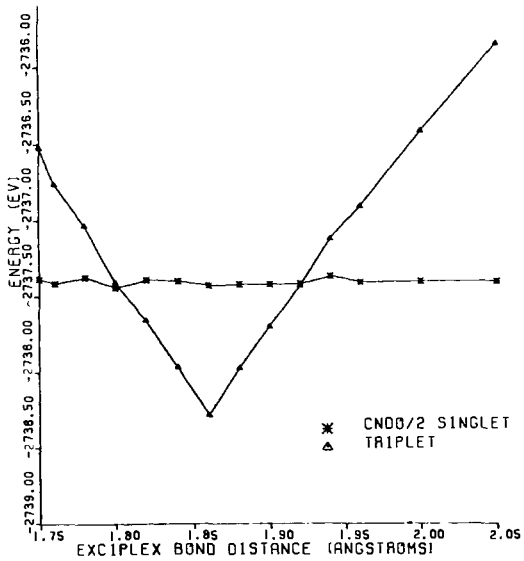


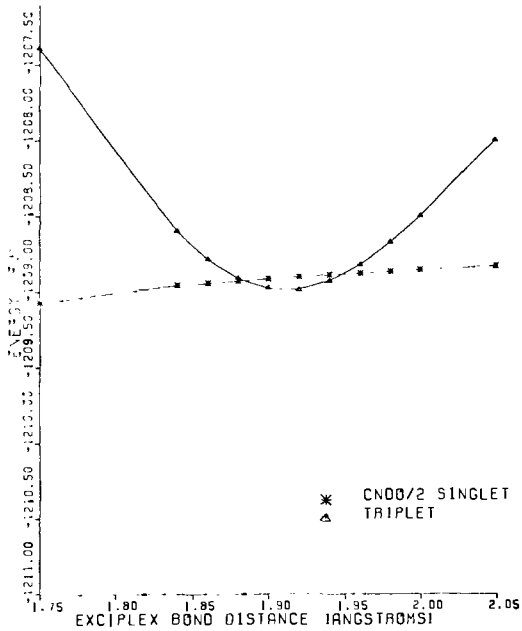
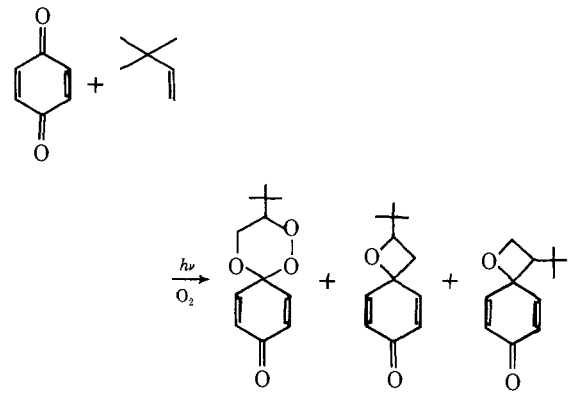


Figure 3. Energy profiles for the lowest singlet and lowest triplet states in a trough cross section for geometry **5:** (a, top) *p*-benzoquinone-ethylene; (b, bottom) formaldehyde-ethylene.

formed from quinones, aldehydes, and presumably other types of carbonyl compounds has a number of intriguing photochemical implications. The singlet-triplet crossings associated with these complexes provide an explanation for the quenching $^3n,\pi^*$ ketones by olefins with high energy triplet states;[2a,c,d,g] the existence of two very similar end-on complexes related to **5** and **6** would account for Singer's observation of what he has termed n and π exciplexes.[2g] In our own work[4,5] the trapping of a *p*-benzoquinone CT complex by oxygen would account for the product distributions in Scheme I. Here the lack of variation of the oxetane isomer ratio (**8**/**9**) with oxygen pressure implies that the oxygen is not trapping the more stable preoxetane biradical, but instead is trapping a species that occurs before partitioning to the two preoxetane biradicals.

Finally it should be noted that carbonyl-olefin excited state complexes may exist at separations of greater than 2.00 Å.[1] While we are currently searching for these long range complexes, we feel that short range complexes such as the CT

Scheme I



	7	8	9	8/9
No O_2	0	25%	75%	0.25
11 atm O_2	43%	13%	44%	0.29

complexes described here will have the greater influence upon excited-state chemistry.

