kcal/mol as calculated from K_s, Figure 4) and athermal ($\Delta H \simeq 0$ kcal/mol) and that this step must undoubtedly be an entropy-driven process. In contrast, the strongly exothermic acylation step which follows binding suggests that, in terms of enthalpy alone, $\Delta G°$ for conversion of E·FAI to EFA and imidazole would be highly favorable. Though we do not know the actual $\Delta G°$ for acylation it is difficult to imagine how $\Delta S°$ for this step could override (negative $\Delta S°$) or promote (positive $\Delta S°$) acylation to an extent greater than the enthalpy contribution. For this reason we believe the acylation step to be primarily enthalpy driven.

Finally, for the process of EFA deacylation we expect that the Gibbs energy change should not be markedly different from the +7.5 kcal/mol reported for deacylation of *N*-acetyl-L-tryptophanylchymotrypsin.[27] Even if this estimate for EFA is in error by several kcal/mol, the process should at least be endergonic to give protonated furylacrylic acid and α-chymotrypsin. Thus, a positive $\Delta G°$ coupled with the enthalpy change of −1.2 kcal/mol clearly distinguishes this last phase of the reaction sequence as an entropy-controlled process.

The enthalpy profile determined by kinetics and calorimetric methods is the first attempt to calorimetrically evaluate enzyme-mediated enthalpy changes consistent with nonenzymic conversion of substrates to products. The kinetics of transformations between intermediates in this system were well suited for calorimetric analysis and no effort was made to flow reatants in the flow microcalorimeter at rates with a resident time (time in measuring chamber) of much less than 30 s. It is quite possible to flow reactants at much faster flow rates and use the techniques developed here. This can potentially extend the scope of such measurements to more specific substrates provided that the elementary steps are themselves reasonably well separated in time.

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Enzyme Immobilization by Condensation Copolymerization into Cross-Linked Polyacrylamide Gels[1]

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Abstract: This paper describes a convenient procedure for immobilization of enzymes based on condensation copolymerization of a water-soluble, functionalized prepolymer (PAN, poly(acrylamide-*co*-*N*-acryloxysuccinimide)), a low molecular weight α,ω-diamine (triethylenetetramine (TET), cystamine), and the enzyme in neutral buffered aqueous solution. This procedure is designed specifically for utility with the relatively delicate and expensive enzymes of interest in enzyme-catalyzed organic synthesis. Inclusion of substrates, cofactors, products, or reversible competitive inhibitors during the immobilization protects the enzyme active site against deactivating acylation. Loosely cross-linked gels present little or no resistance to diffusion of macromolecules into the gel interior, and permit the use of gel-immobilized enzymes to catalyze reactions of soluble, high molecular weight substrates. Tightly cross-linked gels *do* inhibit diffusion of macromolecules into the gel, and can be used to protect immobilized enzymes against deactivation by soluble proteases. Either type of gel protects enzymes against shear deactivation. The gels also protect enzymes included in them from deactivation in aqueous-organic solvent mixtures. Enzyme-containing gels can be used directly in suspension. The gels are too soft to be used alone in columns, but by mixing them with filter aides or by forming them on glass beads, columns having excellent flow characteristics can be assembled. The gels can also be supported on filter cloth or the interior surface of glass tubing. Inclusion of a Ferrofluid—a surfactant-stabilized magnetite colloid—in the gel-forming step results in the formation of a ferrimagnetic gel. Particles of this gel can be manipulated in suspension using high-gradient magnetic filtration techniques. Use of a cross-linking agent containing a disulfide group (cystamine) results in gels which can be dissolved by treatment with a reducing thiol under mild conditions. The factors leading to optimum immobilization yields have been examined in detail for hexokinase. Immobilization yields are summarized for approximately 60 other enzymes.

Introduction

Materials and procedures for protein immobilization are an important component of the developing technology which uses enzymes as catalysts for the in vitro synthesis of complex organic substrates.[3-5] The immobilization of enzymes on insoluble supports serves the primary purpose of facilitating their recovery from reaction mixtures, but it can also slow enzyme deactivation (by inhibiting protease attack and minimizing shear, interfacial, temperature or solvent denaturation) and make possible the manipulation of local concentrations of catalytic species.[6] A large number of immobilization methods have been tested: no single method is (or should be expected to be) superior for every application.

The enzymes of interest for chemical synthesis have several general characteristics which distinguish them from enzymes useful in other areas of applied enzymology and which define the features which should be incorporated into procedures for their immobilization. These enzymes are both relatively difficult to obtain and delicate, and thus expensive. They often contain essential cysteine thiol groups and are deactivated rapidly by oxidizing and alkylating

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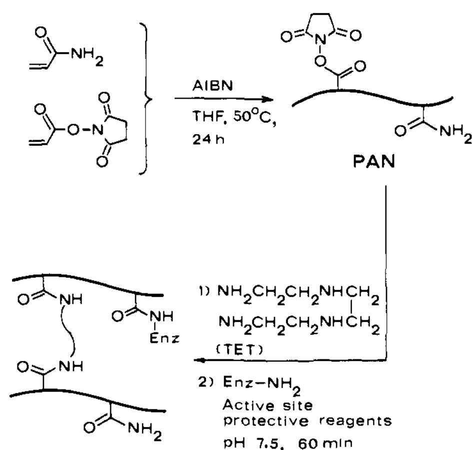
Scheme I. Formation of Gels Containing Immobilized Enzymes



reagents. They require cofactors. A useful immobilization method should thus be applicable to small quantitites of enzyme, give high yields of immobilized activity, and incorporate a link between the enzyme and the immobilizing matrix which is hydrolytically stable. It should involve reagents selective for lysine ϵ-amino groups, and selective against cysteine thiol groups. It should incorporate the enzyme into a hydrophilic medium and isolate it from surfaces and interfaces. It should generate a matrix having high permeability toward low molecular weight substrates and cofactors. In addition, since it will be used by synthetic organic chemists with limited background in enzymology, it should consist of a single experimental protocol which is applicable to a broad range of enzymes with a minimum of empirical modification.

This paper describes a procedure for enzyme immobilization which is based on the simultaneous reaction of three components in neutral buffered aqueous solution at room temperature: a preformed, water-soluble copolymer of acrylamide and *N*-acryloxysuccinimide (PAN), a low molecular weight α,ω-diamine, and an enzyme (Scheme I).[7] This reaction is carried out in the presence of species which occupy the enzyme active site (substrates, cofactors, reversible competitive inhibitors, products) at concentration preferably above their Michaelis constants (K_m) or inhibition constants (K_i). Reaction of the diamine with the active ester groups of the PAN cross-links the polymer chains and forms an insoluble gel connected through amide groups. Reaction of the amino functions of the enzyme with residual active esters covalently links the enzyme to this gel through additional amide linkages. The presence of saturating concentrations of reagents which bind at the active site inhibits reactions between the active ester groups of PAN and nucleophilic groups at or close to the active site, and protects the enzyme against inactivating chemical modifications.

Polyacrylamide was chosen as the starting point for the development of this immobilization procedure because it is hydrophilic[8] and because extensive previous experience has established it as nondenaturing toward many proteins.[9] The *N*-hydroxysuccinimide active ester group is readily introduced into the polymer,[10] has good chemical stability and physical properties, and shows both high reactivity and selectivity toward amine nucleophiles.[11] Because *N*-hydroxysuccinimide has $pK_a = 6$,[12] its release during the gel-forming copolymerization does not overload the buffer systems. By utilizing the reaction of an amine with an active ester to convert the soluble PAN into a cross-linked gel and to connect the enzyme to the gel, the procedure incorporates one of the most reliable and best understood coupling reactions, and avoids the radical and Michael reactions which deactivate enzymes during procedures requiring radical polymerization in enzyme-containing solutions of vinylic monomers.[13,14] Because the enzyme is originally dissolved in a homogeneous solution containing the reactive polymer, and because the entire volume of solution is transformed to gel, the enzyme is completely and uniformly distributed throughout the gel; the kinetic limitations encountered when an enzyme is required to diffuse from solution into a reactive preformed gel (cyanogen bromide activated agarose, functionalized cross-linked polyacrylamide) are thus avoided, and the time required for immobilization is short. The cross-linking reaction generates very little heat and shows no tendency to deactivate enzymes thermally, even when large volumes of gel are formed.

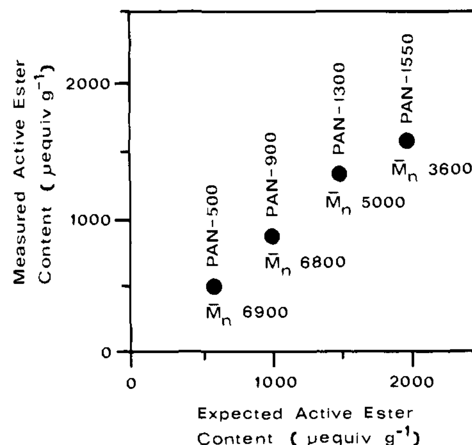


Figure 1. The yield of active ester groups incorporated into PAN decreases as the ratio of *N*-acryloxysuccinimide to acrylamide in the starting reaction mixture increases. The expected active ester content is that calculated assuming that the monomers form a random copolymer. The PAN polymers are named according to the measured active ester content, rounded to the nearest value of 50.

We have used the immobilization method summarized in Scheme I extensively in our program of enzyme-catalyzed organic synthesis, and have found it to be more convenient and generally applicable than other procedures and to give higher immobilization yields.[15-18] It has the disadvantages that it requires an initial synthesis of PAN and that (as with many gel immobilization procedures) the mechanical properties of the enzyme-containing gels are better suited for use suspended in stirred reaction mixtures than packed in columns under pressure. For syntheses generating quantities of products at the scale of gram to kilogram, neither problem is serious. Moreover, by forming the gel on glass beads, filter cloth, or other material, or by mixing it with a diatomaceous earth, it is possible to generate catalysts having properties useful for column operation.

Results

Preparation and Characterization of PAN. PAN was prepared by free-radical polymerization of acrylamide and *N*-acryloxysuccinimide in THF solution, using thermal initiation with azobis(isobutyronitrile) (AIBN).[19] This system was used in order

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Table I. Mechanical Properties of Gels Produced by Cross-Linking PAN-450 with α,ω-Diamines[a]

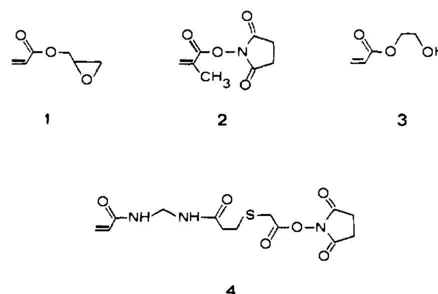
α,ω-diamine	properties
$H_2N(CH_2)_nNH_2$, n = 2, 3, 4	very soft, sticky
$H_2N(CH_2)_nNH_2$, n = 7, 8, 9	medium soft
$H_2N(CH_2)_nNH_2$, n = 5, 6	soft, resilient
$H_2N(CH_2)_2SS(CH_2)_2NH_2$ (cystamine)	soft, resilient
$H_2N(CH_2)_2NH(CH_2)_2NH_2$	soft, resilient
$H_2N(CH_2)_2O(CH_2)_2NH_2$	soft, resilient
$H_2N(CH_2)_3NH(CH_2)_3NH_2$	medium hard, resilient
$H_2N(CH_2)_2NHCONH(CH_2)_2NH_2$	medium hard, resilient
$H_2N(CH_2)_2NH(CH_2)_2NH(CH_2)_2NH_2$ (TET)	medium hard, resilient to brittle[b]
$H_2N(CH_2)_3N(CH_2CH_2)_2N(CH_2)_3NH_2$	medium hard, resilient to brittle[b]

[a] These gels were produced by reaction of 100 mg of PAN-450 dissolved in 0.5 mL of distilled water with 43 μL of a 0.5 M solution of diamine. The mechanical properties were examined after 24 h at room temperature. [b] Brittle gels were obtained with PAN samples containing $\geq$1000 μequiv g^{-1} of active ester groups.

to provide low molecular weight polymers, which, in turn, dissolve quickly and generate aqueous solutions of practical viscosities. Conversion of monomers to polymers was consistently high. These polymers were isolated as white solids. They appear to be indefinitely stable at room temperature in a desiccator under dry air; the active ester groups do, however, hydrolyze on exposure of PAN to undried laboratory air. Aqueous or aqueous buffer solutions containing up to 25% by weight of polymer form readily. The solutions typically used for enzyme immobilization (20% w/w) have a kinematic viscosity of ca. 25 cSt at 25 °C (approximately that of Nujol).

