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A Colorimetric Method for the Quantitative Determination of Reduced Pyridine Nucleotides (NADPH and NADH)

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Spectrophotometric methods for the determination of reduced pyridine nucleotides (NADPH and NADH) have been available for some time. The most widely used method is based on the absorbance at 340 mμ (1), which is characteristic of the reduced pyridine nucleotide; however, more recently fluorescence measurements have been also adopted for the assay of reduced pyridine nucleotides (2).

The present study describes a rapid and convenient method for the colorimetric determination of NADPH and NADH. This determination is based on a reaction between the reduced pyridine nucleotide and *p*-dimethylaminobenzaldehyde (PDAB) under acidic conditions, with a resultant formation of color that is measured spectrophotometrically.

MATERIALS AND METHODS

NADPH and NADH were obtained from Calbiochem and *p*-dimethylaminobenzaldehyde was obtained from Eastman Organic Chemicals.

Reagents. PDAB solution: 90 mg *p*-dimethylaminobenzaldehyde/ml 4.5 *N* H_2SO_4.

Assay procedure. PDAB solution (1 ml)[1] was added to a 4 ml aliquot of a solution containing either NADH or NADPH. After 15–20 min the absorbance of the colored solution was measured at 510 mμ with the Beckman DU monochrometer equipped with a Gilford model 2000 multiple-sample absorbance recorder. The concentration of the reduced pyridine nucleotide was obtained from a standard curve. When the solution to be assayed contained proteins, the suspension resulting from the addition of PDAB was centrifuged at 17,000*g* for 10 min in a Sorvall centrifuge at

[1] When the aliquot of the solution containing the pyridine nucleotide was less than 4 ml, the concentration of PDAB and H_2SO_4 in the PDAB solution was adjusted so that the volume added would contain 90 mg of PDAB and would bring the final volume of the assay solution to 5 ml (0.9 *N* H_2SO_4).

0–4°C. The supernatant solution was allowed to stand at room temperature for an additional 10 min and the absorbance measured as described above.

RESULTS

The reaction of aqueous solutions of NADPH or NADH with PDAB yields a pink colored solution which has an absorption maximum at 510 mμ (Fig. 1). Following the addition of PDAB the absorbance at 510 mμ of the NADPH solution (0.5–2.0 μmoles/ml) increases and reaches a maximal value at 15–20 min (Table 1), however, with higher concentrations of NADPH (4.0 and 6.0 μmoles/ml) maximal absorbance is attained

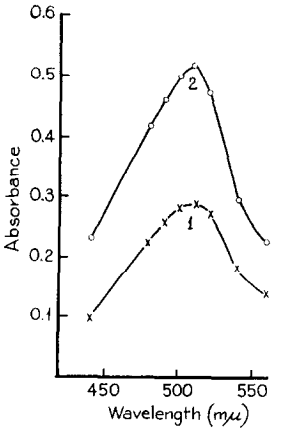

FIG. 1. *Absorbance Spectra of NADPH and NADH Solutions. Curve 1* represents the solution obtained by mixing 4 ml of an aqueous solution of NADPH (0.5 μmole/ml) with 1 ml of PDAB (90 mg/ml of 4.5 *N* H_2SO_4). *Curve 2* represents a similarly treated NADH solution (1 μmole/ml). Measurements were taken 20 min after the addition of PDAB, with a Beckman monochrometer equipped with a Gilford model 2000 multiple-sample absorbance recorder.

at about 12 min. The absorbance of these solutions remains relatively stable for an additional 5–10 min and subsequently declines slowly. Similar results were observed with NADH (Table 2). Figure 2 shows the standard curves obtained with various concentrations of aqueous solutions of NADPH and NADH. The absorbances of the colored solutions of both reduced pyridine nucleotides are proportional to concentration over the range of concentration examined,[2] and the curves obtained with the two

[2] In another experiment with NADPH concentrations up to 8 μmoles/ml, a slight deviation from linearity (ca. 5%) was observed with concentrations above 4 μmoles/ml; the absorbances of these solutions were 2.272 and 2.997 for NADPH solutions of 6 and 8 μmoles/ml, respectively.

