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COMPLEMENTARY USE OF THE REVERSED-PHASE AND ANION-EXCHANGE MODES OF HIGH-PRESSURE LIQUID CHROMATOGRAPHY FOR STUDIES OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

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SUMMARY

The reversed-phase and anion-exchange modes of high-pressure liquid chromatography were used to separate breakdown products and impurities in solutions of the reduced form of nicotinamide adenine dinucleotide (NADH). The two chromatographic modes are compared for studies of NADH. Their use in following the course of acidic breakdown of NADH is described.

INTRODUCTION

The use of high-pressure liquid chromatography (HPLC) with microparticle chemically bonded packings has greatly improved separations of the reduced form of nicotinamide adenine dinucleotide* (NADH) from its breakdown products[1,2]. Reactions involving the breakdown of NADH are difficult to study because a complex mixture of compounds is formed. Although these reactions have been investigated by ultraviolet (UV), fluorescence, circular dichroism, optical rotatory dispersion, and nuclear magnetic resonance spectroscopy, there is conflicting opinion about the nature and number of products, and the mechanism of NADH breakdown. Chromatographic methods (paper, thin-layer, and open column) have helped isolate some NADH breakdown and reaction products[3–7]. These methods, however, are too slow for the analysis of transient species and lack the efficiency needed to separate the complex mixtures formed when NADH degrades.

Products formed in reactions of NADH vary widely in polar and ionic character. In basic solution, oxidation of NADH to NAD can occur as well as cleavage at the sugar or phosphate groups (Fig. 1). In acidic solution, however, the reactions

* Abbreviations used: AMP = adenosine 5′-phosphate; ADP = adenosine 5′-diphosphate; ADPR = adenosine 5′-diphosphoribose; NMN(H) = nicotinamide mononucleotide (reduced); NAD(H) = nicotinamide adenine dinucleotide (reduced) (NADH = β-NADH except where otherwise stated); Ade = adenine; Ado = adenosine; NA = nicotinic acid; (6-HTN)AD = β-6-hydroxytetrahydronicotinamide adenine dinucleotide; NADHX(DPNHX) = NADH breakdown product catalyzed by glyceraldehyde-3-phosphate dehydrogenase (G3PD) [believed to be (6-HTN)AD]; (c-THN)AD = α-O$^{2'}$-6β-cyclotetrahydronicotinamide adenine dinucleotide (Fig. 2); Nic = nicotinamide.

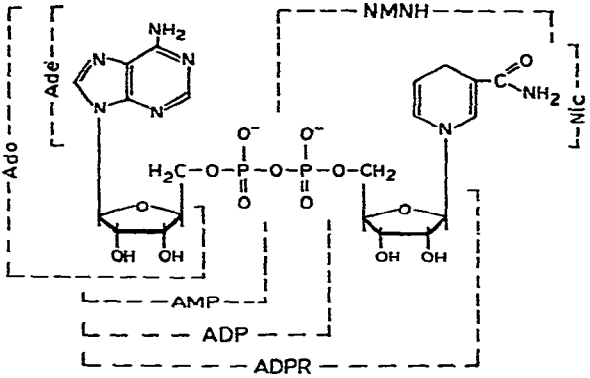


Fig. 1. The molecular structure of NADH and possible cleavage products.

of NADH involve primarily the labile nicotinamide ring. Structures suggested as breakdown products in acid include hydroxy addition products[8,9], a cyclic structure formed between the 6 position of the nicotinamide ring and the 2′ carbon on the adjacent ribose[10,11], aldehydes, dimers, and other addition products[12] (Fig. 2). NADH can be distinguished from NAD, as well as from most of its breakdown products by the characteristic absorbance of NADH at 340 nm. Major breakdown products, which do not have a maximum at 340 nm, have UV spectra similar to each other and to the spectrum of NAD with maxima between 254 and 290 nm. Therefore, in order

