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.

References and Notes

(1) K. Mosbach, Ed., "Methods in Enzymology: Immobilized Enzymes", Vol. XLIV, Academic Press, New York, N.Y., 1976, and references cited therein.
(2) A. Pollak, R. L. Baughn, and G. M. Whitesides, *J. Am. Chem. Soc.*, **99**, 2366 (1977).
(3) Active ester content was estimated by allowing a sample of PAN to react with an excess of ethyl amine in water (pH 7.5, Hepes) for 1 h at 25 °C and measuring the absorbance of the *N*-hydroxysuccinimidyl anion at 259 nm (ϵ 8600 M^{-1} cm^{-1}).[4]
(4) O. Adalsteinsson, A. Lamotte, R. F. Baddour, C. K. Colton, and G. M. Whitesides, *J. Mol. Catal.*, in press.
(5) Preliminary studies have established that the release of *N*-hydroxysuccinimide from PAN in Hepes buffer, pH 7.5, 25 °C, containing 8 mM hexamethylenediamine and 1.5 mM DTT follows pseudo-first-order kinetics, $k_{obsd} = 2.3 \times 10^{-3}$ s^{-1} ($\tau_{1/2} \simeq 5$ min). The active ester groups of PAN appear to react more slowly than those of low molecular weight *N*-hydroxysuccinimide active esters.[4]
(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).

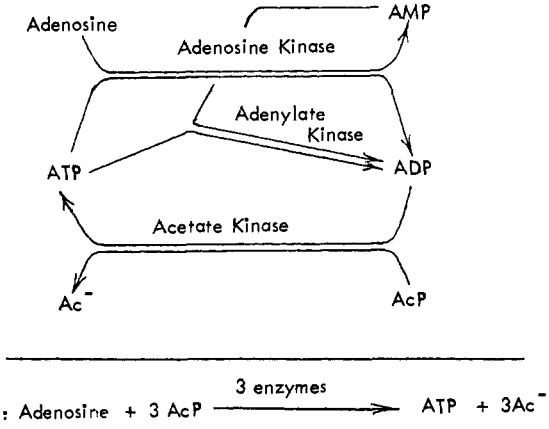
Alfred Pollak, Richard L. Baughn
Örn Adalsteinsson, George M. Whitesides*
Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
Received April 22, 1977

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



0002-7863/78/1500-0304$01.00/0

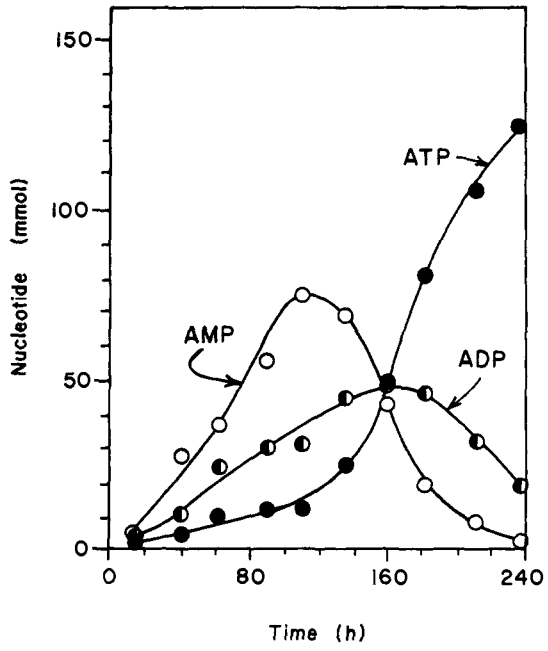


Figure 1. Quantities (millimoles) of nucleosides present during conversion of adenosine to ATP.

sequence establishes the potential of organic syntheses based on ATP regeneration from AMP and adenosine, and illustrates a route for the preparation of nucleotides from nucleosides.