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R. Marshall Wilson,* Russell Outcalt, H. H. Jaffé
Department of Chemistry, University of Cincinnati
Cincinnati, Ohio 45221
Received July 28, 1977

Immobilization of Synthetically Useful Enzymes by Condensation Polymerization

Sir:

We wish to describe a new procedure for the immobilization of enzymes in cross-linked organic polymer gels. This procedure rivals or surpasses in its operational simplicity and generality methods presently widely used (BrCN-agarose, glutaraldehyde, functionalized glass, preformed activated organic polymer gels),[1] and has proved especially valuable in immobilization of reliatively delicate enzymes of interest for enzyme-catalyzed organic synthesis.[2]

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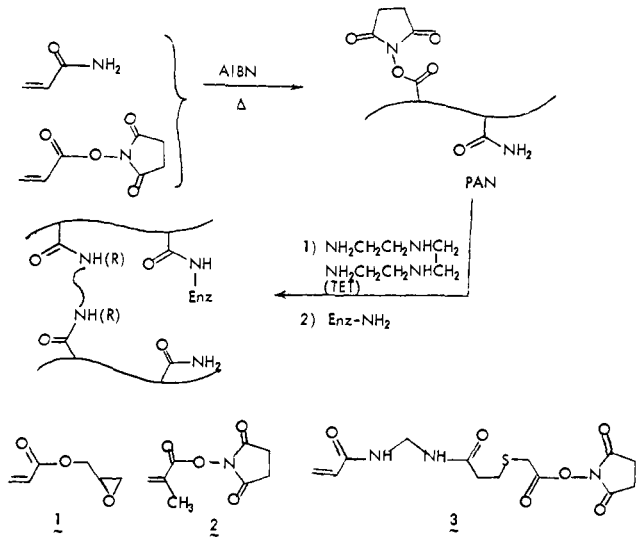
Table I. Yields (Percent) of Immobilized Enzymes and Specific Activities of Enzyme-Containing Gels

E.C. no.	Enzyme	Substrate and/or cofactor (concn, mM)[a]	Yield, %	Specific activity, U/mL of wet gel
3.4.4.4	Trypsin	Benzoyl arginine ethyl ester (1.0)	53	6300[b]
4.1.2.13	Aldolase	Fructose-1,6-P_2 (2.0)	40	15
3.5.4.4	Adenosine deaminase	Adenosine (0.8)	37	2.0
3.2.1.26	Invertase	Sucrose (30)	63[c]	85
5.3.1.9	Phosphoglucose isomerase	Glucose-6-P (6.0), Fructose-6-P (1.5)	56	39
1.11.1.7	Peroxidase	H_2O_2 (4.5)	14[c]	38[d]
1.11.1.17	Lactoperoxidase	H_2O_2 (4.5)	27[c]	1.2[d]
1.1.1.1	Alcohol dehydrogenase, yeast	EtOH (500), NAD^+ (1.0)	22	81
1.1.1.1	Alcohol dehydrogenase, liver	EtOH (850), NAD^+ (3.0)	63	1.0
1.1.2.3	L-Lactate dehydrogenase	Pyruvate (8.5), NADH (1.0)	51	13
1.1.1.49	Glucose-6-phosphate dehydrogenase	Glucose-6-P (6.3), $NADP^+$ (0.6)	35	66
2.7.2.1	Acetate kinase	Acetyl-P (12.5), ADP (20)	55	67
2.7.4.3	Adenylate kinase	ADP (20)	46	92
2.7.1.1	Hexokinase	Glucose (25), ADP (10)	50	105[e]
2.7.1.30	Glycerol kinase	Glycerol (20), ATP (4.0), ADP (12)	70[f]	1.2[g]
2.7.1.40	Pyruvate kinase	PEP (6.7), ADP (25)	46	10
2.7.3.2	Creatine kinase	ADP (10), creatine (40), KNO_3 (100)	45[h]	135
2.7.5.1	Phosphoglucomutase	Glucose-6-P (5.0), glucose-1-P (1.0), glucose-1.6-P_2 (0.01)	67	12
1.2.3.2	Xanthine oxidase	Hypoxanthine (2.2)	80	0.15
2.7.1.20	Adenosine kinase	Adenosine (0.5), ATP (4.5)	42	0.4

[a] Materials present during the immobilizations to protect the enzyme active site. [b] Benzoyl arginine ethyl ester as substrate. [c] The procedure used in immobilizing these enzymes is a modification of that described in the text. The enzyme was added to a solution of PAN in Hepes buffer, stirred for 3–6 min, and then treated with TET and stirred until gel formation had occurred. These enzymes are all glycoproteins and appear to have low reactivity toward the *N*-hydroxysuccinimide active esters of PAN. [d] *o*-Diansidine as substrate. [e] Assayed at pH 6.9. [f] Carried out by V. Rios-Mercadillo. [g] Assayed at pH 7.6. [h] Carried out by Y.-S. Shih.