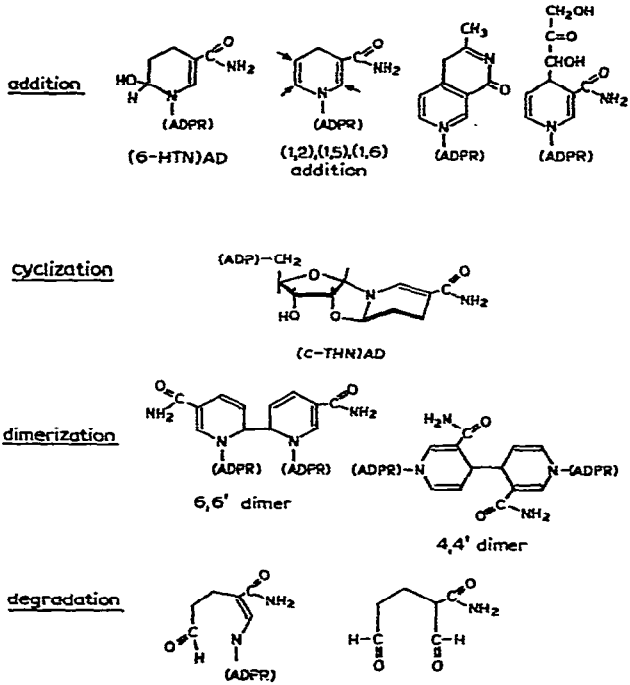


Fig. 2. Molecular structures of possible breakdown and addition products of NADH in acidic solution. Abbreviations of the compounds are taken from the refs. listed.

to identify the breakdown products and study their reactions, it is necessary to separate the compounds present in the reaction mixture.

In the past decade, HPLC has been found to be valuable in the study of nucleotides, nucleosides, and bases[13–16]. Reversed-phase methods are generally used for the polar bases and nucleosides[17] and anion exchange is used for the ionizable nucleotides[13,15,16]. Since NADH is readily ionized and cleavage and oxidation products are ionic in character, ion exchange can be used in the analysis of these compounds. However, the acid breakdown products appear to differ from NADH and from each other mainly in the nicotinamide portion of the molecule. Thus, it was anticipated that reversed phase could be utilized to separate compounds resulting from changes in the nicotinamide ring. Therefore, HPLC procedures for the separation of NADH reaction products using reversed phase and ion exchange were optimized and both of these modes were used to separate impurities and breakdown products in solutions of NADH, to follow the course of the breakdown reactions and to characterize some products of the reactions.

EXPERIMENTAL

Apparatus

A HPLC instrument (Waters Assoc., Milford, Mass., U.S.A.) equipped with a dual wavelength micro UV detector (Model 440), loop injector, dual pumps and a gradient programmer was used for all chromatographic work. A Schoeffel (Westwood, N.J., U.S.A.) variable wavelength monitor (SF 770) was used in series with the Waters instrument. A Hewlett-Packard (Avondale, Pa., U.S.A.) electronic integrator (Model 3380A) was used to obtain peak areas. Reversed-phase columns used were Whatman ODS/2 (Whatman, Clifton, N.J., U.S.A.), μBondapak C_{18} (Waters) and RP 8 (Hewlett-Packard). All ion-exchange work was done on Whatman SAX-10 columns. Columns were approximately 25 cm × 4 mm and the particle size of the packing materials was 5–10 μm. UV spectra were obtained on a Cary 15 (Varian, Palo Alto, Calif., U.S.A.).

Chemicals

Reagents used for buffer solutions were of reagent grade quality, and the solutions were filtered through 0.45-μm filters (Whatman). Nucleotide, nucleoside, and base standards were "Sigma grade", from Sigma (St. Louis, Mo., U.S.A.). The primary acid product (c-THN)AD (Fig. 2) was made according to the method of Oppenheimer and Kaplan[18] and the enzyme-catalyzed product (NADHX) was made according to the method of Rafter *et al.*[19] at pH 5.7.

