CHROM. 5764

QUANTITATIVE DETERMINATION OF OXIDIZED PYRIDINE NUCLEOTIDES BY A PAPER CHROMATOGRAPHIC TECHNIQUE

TAKUO SAKAI*, TOMOFUMI UCHIDA AND ICHIRO CHIBATA

Department of Applied Biochemistry, Chemical Research Laboratory, Tanabe Seiyaku Co., Ltd., 962, Kashima-cho, Higashiyodogawa-ku, Osaka, 532 (Japan)

(Received August 17th, 1971)

SUMMARY

A simplified method for the quantitative determination of oxidized pyridine nucleotides was developed by using a method consisting of a paper chromatographic technique and a fluorometric technique. By this method, oxidized pyridine nucleotides — NAD^+ and $NADP^+$ — were completely separated and determined. The present method is as accurate as the enzymatic method and is believed to be advantageous for the quantitative determination of oxidized pyridine nucleotides in a large number of samples. The practical limit for the quantitative determination of oxidized pyridine nucleotides was found to be 1 nmole, with a recovery of greater than 95%.

INTRODUCTION

There are several methods for the quantitative determination of pyridine nucleotides: (a) the method based on the measurement of the light absorption of reduced pyridine nucleotides at 340 nm (refs. 1 and 2); (b) the method based on the conversion of the oxidized pyridine nucleotides to fluorescent derivatives[2]; (c) the method based on the reaction between the reduced pyridine nucleotides and dyes[3–5]; and (d) the method involving enzyme cycling[6,7]. In these methods, highly purified enzymes are generally required as the reagents for the separative determination of pyridine nucleotides. It is not easy to obtain the highly purified enzymes for the determination of pyridine nucleotides in a large number of samples.

Paper chromatography is used as a simple and convenient technique for both quantitative and qualitative determinations of amino acids and nucleic acid-related compounds. The authors attempted to use paper chromatography for the determination of oxidized pyridine nucleotides.

In the present method, oxidized pyridine nucleotides were separated by paper chromatography and detected and determined by a fluorometric technique after extraction with water from the paper chromatogram.

* Present address: Department of Agricultural Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka, Japan.

EXPERIMENTAL

Materials and apparatus

Toyo Roshi No. 51A filter-paper was used for the paper chromatography. Authentic pyridine nucleotides and enzymes were obtained from C. F. Boehringer & Söhne (Mannheim, G.F.R.), and the other chemicals were obtained from Katayama Chemicals (Osaka, Japan).

An Aminco Bowman spectrophotofluorometer (American Instrument Co. Inc., Silver Spring, Md., U.S.A.) was used to obtain quantitative data.

Method

An outline of the present method is shown in Scheme I.

(A) *Separation and detection*

Paper chromatography
↓
Placed in a mixing vapour of ammoniacal methyl ethyl ketone for 30 min at room temperature
↓
Detected by fluorescence under UV irradiation

(B) *Determination*

Extracted with 1 ml of water
↓ 0.2 ml of methyl ethyl ketone and 0.8 ml of 2.6 *N* NaOH added
Mixed and kept for 5 min at room temperature
↓ 3.0 ml of 0.85 *N* HCl added
Mixed and kept for 5 min at room temperature
↓
Heated on a boiling water bath for 5 min
↓
Cooled under running water
↓
Fluorescent intensity measured

Scheme I. Outline of determination of NAD^+ and $NADP^+$.

To separate the pyridine nucleotides from each other, 50 μl of the sample solution were applied as a band on the original line of a filter-paper, then paper chromatography was carried out.

After being developed, the paper was dried at room temperature. The dried paper was placed in a chamber filled with a mixing vapour of methyl ethyl ketone and ammonia (1:1). By this treatment, the spots containing oxidized pyridine nucleotides on the paper chromatogram became visible as bluish-white fluorescence under ultraviolet irradiation. The area containing the oxidized pyridine nucleotides was cut into pieces, put into a test-tube and extracted with 1 ml of water for a few minutes. After extraction, to each eluent were added 0.2 ml of methyl ethyl ketone and 0.8 ml of 2.6 *N* sodium hydroxide solution. The tube was shaken and left standing for 5 min, then 3 ml of 0.85 *N* hydrochloric acid were added and the tube was well shaken and

left standing for a further 5 min. After the tube had been heated for 5 min on a boiling water bath, it was cooled under running water.