The polymer was assayed for active esters by allowing it to react with aqueous ethylamine solution and measuring the absorbance due to the anion of N-hydroxysuccinimide (ϵ_{259} = 8600 M^{-1} cm^{-1}).[14] The content of active ester groups in a sample of polymer was typically 80–95% of that expected on the basis of the composition of the monomer mixture used in its preparation (Figure 1). Several polymers differing in their content of active ester groups were used in this work. We specify the composition of the polymer in terms of the content (in μequiv/g) of active ester groups: thus, PAN-450 is a polymer which releases 450 (±25) μmol of N-hydroxysuccinimide per gram of dry polymer on treatment with excess aqueous ethylamine solution. To avoid confusion resulting from unnecessary precision, these descriptive numbers are rounded to the nearest 50; thus a polymer assayed to contain 1370 μequiv/g of active ester groups is called PAN-1350. We have not measured the distribution of amide and active ester groups along the polymer, but the copolymerization reactivity indexes for N-acryloxysuccinimide suggest that PAN will be approximately a random copolymer.[20] The number average molecular weight ($\bar{M}_n$) of the polymers was estimated by converting them to polyacrylamide by reaction with an excess of aqueous ammonia and measurement of the viscosity of their aqueous solutions.[21] These data are included in Figure 1 and suggest a composition of 88 acrylamide and 4 N-acryloxysuccinimide monomers for PAN-550, and 37 and 6 for PAN-1550. The end-group composition of the polymers was not examined, but since the amount of AIBN used was much too small to initiate all the chains, most must have initiated by chain transfer; the THF solvent is the most plausible candidate for a chain-transfer agent.[22]

Several copolymers other than PAN were explored for utility in enzyme immobilization. Copolymers of acrylamide and glycidyl acrylate (**1**) and N-methacryloxysuccinimide (**2**) did not produce gels under the conditions used with PAN, and were not explored further. Gels from copolymers of hydroxyethyl acrylate (**3**) were too soft to be useful. Copolymers of acrylamide and **4** formed gels with good mechanical properties, and seemed more reactive than PAN (see below); any advantages of **4** were, however, offset by the requirement that it be synthesized.[23] PAN has remained the most useful of the materials produced.



Formation of Cross-Linked Gels by Reaction of PAN with α,ω-Diamines. PAN dissolves rapidly in water or aqueous buffers at room temperature or 0 °C. The half-time for hydrolysis of the active ester groups in 0.3 M Hepes (pH 7.5) buffer is approximately 20 min at 23 °C, and 95 min at 0 °C. Addition of an α,ω-diamine to a solution of PAN results in an immediate release of N-hydroxysuccinimide which can be followed spectrophotometrically. If the concentrations of reactants and the structure of the diamine are chosen correctly, the solution rapidly (0.1–3 min at 23 °C) sets to a transparent or faintly opalescent gel. The cross-linking reaction is not significantly exothermic and can be carried out in 500-mL scale (50 g of PAN-500) with a temperature rise in the center of the gel of less than 5 °C.

Table I summarizes the mechanical properties of gels prepared by reaction of a 20% w/w solution of PAN-450 with amounts of α,ω-diamine sufficient to provide one primary amino group for each active ester moiety;[24] these quantities correspond to approximately 8.5 mequiv L^{-1} of active ester groups and a concentration of diamine of 40 mM. Based on these observations, TET was chosen as the cross-linking agent which yielded the gel having the greatest mechanical strength. Providing a stoichiometric equivalent of primary amino groups for each equivalent of active ester moieties was important in producing mechanically strong gels; a 30% excess or deficiency of amine groups yielded appreciably softer gels, and a 100% excess (i.e., 2 equiv of amine/equiv of active ester) prevented gel formation completely. The concentration of PAN was also important: concentrations of PAN-450 less than 15% w/w produced very soft gels (or no gel) with even the best concentration of diamine. Concentrations of PAN-450 greater than ~25% w/w became so viscous as to be impractical to work with. Qualitative experiments with PAN-900 and PAN-1550 indicated that the best gels were also formed from these materials, using w/w concentrations of ca. 20%.

Secondary amines react much more slowly with the active ester groups of PAN than primary amines. For example, N,N'-dimethyl-1,6-diaminohexane does not yield a gel when allowed to react with a PAN solution under conditions in which 1,6-diaminohexane, TET, or other substances containing two primary

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(21) The viscosity of polyacrylamide in water is related to its number average molecular weight by $\eta = 6.8 \times 15^{-4} (\bar{M}_n)^{0.66}$: Collinson, E.; Dainton, F. S.; McNaughton, G. *Trans. Faraday Soc.* **1958**, *53*, 476–489.

(22) THF was intentionally used as the solvent to limit the molecular weight of the PAN. We have not established whether the insolubility of the PAN in THF or the chain-transfer activity of the THF is the major factor in this limitation.

(23) Monomer **4** was prepared by Michael addition of thiolglycolic acid to N,N'-methylenebis(acrylamide) (bis) followed by conversion of this acid to **4** using dicyclohexylcarbodiimide and N-hydroxysuccinimide.

(24) The relationship between the physical properties and composition of cross-linked polyacrylamide gels produced by free-radical polymerization has been extensively characterized in terms of two parameters: the concentration of acrylamide in the starting aqueous solution (C, expressed in wt %) and the concentration of cross-linking agent (T, expressed in wt % of the acrylamide monomer). Values of $C > 5$ and $T > 10$ are required to produce gels with significant mechanical strength: Fawcet, J. S.; Morris, C. J. *Sep. Sci.* **1966**, *1*, 9–26. Crambach, A.; Rodbard, D. *Science* **1971**, *172*, 440–450. Rodbard, D.; Crambach, A. *Anal. Biochem.* **1971**, *40*, 95–134, and references cited in each.

Table II. Rate Constants for Release of *N*-Hydroxysuccinimide from Active Ester[a]

active ester	principal nucleophile[b]	$10^2 \times k_{H_2O}^{obsd}$ [c]	$10^{-4} \times k_N$ [c]	$\tau_{1/2}$ [c]
CH_3CONHS	H_2O	6.2		11
	n-$BuNH_2$		12	
$CH_2{=}C(CH_3)CONHS$	H_2O	2.0		35
	n-$BuNH_2$		3.4	
$CH_2{=}CHCONHS$	H_2O	15		4.7
	n-$BuNH_2$		9.5	
4	H_2O	29		2.4
	n-$BuNH_2$		51	
PAN-500	H_2O	3.9		18
	n-$BuNH_2$		0.45	
poly(acylamide-*co*-4)	H_2O	22		3.2
	n-$BuNH_2$		48	

[a] Reactions were carried out at 25 °C in buffered aqueous solution (0.3 M Hepes, pH 7.4) containing *n*-butylamine (16 mM) as nucleophile unless noted otherwise. [b] We have not separated contributions from attack on active ester groups by *n*-butylamine, water, and buffer. The nucleophile listed in this column is believed to account for >90% of the reactivity. [c] $k_{H_2O}^{obsd}$ (min^{-1}), k_N (M^{-1} min^{-1}), and $\tau_{1/2}$ (min) are used. $\tau_{1/2}$ is the half-life for disappearance of NHS esters under the conditions indicated.

amine groups produce resilient gels. In calculating quantities of reactants, only the primary amino groups of substances such as TET and diethylenetriamine were considered.

Qualitative kinetics studies of the reactions of nucleophiles with active ester groups in PAN and related species are useful in defining the characteristics of the coupling reaction responsible for gel formation. The rate of release of *N*-hydroxysuccinimide anion from PAN-500 and several related species by reaction with a large excess of *n*-butylamine was followed spectrophotometrically. These data were analyzed[14] to provide estimates of the rate constants ($k_{H_2O}^{obsd}$ and k_N, eq 1).

$$\frac{d[(NHS^-) + (NHSH)]}{dt} = k_N^{obsd}(RCONHS)[(N:) + (NH)] + k_{H_2O}^{obsd}(RCONHS) \quad (1a)$$

$$\frac{d[(NHS^-) + (NHSH)]}{dt} = k_N(RCONHS)(N:) + k_{H_2O}^{obsd}(RCONHS) \quad (1b)$$

Here N: is the principal nucleophile present in the solution (typically a primary amine) and NH is its protonated form. Three features of these data (Table II) are pertinent to the practical problem of using PAN. First, the reactivity of the active esters of PAN is significantly less than that of low molecular weight NHS-active esters, including esters of acetic and acrylic acids. The origin of the decrease in reactivity is not clear, but may be attributable to a steric effect due to the proximity of these groups to the polymer backbone, since poly(acrylamide-*co*-4), in which the active esters are separated from the chain by a long spacer, has approximately the same reactivity as 4. Second, even though the NHS active esters of PAN are relatively unreactive compared with other NHS active esters, they are still quite reactive; the half-life for reaction of PAN active esters with primary amine groups present at 16 mM (pH 7.5, 23 °C) is approximately 20 min. Third, the rate of amminolysis of PAN under conditions typical of those used in forming a gel should be much faster than the rate of its hydrolysis. As the gel forms, the mobility of polymer-bound amines and active esters may be sufficiently decreased that hydrolysis competes more effectively, but, at least in the initial stages of the reaction, the efficiency of conversion of active ester groups to amides should be high.

The conditions used throughout most of this work were designed to allow gel formation to be completed in approximately 1 h. In the event that it is useful to slow the coupling reaction (especially to lengthen the gel time) the temperature or the pH may be lowered. Thus, a mixture (20% w/w PAN-500, TET, pH 7.5 Hepes buffer) which gels in 3 min at 25 °C, gels in 15 min at 0 °C. If the starting solution is more acidic (pH 6.5, Mops buffer, 25 °C), the gel time is 9 min; if it is more basic (pH 8.5, Taps buffer, 25 °C), the gel time is 1 min.[25] Although we have only briefly explored the gels produced under these conditions, qualitatively it appears that enzyme immobilization yields remain adequate and the mechanical strength of the gel is not markedly changed. The ability to influence the kinetics of gel formation significantly with small changes in solution pH and temperature should prove useful in practical applications of these gels.

Enzyme Immobilization. Studies of gel formation in the absence of protein indicated that reaction of a 20% solution of PAN (0.3 M Hepes buffer, pH 7.5) having 450–1550 μequiv of active ester groups/g with a quantity of TET providing 0.85 equiv of primary amine groups/equiv of active ester yielded a gel with satisfactory mechanical properties. This stoichiometry leaves ca. 15% of the active ester groups unreacted (assuming slow hydrolysis and reaction with the secondary amine moieties of the TET) and available for reaction with other nucleophiles. Addition of a protein to the reacting solution of PAN and TET before it gelled resulted in its covalent incorporation into the cross-linked polymer network. Here we describe a procedure for enzyme immobilization based on this observation, together with the results of experiments which identify points of technique or procedure which are important for preserving enzymatic activity during the immobilization.

In the standard procedure, a quantity of PAN sufficient to give a 20% w/w solution is weighed rapidly in air and dissolved in 0.3 M Hepes buffer[26] containing components intended to protect enzymatic activity. The most important of these are substances—reactants, cofactors, competitive inhibitors, products, metal ions—which occupy the enzyme active site and prevent modification of nucleophiles close to or at the active site by acylation with PAN during the immobilization. The choice of substances to be included is based on practical considerations of availability, binding constant, and reactivity; to be fully effective, the concentration of the active site protecting reagent should be above its Michaelis constant (K_m) or inhibition constant (K_i) so that the active site is saturated. Materials with high Michaelis constants may require such high concentration as to change the solvent properties of the medium. Materials which are themselves nucleophilic may react with the active esters of the PAN to an unacceptable extent. In any event, it is normally not necessary for *all* the components required for the enzyme-catalyzed reaction to be present during the immobilization to provide useful protection for the enzyme active site. A second type of substance routinely included during immobilization of enzymes containing essential catalytic or structural thiol groups is 1,4-dithiothreitol (DTT), 1,3-dithiopropan-2-ol (DTP), or another reducing thiol effective in inhibiting protein autoxidation.[27] Since these thiols are themselves reactive toward the active esters of PAN,[28] they should be used in low concentrations (≲5 mM) and the immobilization should be carried out under argon, using degassed reagents. In a preliminary experiment, the appropriate quantity of TET is added with vigorous stirring to the solution containing PAN, and the gel time (typically 2–3 min) for the mixture is measured.[25] To immobilize the enzyme, the experiment is repeated, with the enzyme added at a convenient time *after* the addition of the TET

(25) The gel time in these systems is experimentally well defined and reproducible, and normally does not depend on active-site protective reagents present in the solution. Operationally we define the gel time as the interval between the time of mixing of the PAN- and diamine-containing solutions and the time at which the viscosity of the reaction mixture becomes so high that the magnetic stirring bar used for mixing stops.

