ANALYTICAL BIOCHEMISTRY 54, 554–560 (1973)

Micro Method for Determination of Borohydride with NAD^+

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Received November 13, 1972; accepted April 24, 1973

A spectrophotometric method for the determination of borohydride is described. It involves the reduction of NAD^+ to a number of isomeric forms of NADH by borohydride. In the standard assay procedure herewith presented, there is a direct proportionality between the absorption at 340 nm and the amount of borohydride in solution over the range 10–100 nmoles, with an effective "molar extinction coefficient" of 12.2×10^3. The method is simple, rapid, and sensitive.

There is need for a rapid and sensitive assay for borohydride, which can be used to (a) measure low concentrations of borohydride, (b) follow the decomposition of borohydride in solution, and (c) determine the rate of reduction of various organic compounds. Several methods have been described for the determination of borohydride (1). The spectrophotometric procedure of Lichtenstein and Mras (2), utilizing the reduction of acetone, is simple, direct, and rapid. However, since acetone has a molar extinction coefficient of only 17.63 at the λ_{max} (265 nm), the method lacks sensitivity. It would appear that a much higher level of sensitivity could readily be achieved for measuring borohydride concentrations by utilizing the well known reduction of NAD^+ to NADH (3), which has a molar extinction coefficient of 6.22×10^3 at 340 nm. The reaction has been studied in great detail by a number of investigators and shown to involve the formation of three isomers of NADH, with different λ_{max} and molar extinction coefficients (4). As far as we are aware, however, this reaction has not been exploited for the quantitative determination of borohydride. A preliminary report of these studies has been described previously (5).

[1] This work was supported by Grant AM 07305 from the National Institutes of Health.

MATERIALS AND METHODS

Materials. Nicotinamide adenine dinucleotide (NAD^+) was obtained from P-L Biochemicals, Inc. Sodium borohydride and Tris were obtained from Sigma Chemical Company.

Methods. The purity of the borohydride was determined by the acetone method (2) using redistilled acetone. It was found to be 93.8% ± 0.06% pure (mean of six determinations).

STANDARD ASSAY PROCEDURE

Reagents. 0.05 M Tris buffer, pH 8.5; 0.05 M KOH; 0.05 M NAD^+, aqueous solution stored at −20°C; 0.001 M $NaBH_4$ in 0.05 M KOH, prepared fresh and stored at 4°C until required.

Procedure. The NAD^+ reagent was a mixture consisting of 0.5 ml of 0.05 M NAD^+ and 8.5 ml of 0.05 M Tris (pH 8.5) prepared just before use. Aliquots of 0.9 ml of this mixture were then distributed into quartz cuvettes. The BH_4^- solution to be assayed and the amount of 0.05 M KOH required to bring the final volume to exactly 1 ml were then added. The necessary amount of KOH was added before the borohydride. The solution was then rapidly mixed, allowed to react for 10 min at room temperature, and the absorbancy measured spectrophotometrically at 340 nm (ΔA) against a reagent blank (Fig. 1). The reagent blank contained all the reaction components except borohydride.

RESULTS

A. A Study of Variables Affecting the Assay

Absorption Spectrum and Stability of NADH Isomers. The absorption spectrum of the NADH was determined in a recording spectrophotometer (Bausch and Lomb, Spectronic 505) as shown in Fig. 2. This figure also shows the change in the absorption spectrum with time.

Effect of Buffer and pH on the Rate and Extent of Reduction of NAD^+ by BH_4^-. The incubation mixtures were made up as in the standard procedure. The concentration of the BH_4^- was the same in each experiment, and Tris buffer (0.08 M) or borate buffer (0.08 M) at varying pH values from 7.0 to 9.0 were used. The rate of the reduction at room temperature in Tris buffers is shown in Fig. 3. Fluorescence of NAD^+ in alkaline solution precluded the use of solutions more concentrated than 0.05 M KOH for the quantitation of the reduction with borohydride.

Effect of Temperature on the Rate of Reduction of NAD^+ with BH_4^- and Stability of Products. Incubation mixtures were prepared as in the standard procedure and the reactions carried out at different temperatures

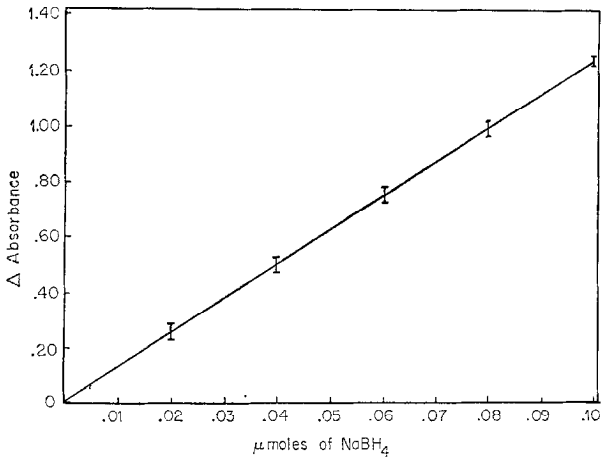

FIG. 1. Standard curve for the assay of borohydride. See text for experimental conditions.