The fluorescence of the supernatant was measured with the spectrofluorophotometer which was standardized against a solution of quinine sulphate at a concentration of 1 μg/ml in 0.1 *N* sulphuric acid.

All treatments, except heating, were carried out at room temperature.

RESULTS AND DISCUSSION

Separation of oxidized pyridine nucleotides

In the present method, paper chromatography was used to separate oxidized pyridine nucleotides. To select a solvent for the chromatography, we paid attention to the following two points. One was the complete separation of NAD^+ and $NADP^+$; the other was the complete separation of nicotinamide ribotide, nicotinamide riboside and N^1-methylnicotinamide from NAD^+ and $NADP^+$.

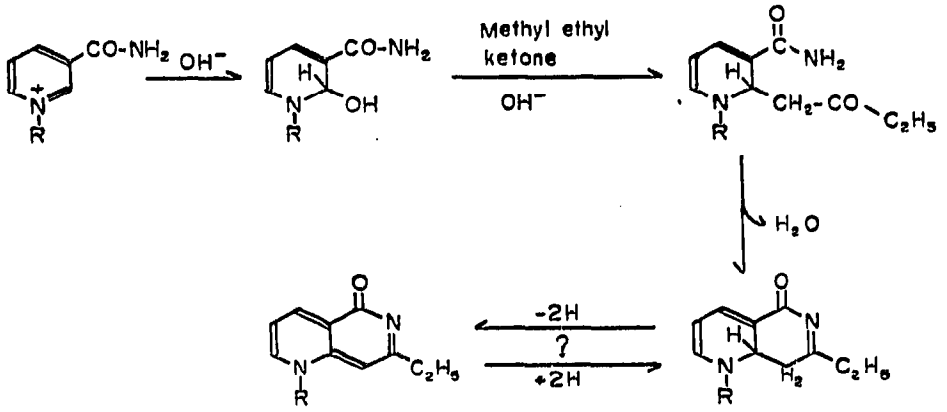


Fig. 1. Probable pathway of the condensation of quaternary pyridine compounds with methyl ethyl ketone.

The detection and determination of oxidized pyridine nucleotides in the present method were based on the conversion of quaternary pyridine compounds having a –CO–NH–R side-chain in the β-position to a fluorescent derivative by means of a carbonyl reagent, such as acetone or methyl ethyl ketone, in alkali (Fig. 1)[8–10]. Naturally-occurring quaternary pyridine compounds are believed to be NAD^+, $NADP^+$, nicotinamide ribotide, nicotinamide riboside, N^1-methylnicotinamide, desamido-NAD, niacin ribotide, niacin riboside and N^1-methylniacin. The first five compounds were detected and determined by the present method, but the last four were not.

For these reasons, we chose the solvent system 60% ammonium sulphate in 0.1 *M* phosphate buffer, pH 6.8, with 2% of *n*-propanol[10], from the various solvent systems tested. R_F values of pyridine nucleotides, nucleic acid-related compounds and amino acids are shown in Table I.

By using this solvent system, NAD^+ was apparently separated from $NADP^+$, and NAD^+ and $NADP^+$ were separated from nicotinamide ribotide, nicotinamide riboside and N^1-methyl nicotinamide.

Detection of oxidized pyridine nucleotides on the paper chromatogram

In the present method, the chromatographic technique plays an important part in the quantitative determination of pyridine nucleotides, and the detectable limit on the chromatogram is the practical lower limit. The pyridine nucleotides are able to be detected as a result of the presence of fluorescent substances formed by the reaction with methyl ethyl ketone and ammonia. Therefore the conditions for the treatment with ammoniacal methyl ethyl ketone were examined to determine

TABLE I

R_F VALUES OF PYRIDINE COMPOUNDS, NUCLEIC ACID-RELATED COMPOUNDS AND AMINO ACIDS

The paper chromatography was carried out for 16 h at room temperature. Solvent system: 60% ammonium sulphate in 0.1*M* phosphate buffer, pH 6.8, with 2% of *n*-propanol.