A typical reaction was run at room temperature in a 5-L flask equipped with a pH electrode and magnetic stirring bar. To 1 L of deoxygenated, doubly distilled water was added ATP, ADP, and AMP (50 mg each as the sodium salts, ~0.1 mmol), adenosine (40 g, 150 mmol, not completely soluble), magnesium acetate (4 g, 19 mmol), and dithiothreitol (DTT, 300 mg, 1.9 mmol). Adenosine kinase (E.C. 2.7.1.20, 40 U), adenylate kinase (E.C. 2.7.4.3, 550 U), and acetate kinase (E.C. 2.7.2.1, 180 U), immobilized in cross-linked polyacrylamide gel particles, were added as a suspension in 550 mL of water.[5-9] An argon atmosphere was maintained in the flask, and the pH was kept between 6.7 and 6.9 by the pH stat controlled addition of 2 M sodium carbonate. Diammonium acetyl phosphate[10] (0.5 M) was pumped in continuously at a rate of 0.1 mol per day. After the first few hours of operation, concentrations of substrates exceeded the Michaelis constants of the enzymes.[11] Additional DTT[12] and magnesium acetate[13] were added during the course of the reaction. An additional 500 U of immobilized acetate kinase was added after 120 h of operation, because the rate of formation of ATP was, at this point, limited by the activity of this enzyme. The quantities of AMP, ADP, and ATP in solution during the course of the reaction are summarized in Figure 1; After 239 h, 125 mmol of ATP, 20 mmol of ADP, and 3 mmol of AMP were present;[14] the final concentration of ATP was 30 mM. The yield of phosphorylated adenosine derivatives was 98% based on adenosine and 38% based on acetyl phosphate added. The equilibrium constants for the reactions in Scheme I are such that conversion of adenosine to ATP should have been complete, and conversions in smaller scale reactions were as high as 94%.[9] The adenosine kinase used in the procedure described was, however, contaminated with ATPase activity, and the observed final concentrations represent a competition between the rates of ATP formation and ATP hydrolysis.

The enzyme-containing gel and a white precipitate (primarily magnesium phosphate) were separated from the solution by centrifugation. ATP was extracted into an organic phase consisting of 5% w:w octadecylamine in 1-pentanol[15] by mixing the phases thoroughly and adjusting the pH of the aqueous phase to 7.4 with glacial acetic acid. The phases were separated, and ATP was reextracted into an aqueous solution whose pH was adjusted to 11.5 with NaOH. This aqueous solution was neutralized with acetic acid, and the dibarium salt of ATP was precipitated by addition of slightly >2 equiv (77 g, 300 mmol) of barium acetate. Isolation[16] of the precipitate yielded 60.9 g of white powder whose ATP content by enzymatic assay[17] corresponded to 77% $Ba_2ATP{\cdot}4H_2O$ (54 mmol). This value represents an isolated yield for ATP of 36% based on adenosine and 15% based on AcP. The activities of recovered enzyme correspond to 93% of the adenosine kinase and 75% of the immobilized acetate kinase added. Adenylate kinase recovery was also good but was not determined accurately.

This synthesis establishes three points pertinent to the use of enzymatic catalysis in organic synthesis. First, the enzymatic regeneration of ATP from AMP proceeds smoothly using acetyl phosphate as the ultimate phosphate donor; enzyme-catalyzed reactions which convert cofactor ATP to AMP can thus be used for practical-scale syntheses. Second, manipulation of a coupled system of three enzymes as part of a synthetic procedure presents no special problems. Third, enzymatic procedures should now be considered as an alternative to conventional chemical or fermentation methods for the large-scale synthesis of ATP and other nucleotides from nucleosides.[18]

