

immobilized pyridoxal 5'-phosphate were synthesized by reaction of pyridoxal 5'-phosphate with a bromoacetyl derivative of Sepharose in 50% (v/v) dimethylformamide and in potassium phosphate buffer, pH 6.0, respectively, for approximately 70 hr at room temperature. The catalytic activities of these derivatives were tested in the nonenzymic cleavage of tryptophan. The N-immobilized pyridoxal 5'-phosphate analog displayed catalytic activity, but the 3-O-immobilized derivative did not exhibit appreciable activity. This behavior, however, is not reflected in the ability of the two derivatives to bind apotryptophanase. Alkylation of the pyridine nitrogen of pyridoxal 5'-phosphate leads to a decreased affinity for the apoprotein, but attachment to Sepharose via the 3-hydroxyl group does not impede binding since this analog maintains all the main functional groups necessary for binding to the apoenzyme. Both derivatives bind apotryptophanase in such a way as to retain 50-60% of the specific activity of the starting material. Tyrosine phenol-lyase (β -tyrosinase) from *Escherichia intermedia* has been similarly immobilized.³⁰ This method of enzyme immobilization was found to be superior to other methods commonly used for preparation of immobilized enzymes.

General Discussion

In summarizing, the following can be said. Two conditions for the practical application of enzyme systems requiring expensive cofactors have to be met, i.e., their retention and regeneration. The regeneration may be accomplished either enzymically as described here, chemically with the participation of artificial electron acceptors or donors,²³ or electrochemically³⁰ (see also chapter [58] this volume). Depending on the type of application, either one of these may be the best choice. Enzymic regeneration appears to be the procedure of choice in the application of enzyme-coenzyme systems in the potential treatment of enzyme deficiency diseases whereby the catalyst system may be used entrapped in microcapsules or polymer beads and which are either placed in extracorporeal shunts or implanted *in vivo*.

With regard to retention, it appears advantageous to have the cofactors immobilized to macromolecular supports although attempts have been made to use unmodified NAD⁺ in hollow fibers as well.²³ However,

³⁰ S. Fukui, S.-I. Ikeda, M. Fujimura, H. Yamada, and H. Kinoshita, *Eur. J. Biochem.* **51**, 155 (1975).

³¹ M. Aizawa, R. W. Coughlin, and M. Charles, *Biochim. Biophys. Acta* **385**, 362 (1975).

the requirement for tight membranes to keep the NAD⁺ entrapped leads to overall poor permeability for substrate/product. Another possibility should be mentioned: for some applications, such as medical and some analytical procedures, which do not require a separation of the catalyst system, including the coenzyme, from the solution in which they are applied, enzymic recycling of the unmodified coenzyme may be the procedure of choice provided the number of cycles is high enough.

Finally an additional possible solution to the problem of coenzyme retention may be found with the following approach. In a previous study glycogen phosphorylase *b* has been immobilized in its allosterically activated form.¹¹ This had been accomplished by coimmobilization of the enzyme and its positive allosteric effector, AMP, using the AMP analog *N*⁶-(6-aminohexyl)-AMP, to CNBr-activated Sepharose. In extending these studies an alcohol dehydrogenase-NAD(H)-Sepharose complex was prepared showing no requirement of soluble coenzyme for its activity, at the same time with the coenzyme susceptible to recycling.¹² This was accomplished in a similar fashion by coupling a preformed enzyme-coenzyme binary complex to an activated matrix.

¹¹ K. Mosbach and S. Gestblom, *FEBS Lett.* **42**, 200 (1974).

¹² S. Gestblom, M. O. Månsson, and K. Mosbach, *Eur. J. Biochem.* **57**, 529 (1975).

[62] Covalent Immobilization of Adenylate Kinase and Acetate Kinase in a Polyacrylamide Gel: Enzymes for ATP Regeneration¹

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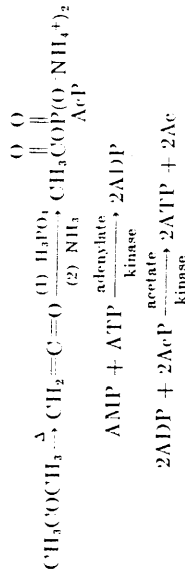
Adenylate kinase (AMP:ATP phosphotransferase, EC 2.7.4.3) and acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) form the basis for a procedure for the regeneration of ATP from AMP and or ADP, using the readily available acetyl phosphate (AcP)² as the ultimate phosphorylating agent.³ Both adenylate kinase and acetate kinase

¹ Supported by the National Science Foundation, (RANN) Grant GI 34284.