Methods

The following compounds were used as standards and were chromatographed under various elution conditions on ion-exchange and reversed-phase columns: α- and β-NAD, and α- and β-NADH, AMP, ADP, ADPR, NMN, Ado, Ade, and nicotinamide. When chromatograms of a solution of partially degraded NADH, or mixtures of standards had symmetrical peaks which were well resolved and the separations were reproducible, conditions were described as optimal.

With ion exchange, buffers of a pH range of 4.0–7.5 were used to avoid

breakdown of NADH on the column. Buffers which were investigated as eluents included the sodium or potassium salts of PO_4^{3-}, SO_4^{2-}, NO_3^-, ClO_4^-, CO_3^{2-}, CH_3COO^-, and Cl^-. Eluents found most suitable for the separation of standard nucleosides and nucleotides were 0.007 *F* KH_2PO_4 for the low concentration eluent and 0.15 *F* KH_2PO_4 with 0.25 *F* KCl as the high concentration eluent. The buffers were adjusted to pH 5.8 and 6.5, respectively, using concentrated KOH. The conditions are summarized in Table I. A gradient delay of 15 min was followed by a linear gradient of 0–100% in 1 h. Most runs were terminated in 45 min. The column was brought back to initial conditions and washed with the low concentration buffer for 15 min before the next chromatographic run. Chromatographic work was done at ambient temperature and constant flow of 2.0 ml/min.

TABLE I

CONDITIONS USED FOR THE SEPARATION OF THE NICOTINAMIDE NUCLEOTIDES AND CLEAVAGE PRODUCTS BY ION-EXCHANGE HPLC

Column	Whatman SAX 10
Eluents	Low: 0.007 *F* KH_2PO_4 (pH 5.8) High: 0.15 *F* KH_2PO_4 + 0.25 *F* KCl (pH 6.5) (adjusted with KOH)
Isocratic	15 min
Gradient	Linear, 0–100% high, 1 h
Flow-rate	2 ml/min
Total run time	Approx. 60 min

For optimal reversed phase conditions, the low strength eluent was 0.02 *F* KH_2PO_4 at pH 7.0. The high strength eluent was methanol–water (60:40, v/v). Initially the eluent was composed of 2% high concentration eluent and 98% low concentration eluent. A concave gradient (Waters' program No. 7) to 30% high concentration eluent was applied over a 30-min period (Table II). The flow-rate was 2.0 ml/min and the chromatographic system was at ambient temperature.

TABLE II

CONDITIONS USED FOR THE SEPARATION OF THE NICOTINAMIDE NUCLEOTIDES AND PRODUCTS FOUND IN ACIDIC SOLUTION BY REVERSED-PHASE HPLC

Columns	Waters Assoc. μBondapak C_{18} (Whatman Partisil 10 ODS; Hewlett-Packard, experimental)
Eluents	Low: 0.02 *F* KH_2PO_4 (pH 7.0) High: 60% methanol
Gradient	0–30% high, 40 min, curve 7 (Waters Assoc. programmer), concave
Flow-rate	2.0 ml/min
Total run time	30 min

NADH solutions were prepared in sodium acetate solutions (0.005–0.05 *M*) with the pH adjusted to values between 4.5 and 6.0 by adding acetic acid. The sodium acetate solutions were filtered through a 0.25-μm membrane filter. NADH solutions were stored in sterile containers and kept dark at 27.0°. Samples of these NADH solutions were chromatographed at intervals on the reversed-phase and ion-

exchange systems. Peak height and peak area measurements from reversed-phase runs were used to calculate the rates of disappearance of NADH.

Breakdown products were isolated by collecting the appropriate fraction from the column, and the UV spectra of these products were obtained. Retention behavior of each of these peaks on ion exchange was determined. Peaks collected from reversed phase were acidified and rechromatographed to determine the stability of each primary breakdown compound.

