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An Ultrasensitive Assay of Nicotinamide Adenine Dinucleotide

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INTRODUCTION

Quantitative assays of pyridine nucleotides play a central role in biochemistry. This is largely due to the fact that many enzymatic reactions may be monitored by changes in the ratio of reduced to oxidized pyridine nucleotide. Actually most methods of enzymatic analysis are based on photometric recording of changes in the concentration of NADH (2). Increased analytical sensitivity may be obtained by chemical amplification with an enzymatic cycling system for NAD (4, 5, 6). The pyridine nucleotide is alternatively oxidized by one enzyme, then reduced back again by another in a cycling fashion. After a sufficient number of cycles, one of the products which has accumulated is measured in an enzymatic indicator reaction based on fluorometric detection of produced NAD or NADH (5).

The present communication describes an assay for NAD in which ATP induced bioluminescence is used as indicator reaction. ATP is produced from ADP equivalent to the amount of NAD present in the reaction mixture by the enzymes glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, GAPDH) and 3-phosphoglycerate kinase (EC 2.7.2.3, PGK):

$$NAD^+ + \text{D-glyceraldehyde-3-phosphate} + P_i \longrightarrow$$
$$\longrightarrow \text{D-glycerate-1,3-diphosphate} + NADH + H^+$$

$$\text{D-glycerate-1,3-diphosphate} + ADP \longrightarrow \text{D-glycerate-3-phosphate} + ATP$$

Glyceraldehyde-3-phosphate dehydrogenase from mammalian tissue or yeast is specific for NAD. Only the oxidized form of the pyridine nucleotide induces production of ATP. The extremely high sensitivity of the firefly luciferase system should make this assay principle useful in applications which have been exclusively restricted to enzymatic cycling systems. In contrast to these systems which are kinetic determinations, uniformity in incubation time and tem-

perature is less critical in the present analytical procedure, thus larger number of samples may be processed within each run. Since the recording of ATP induced bioluminescence may easily be automated using standard equipment (3) the assay described here has the advantage of being much less laborious than previous assays for NAD of comparable sensitivity and specificity.

However, in order to determine the sum of reduced and oxidized NAD or to increase the sensitivity of the assay furthermore, the analytical reagent may be converted to an enzymatic cycling system. An oxidizing step catalyzed by glutamate dehydrogenase (EC 1.4.1.3, GDH) is then included;

$$NADH + \alpha\text{-ketoglutarate} + NH_4^+ \longrightarrow \text{glutamate} + NAD^+$$

The resulting cycling system is principally similar to the one used by Matschinsky (6) although ATP is one of the accumulating products in the present modification. Very high sensitivity may be achieved sine the comparatively low cycling rate of this system (4,6) is compensated for by the sensitivity of bioluminescent detection of ATP.

METHODS

Firefly luciferase was prepared from desiccated firefly lanterns (FFT, Sigma Chemical Co.) as described elsewhere (3). Electrophoretically pure, crystalline bovine serum albumin, GADPH (from yeast), GLDH (from beef liver, solved in glycerol), PGK (from yeast) and all biochemicals were obtained from Boehringer, Mannheim. ADP was purified with ion exchange chromatography to reduce contamination with ATP (8). D-glyceraldehyde-3-phosphate was prepared from the corresponding diethyl acetal compound by acid hydrolysis (1). GADPH was treated with charcoal (4) to reduce the nucleotide content. Chemicals were of analytical grade. Deionized and doubly distilled water was used.

Composition of reagent for NAD determination: This contains 5 mM K_2HPO_4, 3 mM $MgSO_4$, 1 mM EDTA, 0.3 mM D-glyceraldehyde-3-phosphate, 0.02 mM ADP, 1.2 µg/ml GADPH, 0.3 µg/ml PGK and 0.1 % albumin. The reagent is buffered with 100 mM HEPES (N-2-hydroxyethylpiperazine-N=2-ethanesulphonic acid.) adjusted to pH 7.5 with KOH.

Composition of cycling reagent. This reagent consists of 100 mM HEPES (pH 7.5), 5 mM K_2HPO_4, 3 mM $MgSO_4$. 1 mM EDTA, 10 mM α-ketoglutarate, 10 mM ammonium acetate, 0.3 mM D-glyceraldehyde-3-phosphate, 0.02 mM ADP and 0.1 % albumin. The enzymes GLDH, GAPDH and PGK are added to the reagent just before use in order to avoid prolonged cycling of contaminating NAD. The amounts of enzymes are adjusted to give desired cycling rates. The PGK concentration is the same as in the non-cycling reagent (0.3 µg/ml). GAPDH is the rate-limiting enzyme in the enzymatic cycle for NAD. The cycling rate is proportional to the

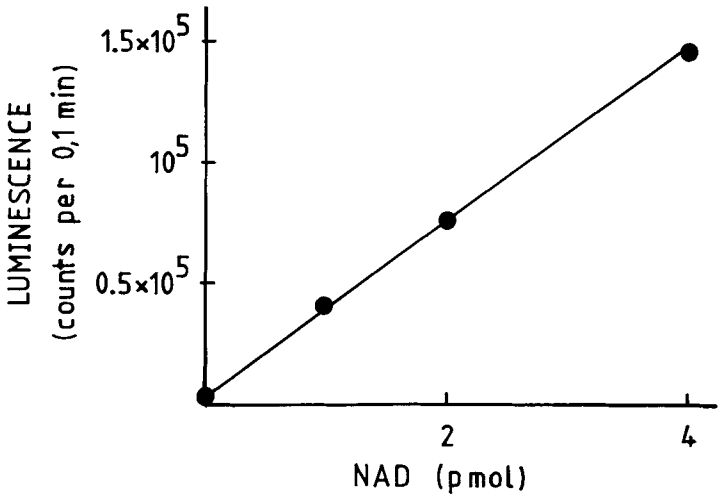

Fig. 1. Standards of NAD assayed with the non-cycling method. The assay was performed as described in the text using an oil well rack. The volume of blanks and standards was 1 µl. The points denote means of triplicate determinations.