(Fig. 4). Constant temperature was maintained with a Haake circulating water bath.

B. An Application of the Assay

Stability of Borohydride in Aqueous Solutions of Different Buffers and at Various pH Values. One-hundred microliters of sodium borohydride,

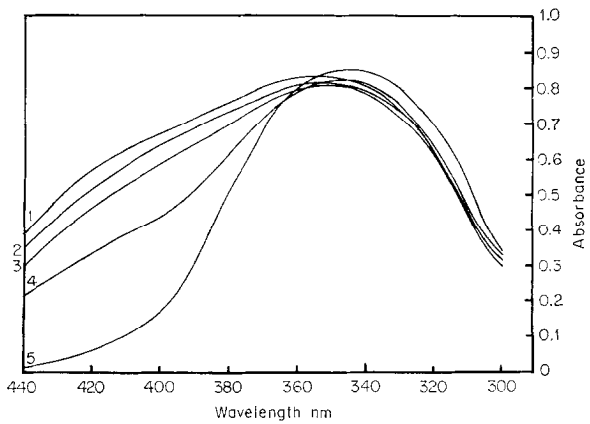

FIG. 2. Absorption curves for the products of reduction of NAD^+ by BH_4^- after various periods of incubating at room temperature. Fifty microliters of 0.05 M NAD^+ were mixed with 850 μl of 0.05 M Tris (pH 8.5), to this was added approximately 0.05, μmoles of borohydride contained in 100 μl of 0.05 M KOH, and the spectrum was recorded after varying periods of time at room temperature; curves (1) 12 min, (2) 30 min, (3) 2 hr, (4) 5 hr, (5) 20 hr. The reference cuvette contained no borohydride.

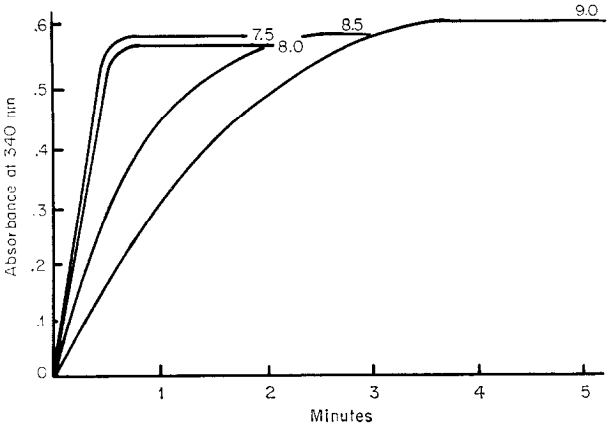

FIG. 3. Effect of pH on the rate and extent of reduction of NAD^+ by BH_4^-. Borohydride (0.5 μmoles) was mixed with 0.1 μmole of NAD^+ in 1 ml of 0.08 M Tris buffer. The change in absorbance at 340 nm with time is shown at the different pH values as indicated.

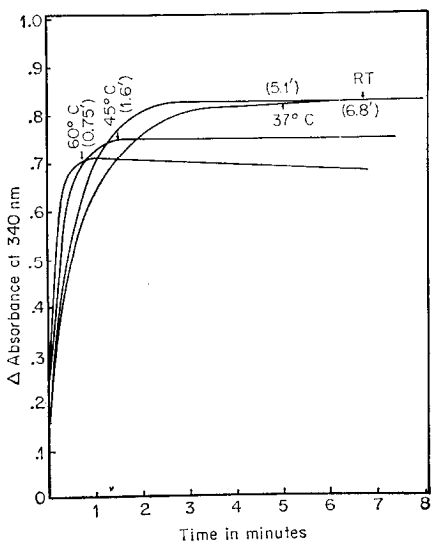

FIG. 4. Effect of temperature on the rate of reduction of NAD^+ by BH_4^-. Fifty microliters of 0.05 M NAD^+ was mixed with 50 μl of 0.001 M $NaBH_4$ in 0.05 M KOH, 50 μl of 0.05 M KOH, 850 μl of 0.05 M Tris buffer (pH 8.5), and incubated at different temperatures as indicated; 60°, 45°, 37°C, and room temperature. The Δ (change in) absorbance at 340 nm with time at the different temperatures is indicated above. The arrows indicate the time required (shown in parentheses) for maximum reduction to NADH.