Scheme I. PAN: Preparation and Use for Enzyme Immobilization



A non-cross-linked, water-soluble polymer bearing active ester groups was prepared by heating acrylamide (13.5 g, 0.19 mol), *N*-acryloxysuccinimide (1.5 g, 8.9 mmol), and azobisisobutyronitrile (80 mg) under argon in THF (distilled from $Ph_2CO^{2-}Na_2^+$ under argon) at 50 ° C for 24 h. The resulting poly(acrylamide-*co*-*N*-acryloxysuccinimide) (PAN) precipitated as a while solid (14.5 g, 96%). It was diluted with 100 mL of THF, separated by centrifugation, resuspended in 100 mL of THF, and recentrifuged. This process was repeated four times, and the final, monomer-free polymer dried in vacuum. This material contained ~500 μmol g^{-1} of active ester groups.[3,4] PAN was stable for longer than 8 months at room temperature under air in a desiccator.

Enzyme immobilization and gel formation were accomplished by the simultaneous reaction of PAN with the enzyme and an α,ω-diamine (as a cross-linking agent) (Scheme I). In a representative immobilization, 200 mg (100 μmol of active ester groups) of PAN was dissolved at 25 °C in 800 μL of 0.3 M Hepes buffer (pH 7.5, containing 30 mM $MgCl_2$, 25 mM glucose, and 10 mM ADP), in a 5-mL glass vial equipped with a small magnetic stirring bar. After 2 min of vigorous stirring, the polymer dissolved completely. Dithiothreitol (DTT, 10 μL of 0.5 M aqueous solution) and triethylenetetramine (TET) (85 μL of 0.5 M solution, 85 μmol of primary amino groups) were added. Sixty seconds later, 200 μL of a solution containing hexokinase was added (210 U, μmol min^{-1} mg^{-1}). In <2 min, the solution set to a transparent, mechanically resilient gel. The gel was allowed to stand for 1 h to complete the coupling,[5] and transferred to a small mortar containing 5 mL of a solution of L-lysine (10 mM in 50 mM Hepes buffer, 10 mM $MgCl_2$, pH 7.5). Brief grinding with a pestle reduced the gel to irregular particles having 30–100-μm diameter. The gel suspension was diluted with 20 mL of the lysine/Hepes solution, transferred into a centrifuge tube, stirred magnetically for 15 min, and separated by centrifugation. This washing procedure was repeated once with the same volume of the buffer containg no lysine. The activity of hexokinase in the gel was 107 U (51%), and 74 U (35%) was detected in the combined washes.[6]

Several features of this procedure deserve comment. First, addition of the enzyme to the solution of PAN after addition of the TET and close to the gel point of the reaction mixture minimized enzyme deactivation. For example, in experiments where hexokinase had been added to the PAN solution first and the TET and DTT solutions were introduced into the reaction mixture 2 min after addition of the enzyme, the enzyme immobilization yield dropped to 30%. Second, the presence of substrate and cofactor in the reaction mixture at concentrations above their Michaelis constants ensured that the majority of enzyme active sites were occupied during the immobilization, and contributed to high immobilization yields; only 12% of immobilized hexokinase was obtained when glucose and ADP were omitted from the polymerizing PAN solution. Third, the DTT added to protect the cysteines of hexokinase is reac-

tive toward *N*-hydroxysuccinimide groups and was used in low concentration. Fourth, the choice of a lysine solution to destroy residual active ester groups at the conclusion of the reaction was arbitrary; ammonium sulfate has also been used successfully. If, however, the residual active ester groups were not destroyed. the yield of active, immobilized enzyme activity decreased by ~5%. Fifth, a number of α,ω-di- (and poly-) amines were surveyed for utility as cross-linking agents. TET was chosen because it is readily available, and because the resulting gel has good physical properties.[7] Sixth, monomers containing other coupling groups (**1–3**) have been substituted for *N*-acryloxysuccinimide in the preparation of the starting, reactive polymer. The polymers derived from these monomers show different reactivity than PAN, and, although the characteristics of these materials may be valuable in particular instances, PAN presently provides the most generally useful combination of ease of preparation and reactivity.

Yields and specific enzyme activities of the gels obtained on immobilization of a number of enzymes using PAN and TET are summarized in Table I: Each entry represents the average of at least two experiments, and reproducibility was good (±5%). Unless otherwise noted, the procedure used for each of these immobilizations was that described for hexokinase, modified only by the substitution of substrates and cofactors appropriate for protection of the active site of the particular enzyme considered. The quantities of protein used in these immobilizations varied between 0.15 mg and 20 mg/g of PAN; enzymes with high specific activity were ordinarily immobilzed using <5 mg of protein/g of PAN. Operations involving oxygen-sensitive enzymes (especially adenylate, acetate, and creatine kinase) were carried out under argon; exclusion of dioxygen was not important in procedures with other enzymes.