(26) Mops, triethanolamine, and phosphate buffers have been used in place of Hepes with no obvious ill effects. The buffer concentration only partly controls pH changes. On adding the diamine to the PAN-containing mixture, the pH changes from 7.5 to 8.0–8.1 and returns to 7.5 over 5–15 min. The final pH of the gel appears to be ca. 7.3–7.4.

(27) Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011–2026. Shaked, Z.; Szajewski, R. P.; Whitesides, G. M. *Biochemistry*, **1980**, *19*, in press, and references cited therein.

(28) DTT (50 mM) will cross-link PAN-500 to a soft gel. This gel is unstable and dissolves, presumably because of hydrolysis of its constituent thioester groups.

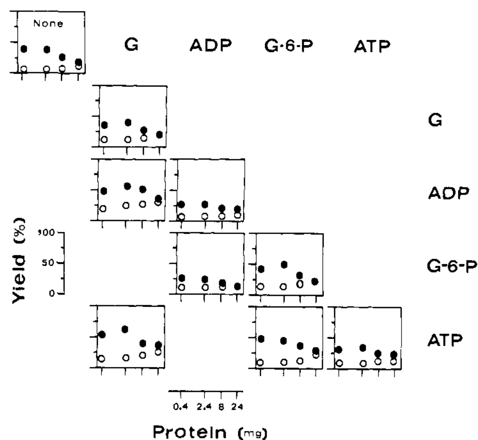


Figure 2. Immobilized yield (●) and yield recovered in solution on washing the gel (○) for hexokinase immobilized in PAN-500. The plots are arranged as a 4 × 4 matrix. The diagonal positions have only the indicated species added; the off-diagonal positions represent data obtained with the two indicated species present: G = glucose (25 mM); ADP (10 mM); G-6-P = glucose 6-phosphate (25 mM); ATP (10 mM). The horizontal axis is mg of hexokinase added per g of PAN. The plot labeled "None" has no added active-site protective species. Species present in all immobilizations are $MgCl_2$ (15 mM), DTT (5 mM), TET (0.5 M), and Hepes buffer (pH 7.5) (0.3 M).

but well *before* (usually 30–60 s) the gel point. The time for addition of the enzyme represents a compromise. In general, the shorter the time the enzyme is exposed to PAN before gel formation, the higher the yield of immobilized activity. If, however, the enzyme is added too close to the gel point, it may not be mixed uniformly into the solution, and the coupling of the protein to the gel may suffer. The enzyme-containing gel is allowed to stand for 60 min to complete the coupling reaction,[29] broken up into ~100-μm particles, washed with aqueous buffer containing ammonium sulfate to convert residual active ester groups to amides, and assayed. The *yield* obtained in the immobilization is defined by eq 2; that is, no correction is made either for enzyme that might

$$\text{yield (\%)} = \frac{\text{units (assayed in enzyme-containing gel)}}{\text{units (assayed in solution before immobilization)}} \times 100 \quad (2)$$

be recovered from washing the gel, or for any pore diffusional limitations which might result in underestimation of the assayed enzyme content in the gel.

Qualitative experience with a number of enzymes has indicated that the most important factors in obtaining high immobilization yields are the presence of active-site blocking species in solution, the loading of enzyme in the gel, the interval between addition of enzyme to the cross-linking solution and the gel point, and the prevention of autoxidation during immobilization. Figure 2 details studies carried out using a representative enzyme, hexokinase, which are pertinent to the first two of these points, and Figure 4 to the third. Autoxidation is prevented by carrying out reactions under argon and maintaining a low concentration of DTT or DTP in solution.[30]

We do not pretend to be able to rationalize the data in Figure 2 mechanistically. All of the species added are above their respective K_m values.[31] It is known that hexokinase undergoes a pronounced conformation change on binding its substrates,[32] and

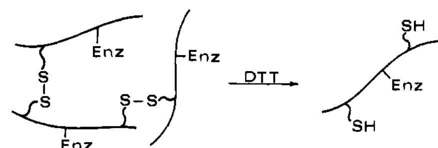
(29) With PAN-500, shorter reaction times, e.g., 20–30 min, resulted in formation of softer gels. PAN-800 or higher did form good gels in ca. 30 min. We did not study explicitly the effect of shorter reaction times on the immobilization yields for enzymes included in Table III. If reaction times less than 10 min are used, the yield of immobilized enzyme activity decreases.

(30) Hexokinase contains four thiols per subunit, of which one is essential for activity: Otieno, S.; Bhargave, A. K.; Barnard, E. A. *Biochemistry* **1977**, *16*, 4249–4255.

(31) $K_G = 1.0 \times 10^{-4}$ M; $K_{MgATP^{2-}} = 1.0 \times 10^{-4}$ M; $K_{Mg^{2+}} = 10^{-3}$ M; Sols, A.; de la Fuente, G.; Villar-Palasi, C.; Asensio, C. *Biochem. Biophys. Acta* **1958**, *30*, 92–101.

(32) Anderson, C. M.; Zucker, F. H.; Steitz, T. A. *Science* **1979**, *204*, 375–380.

Scheme II. Solubilization of a Gel Containing Disulfide Groups in Cross-Linking Moieties by Thiol-Disulfide Interchange



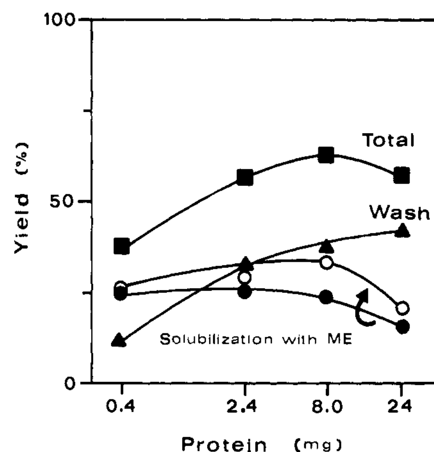


Figure 3. Immobilized (●), recovered (▲), and total (■) activity of hexokinase in PAN-500 gel cross-linked with cystamine $(NH_2CH_2CH_2S)_2$. Treatment of the washed, enzyme-containing gel with mercaptoethanol (ME) resulted in its solubilization; this solution yielded the data represented by the difference (○ − ●) represents the limitation in the measure of the activity of the immobilized enzyme due to pore diffusion.

we speculate that the relative effectiveness of the substrates and products in protecting it against deactivating acylation may reflect this conformational mobility. In any event, it is clear that the correct choice of enzyme loading (ca. 2.5 mg (g PAN)$^{-1}$) and protective reagents (10 mM ATP, 25 mM glucose) can increase the immobilization yield. We have not carried out studies in this detail with other enzymes, but qualitative experience indicates that immobilization yield almost always benefits from the inclusion of substrates, cofactors, and products in the reaction mixture during immobilization. The best combination for any new enzyme can presently only be selected empirically.

The influence of protein loading on yield is puzzling: at high protein loading, both the immobilized and total (i.e., immobilized plus soluble recovered) yields decrease. It seemed possible that this decrease might represent an artifact: pore diffusion limitations might result in an underestimate in the amount of activity actually present in gels containing large amounts of active enzymes. We established that pore diffusional limitations were, in fact, of only minor importance by substituting cystamine for TET as the cross-linking agent in the gel-forming step. Treatment of the resulting gel resulted in rapid cleavage of the disulfide groups and solution of the gel (Scheme II).[33] Comparison of the assayed enzymatic activity before and after solubilization provided an estimate of the importance (<8% at high protein loadings) of mass-transport limitations in determining measured enzymatic activity (Figure 3).

In general, the yield of immobilized activity is highest using quantities of enzyme between 0.5 and 4 mg/g of PAN, although certain enzymes tolerate (e.g., carbonic anhydrase) or require (α-chymotrypsin) larger quantities.[34] PAN containing more than 500 μequiv of active ester groups/g of polymer normally gave lower

(33) Preparation and solubilization of polyacrylamine gels containing disulfide cross-links has been described by Hansen, J. N. *Anal. Biochem.* **1976**, *76*, 37–44.

(34) The best yields (67%) for immobilization of carbonic anhydrase were obtained at loadings of 13 mg of protein (g PAN-1300)$^{-1}$, but it was possible to immobilize this enzyme in 50% yield at loadings up to 85 mg (g PAN-1300)$^{-1}$. By contrast, the immobilization yield for horse liver alcohol dehydrogenase fell from 63 to 39% as the protein leading increased from 2.6 to 5.0 mg (g PAN-450)$^{-1}$.

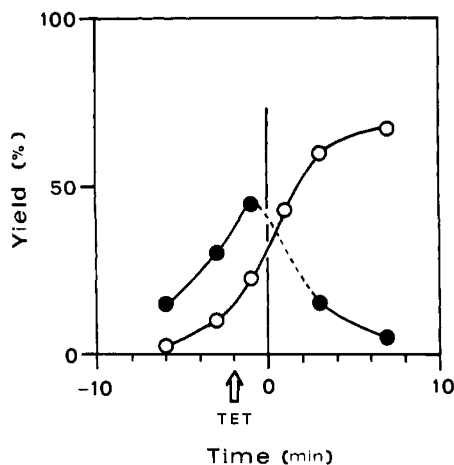


Figure 4. Immobilized (●) and recovered (○) yields of hexokinase as a function of the time of addition of enzyme (2.5 mg (g PAN-500)$^{-1}$) relative to the gel point (t = 0); addition 5 min *before* the gel point is indicated as −5 min. The TET was added 2 min before the gel point (indicated with an arrow on the time axis).

yields than those containing less, although again, exceptions were common. In some instances the improved physical characteristics of the gels produced from PAN containing a high proportion of active ester justified a decrease in immobilization yield.

Figure 4 illustrates the sensitivity of hexokinase to incubation with PAN. The highest yield of the immobilized hexokinase activity was obtained when the enzyme solution had been added to the cross-linking PAN solution about 1 min before its gel point. The yield decreased significantly when the enzyme solution was allowed to react with PAN in the absence of cross-linking agents, or when it was mixed with the gel 1 to 7 min after its gel point. The former observation reflects deactivating acylation of the protein by the large excess of active ester groups present in the PAN. The latter indicates that the physical entrapment of the enzyme in the PAN gel with subsequent covalent immobilization is preferred to diffusion of the enzyme into the preformed gel. Again, we have not explored other systems in detail, but we believe that minimizing the exposure of the enzyme to the PAN is, in most instances, helpful in obtaining good yields (glycoproteins may represent an exception; see below).

At the conclusion of the immobilization, the gel is present as a resilient block. For use in synthetic reactions, it was usually broken into irregular 100–150-μm particles by brief grinding in a mortar or treatment in a Waring blender. The gel still contained residual active ester groups at this stage. Brief treatment (15 min) of the gel suspension with aqueous ammonium chloride solution (0.05 M) converted these groups to amides. Certain enzymes (hexokinase, glucose-6-phosphage dehydrogenase, yeast alcohol dehydrogenase, and chymotrypsin) seemed sensitive to the presence of unreacted active esters, and the activity of gels containing these enzymes would decrease by 5–20% over 24 h if they were not destroyed.