A dual wavelength detector was used to determine peak height ratios at two wavelengths for each product. During the analysis of a solution of NADH which was degrading, the height ratios of peaks being formed were monitored, and inconsistency in ratios was indicative that more than one product was contained under a peak. These ratios enabled us to develop separation conditions which were optimal. Also, the ratios were used to locate peaks under different conditions and columns.

RESULTS AND DISCUSSION

Ion exchange

Of all of the buffers investigated for eluents in the ion-exchange separation of nucleotide and nucleoside standards, the desired separations were obtained only with the carbonate and phosphate buffers. However, it was impractical to use carbonate with silica base packings because the basicity of the solution caused deterioration of the packing. Since NADH breakdown is accelerated by the presence of acid, phosphate buffers in which the pH was close to neutrality were used.

Using the ion-exchange conditions described in *Methods* NMN, AMP, α-NAD, β-NAD, ADPR, NADH and ADP were well separated and some separation of Ado, Ade, nicotinamide and NA was obtained (Fig. 3). Therefore, ion exchange is an excellent method of isolating products which may be formed by oxidation and cleavage. Because a number of these products are formed in basic solutions of NADH, it is important to be able to detect their presence in order to assess the purity of NADH standards.

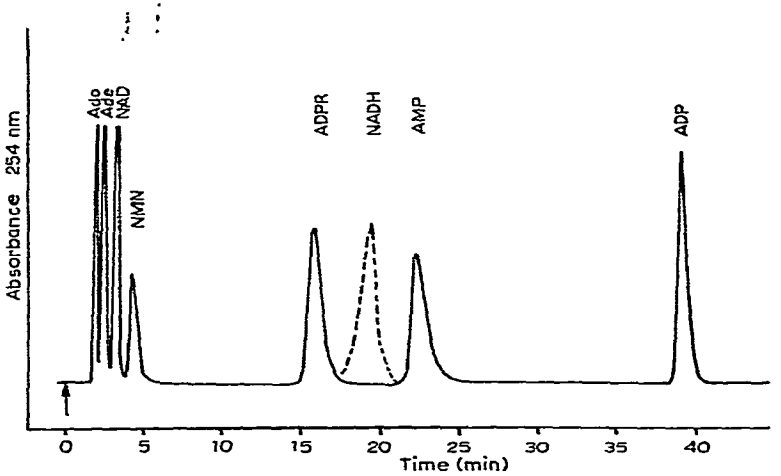
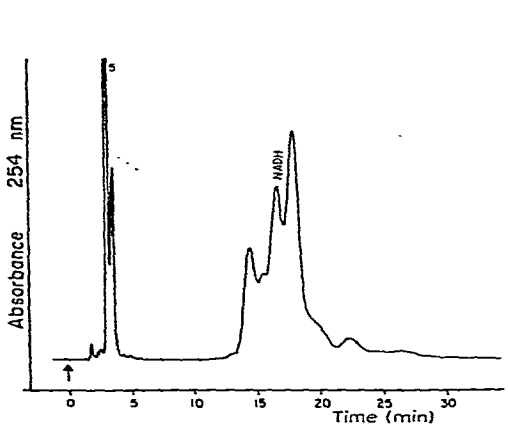


Fig. 3. The separation of nicotinamide nucleotides and standards of cleavage products by ion exchange (conditions given in Table I).

Although two products formed by the decomposition of NADH in acid were well separated from the others using ion exchange, many rearrangement products of the nicotinamide ring of NADH were not resolved (Fig. 4). Product 5 (Figs. 4 and 5) was well separated from other acid breakdown products by ion exchange and poorly separated by reversed phase. Because of its retention behavior on both columns, the structure of this product is thought to be significantly different from the other acid breakdown products.