Compound	R_F	*Compound*	R_F
Pyridine compounds		*Nucleic acid-related compounds*	
NAD+	0.27	Adenine	0.12
NADP+	0.40	Guanine	0.15
Nicotinamide ribotide	0.76	Xanthine	0.26
Nicotinamide riboside	0.75	Hypoxanthine	0.25
N^1-Methylnicotinamide	0.74	Cytosine	0.55
NADH	0.11	Uracil	0.50
NADPH	0.22	Adenosine	0.13
Nicotinamide	0.29	Guanosine	0.26
Niacin	0.33	Inosine	0.36
		Cytidine	0.56
Amino acids		Uridine	0.55
L-Ala	0.92	5'-AMP	0.24
L-Cys	0.83	5'-GMP	0.40
Gly	0.93	5'-IMP	0.55
L-Lys	0.98	5'-CMP	0.67
L-Met	0.76	5'-UMP	0.65
L-Phe	0.59	ADP	0.34
L-Trp	0.24	ATP	0.37
L-Tyr	0.58		

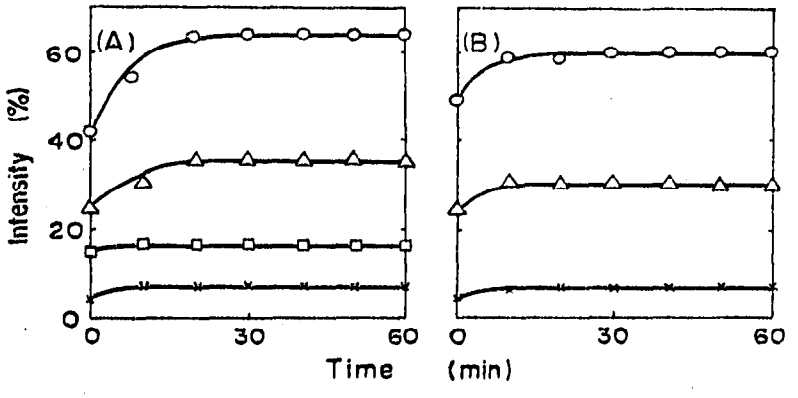


Fig. 2. Effect of ammoniacal methyl ethyl ketone on the development of fluorescence. The dried paper chromatogram was placed in a chamber filled with a mixing vapour of methyl ethyl ketone and ammonia (1:1) for the indicated time at room temperature. After the development of fluorescence as described in the text, the fluorescent intensity of NAD+ or NADP+ in each spot was measured. (A) 11 nmoles (○), 6 nmoles (△), 3 nmoles (□) or 1 nmole (×) of NAD+ were used; (B) 10 nmoles (○), 5 nmoles (△) or 1 nmole (×) of NADP+ were used.

the practical detection limit of the present method. As shown in Fig. 2, the fluorescent intensity reached a constant value after treatment for 30 min. Therefore, 30 min was chosen for the treatment time in the present method. Under this condition, the practical lower detection limit was 1 nmole of each pyridine nucleotide in a spot.

Fluorometric determination of oxidized pyridine nucleotides

To determine each oxidized pyridine nucleotide separated by paper chromatography, the fluorometric method of TABUCHI *et al.*[11] was used with some modification.

The optimal conditions for the alkali-, acid- and heat-treatment steps were examined. The optimal concentrations of alkali and acid were 2.6 *N* and 0.85 *N*, respectively. As shown in Fig. 3, the efficient treatment times with alkali, acid and heat were each found to be 5 min.

The derivatives formed in this method gave the most intense fluorescence with excitation at 375 nm and emission at 460 nm (Fig. 4) and were stable for at least 2 h at room temperature (Fig. 5).

Interference of various compounds with the development of fluorescence

When oxidized pyridine nucleotides were separated by paper chromatography, it is possible that some compounds overlapped with NAD^+ or $NADP^+$ on the paper chromatogram and interfered with the development of fluorescence. The influence of various compounds on the development of fluorescence was therefore examined. As shown in Table I, some of nucleic acid-related compounds showed R_F values close

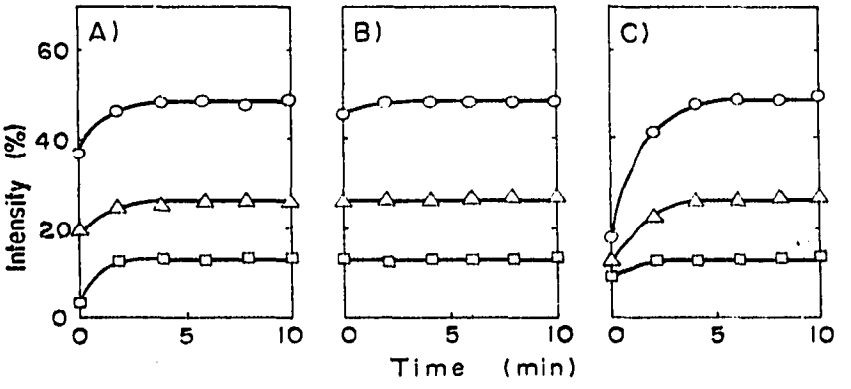


Fig. 3. Effect of treatment with (A) NaOH, (B) HCl and (C) heat on the development of fluorescence; 8 nmoles (○), 4.5 nmoles (△) or 2 nmoles (□) of NAD^+ were determined as described in the text except that the treatment periods were changed as indicated.