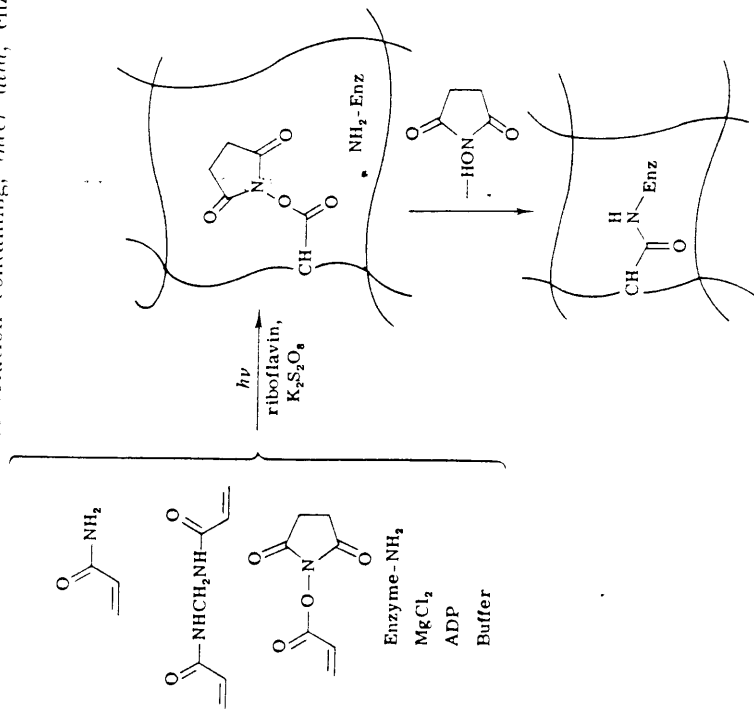
² G. M. Whitesides, M. Siegel, and P. Garrett, *J. Org. Chem.* **40**, 2516 (1975).

³ C. R. Gardner, C. K. Colton, R. S. Langer, B. K. Hamilton, M. C. Archer, and G. M. Whitesides, in "Enzyme Engineering" (E. K. Pye and L. B. Wingard, Jr., eds.), Vol. 2, p. 209, Plenum, New York, 1974; G. M. Whitesides, A. Churnny, P. Garrett, A. Lamotte, and C. K. Colton, *ibid.*, p. 217.

contain structurally important, reactive, cysteine residues close to their



active sites,¹ and their successful immobilization depends primarily on the use of a procedure that permits these residues to emerge from the immobilization unmodified. A solution containing, *inter alia*, enzyme,



Scheme 1

¹ L. Noda, in "The Enzymes" (P. Boyer, ed.), 3rd ed., Vol. 8, p. 279. Academic Press, New York, 1973; O. H. Callaghan, *Biochem. J.* **67**, 651 (1957); G. E. Schulz, M. Elzinga, F. Marx, and R. H. Schirmer, *Nature (London)* **250**, 120 (1974); L. A. Rose, M. Grumberg-Mannago, S. T. Korey, and S. Ochoa, *J. Biol. Chem.* **211**, 734 (1954).

acrylamide monomer, cross-linking agent, *N*-acryloxysuccinimide, and a photochemical free-radical initiation system is irradiated. Copolymerization of the vinyl monomers results in initial physical entrapment of the enzyme in a polyacrylamide gel containing active ester functionalities. A subsequent, slower step results in covalent coupling of the enzyme to the polymer backbone by reaction of nucleophilic groups on the enzyme (particularly lysine γ -amino groups) with these active esters (Scheme 1).

