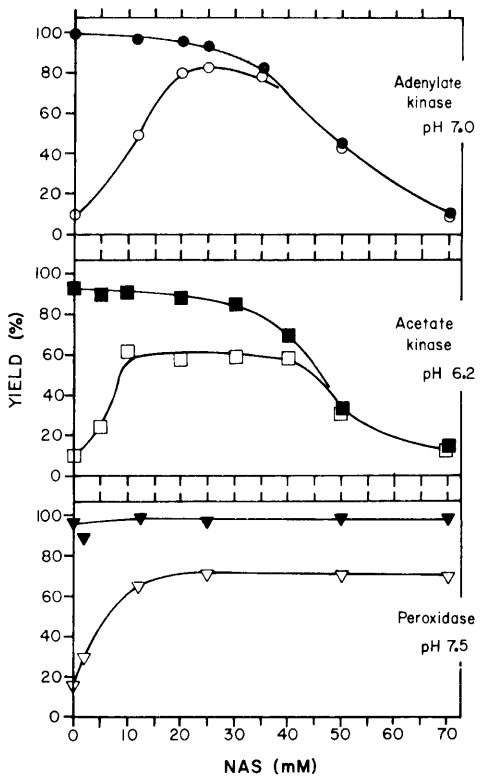


Fig. 6. The yields on immobilization of enzymes in magnetic and non-magnetic polyacrylamide particles (obtained by breaking up gel blocks) are similar: ○, immobilized yield; ●, total enzyme activity accounted for in the particles and in the washings. The immobilizations were run in Hepes buffer for adenylate kinase and peroxidase, and in Mops buffer for acetate kinase. The enzyme loading in the gel varied from 10 mg to 50 mg per ml of gel.

The ferrofluid-containing gels could be broken into small particles by brief grinding at −10 °C in a chilled mortar. These particles could be separated rapidly from suspension simply by holding a permanent magnet having a field strength of 4 kG near the side of the flask. Very rapid separations were possible using high-gradient magnetic separation techniques [43 - 45]. In this technique, a fine, chemically inert ferromagnetic material (*e.g.*, stainless steel wool) was loosely packed in a tube between the pole faces of a magnet (*ca.* 14 kG field strength), and the solution containing the suspension of magnetic gel particles was passed through this filter. Magnetic saturation of the steel wool strands results in very intense magnetic field gradients close to their surfaces. These gradients will retain magnetic polyacrylamide particles quantitatively at very high rates of flow. Although high-gradient magnetic filtration techniques are not necessary for manipulations of small-volume suspensions of polyacrylamide particles, they should be valuable for large volume separations, or for the construction of magnetized fluidized bed reactors.

Preparation of polyacrylamide beads by suspension polymerization is best accomplished in a thixotropic medium

A useful configuration for the enzyme-containing, magnetic polyacrylamide beads would be small spheres. The preparation of polymer beads is usually accomplished by suspension polymerization [26]. An aqueous solution of vinyl monomers is suspended, usually with a surfactant, with vigorous stirring, in an immiscible hydrocarbon phase. The stirring is stopped, interfacial tension permitted to restore the suspended drops to spherical form, and polymerization carried to the gel point. After polymerization is completed, the resulting beads are separated and washed. This technique is complex in practice, particularly in a system containing a sensitive biochemical component. Six considerations must be satisfied in this instance. First, the hydrocarbon phase must be immiscible with the aqueous phase. A hydrocarbon phase that would partition significantly into the water would probably denature and precipitate proteins. Second, the oil phase must have the same density as the aqueous phase. If the densities are unequal, separation of the phases causes the drops to coalesce. Third, the oil phase must not dissolve any of the vinyl monomers. If any component susceptible to polymerization is soluble in the oil phase, the resulting beads have a "sticky" surface, and aggregate. Fourth, the surfactant used to disperse the aqueous phase in the hydrocarbon phase must inhibit interfacial denaturation of proteins. Fifth, the viscosity of the hydrocarbon phase must be high enough to prevent the dispersed aqueous droplets from merging on collision [59, 60]. Sixth, it is essential to prevent contact between the drops during the polymerization process. After *ca.* 20% conversion of monomer to polymer, the aqueous drops consist of a fairly concentrated solution of polymer in monomer, and become tacky. Coalescence of droplets at this stage is usually irreversible [61].

The system developed for suspension polymerization consisted of three important parts. The composition of the hydrocarbon phase — a mixture of

carbon tetrachloride and n-heptane — was adjusted so that this phase had the same density as the aqueous phase. Acrylamide, Bis, and NAS all partitioned essentially exclusively into water in the presence of this mixture. Both heptane and carbon tetrachloride are sufficiently insoluble in water for them not to cause precipitation or denaturation of AdK or AcK. Lecithin was used as the surface-active component. This material caused little or no interfacial deactivation of the proteins we have examined, but, by itself, it generates a system in which irreversible aggregation is important during polymerization. To prevent aggregation, the hydrocarbon phase was made thixotropic by addition of 10% by weight of silylated, fumed silicon dioxide. With this addition, the aqueous phase could be suspended in the hydrocarbon phase with stirring in the usual way. When stirring was stopped, the hydrocarbon phase immediately set to a gel. Polymerization in this phase did not lead to aggregation, since the droplets of the aqueous phase did not have sufficient motional freedom to collide during the polymerization. When polymerization was complete, carbon tetrachloride was added. The beads were separated by flotation, washed in mild aqueous detergent (1% Tween 20) to remove hydrocarbons, and then suspended in buffer, Yields of immobilized enzymes obtained by this procedure were approximately half of those observed for immobilization in gel blocks. Figure 7 shows a representative sample of beads prepared by this procedure, together with pictures of the irregular particles formed by fracturing the gel blocks.

Kinetic behavior of immobilized enzymes

Irregular particles of gel containing immobilized adenylate kinase and acetate kinase have proved useful in large-scale ATP regeneration schemes, and the kinetic behavior of these enzyme preparations has been analyzed and described elsewhere [62].

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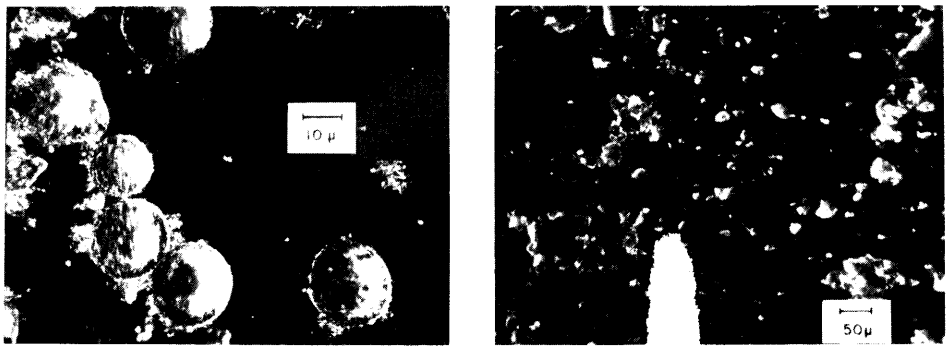

Fig. 7. Upper. Polyacrylamide beads containing covalently im ›bilized AdK and physically entrapped magnetite particles, prepared by suspension polymerization in a thixotropic medium. Lower: Polyacrylamide particles containing covalently immobilized AdK, prepared by fracturing a larger polyacrylamide gel block in a mortar at —10 °C.

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