References and Notes

(1) Supported by the National Science Foundation, Grant GI 34284. Ö.A. acknowledges partial financial support from the Icelandic Science Foundation. R.L.B. was a postdoctoral trainee under NIH Training Grant 5 T32 CA 09112 CT.
(2) A. Pollak, R. L. Baugh, and G. M. Whitesides, *J. Am. Chem. Soc.*, **99**, 2366 (1977).
(3) Y.-S. Shih and G. M. Whitesides, *J. Org. Chem.*, **42**, 4165 (1977).
(4) "The Enzymes", Vol. 8, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1973: E. R. Stadtman, Chapter 1: S. H. Mudd, Chapter 4. G. L. Cantoni, *Ann. Rev. Biochem.*, **44**, 435 (1975).
(5) Adenosine kinase was partially purified from brewer's yeast (Sigma) according to a literature scheme.[6] The yeast extract was subjected to ammonium sulfate fractionation, DEAE-Sephadex chromatography, and Sephadex G-75-120 chromatography to yield a preparation of specific activity 0.1 U/mg (1 U = 1 μmol/min·mg). Acetate kinase and adenylate kinase (both from Sigma) had specific activities of 220 and 2250 U/mg, respectively, following activation with DTT.
(6) T. K. Leibach, G. I. Speiss, T. J. Neudecker, G. Peschke, G. Puchwein, and G. R. Hartmann, *Z. Physiol. Chem.*, **352**, 228 (1971).
(7) Enzymes were immobilized using the procedure described in A. Pollak, R. L. Baughn, Ö. Adalsteinsson, and G. M. Whitesides, *J. Am. Chem. Soc.*, preceding paper in this issue. The immobilization yields and gel volumes employed were as follows: adenosine kinase (25%, 300 mL), adenylate kinase (40%, 25 mL), and acetate kinase (40%, 25 mL).
(8) The enzymes must be mixed, rather than used separately as catalysts in sequential reactions. Adenosine kinase and acetate kinase must be present simultaneously to catalyze the phosphorylation of adenosine with AcP as the ultimate phosphate donor in a reaction regenerating ATP. The conversion of ADP to ATP by adenosine kinase is driven by the high equilibrium constant for the acetate kinase catalyzed phosphorylation of AMP.[9]
(9) R. Cuputto in "The Enzymes", Vol. 6, 2nd ed, P. D. Boyer, H. Lardy, K. Myrback, Ed., Academic Press, New York, N.Y., 1962, p 133; R. S. Langer, C. R. Gardner, B. K. Hamilton, and C. K. Colton, *AIChE J.*, **23**, 1 (1977).
(10) G. M. Whitesides, M. Siegel, and P. Garrett, *J. Org. Chem.*, **40**, 2516, (1975). The material used was 82–89% pure, with ammonium acetate, ammonium phosphate, and acetamide as the principal impurities. The AcP solution was kept at 4 °C before addition to minimize hydrolysis
(11) For acetate kinase, K_{AcP} = 0.34 mM and K_{ADP} = 1.1 mM (C. A. Janson and W. W. Cleland, *J. Biol. Chem.*, **249**, 2567 (1974)). For adenylate kinase, K_{ADP} = 1.58 mM, K_{AMP} = 0.5 mM, and K_{ATP} = 0.3 mM (P. DeWeer and A. G. Lowe, *J. Biol. Chem.*, **248**, 2829 (1973); J. M. Blair, *Eur. J. Biochem.*, **13**, 384 (1970)). For adenosine kinase, K_A = 0.001 mM (T. E. Barman, "Enzyme Handbook", Vol. I, Springer-Verlag, New York, N.Y., 1969, p 394).
(12) Adenosine kinase is inhibited by DTT. The DTT concentration was initially maintained at 1–2 mM to minimize this inhibition. When all of the adenosine had been phosphorylated, the DTT concentration was increased to 5–10 mM. The total quantity of DTT used was 5.5 g (36 mmol).
(13) Magnesium acetate was added as necessary to maintain a level 20–40 mmol higher than the assayed amount of ATP to ensure that free magnesium(II) would be present for complexation with ADP and AMP. The total quantity of magnesium acetate added was 36 g (168 mmol).
(14) Concentrations were assayed by standard enzymatic methods: Hans Ulrich Bergmeyer, Ed., "Methods of Enzymatic Analysis", Verlag Chemie, Weinheim/Bergstr., Germany, Academic Press, New York and London, 1974.
(15) G. W. E. Plaut, S. A. Kuby, and H. A. Lardy, *J. Biol. Chem.*, **243**, 184 (1950). The basic procedure described in this reference for the extraction of ATP