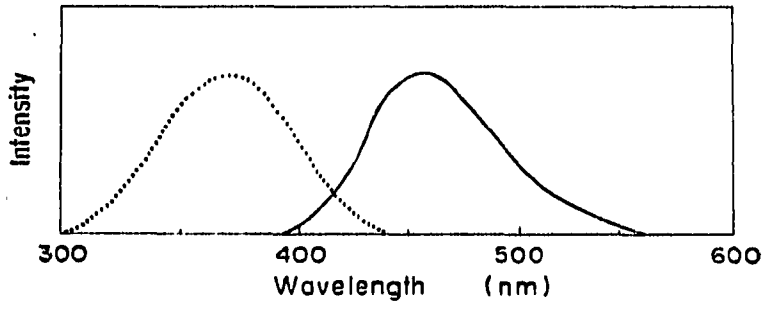


Fig. 4. Excitation and emission spectra of the reaction product of NAD^+ and methyl ethyl ketone. Excitation spectrum (. . . . .) emitted at 460 nm; emission spectrum (——) excited at 375 nm.

to those of NAD^+ or $NADP^+$. However, they did not interfere with the development of fluorescence of NAD^+ in the present method (Table II).

Sugars, such as D-mannose and D-xylose, interfered with the development of fluorescence, but the interference occurred at comparatively higher concentrations than those in common culture media (Table III).

Among the amino acids tested in this study, L-cysteine caused a decrease in the fluorescent intensity, and glycine, L-lysine and L-tryptophan caused an increase in the fluorescent intensity (Table IV). However, these amino acids were separated

TABLE II

EFFECT OF NUCLEIC ACID-RELATED COMPOUNDS ON THE DEVELOPMENT OF FLUORESCENCE

Ten nmoles of NAD^+ were added to assay tubes in the presence of each nucleice acid-related compound and the fluorescent intensity was measured.

Compound	*Relative intensity*[a]	
	0.1 μmole added	*0.3 μmoles added*
Nicotinamide	96	104
Niacin	100	96
Adenine	99	98
Adenosine	100	98
5′-AMP	99	97
ADP	100	101
ATP	99	98
5′-GMP	97	96
5′-IMP	101	97
5′-CMP	98	96
5′-UMP	102	96

[a] Relative intensity: the fluorescent intensity of NAD^+ in the absence of compounds was taken as 100%.

TABLE III

EFFECT OF SUGARS ON THE DEVELOPMENT OF FLUORESCENCE

The determination of the fluorescent intensity was performed as described in Table II except that sugars were added instead of nucleic acid-related compounds.

Sugar	*Relative intensity*	
	1000 μmoles added	*5 μmoles added*
Raffinose	108	99
Sucrose	102	100
Lactose	100	98
D-Glucose	94	102
D-Galactose	95	96
D-Mannose	149	99
D-Fructose	96	100
L-Sorbose	106	102
Sorbitol	100	98
D-Ribose	102	100
D-Xylose	120	102
L-Arabinose	105	98

from NAD^+ or $NADP^+$ on the paper chromatogram (Table I) and did not interfere with the determination of NAD^+ or $NADP^+$ in the present method.

Metal ions, such as Cu^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Fe^{3+}, showed interference with the development of fluorescence (Table V). However, the effective concentrations

TABLE IV

EFFECT OF AMINO ACIDS ON THE DEVELOPMENT OF FLUORESCENCE

The determination of the fluorescent intensity was performed as described in Table II except that amino acids were added instead of nucleic acid-related compounds.