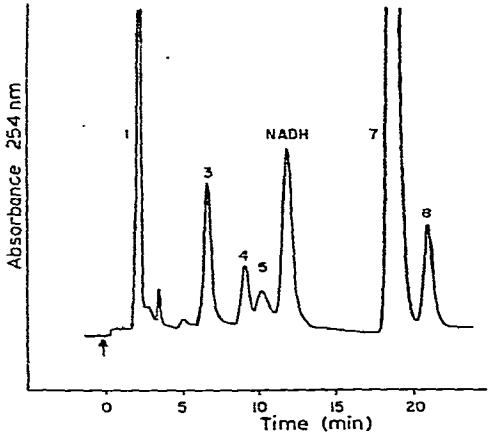


Fig. 4. The separation of NADH and acidic breakdown products by ion exchange HPLC (1 m*M* NADH stored in 0.05 *F* KH_2PO_4, pH 5.2, 1 day).

Fig. 5. The separation of NADH and acidic breakdown products by reversed-phase HPLC (1 m*M* NADH, stored in 0.05 *F* sodium acetate, pH 5.41, for 19 h).

Reversed phase

Using the reversed-phase partition mode with the conditions described in *Methods*, Ade, Ado, nicotinamide, β-NAD, α-NAD, and NADH were resolved. AMP, ADP, and ADPR, however, did not separate (Fig. 6). With the exception of α-NADH and β-NADH, which have the same retention time, major acidic breakdown products were well resolved from each other and from NADH (Fig. 5). Although a minor product, 5, is not well resolved from product 4 with reversed phase, it can be isolated using ion exchange (Fig. 4). Since the NADH peak on reversed phase was shown to be the pure compound by Margolis *et al.*[2], reversed phase is well suited for mesaurement of the rate of breakdown on NADH in acid. It is an excellent method for isolating the acid breakdown products, since they are eluted only with low concentrations of salt and methanol; thus characterization of collected fractions by other methods is simplified.

Acidic breakdown of NADH

Six major, and a number of minor products produced by the breakdown of NADH in the presence of acid were observed using the reversed phase system. Formation of 4 products of NADH, as shown in Fig. 7, fit an A $\rightarrow$ B $\rightarrow$ C $\rightarrow$ D reaction mechanism. A similar mechanism was suspected by Alivisatos *et al.* using UV spectroscopy[20]. When NADH breaks down, peaks 3, 4, and 5 are initially formed.

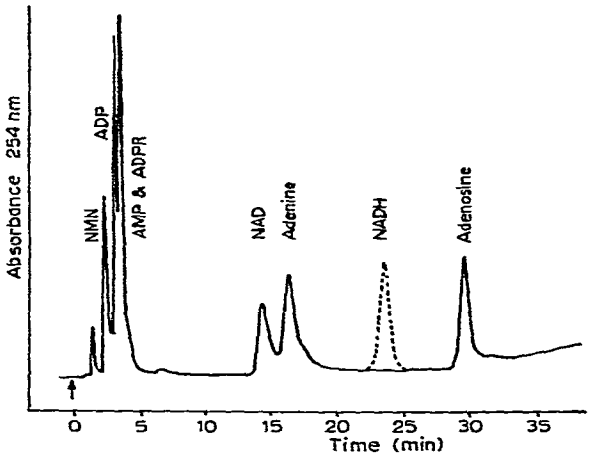


Fig. 6. The separation of nicotinamide nucleotides and standards of cleavage products by reversed-phase HPLC (conditions given in Table II, μBondapak C_{18} column).