should result in a distribution coefficient of 11. It was, however, found that the presence of high concentrations of other salts in the reaction mixture interfered with ATP extraction. As a result, the reaction mixture was diluted with 50% of its volume of water and then extracted twice at 45 °C (to maintain homogeneity) with volumes of organic phase equal to the original volume. This treatment resulted in the separation of 75% of the ATP from the aqueous phase; 44% of this ATP was subsequently isolated.
(16) G. A. LePage in "Biochemical Preparations", Vol. I, H. E. Carter, Ed., Wiley, New York, N.Y., 1949, p 5.
(17) The preparation also contained 7% ADP, but no detectable AMP. The remainder was presumably barium phosphate.
(18) The specificity of adenosine kinases is broad; cf. ref 6 and B. Lindberg, H. Klenow, and K. Hansen, *J. Biol. Chem.*, **242**, 350 (1967). A large number of other nucleosides and nucleotide kinases are also known; cf. E. P. Anderson in "The Enzymes", Vol. 9, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1973, Chapter 2. Cell-free enzymatic synthesis of ATP might be especially valuable when alternative synthetic routes yield product mixtures containing difficulty removed impurities (for example, GTP in fermentation processes).

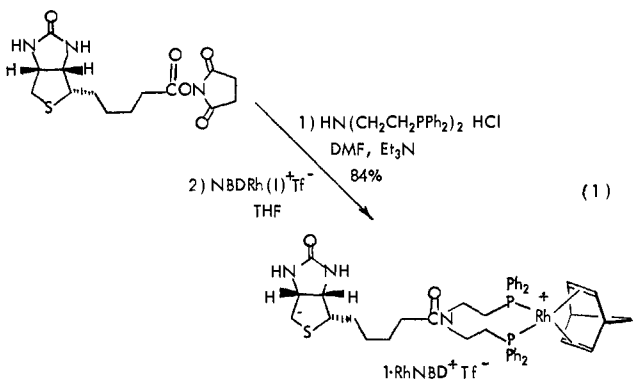
Richard L. Baughn, Örn Adalsteinsson
George M. Whitesides*
Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
Received September 29, 1977

Conversion of a Protein to a Homogeneous Asymmetric Hydrogenation Catalyst by Site-Specific Modification with a Diphosphinerhodium(I) Moiety[1]

Sir:

We wish to describe an approach to the construction of asymmetric hydrogenation catalysts based on embedding an (effectively) achiral diphosphinerhodium(I) moiety at a specific site in a protein: the protein tertiary structure provides the chirality required for enantioselective hydrogenation. Although it is presently difficult to predict the enantioselectivity of any hydrogenation from knowledge of the structures of catalyst and substrate, phosphine–rhodium(I) complexes having rigid, conformationally homogeneous structures seem generally to be more effective catalysts than those which are conformationally mobile.[2] A globular protein modified by introduction of a catalytically active metal at an appropriate site could, in principle, provide an exceptionally well-defined steric environment around that metal, and should do so for considerably smaller effort than would be required to construct a synthetic substance of comparable stereochemical complexity.

Our initial efforts have focused on avidin. This well-characterized protein is composed of four identical subunits, each of which binds biotin and many of its derivatives sufficiently tightly that association is effectively irreversible (K_d = 10^{-12}–10^{-15} M).[3,4] Biotin was converted to a chelating diphosphine and complexed with rhodium(I) by the sequence outlined in eq 1 (NBD = norbornadiene, Tf = triflate).[5,6] The intermediate *N*,*N*-bis(2-diphenylphosphinoethyl)biotinamide (**1**) was fully characterized;[5] the rhodium complex **1**·RhNBD$^+$Tf$^-$ was prepared in situ and used without characterization.[6] The enantioselectivity of catalysis by complexes of avidin with **1**·RhNBD$^+$Tf$^-$ was tested by the reduction of α-acetamidoacrylic acid (**2**) to *N*-acetylalanine (**3**) (eq 2). This reduction has been used frequently in estimating the enantioselectivity of other asymmetric hydrogenation catalysts.[7]

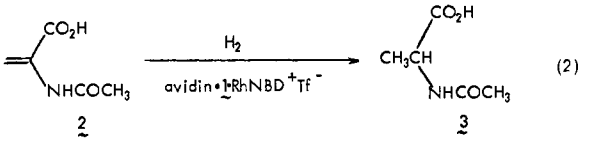


(1)