Table III lists immobilization yields obtained by applying this procedure to several enzymes. Activities are given in units of μmol/min (for reference, a total of 700 U of enzymatic activity is required to transform 1 mol of product per day). Each entry in the table represents the average of two or more experiments having reproducibility within 5%. Only a fraction of these immobilizations are optimized yields. The immobilization procedure used for the immobilization of all but the three glycoproteins (horseradish peroxidase, lactoperoxidase, and invertase) was the standard one described in detail for hexokinase in the Experimental Section, modified only by the substitution of substrates or cofactors necessary for protection of the active site of the particular enzyme immobilized. Glycoproteins appear to be relatively unreactive toward the active ester groups of PAN, and were immobilized using a modified procedure: the enzyme was added to the buffered solution of PAN, stirred for 3 to 6 min, and the polymer then cross-linked by addition of TET. In this technique, the protein is exposed for a much longer time to the active ester groups.

Most of the experiments summarized in Table III were carried out on small scale (0.02–16 mg of protein, 200 mg of PAN, 1 mL of solution). Immobilizations of hexokinase, acetate kinase, and adenylate kinase have been carried out at 20 times this scale, and we have immobilized 400 mg of horse liver alcohol dehydrogenase in 50 g of PAN-500 (72% yield, 688 U of immobilized activity) without difficulty or loss in yield. The negligible exothermicity of the cross-linking reaction is the major reason why the immobilization can be easily increased in scale.

We have also been successful in immobilizing live cells in PAN gel. For example, a suspension of live Baker's yeast (*Saccharomyces cerevisiae*, ca. 0.5 g) was immobilized in PAN gel using the standard procedure for enzymes. The gel particles, after being carefully washed, were suspended in a glucose-containing solution (20 g/L) thermostated at 30 °C under anaerobic conditions. In a medium containing glucose and water (but no other nutrients), ethanol production continued over a period of 36 h and then ceased.

Supported Gels. Our usual method of using gel-immobilized enzymes is to break the gel into small particles and to suspend the particles in the reaction mixture with stirring.[13-17] The reaction solution is separated from the gel by decanting. In certain circumstances (especially when large volumes of liquid are to be used or when rapid or complete separation of gel and reaction mixture is required), this procedure may prove less satisfactory than use of the gel in a column. The gel particles cannot be used undiluted in a column; they fuse under pressure into a mass having very poor flow characteristics. The gel can, however, be formed on or in a variety of supporting structures, or diluted with filter aids; these configurations can lead to columns having excellent flow characteristics.

The simplest procedure of this type involved formation of the polymer in a thin film on the outside of 1-mm glass beads. Typically, the cross-linking PAN solution (1 min before its gel point) was mixed with the glass beads and mechanically stirred until the beads were covered with a layer of the PAN solution. This solution gelled on the glass surface. The resulting composite material was mechanically stable against deformation under pressure, and columns filled with it showed excellent flow characteristics. Alternatively, enzyme-containing gels could be formed on a sheet support by mixing all the components required for enzyme immobilization, pouring the fluid solution onto a sheet of, e.g., filter paper, and allowing gel formation to occur in the paper. The resulting sheet had good mechanical strength and could be used rolled into a coil (with suitable spacer interleaved to ensure adequate mass transport) or in other geometries. Similarly, by forcing the immobilization mixture quickly through a length of glass or tubing while still fluid and allowing the residual film to polymerize on the walls of the tubing, a thin polymer coating was formed which showed excellent adhesion to the wall. Distributing a small quantity of solution around the inside of a test tube by swirling again produced a thin, coherent film.

Useful column operation could also be achieved by mixing gel particles with a filter aid. The flow characteristics of these columns were sensitive to the relative quantities of gel and filter aid used (Figure 5). So long as the gel contained less than approximately 8% w/w of PAN in Celite 545 (dry weights for each), the flow characteristics were good.

Of these solutions to the problem of using the mechanically soft PAN gels in column operation, mixing with Celite proved the most practical. Figure 6 shows a test of the long-term behavior of a column based on this procedure. This figure represents the change in activity with time of a column originally containing 2630 U of invertase immobilized in 15 mL (ca. 3 g dry weight) of PAN-500, mixed with 50 g of Celite. Over the course of the experiment (25 days), the column hydrolyzed approximately 23 kg of sucrose to glucose and fructose (corresponding to a turnover number for the enzyme of ca. 10^9). The flow characteristics of the column did not change in this period. Its smooth performance indicates that this method of operation provides a practical solution to the problem of using the mechanically weak PAN gels in columns.

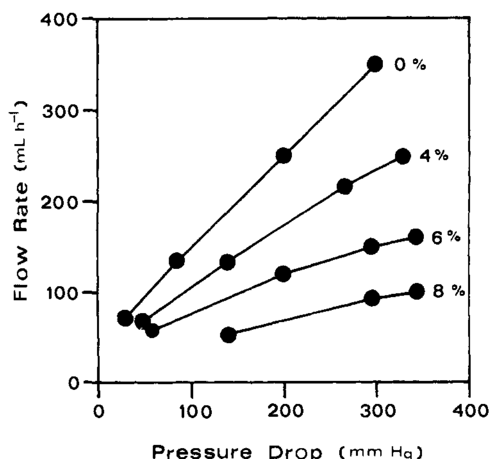


Figure 5. Pressure drop across a column (1.5 cm × 28 cm) containing mixtures of Celite 545 and PAN-500 (ca. 150-μm particles). The weight of Celite used in each column was 9.2–10.0 g; the proportion of PAN gel included (calculated as wt % based on the dry weight of starting, soluble, polymer) is shown on the plot.

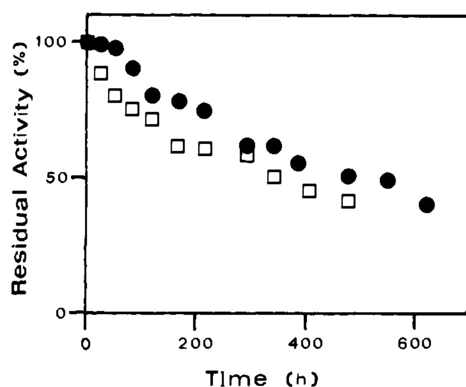


Figure 6. Activity of a column of invertase immobilized in PAN-500 and mixed with Celite (●), as a function of time (45 °C, pH 4.8, 0.5 M sucrose solution, 25 mM sodium acetate, 9.0–10.6 L day^{-1}). On days 5, 11, and 16 the column was flushed briefly with aqueous sodium azide solution (100 mg in 10 mL) to inhibit microbial growth, but otherwise no special effort was made to ensure sterility. Activity of a column of invertase immobilized in PAN-500 containing a Ferrofluid and held on a ferrimagnetic stainless steel wool matrix in a ~10 kG applied external magnetic field (□).

This experiment also indicates that the linkage of the enzyme to the gel is hydrolytically stable, since the column was operated at pH 4.8 and 45 °C.

Magnetic Gels. Another potentially useful material was obtained by including ca. 5% v/v of an aqueous ferrofluid[35] (a surfactant-stabilized magnetite colloid) in the immobilization mixture before gel formation.[36] The resulting brown, opaque gels showed the high responsiveness toward magnetic field gradients expected on the basis of their ferrimagnetic component. These gels could be separated from suspension rapidly using conventional or high-gradient magnetic filtration,[37] or contained in one region of a flowing stream (a "magnetic fluidized bed") using relatively weak magnetic fields. We tested a column-type reactor containing invertase (ca. 215 U) immobilized in magnetically responsive PAN-500 gel particles held in the column by exploiting high-gradient magnetic filtration techniques. The magnetically responsive gel particles were held on the surface of stainless steel wool in a tube in between the poles of an electromagnet, and the reaction solution was passed through the steel wool/magnetic gel matrix (Figure 7). This column was continuously operated for ca. 480 h at 45 °C and pH 4.8. It showed excellent flow characteristics and no detectable loss of the magnetically responsive gel particles. The enzymatic activity of the column decreased at about the same rate as the previous experiment with the PAN gel/Celite mixture (Figure 6).

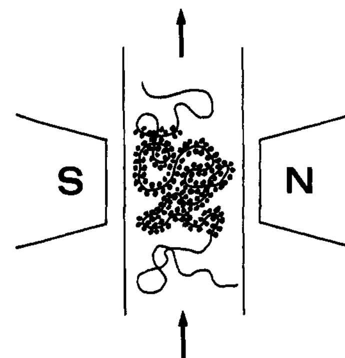


Figure 7. Schematic illustration of a column containing particles of a Ferrofluid- and enzyme-containing PAN gel immobilized on a stainless steel wool matrix in a region of applied external magnetic field.

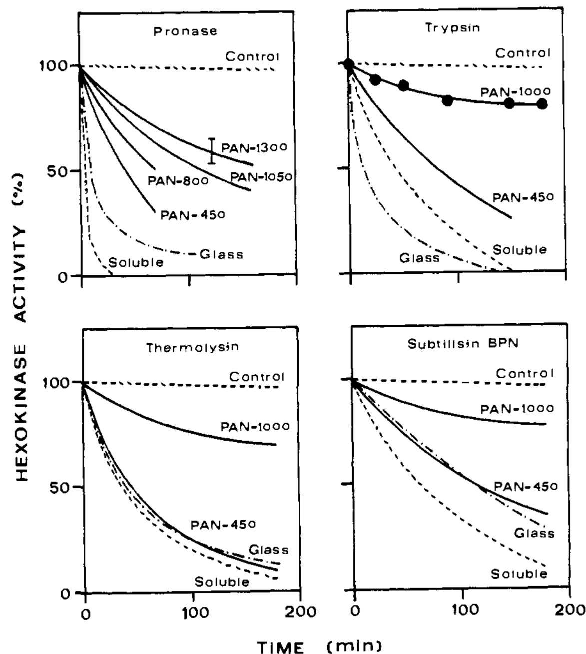


Figure 8. Deactivation of hexokinase by proteolysis (---), soluble enzyme (-····-), immobilized on silanized glass; (—) immobilized in PAN gels; (---) soluble enzyme with no protease. Reactions were carried out at 25 °C, pH 8.0 (0.05 M Hepes buffer). The initial concentrations (μg mL^{-1}) of proteases were: pronase, 7.5 μg mL^{-1}; trypsin, 7.8 μg mL^{-1}; thermolysin, 12.5 μg mL^{-1}; subtilisin BPN′, 12.5 μg mL^{-1}. To minimize confusion in these plots, experimental points have been omitted. Typical data are shown for the plot for PAN-1000/ trypsin, and an estimated error by the bar for PAN-1300/Pronase. Hexokinase activity is expressed as a percentage of its value at time = 0. Reactions involving Pronase were carried in solutions containing 5 mM Ca(II).

Protection of Enzymes against Attack by Proteases and Shear. Proteolytic degradation may contribute significantly to loss in activity during use of enzymes. We have explored the possibility that gel immobilization might afford protection of enzymes against attack by proteases (usually encountered in enzymatic synthesis as extracellular proteases elaborated by contaminating microorganisms) by inhibiting diffusion of the proteases. Experiments summarized in Figure 8 compare the rate of deactivation of hexokinase (taken as a representative enzyme) in solution, immobilized on the surface of glass beads using a standard silane

(35) Ferrofluids: Khalafalla, S. E.; Reimers, G. W. *Sep. Sci.* **1973**, *8*, 161–178. *CHEMTECH* **1975**, 540–546.

(36) For previous applications of magnetic gels in biochemical separations, see Robinson, P. J.; Dunnill, P.; Lilly, M. D. *Biotech. Bioeng.* **1973**, *15*, 603–606. Mosbach, K.; Anderson, L. *Nature (London)* **1977**, *270*, 259–261. Guesdon, J. L.; Avrameas, S. *Immunochemistry* **1977**, *14*, 443–447. van Leemputten, E.; Horrisberger, M. *Biotech. Bioeng.* **1974**, *16*, 385–396; and ref 14.

(37) Kolm, H.; Oberteuffer, J.; Kelland, D. *Sci. Am.* **1975**, *233* (5), 46–54; Oberteuffer, J. *IEEE Trans. Magn.* **1974**, *10*, 223–238.