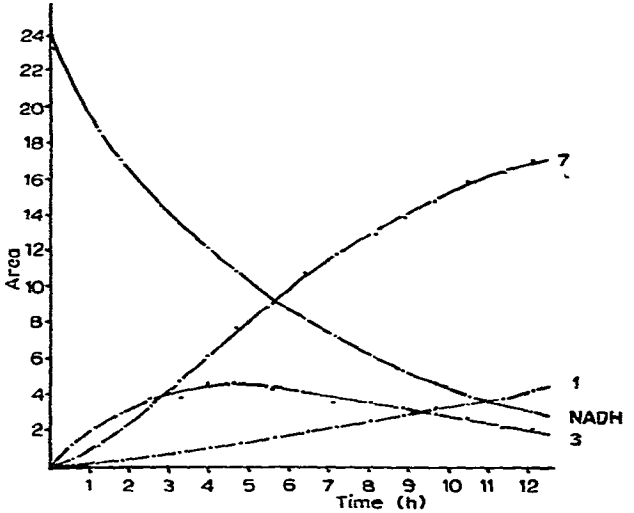


Fig. 7. Graph of the formation of the primary and secondary acid products of NADH *vs.* time of storage (1 m*M* NADH in 0.05 *F* sodium acetate, pH 5.22 ± 0.02, 27.0°).

Subsequently, peaks 7 and 8 are produced. By collecting, acidifying and chromatographing products 7 and 8, it can be shown that peak 1 is formed from products 7 and 8. Chromatograms illustrating the breakdown sequence are shown in Fig. 8.

Tentative identification and characterization of breakdown products have been made by UV analysis and by reference to literature spectral information. Peak 7 has a retention time and an absorbance spectrum which corresponds to the primary acid product, (c-THN)AD[10,11]. When produced by the method of Rafter *et al.*[19], NADHX also shows the same breakdown pattern, with the major product having the retention time of peak 7. Peak 1 has an absorbance spectrum with a UV maximum at 260 nm and is assumed to be the product others have referred to as the secondary acid product[12]. It has been observed by the collection and chromatography of inter-

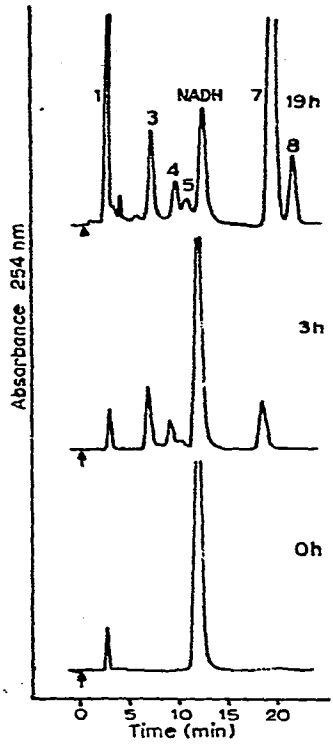


Fig. 8. Chromatograms of a 1 m*M* NADH solution in 0.05 *F* sodium acetate (pH 5.41) when stored for 0, 3, and 19 h.

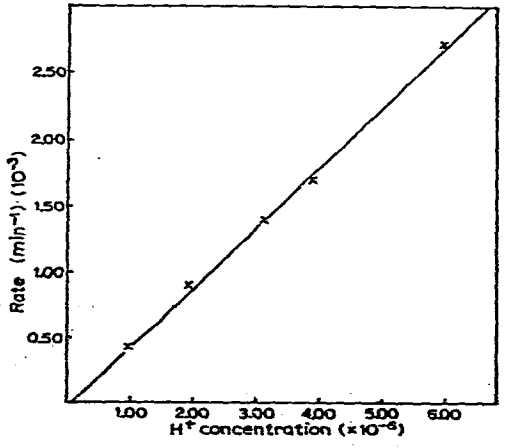


Fig. 9. Dependence of NADH degradation on $[H^+]$. Graph of rates k (min^{-1}) of degradation of a 0.05 *F* sodium acetate solution of 1 m*M* NADH at 27.0° versus H^+ concentration. Intercept = 0.00020, $k/[H^+] = 450 \pm 6$. Regression coefficient = 1.000.

mediate products 3 and 4, that there is a reversible reaction between these products. Products 3 and 4 represent the second step of the 3 part sequential mechanism shown in Fig. 7. Products comprising this part of the mechanism were not previously observed. The UV spectra of products 3 and 4 are broad and similar to each other, having UV maxima at about 265 nm.