Immobilization Procedures

Enzymes. Adenylate kinase (porcine muscle) is commonly purchased as a suspension in 3.2 *M* ammonium sulfate (Sigma). Its specific activity after treatment with dithiothreitol (DTT) is 2050 IU. Activation is carried out by centrifuging 1 ml of the commercial suspension (5 mg of adenylate kinase per milliliter) for 20 min at 27,000 *g*. The supernatant is discarded and the precipitate is resuspended in degassed HEPES buffer (50 mM, pH 7.50) with final volume adjusted to 1 ml. This suspension is added to 9 ml of degassed HEPES buffer (50 mM) containing 20 mM DTT (pH 7.80); after mixing, the pH drops to 7.50. The enzymic activity is monitored during activation at 25°. It normally increases to a stable plateau in 2 hr. This solution is dialyzed using a hollow-fiber dialysis unit under argon at 4° against two 250-ml charges (1 hr each) of degassed HEPES buffer (0.05 *M*, pH 7.5) to remove ammonium sulfate. The dialyzed adenylate kinase is transferred under argon to a storage tube and kept at 4°. Typically this solution contains about 2400 U of adenylate kinase per milliliter. The enzyme is stored at 4°C.

Acetate kinase (*Escherichia coli*) is also ordinarily obtained as a suspension in 3.2 *M* ammonium sulfate (Sigma). Its specific activity after treatment with DTT is 330 IU. Acetate kinase is activated using a procedure analogous to that described for adenylate kinase except that MOPS buffer (0.05 *M*, pH 6.2) is used throughout and that the mixture of acetate kinase and DTT requires approximately 4 hr at 25° to reach constant activity. Typically the solution resulting from activation with DTT and dialysis contains about 170 U of acetate kinase per milliliter.

Reagents Solutions for Immobilization of Adenylate Kinase. Seven stock solutions (S) are required.

- S1:** HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] 0.2 *M*, pH 7.0, containing acrylamide (0.475 g/ml) and *N,N'*-methylenebisacrylamide (0.025 g/ml)
- S2:** Water, containing riboflavin (2 mg/ml as a fine suspension)

- S3:** Water, pH 7.6, containing potassium persulfate (50 mg/ml)
S4: *N*-Acryloyloxysuccinimide (5 *M* in dimethyl sulfoxide, 845 mg/ml) (preparation follows)
S5: HEPES buffer, 0.05 *M*, pH 7.5, containing dithiothreitol (10 mM), MgCl₂ (30 mM), and ADP (10 mM)
S6: HEPES buffer, 0.05 *M*, pH 7.5, containing dithiothreitol (10 mM) and ammonium sulfate (0.5 *M*)
S7: Adenylate kinase (ca 2400 U/ml) in HEPES buffer, 0.05 *M*, pH 7.5

Immobilization of Adenylate Kinase. **S5** (1.4 ml) and **S2** (100 μ l) are placed in a 5-ml beaker containing a small magnetic stirring bar; **S1** (500 μ l) and **S3** (50 μ l) are transferred into two separate 15-ml centrifuge tubes; **S4** is stored in a 1-ml test tube.⁵ Containers are capped with serum stoppers and swept with a stream of argon for 20 min to remove molecular oxygen. **S1**, **S2**, and **S5** are stored at room temperature; **S3** and **S4** are stored at 0°. **S4** is warmed briefly on a steam bath to room temperature to dissolve precipitated *N*-acryloyloxysuccinimide, and 10 μ l is added to the beaker containing the vigorously stirred mixture of **S2** and **S5**. A portion of the *N*-acryloyloxysuccinimide initially precipitates as a fine powder on addition to the aqueous solution, but redissolves in about 30 sec.⁶ When the ester has dissolved, the solution is cooled to 0° by immersion in an ice-salt bath. **S1** and **S3** are rapidly transferred to the

⁵ Transfers of less than 10 μ l are usually accomplished with a syringe; larger volumes are transferred by forced siphon through a stainless steel cannula under argon. The degassed solutions must be carefully protected from contamination by atmospheric oxygen in order for polymerization behavior, gel times, and gel properties to be reproducible. Even small quantities of oxygen introduced into these solutions by accident can result in unacceptably long gel times and poor gel physical characteristics. Techniques useful in anaerobic transfers using cannulas and serum stoppers are described in useful detail in H. C. Brown, G. W. Kramer, A. B. Levy, and M. M. Midland, in "Organic Syntheses via Boranes," Wiley, New York, 1975.