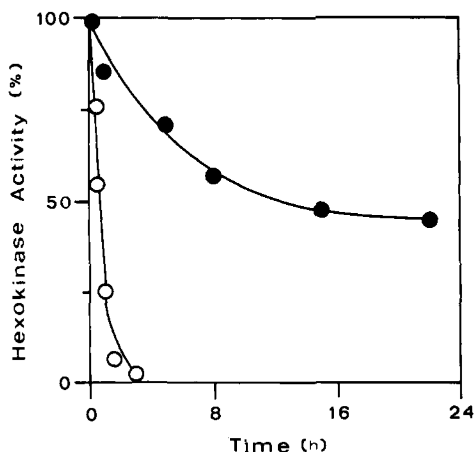


Figure 9. Degradation of hexokinase (15 U) by trypsin (26 U against *p*-tosyl-L-arginine methyl ester): (O) both enzymes are in solution (6.5 mL Taps buffer, pH 8); ● enzymes are coimmobilized in PAN-500 (ca. 0.2 mL of wet gel) suspended in a solution of total volume 8.0 mL. Residual hexokinase activity is expressed as a percentage of its value at time = 0.

coupling sequence,[38] and immobilized in the interior of a cross-linked PAN gel, by four soluble proteases. Two qualitative conclusions follow from these plots: first, loosely cross-linked gels (PAN-450) offer only modest protection against Pronase, trypsin, and subtilisin BPN' and none against thermolysin. Tightly cross-linked gels (PAN-1000 or PAN-1300) offer significant protection (for Pronase, a factor of ca. 50).

These studies suggest that loosely cross-linked PAN gels offer relatively little resistance even to the diffusion of macromolecules and are in agreement with studies of the hydrolysis of dextrans described below. They establish that PAN gel immobilization can, however, provide useful protection against attack by soluble proteases provided that PAN-1000 or other polymers containing a high proportion of active ester groups are used as starting materials and the gel-forming reaction is carried out in a way that yields a tightly cross-linked material.

Proteases may, of course, also be present as contaminants in the original mixture of proteins to be immobilized. Figure 9 illustrates the effectiveness of immobilization in PAN gel in inhibiting attack of trypsin on hexokinase when *both* enzymes are immobilized. It is clear from this plot that immobilization affords significant isolation of hexokinase from trypsin. The degree of protection suggested by this plot is probably an underestimate for two reasons. First, the concentration (in terms of activities) of enzymes in the gel is higher by a factor of ca. 5 than in the soluble model. Second, work by Tanaka and colleagues has established a high degree of flexibility of individual segments of cross-linked polyacrylamide gels.[39] The immobilized protease would be expected to attack immobilized hexokinase in its immediate vicinity, but after exhausting this local enzyme it should not be able to reach more distant, immobilized enzyme. Thus, the activity of hexokinase might be expected to fall to a plateau and not decrease beyond this value. Figure 9 shows evidence of this behavior. With more dilute protease, the plateau should be higher, and the protection afforded by coimmobilization more complete.

Shear can also provide an important contribution to the deactivation of susceptible enzymes in vigorously stirred reaction mixtures. Figure 10 compares the deactivation of soluble and gel-immobilized hexokinase in a Waring blender, and indicates that the gel provides excellent protection against shear. The initial increase in the activity of the immobilized enzyme is real, and presumably reflects the reduction in size of the gel particles during agitation. It suggests that the measured enzymatic activity in this system is partially limited by pore diffusion, and is in qualitative

(38) Weetall, H. H., "Methods in Enzymology", Vol. XLIV, Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1976; pp 134-148.
(39) Tanaka, T.; Fillmore, D. J. *J. Chem. Phys.* **1979**, *70*, 1214-1218. Tanaka, T. *Phys. Rev. A* **1978**, *17*, 763-766.

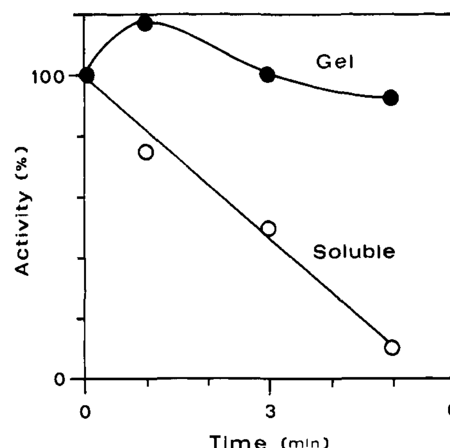


Figure 10. Gel immobilization of hexokinase (PAN-500) affords protection against shear deactivation in a Waring blender. The residual activity of the enzyme is represented as a percentage of its value before being subjected to shear in the blender: the blending time is given in minutes.

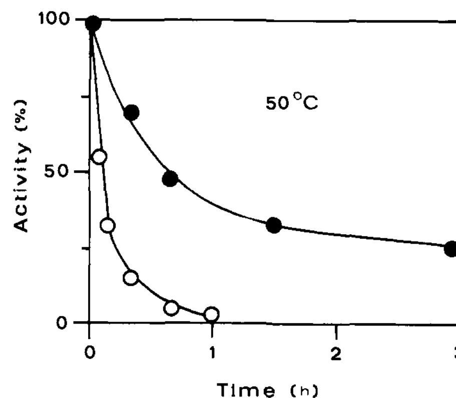


Figure 11. Deactivation of hexokinase: (O) soluble enzyme at 50 °C; (●) immobilized in PAN-450 gel at 50 °C. Reactions were carried out at pH 7.5 (0.05 M Hepes buffer). Hexokinase activity is expressed as a percentage of its value at time = 0.

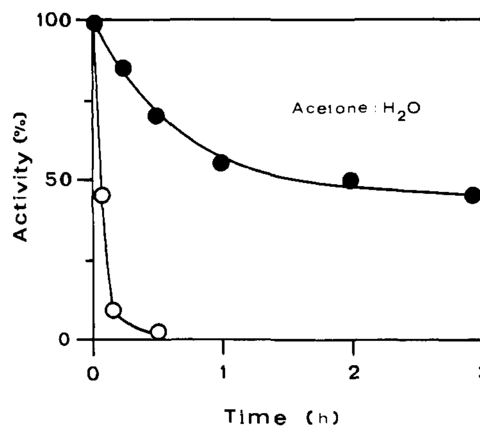


Figure 12. Deactivation of hexokinase: (O) soluble enzyme in 26% acetone at 25 °C; (●) immobilized in PAN-450 gel in 26% acetone at 25 °C. Reactions were carried out at pH 7.5 (0.05 M Hepes buffer). Hexokinase activity is expressed as a percentage of its value at time = 0.

agreement with earlier experiments suggesting a 5-10% pore diffusion limitation to the measured activity of enzymes in gel particles of the size used here.

The PAN gel appears to protect hexokinase significantly against denaturation at high temperature and by organic solvent. Figures 11 and 12 compare the rates of deactivation of soluble hexokinase and PAN-immobilized hexokinase at 50 °C in an aqueous buffer at pH 7.5, and in a buffer containing 26% of acetone at 25 °C,

Table III. Yields (%) of Immobilized Enzymes and Specific Activities of Enzyme-Containing Gels[a]

E.C. no.	enzyme	c^b (mg g^{-1} PAN)	M^c (concn, mM)	yield[d] (%)	activity (U mL^{-1})
1.1.1.1	alcohol dehydrogenase, yeast	2.4	EtOH (500), NAD (1.0)	22*	81
1.1.1.1	alcohol dehydrogenase, liver	4.3	EtOH (850), NAD (3.0)	63	1.0
1.1.1.8	α-glycerophosphate dehydrogenase	0.1	dihydroxyacetone-P (5.0), NADH (5.0)	66	0.7
1.1.1.27	L-lactate dehydrogenase	0.2	pyruvate (8.5), NADH (1.0)	51	13
1.1.1.28	D-lactate dehydrogenase	2.0	pyruvate (8.5), NADH (1.0)	37[e]	45
1.1.1.37	L-malic dehydrogenase	0.7	oxaloacetate (10), NADH (5.0)	61	102
1.1.1.42	isocitric dehydrogenase	1.9	D, L-isocitrate (50), NADP (10)	53	0.9
1.1.1.44	6-phosphogluconic dehydrogenase	10	6-P-gluconate (2.0), NADP (0.5)	38[f]	35
1.1.1.48	β-D-galactose dehydrogenase	6.6	galactose (100), NAD (15)	29	1.9
1.1.1.49	glucose-6-dehydrogenase, yeast	2.5	glucose-6-P (6.3), NADP (0.6)	35*	66
1.1.1.49	glucose-6-P dehydrogenase *Leuconostoc mesenteroides*	4.8	glucose-6-P (6.0), NADP (0.6)	29 (23)	140[g] (72)[h]
1.1.1.49	glucose-6-P dehydrogenase bovine adrenals	0.7	glucose-6-P (6.0), NADP (1.0)	29	3.7
1.1.3.4	glucose oxidase	5.0		30	68
1.2.1.2	formate dehydrogenase	8.0	formate (100), NAD (4.0)	47[i]	0.24
1.2.1.12	glyceraldehyde-3-P dehydrogenase	2.6	glyceraldehyde-3-P (10), NAD (10)	31	22
1.2.3.2	xanthine oxidase	0.5	hypoxanthine (2.2)	80	0.15
1.4.1.1	L-alanine dehydrogenase	4.5	L-alanine (20), NAD (5.0)	26	7.0
1.4.1.3	L-glutamate dehydrogenase	3.4	ketoglutarate (15), ADP (2.0), NADH (5.0), NH_4Cl (20)	42	34
1.6.4.3	diaphorase	3.9	2,6-dichloroindophenol (10), NADH (10)	64	3.0
1.11.1.6	catalase	10		27	20,000
1.11.1.7	peroxidase, horseradish	0.6	H_2O_2 (4.5)	14	38[j]
1.11.1.17	lactoperoxidase	2.5	H_2O_2 (4.5)	27	1.2[j]
2.4.2.1	nucleoside phosphorylase	2.5	inosine (10), glycerol (10), P (10)	65[k]	6.5
2.5.1.6	ATP: L-methionine-S-adenosyl-transferase	0.09	1-Meth (2.0), ATP (2.0), SAM (1.0), KCl (200)	40	0.02[l]
2.7.1.1	hexokinase	2.5	glucose (25), ADP (10)	50*	105
2.7.1.19	phosphoribulokinase	2.8	ATP(15), ribulose-5-P (15)	30[f]	4.0
2.7.1.20	adenosine kinase	5	adenosine (0.5), ATP (4.5)	42	0.4
2.7.1.30	glycerol kinase	0.2	glycerol (20), ADP (12), ATP (4.0)	70[k]*	0.2[m]
2.7.1.40	pyruvate kinase	0.3	PEP (6.7), ADP (25)	46	10
2.7.2.1	acetate kinase	0.9	acetyl P (12.5), ADP (20)	55	67
2.7.2.3	3-phosphoglycerate kinase	0.4	glycerate-3-P (10), ATP (10)	15	19
2.7.3.2	creatine kinase	30	creatine (40), ADP (10), KNO_3 (100)	45[n]*	135
2.7.4.3	adenylate kinase	0.5	ADP (20)	46*	92
2.7.4.4	nucleoside monophosphate kinase	5.3	CMP (5.0)	40[o]	0.25
2.7.4.8	guanylate kinase	1.4	GMP (5.0)	42[o]	0.6
2.7.5.1	phosphoglucomutase	0.2	glucose-6-P (5.0), glucose-1-P (1.0), glucose 1,6-di-P (0.01)	67	12
2.7.6.1	phosphoribosyl pyrophosphate synthetase	3.0	PRPP (4.0), ATP (3.0), AMP (4.0), ribose-5-p (5.0)	74[p]	40
2.7.7.1	NMN-ATP adenylyl transferase	10	ATP (8.0), NMN (1.5), NAD (1.0), nicotinamide (2.0), PP (1.5), glycerol (10)	95[k]	4.4
2.7.7.9	UDPG pyrophosphorylase	3.0	UDPG (1.3), PP (2.0)	50[o]	0.55
	riboflavin kinase-FAD synthetase	47		69[q]	0.002
3.1.1.4	phospholipase A_2	1.5	dihexanoyl PC (6.0), Ba(II) (5.0)	90[r]	
3.1.2.6	glyoxalase II	8.1	glutathione (10)	31[s]	58
3.1.4.3	phospholipase C	0.9		5[r]	
3.1.3.1	phosphatase, alkaline	0.2	glucose-6-P (25.0)	64	9.2
3.1.3.2	phosphatase, acid	2.8	*p*-nitrophenyl phosphate (25.0)	83	0.4
3.2.1.11	dextranase	13	dextran (MW 500 000, 2%)	16	24
3.2.1.20	α-glucosidase	9.6		43	1.3
3.2.1.21	β-glucosidase	9.7		30	2.1
3.2.1.26	invertase	4.0	sucrose (30)	63	85
3.4.4.4	trypsin	5.0	benzoylarginine ethyl ester (1.0)	53	6300[t]
3.4.4.5	α-chymotrypsin	50		21	168
3.4.4.16	subtilisin BPN′	64		32	2.0
	Pronase P	10		8	5.5
3.5.4.4	adenosine deaminase	1.3	adenosine (0.8)	37	2.0
4.1.1.32	phosphoenolpyruvate carboxy-kinase	38	IDP (0.5), PEP (5.0)	79[u]	1.0
4.1.2.13	aldolase	21	fructose-1,6-di-P (2.0)	40	15
4.2.1.1	carbonic anhydrase	10		44	0.8[v]
4.4.1.5	glyoxalase I	0.6	S-*p*-bromobenzylglutathione (0.55), glutathione (0.55), methylglyoxal (0.55)	40[s]	16
5.3.1.6	ribose-5-P isomerase	10	ribose-5-P (200)	36[f]	67
5.3.1.9	phosphoglucose isomerase	0.8	glucose-6-P (6.0), fructose-6-P (1.5)	56	39
6.2.1.4	succinic thiokinase	4.0	GTP (2.0), CoA (10), succinate (100), KCl (100)	46	0.6