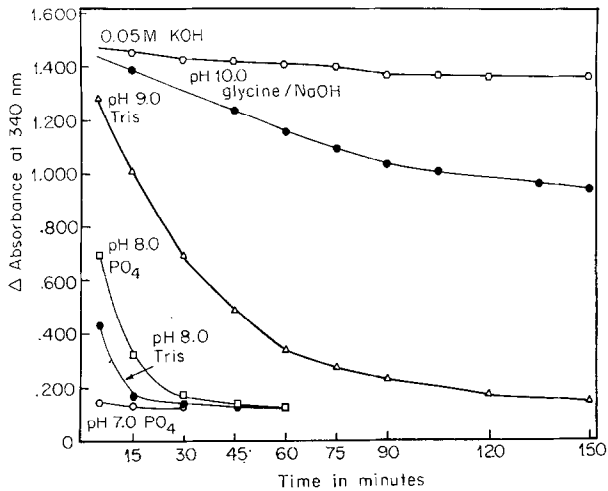

FIG. 5. Effect of buffer and pH on the stability of borohydride in aqueous solution. See text for experimental details.

0.01 M solution in 0.05 M KOH, was added to 900 μl of 0.05 M buffer, well mixed, and kept at room temperature. At predetermined time intervals, aliquots of 100 μl were removed, and the borohydride content determined according to the general procedure outlined above. The results are shown in Fig. 5.

DISCUSSION

Chaykin *et al.* (4) demonstrated that the reduction of NAD^+ by BH_4^- (3) is a complex reaction and that several isomeric reduced products are formed. We have reinvestigated the reaction primarily to develop an analytical method for the determination of borohydride. A number of variables, such as buffer, pH, and temperature have been tested, and the effect each had on the rapidity of reduction and the stability of the products was observed. The optimal conditions are described in the Standard Assay Procedure.

Tris buffer is the most effective in giving a stable reduction product. Phosphate buffers are unsuitable as has already been reported (4). From the results shown in Fig. 3, it is readily apparent that reduction in Tris buffer is most rapid at lower pH values. Borate buffers gave a similar set of curves. In both cases, however, the extent of reduction of NAD^+ is slightly greater at the higher pH values. The relative instability of $NaBH_4$ near neutrality makes quantitation of the reagent difficult; this is the reason why a higher pH value was selected for a routine assay

procedure. A pH of 8.5 is chosen as optimal because at higher pH values NAD^+ shows an undesirable amount of fluorescence.

The reduction is very rapid as shown in Fig. 4, and the extent of reduction is inversely dependent upon the temperature. The reduction is essentially complete in 5 min at both room temperature and 37°C, but a 10-min reaction time is used since there is no change in the absorption at 340 nm over a period of 1 hr.

As would be expected in a bimolecular reaction, the greater the ratio of NAD^+–BH_4^-, the greater the rate and extent of reduction of NAD^+ with a limited amount of BH_4^-. Under such conditions, the competing hydrolytic degradation of BH_4^- is minimized, and the conversion of NAD^+ to NADH becomes a more quantitative measure of the amount of BH_4^- present in solution. However, the maximum amount of NAD^+ that can be present in excess is set by the limitations of the amount of reference energy that can go through the solutions in the spectrophotometer.

All the variables that affect the rate and extent of reduction of NAD^+ to NADH empirically define the conditions optimal for the determination. Figure 1 shows a typical calibration curve employing these conditions for the determination of 10–100 nmoles of borohydride at 340 nm. The choice of 340 nm is empirical, as it represents the wavelengths at which there is minimal change in absorption with time after the initial 10-min incubation period.

The spontaneous decomposition of borohydride in water with release of hydrogen must always be considered in any quantitative use of borohydride as a reducing agent. The conditions chosen for this assay attempt to maximize the reduction of NAD^+ and to minimize the competitive decomposition of BH_4^-. The effectiveness of the system is demonstrated by the stoichiometry of the reaction. The molar extinction coefficient of BH_4^- in this assay procedure was found to be 12.2×10^3, whereas the known molar extinction coefficient of enzymatically obtained 1, 4-NADH is 6.22×10^3.

A "molar extinction coefficient" of 12.2×10^3 for BH_4^- in this NAD^+ assay is approximately 200-fold greater than the effective "molar extinction coefficient" of BH_4^- in the acetone assay (2). In the latter assay, one mole of BH_4^- effectively reduces 4 moles of acetone with a molar extinction coefficient of 17.63.

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