Table I. Catalytic Reduction of α-Acetamidoacrylic Acid (**2**) to *N*-Acetylalanine (**3**) using **1**·RhNBD$^+$Tf$^-$ (Alone and Mixed with Proteins) as Catalyst[a]

Protein added (mg)	Turnover no.[b] **2** → **3**[c]	Turnover no.[b] **4** → **5**[d]	Enantiomeric excess (**3**)[e] Polarimetric (±5%)[f]	Enantiomeric excess (**3**)[e] NMR (±10%)
None	475	10.3	<2	<2
Lysozyme (15)	450		<1	<2
Bovine serum albumin (15)	150		<5	
Carbonic anhydrase (15)	50	3.3	<10	
Avidin (10; 1 equiv[g])	>500[h]	12.6	41	44
Avidin (20; 2 equiv)	>500[h]		35	33
Avidin (10)·biotin[i]	200	3.5		<4
Avidin (10)·biotin[j]	160			<5
Avidin (10) + bovine serum albumin (15)[k]	480		34	34

[a] All hydrogenations were run with 0.50 μmol of **1**·RhNBD$^+$Tf$^-$ in 6.0 mL of water (0.1 M Na_2HPO_4 buffer, pH 7.0) at 0 °C for 48 h under 1.5-atm pressure of H_2. [b] Molecules of **2** or **4** hydrogenated per rhodium atom. [c] All experiments were run with 0.25 mmol of **2** and 0.25 mmol of Na_2HPO_4. [d] All experiments were run with 0.25 mmol of allyl alcohol. [e] The *S* enantiomer was in excess. [f] Calculated on the basis of the reported values for optically pure *N*-acetyl-(*R*)-alanine: $[\alpha]^{25}_D$ +66.5° (*c* 2, H_2O) (S. M. Birnbaum, L. Levitow, R. B. Kingsley, and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952)). No rotations were observed for values denoted (<): low turnover numbers for CA and BSA led to the large experimental uncertainty. [g] The quantity of avidin added was 125 U, and was sufficient to bind 0.50 μmol of biotin.[8] [h] This value represents complete hydrogenation: these turnover numbers are thus lower limits. [i] The avidin was incubated with a 10% excess of biotin (0.55 μmol, 0.13 mg) before exposure to the solution containing **1**·RhNBD$^+$. The excess of biotin precluded polarimetric assay. [j] The avidin was incubated with a 10-fold excess of biotin (1.2 mg). [k] The avidin and bovine serum albumin were mixed before adding to the solution of **1**·RhNBD$^+$.



(2)

2 **3**

A representative hydrogenation was conducted as follows. α-Acetamidoacrylic acid (32 mg, 0.25 mmol) and Na_2HPO_4 (36 mg, 0.25 mmol) were degassed in a 20-mL pressure reaction bottle (Lab Glass) with argon, and 5 mL of aqueous 0.1 M Na_2HPO_4 buffer (pH 7.0) was added. The solution was cooled to 0 °C and 1.0 mL of a similarly buffered solution of avidin (~10 mg, 125 U, binds 0.50 μmol of biotin[8]) was added. The bottle was swept with dihydrogen (welding grade) and the pressure adjusted to 1.5 atm with dihydrogen. A solution of **1**·RhNBD$^+$Tf$^-$ in THF (25 μL of a 20 mM solution, 0.50 μmol) was injected: the resulting solutions were pale yellow and homogeneous. The reaction was stirred for 48 h (0 °C, 1.5 atm of H_2). The reaction was worked up by adjusting the pH to 2.0 with 2.0 N aqueous HCl solution and filtered through Celite to remove any precipitated **1** and through an Amicon Diaflo PM 10 ultrafiltration membrane (10 000 mol wt cutoff) to separate avidin and avidin·**1** complexes from **2** and **3**. The optical rotation of the resulting clear colorless filtrate was combined with an NMR measurement of the extent of con-

0002-7863/78/1500-0306$01.00/0