Footnotes for Table III

[a] Immobilizations were carried out using PAN-450, 25 °C, 0.3 M Hepes buffer, pH 7.5, containing 15 mM $MgCl_2$. [b] Quantity of protein (mg) allowed to react/g of PAN. This quantity is approximate, since the purity of most of the enzymes was only approximately known. [c] Materials present during the immobilization to protect the enzyme active site. [d] Defined in the text by eq 2. Yields indicated with an asterisk represent the result of programs involving significant efforts to improve yield. Other yields reflected only two to five (in four instances only one) experiments, and could probably be improved. [e] Carried out by O. Abril. [f] Carried out by C. H. Wong. [g] NAD as substrate. [h] NADP as substrate. [i] Carried out by Ze'ev Shaked. [j] o-Dianisidine as substrate. [k] Carried out by V. Rios-Mercadillo. [l] Carried out by Shimona Geresh. [m] Assayed at pH 7.6. [n] Carried out by Y.-S. Shih. [o] Carried out by S. L. Haynie. [p] Carried out by J. Lewis. [q] Carried out by D. Light and F. Jacobson. [r] Carried out by M Roberts. [s] Carried out by A. K. Patterson. [t] Benzoylarginine ethyl ester as substrate (units not in μmol min^{-1}). [u] Carried out by Hans-Jürgen Leuchs. [v] p-Nitrophenylacetate as substrate.

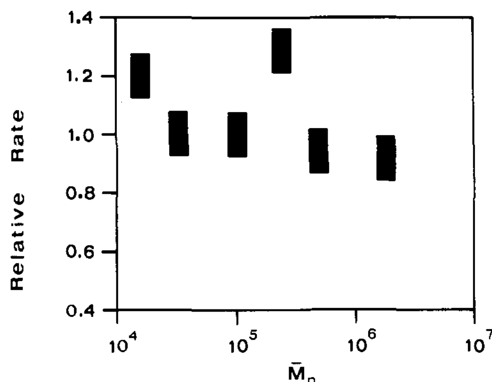


Figure 13. Relative rates of hydrolysis of dextrans by dextranase immobilized in a gel formed from PAN-500 and TET.

respectively. In both cases, the hexokinase immobilized in PAN gel significantly exceeds the stability of the soluble enzyme. We did not study the behavior of other enzymes under similar conditions, but as mentioned earlier, we did observe excellent stability for the PAN-immobilized invertase at 45 °C over a period of about 4 weeks.

Permeability of PAN Gels to High Molecular Weight Substrates. The pattern of deactivation of immobilized hexokinase by soluble proteases suggested that only highly cross-linked gels (PAN ≥ 1000) significantly hindered diffusion of the proteases, and that loosely cross-linked gels (PAN-500) afforded little limitation to diffusion. To check this latter conclusion and to determine the ability of an enzyme immobilized in PAN-500 gel to catalyze transformations of high molecular weight substrates, we have examined the hydrolysis of dextrans having a range of molecular weights by dextranase immobilized in PAN-500 (Figure 13). The small variation in rates observed indicates that diffusional limitations are unimportant in this system. We have not investigated the influence of substrate size on gel permeability in sufficient detail to establish whether the molecular weight independence of the rates reflects the random coil conformation of the polysaccharide, the open gel structure, or both. In any event, it is clear that gel-immobilized enzymes can be effective catalysts for transformations of at least some very high molecular weight soluble substrates.

Conclusions

This immobilization method is directed specifically toward the relatively delicate and expensive intracellular enzymes of interest as catalysts in organic synthesis. The major advantages of this technique are experimental convenience and generality. Once the starting PAN is prepared, it is faster to immobilize an enzyme by this procedure than using cyanogen bromide – agarose,[40] the technique now most commonly used for research-scale immobilizations. Further, the use of PAN avoids the danger of modification of thiol functions which accompanies procedures which leave traces of cyanogen bromide in the activated gel, generates a hydrolytically stable amide link between the matrix and the enzyme, and incorporates the enzyme into a gel which is a poor substrate for microbial growth and which is resistant to biodegradation. The ability of the gels to provide protection against deactivation due to proteases present as impurities in the starting preparation is helpful when working with relatively crude enzyme preparations. The requirement for N-acryloxysuccinimide as a starting material makes the PAN gels modestly expensive, but the cost of the gel is usually minor compared with the cost of the enzymes. The relatively low specific activities obtained with certain of the enzymes (U mL^{-1}, Table III) can be a disadvantage in some applications.

Although this technique is not intended to compete with immobilization techniques such as ion-exchange adsorption or glutaraldehyde cross-linking whose economy makes them attractive for very large scale applications, the principle of enzyme immobilization by condensation polymerization into organic polymer-based hydrogels should find wide application. The ability to modify the mechanical properties of the gel, to adjust its hydrophilicity, and to incorporate other functional groups into the matrix are all potentially useful for particular applications in applied biochemistry.

Experimental Section

General. All chemicals and biochemicals were obtained from Sigma or Aldrich, unless otherwise mentioned. Dextrans were purchased from Pharmacia, and alkylamine-functionalized porous glass beads (CPG 550) from Pierce Chemical Co. Organic solvents were AR grade. THF was distilled from calcium hydride. Water was deionized and distilled using a Corning Model 3B still. Argon and nitrogen were prepurified grade. Volumetric transfers were accomplished using Hamilton syringes, Eppendorf pipets, and disposable glass micropipets (Fisher).

Enzymes. Those obtained as suspensions in ammonium sulfate solution were dialyzed twice against 150 volumes of 50 mM Hepes buffer (pH 7.5) at 4 °C before use; small quantities of ammonium ion left in solution competed with the α,ω-diamine for active ester groups on the PAN, and the cross-linking reactions either failed to produce gels or produced mechanically weak, sticky, gels. Enzymes obtained as solutions in aqueous glycerol could be used in quantities up to 100 μL/200 mg of PAN; larger quantities of glycerol resulted in soft gels. Enzymes sensitive to dioxygen were dialyzed under argon against Hepes buffer containing 5 mM DTT or DTP.

Enzymes were purchased from Sigma or Behringer-Mannheim, or prepared following literature procedures: adenosine kinase,[41] phosphoribosylpyrophosphate synthetase,[42] riboflavin kinase,[43] phosphoenolpyruvate carboxykinase,[44] ATP (L-methionine-S-adenosyltransferase).[45] All activities are reported in units of μmol min^{-1}.

Assays of enzymes in homogeneous solution were performed spectrophotometrically following the standard assay procedures in Bergmeyer.[46] Immobilized enzymes were assayed using the same procedures, with care taken that the enzyme-containing gel particles were sufficiently small (30–50 μm) that diffusional effects were negligible. A dilute suspension containing gel particles was added to the assay solution in the spectrophotometer cuvette. The cuvette was stoppered with a Teflon stopper, shaken for a few seconds, and put into the cell compartment, and the increase or decrease of the absorbance was recorded for 30 s; during this 30-s interval the gel particles did not settle appreciably, and because the particles are transparent they did not interfere with spectrophotometric analysis. After the 30-s interval, the cuvette was taken out of the cell compartment and shaken again for a few seconds, and the absorbance was recorded for another 30 s. Usually, this assay procedure was ex-

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tended over a period of a few minutes. Almost linear assay responses were obtained in all instances. Although assay conditions were chosen to avoid pore diffusional effects on the assays, these assay procedures were not explicitly demonstrated to be independent of diffusion. The yield of an immobilization was defined by eq 2.

Kinetics Experiments. Stock solution containing species including *N*-hydroxysuccinimide active ester groups were prepared in dry, distilled Me_2SO shortly before the kinetic runs and were stored at 0 °C; the stock solutions were ca. 10 mM in active ester groups. Aliquots (30 μL) were removed using an Eppendorf pipet and added to 3 mL of assay solution (0.3 M Hepes buffer, pH 7.4) containing *n*-butylamine (16 mM in some experiments) in a quartz cuvette (the buffer-containing cuvette was equilibrated prior to the addition of the Me_2SO solution of the substrate (10 min) in the sample compartment of the spectrophotometer thermostated with circulating water (25 °C)). The absorbance of the solution was monitored at 259 nm and an extinction coefficient of 8600 M^{-1} cm^{-1} was used for *N*-hydroxysuccinimide anion at pH 7.5. The pseudo-first-order rate constants for the release of *N*-hydroxysuccinimide anion were calculated using standard procedures.[14]

***N*-Acryloxysuccinimide.** *N*-Hydroxysuccinimide (115 g, 1.0 mol) and triethylamine (110 g) were dissolved in 1500 mL of chloroform at 0 °C. Acryloyl chloride (Aldrich, 100 g, 1.1 mol) was added dropwise over a 20-min period to the stirred reaction mixture. After being stirred an additional 20 min at 0 °C, the solution was washed with 800-mL portions of ice-cold water and saturated brine, dried with $MgSO_4$, and filtered; 50 mg of 2,6-di-*tert*-butyl-4-methylphenol (polymerization inhibitor) was added to the chloroform solution which was concentrated to a volume of 300 mL in vacuo using a rotary evaporator and filtered. Ethyl acetate (30 mL) and 200 mL of *n*-hexane were added slowly with stirring to the chloroform solution which was left to stand at 0 °C for several hours. The precipitated, colorless crystals were separated by filtration and washed with an ice-cold 100-mL portion of a mixture of *n*-hexane and ethyl acetate (4:1), then with another 100-mL portion of *n*-hexane and ethyl acetate (9:1), and finally with two 100-mL portions of *n*-hexane. The crystals were dried in vacuo at ambient temperature to constant weight; 118 g (70%) was obtained at this stage, mp 69.5–71.0 °C (lit.[10] mp 67 °C). This material is pure enough for the PAN preparation. A slightly purer product (mp 70.5–71.5 °C) could be contained by the recrystallization from a mixture of *n*-hexane and ethyl acetate. The product has the expected spectral characteristics: NMR ($CDCl_3$) 2.85 (s, 4 H), 6.0–7.0 (mult, 3 H); IR (Nujol mull) 1800, 1775, 1735, 1260, 995, 870 cm^{-1}.

Poly(acrylamide-*co*-*N*-acryloxysuccinimide) (PAN). A 5000-mL, round-bottomed flask, equipped with a Teflon-coated magnetic stirring bar (0.5 × 2.0 in.) and a reflux condenser, was charged with acrylamide (275 g, 3.85 mol), *N*-acryloxysuccinimide (30 g, 178 mmol), AIBN (1.75 g, 11 mmol), and 2500 mL of THF (AR grade, distilled from CaH_2). The reflux condenser was capped with a serum stopper and the flask degassed with nitrogen for 30 min with vigorous stirring to remove dioxygen. The flask was maintained in a constant-temperature water bath at 50 °C under slight positive pressure of nitrogen for 24 h. After 24 h, 1000 mL of THF was added to the flask and the contents were stirred for 10 min. The precipitated white polymer was separated by filtration on a large Büchner funnel. The polymer was washed on the funnel four times with 1000-mL aliquots of dry THF, transferred to a vacuum desiccator, and dried under vacuum (0.02 Torr) for 24 h at room temperature; 325 g (106%) of a white, very fluffy product was obtained. An assay of this polymer showed that it contained 512 μmol of active ester groups per gram: IR (Nujol mull) 3340, 3200, 1730, 1660, 1210, 1070 cm^{-1}.