TABLE 1
Rate of Development and Stability of the Colored Product of NADPH

Time,[b] min	Concentration of NADPH, μmoles/ml[a]				
	0.5	1.0	2.0	4.0	6.0
3				1.211	1.735
6	0.239			1.496	2.121
9	0.262			1.606	2.249
10		0.387			
12	0.273			1.648	2.272
15	0.280	0.410	0.822	1.632	2.227
18	0.290			1.604	2.170
20		0.411	0.801		
21	0.290			1.558	2.095
24	0.291			1.514	
25		0.395	0.771		2.021
27	0.290			1.466	1.948
30	0.288	0.375	0.730	1.421	1.872
36	0.282				
42	0.262				
60	0.217				
70	0.191				

[a] To 4 ml of an aqueous solution of NADPH, at the indicated concentration, was added 1 ml of PDAB solution (90 mg in 4.5 N H_2SO_4). Values represent absorbance at 510 mμ.

[b] Time after addition of PDAB.

nucleotides are almost identical. For comparison purposes, the absorbance at 340 mμ of aqueous solutions of NADPH and NADH (not reacted with PDAB) was measured.

There was no color formation by solutions containing NAD, NADP,

TABLE 2
Rate of Development and Stability of the Colored Product of NADH[a]

Time, min	Concentration of NADH, μmoles/ml		
	0.375	0.75	1.5
3	0.072	0.194	0.444
6	0.110	0.272	0.591
9	0.133	0.311	0.651
12	0.147	0.330	0.673
15	0.150	0.338	0.674
18	0.150	0.338	0.662
21	0.148	0.333	0.652
24	0.144	0.323	0.628
27	0.132	0.307	0.608
30	0.122	0.293	0.586

[a] Conditions described in Table 1.

nicotinamide, ADP, or ATP at concentrations of 0.75 μmole/ml, demonstrating the specificity of the color reaction for the reduced pyridine ring.

The present method has been applied to enzymic reactions which are known to utilize NADPH: (*a*) demethylation of *p*-chloro-*N*-methylaniline by 9000*g* supernatant from rat liver (Table 3) and (*b*) ring A reduction of hydrocortisone by female rat liver microsomes (Table 4).

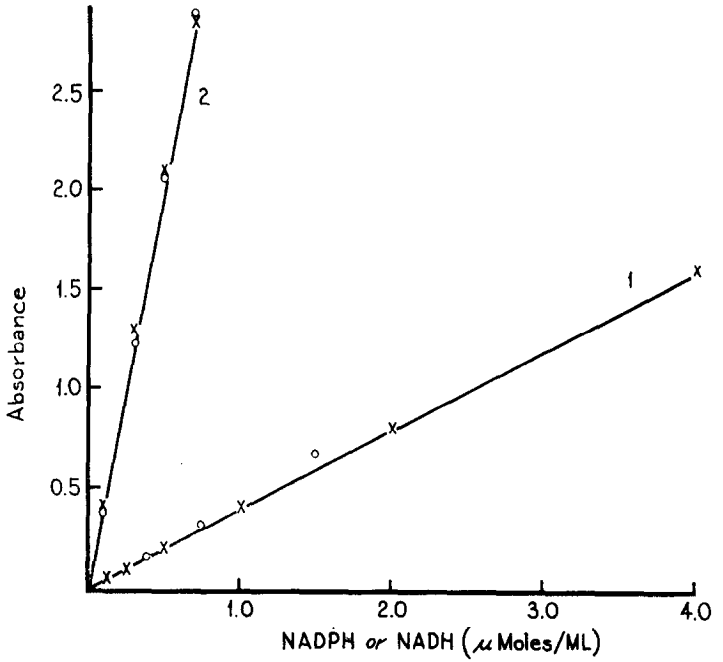

FIG. 2. *Absorbance as a Function of Concentration of Reduced Pyridine Nucleotides.* NADPH (x); NADH (o). Aqueous solutions of NADPH and NADH were freshly prepared. A portion of each solution was immediately read at 340 mμ and the remainder solution was used for mixing with PDAB as described: *Curve 1* represents the absorbance of the colored solutions obtained by mixing 4 ml of aqueous solutions of the respective pyridine nucleotide with 1 ml PDAB (90 mg/ml of 4.5 *N* H_2SO_4). Absorbance readings at 510 mμ were taken 20 min after the addition of PDAB. *Curve 2* represents absorbance at 340 mμ.