⁶ All steps after addition of **S4** to the aqueous solution must be carried out rapidly and in a reproducible way, to minimize destruction of the active ester by hydrolysis. The pseudo first-order rate constant for hydrolysis of *N*-acryloyloxysuccinimide at pH 7 at 24.5° is 0.002 min⁻¹ (half-life for hydrolysis (24.5°) \approx 220 min); at 0° it can be estimated to be approximately 0.00013 min⁻¹ (half-life for hydrolysis (0°) \approx 5500 min). These hydrolyses are accelerated to a small extent by amine-containing buffers; for a discussion of the related hydrolysis of *p*-nitrophenyl acetate, see H. J. Goren and M. Fridkin, *Eur. J. Biochem.* **41**, 263 (1974). *N*-Acryloyloxysuccinimide reacts rapidly with primary and secondary amines. Reagents containing these functional groups, and species containing ammonium ion, will compete with enzymes for *N*-hydroxysuccinimide active ester moieties, and should be avoided if possible.

beaker. As soon as the mixture in the beaker has reached 0° (about 1 min) polymerization is initiated by irradiation. In this work, irradiation was accomplished using a high-intensity ultraviolet polymerization lamp (Polysciences (atalog No. 0222) delivering 8.4 mW/cm² (measured at a distance of 18 inches) in the active region for initiation (360 nm). An aliquot of the solution of adenylate kinase (22 μ l, 54 U) is added to the polymerizing mixture from a syringe 5 sec before the gel point (in our experiments, 32 \pm 4 sec).⁷ The total time the mixture is irradiated is 60 sec.

The beaker, containing a yellow gel (ca. 2 ml), is removed from the ice bath and permitted to stand at room temperature for 10 min to allow the enzyme to couple with the polymer. The gel is then transferred to a mortar, which has been precooled to -15°, and is broken up rapidly by grinding with a pestle. Two minutes of vigorous grinding give irregular particles having an average particle size of approximately 20-30 μ m. These gel particles are immediately washed into a 50-ml centrifuge tube using a total of about 10 ml of **S6**. The tube is capped and stirred for 15 min at room temperature. The gel particles are separated by centrifugation and resuspended in a second 10-ml aliquot of **S6**. The washing procedure is repeated twice, or until no enzymic activity is detected in the washes. Assay of the gel indicates the presence of 27 U of adenylate kinase in the gel (50%). With experience, higher yields can be obtained.

Reagent Solutions for Immobilization of Acetate Kinase

- S1:** MOPS buffer (4-morpholinepropanesulfonic acid), 0.2 *M*, pH 6.2, containing acrylamide (0.475 g/ml) and *N,N'*-methylene-bisacrylamide (0.025 g/ml)
S2: Water, containing riboflavin (4 mg/ml as a suspension)
S3: Water, pH 7.6, containing potassium persulfate (50 mg/ml)
S4: *N*-Acryloyloxysuccinimide (5 *M* in dimethyl sulfoxide) (preparation follows)
S5: MOPS buffer, 0.05 *M*, pH 6.2, containing MgCl₂ (30 mM), ADP (5 mM), acetyl phosphate (5 mM)
S6: HEPES buffer, pH 7.5, 0.05 *M*, containing dithiothreitol (10 mM), and ammonium sulfate (0.5 *M*)
S7: Acetate kinase (ca. 170 U/ml) in MOPS buffer, 0.05 *M*, pH 6.2, containing DTT (2 mM)

⁷ The gel point is defined as the point at which the polymerization has proceeded to the stage at which the stirring bar stops turning. With care it is reproducible to \pm 10% (i.e., ca. \pm 3-4 sec).

Immobilization of Acetate Kinase. The sequence of steps is analogous to that described for the immobilization of adenylate kinase. Each solution is degassed by sweeping for 20 min with a stream of argon, and stored under argon. *S5* (1.4 ml), *S2* (50 μ l), and *S1* (500 μ l) are added to a capped 5-ml beaker. *S4* (10 μ l) is added, and, as soon as the *N*-acryloyloxysuccinimide has dissolved, the solution is rapidly transferred to an ice-salt bath and cooled to 0°. *S3* (50 μ l) is added by syringe, and the resulting solution is stirred for 2 min at 0°. Polymerization is initiated by irradiation, and the enzyme-containing solution (*S7*, 30 μ l, 5.40 U) is injected into the solution 5 sec before the gel point. Irradiation is continued for 25 sec. The beaker containing the resulting gel (about 2 ml) is removed from the ice bath and allowed to stand at room temperature for 10 min. The gel is broken up by grinding in a mortar precooled to -15°, transferred to a centrifuge tube with about 10 ml of *S6*, and washed with 10-ml aliquots of *S6*. Assay of the gel typically indicates activity of 1.14 U (21%).