Assay for the Active Ester Content of PAN. PAN (~50 mg, ~50 μmol of active ester groups, dried under vacuum at 0.01 Torr and 45 °C for 24 h) was dissolved and made up to volume in distilled water in a 5-mL volumetric flask. A 50-μL aliquot of this solution was added into a 5-mL quartz cuvette containing 3000 μL of Hepes buffer (0.1 M, pH 7.5), 50 μL of 1 M ethylamine solution, and 10 μL of a 1 M solution of mercaptoethanol; the rate of the appearance of *N*-hydroxysuccinimide was followed spectrophotometrically at 259 nm at 25 °C; after the reaction was completed (ca. 90 min) and the increase of the absorbance leveled off, the active ester concentration was calculated. When required, the total concentration of neutral (NHSH) and deprotonated *N*-hydroxysuccinimide (NHS^-) was estimated from the observed concentration of the anion using eq 3. At pH 7.5, for $pK_a^{NHSH} = 6.0$, this

$$\frac{(NHS^-)}{(NHSH) + (NHS^-)} = (1 + 10^{pK_a^{NHSH} - pH^{-1}}) \qquad (3)$$

correction is approximately 3% ((NHS^-)/(NHSH) + (NHS^-) = 0.969), and was ignored.

Poly(acrylamide-*co*-*N*-acryloxysuccinimide) (PAN): Small Scale. Acrylamide (13.5 g, 0.19 mol) and *N*-acryloxysuccinimide (1.50 g, 8.9 mmol) were added to a 200-mL centrifuge tube equipped with a Teflon-coated magnetic stirring bar. The tube was capped with a serum stopper. Purified THF (150 mL distilled from $Ph_2CO^{2-}Na_2^+$ under argon) was introduced into the tube through a cannula. A solution of AIBN (80 mg) in dry THF (5 mL) was introduced into the reaction solution by a syringe, and the reaction mixture degassed in a stream of argon for 10 min. The centrifuge tube was held under positive pressure of argon in a constant-temperature water bath at 50 °C for 24 h. The resulting PAN precipitated as a white solid. Purified THF (100 mL) was added to the tube by cannula. The white precipitated product was resuspended by mechanical shaking and separated by centrifugation at 3000 rpm for 5 min; the supernatant liquid phase was removed by help of a cannula and positive argon pressure, and the product resuspended in 100 mL of THF and recentrifuged. This process was repeated four times, and the final monomer-free polymer dried under vacuum (0.1 Torr) at room temperature for at least 24 h or to the constant weight of the product: 15.5 g (103%) of a white, fluffy material was obtained. This polymer should theoretically have contained 594 μmol/g of active *N*-hydroxysuccinimide active ester groups: 496 were assayed spectrophotometrically.

Other PAN polymers containing up to 1550 μmol/g of active ester group were prepared similarly.

Determination of the Molecular Weight of PAN. The number average molecular weights of aminolyzed PAN samples were calculated from their intrinsic viscosities, determined as described by Flory.[47] Aminolysis of PAN was accomplished by treatment of the polymer (ca. 2 g/100 mL) with 25% ammonium hydroxide solution, using a 50% excess calculated with respect to the estimated number of active ester groups of the PAN sample.

Cross-Linking of Reactive Polymers. Determination of the Gel Time. A 5-mL beaker was charged with 100 mg of the reactive polymer and a $^3/_8$-in. magnetic stirring bar. Distilled water or the appropriate buffer (500 μL) was added to the beaker, and the polymer was brought into solution within 1 min by rubbing it against the wall of the beaker with a small glass rod. The solution of the cross-linking diamine was added to the stirred solution using an Eppendorf pipet. A stopwatch was started at the same time. The polymer solution rapidly became more viscous, and after 1–3 min the magnetic stirring bar stopped turning. The stopwatch was stopped: the interval was taken as the gel time. (More reactive polymers and polymers with high contents of active ester groups had gel times as short as 10–15 s; polymerizations carried out in more acidic solutions or at lower temperatures had much larger gel times.) The gel hardness and mechanical properties were determined after 24 h in those experiments intended to determine the influence of the structure of the cross-linking agent on these characteristics of the gel. The gels adsorb large quantities of water, and swell by ca. 100% on soaking. The swollen gel is colorless and transparent.

Immobilization of Hexokinase: Small-Scale Procedure. PAN-500 (200 mg, ca. 99 μmol of active ester groups) was placed in a 5-mL beaker containing a small stirring bar, and 800 μL of 0.3 M Hepes buffer, pH 7.5, containing 30 mM $MgCl_2$, 25 mM glucose, and 10 mM ADP, added. The polymer was dissolved within 1 min by mixing and rubbing against the beaker walls with a glass rod. (Times for dissolving PAN longer than 5 min lead to formation of soft gels because of the hydrolysis of the active ester groups.) The polymer solution was stirred magnetically for 30 s at room temperature to ensure complete solution and 10 μL of a 0.5 M aqueous solution of DTT or DTP and 85 μL of a 0.5 M aqueous solution of TET were added; 60 s later, 200 μL of a solution containing hexokinase (210 U) was added. In less than 2 min, the solution set to a transparent, mechanically resilient gel. The gel was allowed to stand for 1 h at room temperature to complete the coupling of enzyme and transferred to a small mortar. The gel at this point was usually opalescent and filled with trapped air bubbles; it was occasionally colored yellow. This yellow coloration appeared to have no correlation with the ultimate immobilization yield. The gel was ground with a pestle for 2 min, 5 mL of Hepes buffer (50 mM, pH = 7.5, containing 50 mM ammonium sulfate and 10 mM $MgCl_2$) added, and grinding continued for an additional 2 min. This grinding reduced the gel to irregular particles having 30–100 μm diameter. The gel suspension was diluted in 10 mL of the ammonium sulfate/Hepes buffer and transferred into a centrifuge tube; the mortar and pestle were washed with another 5 mL of the ammonium sulfate/Hepes buffer which was also transferred into the centrifuge tube. The gel suspension was stirred magnetically for 15 min and separated by gentle centrifugation (~3000 rpm). The supernatant buffer was assayed for the nonimmobilized hexokinase activity. The washing procedure of the gel particles was repeated once with the

(47) Flory, P. J. "Principles of Polymer Chemistry"; Cornell University Press: Ithaca, N.Y., 1953; pp 308–314.

same volume of the buffer containing no ammonium sulfate. The gel particles were then resuspended again in the same volume of the Hepes buffer and assayed for enzymatic activity. The activity of hexokinase gel was 107 U (51%), and 74 U (34%) was detected in the combined washes.

A number of modified immobilization procedures have been carried out on hexokinase, but consistently the best immobilization yields were obtained using the procedure described above. This procedure, using the same amounts of PAN, DTT, TET, and Hepes buffer, was used for the immobilization for many of the enzymes included in Table II. The enzymes which were sensitive to dioxygen (e.g., acetate kinase) were manipulated under an inert atmosphere. Two variants of a modified procedure were used. In the first, the entire procedure was carried out in an inert atmosphere enclosure under nitrogen or argon, and the Hepes buffer used for washing contained DTT in a 5 mM concentration. In the second, the 5-mL beaker in which the PAN polymer solution had been prepared in air was quickly capped with a serum stopper and flushed for 30 s with a vigorous stream of argon, and DTT, TET; the enzyme solutions were introduced into the well-stirred solution using Hamilton syringes. The gel was ground in a mortar under air, but the washing Hepes buffer contained 10 mM DTT. Immobilization yields following the second procedure were slightly lower (less than 5%) than those in the first. The gel particles containing immobilized dioxygen-sensitive enzymes must be stored in a serum stopper-capped centrifuge tube in Hepes buffer containing 5–10 mM DTT or DTP under a positive pressure of argon in order to assure their long-term storage stability.

Enzyme Immobilizations Using PAN > 500. These procedures followed the general procedure outlined for immobilizations with PAN-500, modified by adding the enzyme immediately after (PAN-800) or immediately before (PAN-1000 or PAN-1300) adding the TET, in order to compensate for the very short gel times of these solutions (5–60 s).

Immobilization of Hexokinase: Large-Scale Procedure. PAN (3.0 g, 450 μmol/g, 1350 μmol of active ester groups) was placed in a 50-mL beaker and 12.0 mL of 0.3 M Hepes buffer, pH 7.5, containing 15 mM $MgCl_2$, 60 mM glucose, and 30 mM ADP, was added. The polymer was quickly (within 1 min) dissolved by mechanical grinding with a glass rod against the glass wall of the beaker and a 1-in. magnetic stirring bar was dropped into the polymer solution. The solution was stirred magnetically for 30 s, and 150 μL of 0.5 M DTT solution and 1.275 mL of TET (1275 μmol of primary amino groups) were added with vigorous stirring; 30 s later, 1000 μL of a solution of hexokinase (1160 U) was added. In less than 2 min, the solution set to a transparent, resilient gel. The gel was allowed to stand for 1 h at ambient temperature, and then transferred to a Waring blender containing 185 mL of Hepes buffer (50 mM, pH 7.5, containing 50 mM ammonium sulfate and 10 mM $MgCl_2$). Blending at low speed for 3 min followed by 30 s at high speed reduced the gel to a suspension of particles having ~100 μm diameter. The suspension was transferred to a 200-mL centrifuge tube, stirred magnetically for 15 min, and separated by centrifugation. This washing procedure was repeated twice with buffer containing no ammonium sulfate, and the suspension was diluted to 150 mL, using the same Hepes buffer. The activity of hexokinase in the gel was 710 U (61%), and 90 U (8%) was detected in combined washes.

The immobilization was repeated with PAN-800 (3 g, 2400 μmol of active ester groups), 250 μL of 0.5 M DTT, 2.200 mL of TET (2200 μmol of primary amino groups), and 100 μL (1150 U) of hexokinase solution. The hexokinase solution was added immediately after the TET, which was added to the vigorously stirred PAN solution slowly (within 5 s) to avoid local gelling. Otherwise the procedure was the same. The activity of the hexokinase in the gel was 536 U (47%), and 105 U (9%) was detected in the combined washes.

Immobilization of Horse Liver Alcohol Dehydrogenase: Large-Scale Procedure. PAN-500 (50 g, 25 mmol of active ester groups) was placed in a 600-mL beaker; 12.0 mL of 96% ethanol (200 mmol) and 8.0 mL of 0.1 M NAD (0.8 mmol) solution were added to 200 mL of Hepes buffer, 0.3 M, pH 7.5, containing 15 mM $MgCl_2$. This solution was introduced into the beaker and the polymer was dissolved quickly (within 1 min) with mechanical grinding against the beaker wall, using a 50-mL syringe plunger. A large (2 × $^1/_2$ in.) Teflon-coated magnetic stirring bar was added to the polymer solution and magnetic stirring initiated. DTT (1.25 mL of a 1.00 M solution, 1.25 mmol) and 21.5 mL (21.5 mmol of primary amino groups) of a 0.5 M TET solution were added to the PAN solution with vigorous magnetic stirring. (A very good magnetic stirrer is needed to provide efficient agitation of the viscous polymer solution; a motor-driven stirrer is probably a better choice for most preparations at this scale.) Thirty seconds later, 28 mL of an aqueous solution containing ca. 400 mg of alcohol dehydrogenase (950 U, horse liver, Boehringer) was added and stirring continued until the solution set to a gel (in less than 2 min). A thermometer inserted in the gelling solution recorded a 3 °C temperature rise during the gel formation. After 1 h, the gel was processed as described for the immobilization of hexokinase carried out on the 3-g (of PAN) scale. The gel particles contained 688 U (72%) of activity, and the collected wash solutions 162 U (17%).

Immobilizations of Horseradish Peroxidase, Lactoperoxidase, Invertase, and Carbonic Anhydrase. When these enzymes were subjected to the procedure described for hexokinase, most of the enzymatic activity was recovered in the wash solution. The inference that the amino groups of these enzymes were unreactive was tested by modifying the immobilization procedure. The enzyme and the aqueous PAN solution were allowed to react in the absence of cross-linking agent (for peroxidase, 6 min; for lactoperoxidase, 3 min; for invertase, 3 min; for carbonic anhydrase, 1 min). TET was then added, and the further course of the immobilization followed the course described for hexokinase.