None of the acidic products formed from NADH have an absorbance at 340 nm. Therefore, as was reported in earlier UV studies[21,22], the rate of breakdown of NADH can be determined accurately using the disappearance of the 340 nm absorbance peak. The rate of breakdown of NADH, found by measuring the heights and/or areas of the NADH peaks, was linearly dependent on H^+ and concentration of the solution (Fig. 9), and was found to be 450 ± 10 min^{-1} between pH 5 and 6 at 27°*. Rates of formation of the products did differ when NADH was stored in phosphate or phthalate, rather than acetate buffers. However, the retention times and the peak height ratios of the products were the same when the NADH was stored in each of these buffers, indicating that no anionic addition products were being formed.

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REFERENCES

1 J. R. Miksic and P. R. Brown, *Examination of Nicotinamide Nucleotides by High Pressure Liquid Chromatography, Pittsburgh Conference for Analytical Chemistry and Applied Spectroscopy, Cleveland, Ohio, March 1975*, Paper 330.
2 S. A. Margolis, B. F. Howell and R. Schaffer, *Clin. Chem.*, 22 (1976) 1322.
3 T. Sakai, T. Uchida and I. Chibata, *J. Chromatogr.*, 66 (1972) 111.
4 G. Disabato, *Biochim. Biophys. Acta*, 167 (1968) 646.
5 Stanjord and Clayson, *J. Lab. Clin. Med.*, 67 (1966) 144.
6 E. Silverstein, *Anal. Biochem.*, 12 (1965) 199.
7 F. Pastore, *J. Biol. Chem.*, 236 (1961) 2314.
8 A. Stock, E. Sann and G. Pfleiderer, *Justus Liebigs Ann. Chem*,. 647 (1961) 188.
9 N. J. Oppenheimer and N. O. Kaplan, *Biochemistry*, 13 (1974) 4685.
10 N. J. Oppenheimer and N. O. Kaplan, *Biochemistry*, 13 (1974) 4675.
11 T. J. Williams, P. D. Ellis, T. A. Bryson, R. R. Fisher and R. B. Dunlap, *Arch. Biochem. Biophys.*, 176 (1976) 275.
12 T. C. Bruice and S. Benkovic, *Bioorganic Mechanisms*, Vol. II, Benjamin, New York, 1966.
13 C. G. Horvath, B. Preiss and S. R. Lipsky, *Anal. Chem.*, 39 (1967) 1922; 41 (1969) 1227.
14 P. R. Brown, S. Bobick and F. L. Hanley, *J. Chromatogr.*, 99 (1974) 587.
15 M. Uziel, C. Koh and W. Cohn, *Anal. Biochem.*, 25 (1968) 77.
16 A. Burtis, M. Munk and F. MacDonald, *Clin. Chem.*, 16 (1970) 667.
17 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 126 (1976) 679.
18 N. J. Oppenheimer, *Biochem. Biophys. Res. Commun.*, 50 (1973) 683.
19 G. W. Rafter, S. Chaykin and E. G. Krebs, *J. Biol. Chem.*, 208 (1954) 799.
20 S. G. A. Alivisatos, F. Ungar and G. J. Abraham, *Biochemistry*, 4 (1965) 2616.
21 O. H. Lowry, J. V. Passonneau and M. K. Rock, *J. Biol. Chem.*, 236 (1961) 2756.
22 C. C. Johnston, J. L. Gardner, C. H. Suelter and D. E. Metzler, *Biochemistry*, 2 (1963) 689.

* Lowry *et al.*[21] give a value of 380 (H^+) min^{-1} at 23° and Johnston *et al.*[22] found the rate to be 462 (H^+) min^{-1} at 25°.