DISCUSSION

The method described here is based on the formation of a colored product when a solution of reduced pyridine nucleotide (NADPH or NADH) is allowed to react with PDAB. At low concentrations of the pyridine nucleotide this method is substantially less sensitive than the procedure that utilizes absorbance at 340 mμ (Fig. 2). However, in contrast to the latter procedure, the present method is applicable for the

TABLE 3
Determination of Changes in NADPH Concentration in the Course of Demethylation of *p*-Chloro-*N*-methylaniline by 9000*g* Liver Supernatant from Male Rats

A. NADPH added

Incubation,[a] min	NADPH, μmoles		*p*-Chloroaniline, μmoles formed
	Found	Disappeared	
0	1.77	−0.11	
12	0.46	1.31	0.21
24	0.23	1.54	0.31

B. NADPH generated *in situ*

Incubation,[b] min	NADPH, μmoles found	*p*-Chloroaniline, μmoles formed
0	1.96	
12	1.92	0.22
24	2.10	0.57

[a] Incubation conditions and assay of *p*-chloroaniline were essentially as previously described (8). In a 1 ml incubation: sodium phosphate buffer, pH 7.4 (50 μmoles); nicotinamide (10 μmoles); *p*-chloro-*N*-methylaniline (1.5 μmoles); NADPH (0.83 μmole) and 9000*g* liver supernatant (49.5 mg liver prepared in 0.01 *M* sodium phosphate/0.0014 *M* $MgCl_2$, pH 7.6). At the indicated time intervals a 2 ml aliquot was taken and was added to a 3 ml PDAB solution (90 mg PDAB in 1.5 *N* H_2SO_4). Following centrifugation, the clear supernatant was read at 445 mμ (*p*-chloroaniline) and at 510 mμ (NADPH) in the Beckman DU monochrometer equipped with a Gilford model 2000 multiple-sample absorbance recorder.

[b] Incubation constituents as described in the above footnote except for the absence of NADPH and for the following additions: NADP (0.8 μmole); $MgCl_2$ (15 μmoles); glucose 6-phosphate (8 μmoles); glucose-6-phosphate dehydrogenase (60 Bücher units).

measurement of a wide range of concentrations (ca. 20-fold) without the necessity of performing serial dilutions.

In assaying enzymic reactions, the color reagent (PDAB) performs a dual function, i.e., stops the enzymic reaction by precipitating the protein and reacts with the reduced cofactor to form the desired color. This property of the color reagent facilitates the analysis of fast enzymic reactions.

The present method was applied to the determination of changes in NADPH concentration in enzymic reactions known to utilize this cofactor (Tables 3 and 4). The proper function of the NADPH-generating system was demonstrated; NADPH remained constant within the time examined (Table 3B). Since, in these experiments, controls were not used, stoichiometry between NADPH loss and product formation cannot be deduced.

Previous investigations demonstrated that PDAB, often referred to as

TABLE 4
Determination of Alteration in NADPH Concentration in the Course of Ring A Reduction of Cortisol by Liver Microsomes from Female Rats

Incubation,[a] min	NADPH, μmoles		Cortisol, μmoles	
	Found	Disappeared	Ring A intact	Ring A reduced
0	1.08	0.02	0.37	
10	0.59	0.51		
20	0.17	0.93	0.17	0.20

[a] Conditions of incubation and determination of reduction of ring A of cortisol were essentially as previously described (9). In a 1 ml incubation: cortisol (0.44 μmole); Tris (10 μmoles, pH 7.4); KCl (50 μmoles); microsomes (100 mg liver equivalent); NADPH (1.23 μmoles). Incubations were at 37°C in an atmosphere of air. At the indicated time intervals, two 0.9 ml aliquots were taken, one of which was added to 4.0 ml of PDAB solution (90 mg PDAB in 1.13 N H_2SO_4) for NADPH assay and the other aliquot was extracted with 5 ml methylene chloride and read at 240 mμ for cortisol determination. NADPH determination as described in "Materials and Methods."

Ehrlich's reagent, reacts with a variety of compounds to yield colored products (3–5). In fact, based on such findings assays have been developed that permit the quantitative determination of various compounds, such as indole derivatives (6, 7) and certain aromatic amines (7, 8). With these substances the colored products usually absorb at wavelengths which do not interfere with the present assay. However, the possibility that certain substances may be present whose colored product will interfere with the measurement of reduced pyridine nucleotides cannot be ruled out.

Lastly, although the present method is considerably less sensitive than some of the classic procedures, its simplicity and the possible use of a simple colorimeter will permit its utilization by laboratories that lack the more costly spectrophotometers.

SUMMARY

A method has been developed for the determination of NADPH and NADH in aqueous solutions. The method is based on a reaction of the reduced pyridine nucleotide with *p*-dimethylaminobenzaldehyde in weak acid solutions and the intensity of the color formed is determined spectrophotometrically. Compounds such as NAD, NADP, nicotinamide, ADP, or ATP do not form color under these assay conditions, demonstrating the specificity of the reagent for the reduced pyridine moiety. The usefulness of this method is discussed.

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