Assay Procedures

Adenylate kinase is assayed in homogeneous solution by coupling production of ATP from ADP to the production of NADPH, by first phosphorylating glucose with this ATP using hexokinase, and then oxidizing the resulting glucose 6-phosphate using glucose 6-phosphate dehydrogenase (G-6-PDH) and NADP⁺. The following stock solutions are required.

Solution I: 0.2 M Tris-HCl buffer, pH 7.5, containing 5 mM glucose, 30 mM MgCl₂, hexokinase (2500 U/liter); and G-6-PDH (1250 U/liter). The buffer, glucose, and MgCl₂ are mixed, the pH is adjusted to 7.5, and the enzymes are added. The resulting solution is stable at 0° for several months.

Solution II: ADP (disodium salt), 0.5 M in water, pH 6.8. This solution is stable at <5° for several weeks.

Solution III: 62.5 mM NADP⁺ (sodium salt) in water, no pH adjustment. This solution is also usable after storage at <5° for several weeks.

In a typical assay, 1 ml of solution I is mixed with 20 μ l of solution II and 10 μ l of solution III. The mixture is equilibrated for 3 min at 25° to destroy ATP present as an impurity in the ADP. An aliquot of the solution to be assayed is then added; the size of this aliquot is adjusted so that the final solution contains less than 0.01 U per milliliter of adenylate kinase. The solution is mixed and poured into a 1-cm quartz cuvette, and

the rate of appearance of NADPH is followed spectrophotometrically at 340 nm (30°).

This assay is a compromise between accuracy and economy. The Michaelis constant for binding of ADP to porcine adenylate kinase is $K_{m,ADP} = 1.58$ mM.^{1,2} In these solutions, the concentration of ADP is 10 mM; this concentration gives rates of approximately 0.9 V_{max} . Values closer to V_{max} would be obtainable at higher ADP concentrations, but at greater expense. Enzyme concentrations for adenylate kinase, hexokinase, and G-6-PDH are chosen so that the adenylate kinase-catalyzed reaction is overall rate-limiting. Experimentally, the minimum ratio of hexokinase to adenylate kinase activities for which adenylate kinase activity is rate-determining is 100; in this assay, hexokinase/adenylate kinase = 250, to provide for losses on storage. The optimum ratio of activities for hexokinase and G-6-PDH is 2. For a sample aliquot such that the final activity of adenylate kinase in the assay solution is less than 0.01 U/ml, less than 1% of the total ADP is converted to ATP per minute, and the change in absorbance is linear with time. The assay is reproducible to $\pm 5\%$, and its accuracy is approximately -10% .

Immobilized adenylate kinase is assayed using the same procedure, with care taken that the enzyme-containing polyacrylamide particles are small (<25 μ m average). The washed, enzyme-containing gel particles, suspended in a known volume of HEPES buffer from their preparation, are stirred vigorously. An aliquot of this suspension (10 μ l, about 0.005 U) is withdrawn using an Eppendorf pipette, and used for the assay. If the gel particles are small and the stirring vigorous, this aliquot is representative of the total suspension. This aliquot of suspended gel is added to the assay solution, mixed, and poured into the spectrophotometer cuvette, as described for the homogeneous assay. The size and density of the particles are such that they do not settle appreciably during the time of the assay. Diffraction does not interfere with the assay both because the size and concentration of the particles are small, and because they are transparent.

Acetate kinase is assayed using a modification of the adenylate kinase assay. A fourth stock solution is prepared (Solution IV: acetyl phosphate in water, 0.5 M, without pH adjustment, usable for several weeks after storage at 0°).³ Solutions I, II, and III are mixed and incubated as described above, and 10 μ l of Solution IV is added. An aliquot of the acetate kinase-containing solution is added, such that the final activity in the assay solution is less than 0.01 U/ml. This procedure gives

³ P. DeWeer and A. G. Lowe, *J. Biol. Chem.* **248**, 2829 (1973) and references cited therein.