Immobilization of Hexokinase on Functionalized Porous Glass. *N*-Hydroxysuccinimide active esters were introduced onto the surface of controlled pore glass beads (Pierce, CPG-550, alkylamine functionality) using a standard procedure.[38] Hepes buffer (4.0 mL of 0.3 M solution, pH 7.5, containing 15 mM $MgCl_2$, 15 mM ADP) and 500 μL (688 U) of hexokinase solution were added to a 10-mL round-bottomed flask containing a small magnetic stirring bar. The functionalized beads (500 mg) were added and stirred magnetically under vacuum at ambient temperature for 5 min and then at ambient pressure for 2 h. The glass beads were washed twice with 40-mL portions of 50 mM Hepes buffer (pH 7.5, containing 15 mM $MgCl_2$) and finally diluted to 16 mL with the same buffer. The activity of the hexokinase on the glass beads was 167 U (24%), and 194 U (27%) was detected in the wash.

Cystamine-Cross-Linked Gels. Enzyme immobilizations were carried out using the same procedure described for gels cross-linked with TET. Treatment of a suspension of cystamine-cross-linked gel particles with 0.05 M mercaptoethanol for 1 h at room temperature under argon resulted in smooth dissolution of the gel. If this solution was dialyzed and exposed to air with stirring for 48 h, the gel re-formed. This second gel was weaker than the initial gel, suggesting a lower density of interchain cross-links.

Glucose-6-phosphate Dehydrogenase/PAN Coated Tubing. Glucose-6-phosphate dehydrogenase (yeast, 176 U), PAN-500 (0.200 g), TET, and DTT were mixed in 0.800 mL of Hepes buffer (0.3 M, containing 0.6 mM NADP and 6.0 mM glucose-6-P, pH 7.5). A portion (ca. 0.2 mL) of this solution was forced quickly through the length of a glass tube (2.5 × 150 mm) by applying light vacuum. The tube was rotated in horizontal position for ca. 5 min during which a uniform gel coating was formed on the inner surface of the tubing. After careful washing, this tubing showed an activity of ca. 0.025 U/cm.

Hexokinase/PAN Gel Supported on Filter Paper. Hexokinase (2100 U), PAN-1000 (3.0 g), DTT (0.5 M, 50 μL), and TET (0.5 M, 2.20 mL) were mixed in 12.0 mL of 0.3 M Hepes buffer, pH 7.5, containing 15 mM $MgCl_2$, 60 mM glucose, and 30 mM ADP. The fluid solution was quickly poured on a sheet of filter paper supported on a glass plate (Whatman No. 3 MM, 25 × 35 cm) and dispersed uniformly into the paper structure by using a rubber roller. The filter paper sheet was kept in a glass chamber with 100% humidity for ca. 1 h and washed extensively to remove the nonimmobilized hexokinase. This filter paper/PAN composite material showed an activity of ca. 0.2 U/cm^2.

Column Operation. Immobilized Invertase Mixed with Celite. Invertase (20 mg, ca. 12 000 U) was immobilized in 12 mL of 0.3 M Hepes buffer, pH 7.5, containing 0.1 M sucrose with 3 g of PAN-500. The washed gel particles had an activity of 4800 U (40% yield). These gel particles were suspended in 600 mL of Mops buffer (50 mM, pH 7.5) containing 50 g of Celite 545 and stirred for 2 h. A jacketed column (Pharmacia, 3 × 43 cm) was filled with ca. 75% of this PAN/Celite slurry. The column was thermostated at 45 °C by circulating water through the column jacket employing a circulating thermostat. A 0.5 M solution of sucrose, pH 4.8, containing 25 mM acetate (neutralized with sodium hydroxide to pH 4.8) was pumped continuously through the column for a period of over 600 h (26 days) at flow rates between 9.00 and 10.6 L/day. The average activity of the column was calculated by estimating the residence time of the sucrose sample in the column at a particular flow rate and measuring the conversion of sucrose to glucose and fructose. Combination of these quantities allowed the calculation of the quantity (mmol of sucrose consumed/min) required for estimation of the column activity.

Initially the column had an activity of 2630 U at 45 °C and pH 4.8. On days 5, 11, and 16 an aqueous solution of sodium azide (100 mg in 10 mL) was introduced into the column to inhibit growth of mold. The sucrose solution was sterilized by boiling it for 15 min, but otherwise no special precautions were taken to assure strict sterility. The activity of the column decreased slowly, as shown in Figure 6, and dropped to ca. 1000 U (40%) in 624 h. The pressure drop across the column was 160 mm Hg at 10 L/day flow rate, and remained constant over the entire run.

Column Operation: Invertase/PAN Coated on Glass Beads. Invertase (5.0 mg, ca. 3100 U) was mixed with 2.0 g of PAN-500 in 8.0 mL of

Hepes buffer (0.3 M, containing 0.1 M sucrose, pH 7.5). After 3 min, DTT (0.5 M, 0.100 mL) and TET (0.5 M, 0.85 mL) were added and the solution was mixed thoroughly and poured onto 50 mL of glass beads (B. Braun Melsungen Apparatebau) (1.00 mm). The beads were mechanically stirred for ca. 6 min to provide a uniform coating with the PAN solution as well as to prevent their clumping as the solution gelled. After 1 h, the invertase/PAN coated glass beads were suspended in Hepes buffer (0.1 M, pH 7.5, containing 50 mM ammonium sulfate), and gentle stirring was continued for 20 min. A jacketed column (Pharmacia, 1.5 × 30 cm) was filled with ca. 85% of these beads. The column was thermostated at 45 °C and a 0.5 M solution of sucrose, pH 4.8, containing 25 mM acetate, was pumped continuously through the column for a period of 350 h at a flow rate of ca. 2.4 L per day. The initial column activity was ca. 960 U at 45 °C and pH 4.8. At the end of the experiment, the column activity dropped to ca. 700 U.

Magnetically Responsive Invertase/PAN Gel. Invertase (20 mg, 12,000 U) was mixed with 3.0 g of PAN-500 in 12.0 mL of Hepes buffer (0.3 M, containing 0.1 M sucrose, pH 7.5). After 3 min, DTT (0.5 M, 0.100 mL), TET (0.5 M, 1.275 mL), and aqueous ferrofluid (Ferrofludics Corp., Burlington, Mass., magnetic saturation of 200 G, 0.5 mL) were added, and the solution was mixed until it had gelled. The brown-colored gel particles, obtained following the standard work-up procedures, contained 4500 U (37%) of the invertase activity.

Column Operation: Invertase/Magnetically Responsive PAN Gel. A jacketed column (Pharmacia, 3 × 43 cm) was placed between the poles of an electromagnet (Varian, V-4004, 2 A, ~10 kG). A 10-cm plug of stainless steel wool (International Steel Wool Corp., fine grade) was placed in the magnetic field zone and a suspension of the magnetically responsive invertase/PAN-500 gel particles (ca. 0.5 g of PAN-500) was filtered through the plug. The particles immediately adhered to the plug. The column was thermostated at 45 °C and a 0.5 M solution of sucrose, pH 4.8, containing 25 mM acetate, was pumped continuously through the column for a period of 20 days at a rate of ca. 1.8 L/day. The initial column activity of ca. 215 U at 45 °C and pH 4.8 dropped to ca. 90 U.

Treatment of Gel- and Glass-Immobilized Hexokinase with Soluble Proteases. The experimental protocols used in these experiments were unexceptional extensions of those already described. Exact analysis of the rate of loss of hexokinase activity is unprofitable, since autoproteolysis also significantly lowered the activity of the proteases during the course of the experiments. Separation of the gel particles from the reaction mixtures and analysis of the solution established that most of the protease activity was present in the solution, rather than associated with the gel.

Coimmobilization of Hexokinase and Trypsin. Hexokinase (2.0 mg, 510 U) and trypsin (2.0 mg, 780 U (*p*-tosyl-L-arginine hydrochloride as substrate)) were coimmobilized in PAN-500 (0.200 g) dissolved in 0.3 M Hepes buffer (pH 7.5, containing 15 mM $MgCl_2$, 10 mM ADP, 25 mM glucose, and 5 mM benzamidine hydrochloride) following the small-scale immobilization procedure for hexokinase. After the standard washing procedure (three washes), an assay of 200 U (39%) of hexokinase activity and 351 U (45%) of trypsin activity was determined in the gel particles. To decrease the concentration of the benzamidine, a potent trypsin inhibitor ($k_i = 1.84 \times 10^{-5}$ M),[48] the gel particles were washed five more times with 0.05 M Mops buffer, pH 7.0 (gel particles volume to total volume ratio ca. 5) at 0 °C.

Acknowledgment. Dr. Orn Adalsteinsson carried out initial experiments in this project. Measurements of gel particle sizes used microscopes in the M.I.T. Materials Research Laboratory. A number of individuals in the group—especially Mr. Victor Rios-Mercadillo, Ms. Sharon Haynie, Dr. Hans-Jürgen Leuchs, Dr. Shimona Geresh, and Dr. Yen-Shiang Shih—have used this immobilization method in their work, and their experience has contributed to its development. We thank our colleague, Professor Mary Roberts, for her collaboration in the immobilization of several phospholipases.

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Communications to the Editor

Free-Radical Dynamics in Organized Lipid Bilayers

Sir:

Organized lipid bilayers are the principal structural units of most cell membranes and a major site for enzymes important in cellular metabolism. Destruction of membrane lipids and other cell components by adventitious free-radical oxidants is believed to constitute one component of the aging process in higher organisms, including man. The presence of vitamin E, a powerful antioxidant, in most cell membranes is cited as evidence of the damaging effects of free-radical oxidants on biological systems.[1-3] Quantitative studies of radical oxidants in lipid bilayers are virtually unknown although a qualitative literature exists.[4-8]

This report describes kinetic experiments with a lipophilic peroxy radical source, azobis[(2-*n*-butylcarboxy)propane],[9] $[Me_2C(CO_2Bu)]_2N_2$ (ABCP), and a phenolic antioxidant, 2,6-di-*tert*-butyl-4-methylphenol (BHT), both in model cell membranes and homogeneous solution. From these data, we are able to deduce some quantitative effects of lipid bilayers on free-radical dynamics.

Table I. Effect of Solvent on Decomposition of ABCP at 50.0 °C

solvent	10^2-$[ABCP]_0$, M	$10^6 k_d$, s^{-1}	$t_{1/2}$, h[a]
dodecane	3.0	1.38 ± 0.08	140
Nujol	3.0	1.56 ± 0.08	123
methyl laurate	2.0	1.82 ± 0.02	106
1-butanol	3.0	3.02 ± 0.02	64
CH_3CN/H_2O, 1:1	3.0	3.7 ± 0.5	53
DLPC bilayer	2.0[b]	2.2 ± 0.3	89
DSPC bilayer	2.0[b]	1.7 ± 0.05	111

[a] Half-life for decomposition. [b] Concentration in lipid phase of a 1% w/v suspension, pH 7.

In several solvents, ABCP (10^{-2} M) decomposes with clean first-order kinetics with a half-life of 53–140 h at 50.0 °C, depending on solvent polarity, but not viscosity. As shown in Table I, decomposition is faster in more polar media, increasing by a factor of three upon going from dodecane to CH_3CN/H_2O. Increased solvent viscosity, however, is not expected to retard the decomposition of ABCP because symmetrical azoalkanes decompose by concerted two-bond cleavage.[10,11] We find a slight increase in k_d upon going from dodecane (0.92 cP) to Nujol (37 cP), indicating that viscosity is not a controlling factor.

In synthetic bilayer suspensions of L-α-dilauroylphosphatidylcholine (DLPC) or L-α-distearoylphosphatidylcholine

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(9) ABCP was synthesized by HCl-catalyzed butanolysis of azobis(2-cyanopropane) (AIBN) by using the procedure of Mortimer, G. *J. Org. Chem.* **1965**, *30*, 1632. The viscous liquid product was purified by recrystallization from pentane at −50 °C. NMR and UV spectral data confirmed the structure, and high-pressure liquid chromatography (high-pressure LC) showed less than 5% contamination by AIBN. We thank C. Gould for the synthesis.

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