The procedure summarized in Scheme I has a number of features which recommend it for immobilizations of enzymes (especially those to be used in organic synthetic procedures) and other biochemicals. (1) It is simple and general. PAN is easily prepared and stable to storage. The manipulation involved in gel formation are straightforward. The enzymes are not exposed to deactivating reagents or reaction conditions. The procedure is especially useful for small quantities and low concentrations of enzymes, and should be directly applicable to the immobilization of whole cells and organelles. (2) The amide-forming reactions that provide the basis for gel production and enzyme coupling are chemically well-defined and susceptible to rational modification and control. These amide linkages are hydrolytically stable under conditions in which the gels would be used. (3) The organic polymer gel is a useful matrix, and amenable to a range of types of modification. It is not biodegradable. Its hydrophilicity and charge can be controlled by inclusion of appropriate monomers in the initial copolymerization, or by reaction of PAN with nucleophilic modifying groups. Covalent incorporation of the enzyme into the gel provides some protection against proteases. Formation of gel on supporting structures (porous glass, filter paper, the inner wall of glass tubing) is accomplished readily, and the resulting composite materials have useful physical characteristics for applications in large-volume enzymatic reactors. The gel can be rendered susceptible to magnetic filtration by including a ferrofluid in the gel formation step.[4]

Acknowledgments. The research was supported by the National Science Foundation by Grant GI 34284 and through grants to the M.I,T. Materials Research Laboratory. Certain of the immobilizations reported in Table I were carried out by Y.-S. Shih and V. Rios-Mercadillo. R. L. Baughn was an NIH Postdoctoral trainee (Grant 1 T32 CA 09112). Ö. Adalsteinsson acknowledges support from the Icelandic Science Foundation.

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(6) Suspensions of particles of immobilized enzymes were assayed by standard procedures for soluble enzymes (H. U. Bergmeyer, Ed., "Methods of Enzymatic Analysis", Verlag Chemie, Weinheim/Bergstr., Academic Press, New York and London, 1974). Because aqueous solutions of gel particles were transparent, these particles did not interfere with spectrophotometric analyses. Assay conditions were chosen to avoid pore diffusional effects on the assays, but the assay procedures were not explicitly demonstrated to be independent of diffusion. The yield of an immobilization is defined as yield = 100 × (enzymatic activity of enzyme-containing gel particles)/(activity of starting soluble enzyme).
(7) Gels cross linked with other diamines are useful in special applications. For example, a gel formed using cystamine hydrochloride ($(NH_2CH_2CH_2S)_2$) in place of TET dissolves readily in 20–50 mM DTT. Comparison of the activity of a suspension of hexokinase immobilized in this gel (U mL^{-1}) with that of the solution obtained after its reduction shows no significant change in total enzymatic activity, and demonstrates directly that pore diffusional limitations are not important in this system. Related gels might also find applications in PAGE; cf. J. N. Hansen, *Anal. Biochem.*, **76**, 37 (1976).

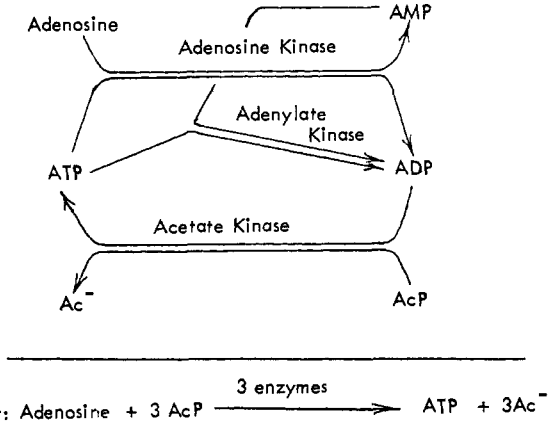
Alfred Pollak, Richard L. Baughn
Örn Adalsteinsson, George M. Whitesides*
Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
Received April 22, 1977

Large-Scale Enzyme-Catalyzed Synthesis of ATP from Adenosine and Acetyl Phosphate. Regeneration of ATP from AMP[1]

Sir:

In previous reports, we have described large-scale enzyme-catalyzed organic syntheses requiring the enzymatic regeneration of ATP from ADP and acetyl phosphate.[2,3] Many important biosynthetic reactions transform ATP to AMP rather than ADP; a few generate adenosine.[4] Here we summarize the operation of a three-enzyme sequence which converts adenosine to ATP (Scheme I): In this scheme, AMP and ADP are involved both as intermediates in the phosphorylation of adenosine to ATP, and as parts of the catalytic cofactor utilization cycle which consumes and regenerates ATP. This

Scheme I. Conversion of Adenosine to ATP



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