[AcP] \approx 5 mM in the assay solution, in adequate excess over $K_{M,AcP}$ = 0.34 mM.⁹

*N-Acryloyloxysuccinimide.*¹⁰ *N*-Hydroxysuccinimide (11.5 g; 0.10 mole) and sodium bicarbonate (16.8 g; 0.20 mole) are dissolved in 200 ml of water, and the solution is stirred vigorously in an ice bath at 0°. Acryloyl chloride (9.1 g; 0.10 mole) is added in drops over a 10-min interval, and subsequent stirring for 3 min at 0° leaves no odor of acryloyl chloride. Cooling the flask for 5 min in a -5° ice-salt bath completes the precipitation of the acrylate ester. The resulting white precipitate is filtered with suction, washed with 50 ml of ice-cold water, and dried at reduced pressure (ca. 30 mm). The product is dissolved in 40 ml of ethyl acetate, dried with a small amount of MgSO₄, and filtered into a clean, dry flask. The remaining precipitate is extracted with two 20-ml portions of ethyl acetate and filtered into the flask to give a clear, colorless solution. Hot hexane (150 ml) is added, and the solution is warmed gently until it is clear. Slow cooling to 0° gives a white precipitate, which is filtered and washed with a small amount of hexane, yielding 8.2 g of thin plates, mp 60°-62°. Concentration of the mother liquor affords an additional 3.0 g of product (66% combined yield). A further recrystallization from ethyl acetate:hexane (20:80) gave 8.7 g (52%) of large, thin, colorless plates, mp. 69-70°, having NMR (CDCl₃) δ 2.87 (s, 4H), 6.0-7.0 (mult., 3H).

Discussion

Polyacrylamide has many advantages as a matrix for enzyme immobilization (see also chapters [12] [15]). It is inexpensive and resistant to biological attack. Its low mechanical strength can be increased, and other physical properties modified, by varying the extent of cross-linking,¹¹ or by incorporating other, more hydrophobic, vinyl monomers. It can be formed into beads by suspension polymerization¹² (see also chapter [5], [6] and [14] or rendered susceptible to recovery by magnetic

⁹ C. A. Janson and W. W. Cleland, *J. Biol. Chem.*, **249**, 2567 (1974).

¹⁰ This preparation was developed by Mr. Michael Wilson. The properties of the *N*-acryloyloxysuccinimide prepared in this procedure are similar to those reported by R. L. Schnaar and C. Y. Lee, *Biochemistry* **1975**, 1535 (1975).

¹¹ D. Rodbard and A. Crambach, *Anal. Biochem.* **40**, 95 (1971); A. Crambach and D. Rodbard, *Science* **172**, 440 (1971).

¹² K. E. J. Barrett, in "Dispersion Polymerization in Organic Media," Wiley, New York, 1975.

filtration by including colloidal magnetite in the gel.^{3,13} The procedure illustrated here is designed to circumvent disadvantages sometimes encountered in other polyacrylamide gel immobilization procedures. Incorporation of active ester groups into the polymer permits covalent coupling of enzyme to the gel and prevents the slow leakage of enzyme from gel characteristic of noncovalent physical entrapment. Since the gel is formed with enzyme initially uniformly distributed throughout, the low immobilization yields sometimes encountered on reaction of a preformed, reactive gel with enzyme can, in some instances, be avoided.

Although this procedure is versatile, it requires careful attention to certain details to be successful. The two major concerns are protection of enzyme from deactivation during polymerization, and choice of the optimum concentration of active ester functionalities in the gel. Studies of the stability of adenylate kinase and acetate kinase in polymerizing polyacrylamide have established that four reactions are responsible for most of the deactivation observed¹⁴: first, Michael addition (1,4-conjugate addition) of enzyme -SH and -NH₂ groups to acrylamide; second, autooxidation of -SH groups by molecular oxygen, in a reaction strongly catalyzed by transition metal ions; third, oxidation of the enzyme by singlet oxygen (¹O₂), generated by energy transfer from excited riboflavin to ³O₂ during irradiation; fourth, attack on enzyme by SO₂ and other free radicals. These reactions are controlled by excluding oxygen, by carrying out reactions at 0° and relatively low pH, by minimizing contact of the enzyme with acrylamide and *N*-acryloyloxysuccinimide, and by protecting the enzyme active site with saturating concentrations of substrate. The only one of these considerations whose experimental implementation is not obvious is the third; it is an important initial part of the procedure for developing immobilized conditions for a new enzyme, or for reproducing an established procedure, to establish a reproducible polymerization procedure and to establish a gel point (\pm 10%) in the absence of enzyme. The enzyme can then be immobilized, using this procedure, by injecting enzyme into the polymerizing mixture just before (about 5 sec) this previously established gel point. This procedure effectively eliminates deactivation of the enzyme by reaction with the acrylic monomers. Experimentally, the gel point is not significantly influenced by the presence of the enzyme in solution.