concentration of this enzyme at least up to 30 µg/ml which is the highest concentration tested in this study. However, very little sensitivity is gained by increasing the GAPDH concentration to more than 10 µg/ml. This amount yields 500 - 800 cycles during one hour at 25 C. GLDH should be added in excess, i. e. two to three times the concentration of GAPDH. The GLDH preparation supplied in glycerol was found to be the most satisfactory since its contribution to the blank is rather small.

Analytical procedures. There is much to be gained by reducing the reagent volume when determining small amounts of NAD. The blank decreases and the concentration of sample NAD is kept higher. Volumes of 1µl or less are conveniently handled with the oil well technique (7) which is used in the procedure outlined here. The description is relevant both for the non-cycling and the cycling method. Reagent (1 µl) is added to the samples (1 µl or less) in the oil wells at room temperature. After 30 min incubation 20 µl HEPES buffer (25 mM) pH 7.5 is added to each oil well and the rack is then heated for 15 min in an oven at 100 C. (When using the cycling method it is recommendable to incubate for at least one hour to obtain higher amplification and better precision). The rack is cooled and 10 µl aliquots are transferred to micro test tubes for determination of ATP as described below.

If the assay is performed in test tubes using a larger reagent volume the dilution step may be omitted. Furthermore, it is sufficient to heat the test tubes for 3 to 5 min only in boiling water after the incubation period. Instead of transferring an aliquot of the sample-reagent mixture to new tubes for ATP determination, a larger volume of the ATP reagent (see below) may then be added

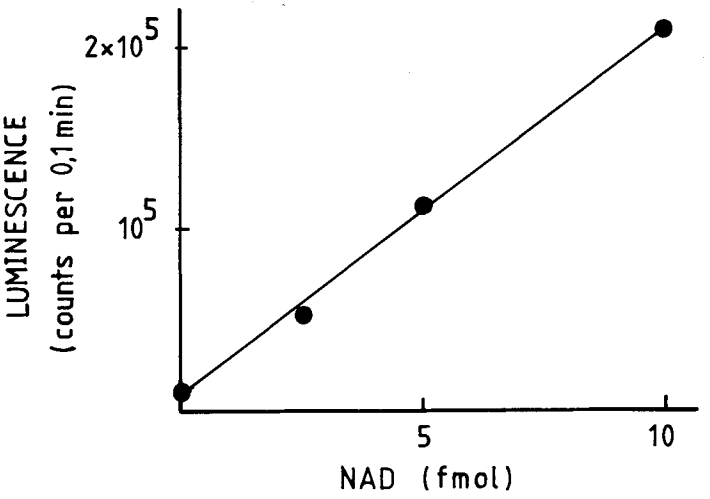

Fig. 2. Determination of NAD standards with the cycling method. The volumes of standards and cycling reagent were 1 µl. The cycling reagent contained 10 µg/ml GAPDH and 20 µg/ml GLDH. The procedure outlined in the text was followed. The cycling reaction was allowed to proceed for 60 min (apparent cycling rate 500/h). The points denote means of triplicate determinations.

to the test tubes after the heating step.

Bioluminescent assay of ATP. Determination of the produced ATP is performed as described earlier (3). The reagent contains 25 mM HEPES buffer pH 7.5, 5 mM $MgSO_4$, 0.1 mM EDTA, 26 µM D-luciferin and 0.05 % albumin. Purified luciferase is added to yield about 100,000 counts per 0.1 min in the presence of 1 pmol ATP. The samples are mixed with 75 µl reagent in polypropylene micro test tubes. The tubes are then transferred to glass counting vials in the refrigerated (+4 C) sample changer of a Packard Tri-Carb Liquid Scintillation Spectrometer (model 3310). At least 30 min are allowed before automatic recording of the luminescence is started (preset time 0.1 min, discriminator setting 30 - 400, gain 100 % and coincidence off). Blanks and standards of ATP may be included.

RESULTS AND DISCUSSION

The sensitivity of the ATP detecting step is very high and the blank of the ATP reagent is very low (equivalent to 0.02 - 0.04 nM ATP). This means that the analytical limit for detection of NAD by the direct, non-cycling method described here should be less than 5 fmol provided that the blank contribution of the initial reagent is negligable. This is not the case. At present the blank corresponds to about 50 nM NAD (i.e. 50 fmol in 1 µl reagent). Most of this blank comes from contamination of ADP with ATP. Thus further purification of the ADP preparation is the mean to increase the sensitivity. The ATP contamination is on the other hand less important in the cycling method, since only the pyridine

nucleotide content is amplified. The optimum number of cycles seems to be about 400 - 800. At this amplification the blank contribution of contaminating ATP is insignificant and most of the blank (equivalent to 0.5 nM NAD in the cycling reagent) depends on the pyridine nucleotide content of one of the cycling enzymes, namely GAPDH.

The direct, non-cycling method measures the sum of NAD and ATP. Therefore ATP has to be removed from biological samples before they are assayed. Treatment of the sample extracts with apyrase (3) is effective in reducing the ATP concentration to very low levels without affecting the NAD content. Apyrase treatment may not be necessary when using the cycling method because of the selective amplification of the NAD content in this method.

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