¹³ A. Lamotte, O. Adaksteinsson, R. F. Baddour, C. K. Collon, and G. M. Whitesides, unpublished results; A. Lamotte, Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, Massachusetts, 1975.

The presence of DTT or other mercaptans in the polymerization mixture helps to protect the enzyme against attack by radicals, but increases the gel times and requires longer irradiation. Certain enzymes are immobilized in higher yield in the presence of DTT, certain others in its absence.

For a new enzyme, it is also necessary to determine an optimum *N*-acryloylsuccinimide concentration for a given set of conditions. Concentrations either higher or lower than an optimal concentration lead to reduced immobilization yields for adenylate kinase and acetate kinase (Fig. 1). Certain enzymes (e.g., horseradish peroxidase) are, however, relatively insensitive to high concentrations of active ester, and plots

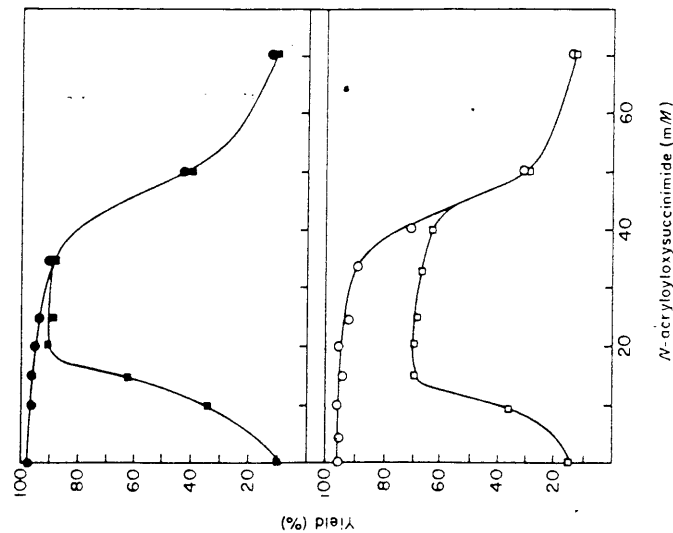


FIG. 1. Immobilized (●) and total recovered yields (○) for adenylate kinase as a function of the starting concentration of *N*-acryloylsuccinimide. The latter yield represents the sum of the enzymic activity immobilized in the gel and that free in the wash solutions. Reaction conditions are those described in the experimental procedure. Analogous data for acetate kinase (□, ○) are also summarized. The maximum yields obtained in these experiments are higher than those described in the experimental procedure, and represent optimized data collected by an experienced individual. The lower yields described in the procedure are those expected with only routine experience in this technique.

analogous to Fig. 1 for these enzymes show either a plateau or a broad maximum instead of the relatively sharp maximum observed for adenylate kinase and acetate kinase.

This procedure is the most efficient one presently available for regeneration of ATP from ADP or AMP.¹¹ The specific activities of the acetate kinase and adenylate kinase preparations used here (about 300 and 2000 IU, respectively) are such that, with the immobilization yields reported, a reactor utilizing the immobilized enzyme preparations obtained starting from 30 mg of acetate kinase and 2 mg of adenylate kinase should in principle be capable of regenerating about 2 moles of ATP (1 kg) from AMP and acetyl phosphate per day. The actual productivity of such a reactor depends on the success with which its design permits the enzymes to operate close to V_{max} .

¹¹ An alternative procedure for transformation of ADP to ATP depends on carbonyl phosphate ($H_2NCO_2PO_3$) as phosphate donor, and carbamylphosphokinase as catalyst. Although carbamyl phosphate is readily prepared *in situ*, the equilibrium constant for phosphate transfer to ADP is less favorable than that for acetyl phosphate. See D. L. Marshall, *Biotechnol. Bioeng.*, **15**, 447 (1973).