Amino acid	*Relative intensity*		
	2 μmoles added	*0.2 μmole added*	*0.02 μmole added*
L-Ala	97	100	98
L-Arg	98	102	99
L-Asp	98	102	100
L-Cys	81	90	99
Gly	111	100	101
L-Glu	100	99	100
L-His	98	100	96
L-Ile	98	102	100
L-Leu	98	100	99
L-Lys	111	102	99
L-Met	100	99	100
L-Phe	98	100	102
L-Pro	100	98	100
L-Ser	100	99	103
L-Thr	100	102	98
L-Trp	134	123	100
L-Tyr	100	100	102
L-Val	100	99	100

TABLE V

EFFECT OF METAL IONS ON THE DEVELOPMENT OF FLUORESCENCE

The determination of the fluorescent intensity was performed as described in Table II except that metal ions were added instead of nucleic acid-related compounds.

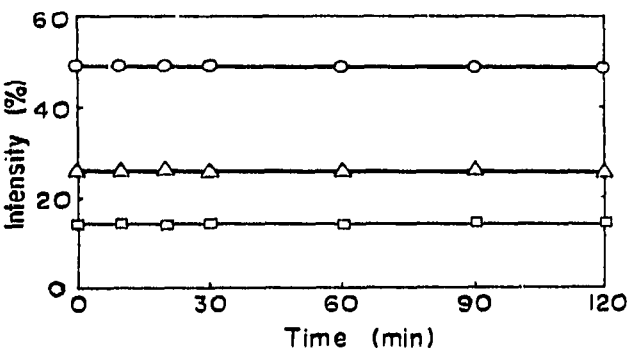
Metal ion	*Relative intensity*		
	500 nmoles added	*5 nmoles added*	*0.5 nmole added*
Na^+	100	100	99
K^+	100	100	100
Li^+	99	101	98
Mg^{2+}	101	103	99
Ca^{2+}	99	96	98
Ba^{2+}	99	99	100
Zn^{2+}	98	100	99
Sr^{2+}	102	95	98
Cd^{2+}	98	100	100
Cu^{2+}	111	99	98
Mn^{2+}	33	100	102
Ni^{2+}	110	100	101
Co^{2+}	140	111	98
Fe^{3+}	116	100	100

of these heavy metal ions were much higher than those found in the common biological materials or culture media.

The interference of the above compounds with the determination of oxidized pyridine nucleotides was negligible in the present method because effective compounds were separated from NAD^+ or $NADP^+$ on the paper chromatogram and effective concentrations were comparatively high.

Standard assay curves

By using the method described above, contents of NAD^+ and $NADP^+$ at various concentrations in solutions were determined. Fig. 6 shows the linearity of the graph of fluorescent intensity against concentration over the range 0–10 nmole of NAD^+ or $NADP^+$. The molecular fluorescent intensity of NAD^+ was the same as that of $NADP^+$.



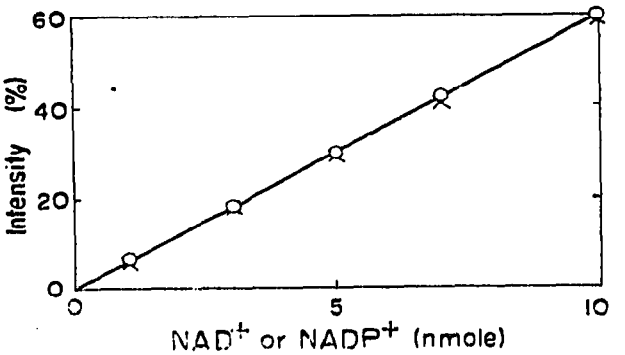


Fig. 5. Stability of fluorescent substance. Fluorescent substance derived from NAD^+ by the present method was left standing at room temperature for the indicated time, and the fluorescent intensity was measured at intervals; 8 nmoles (○), 4.5 nmoles (△) or 2 nmoles (□) of NAD^+ were used.

Fig. 6. Standard curves for the determination of NAD^+ (○) and $NADP^+$ (×).

TABLE VI

RECOVERIES OF NAD^+ AND $NADP^+$ ADDED TO THE CULTURE MEDIUM

Amounts of NAD^+ or $NADP^+$ were determined by the present method in 50 µl of the culture medium, which contained 5.0% of glucose, 1.5% of casein hydrolysate, 1.5% of yeast extract, 0.5% of NaCl, 0.1% of KH_2PO_4, 0.1% of K_2HPO_4 and 0.03% of $MgSO_4 \cdot 7H_2O$ (pH 6.5).

Nucleotide	*Amount added*[a] *(nmoles)*	*Amount found (nmoles)*	*Recovery (%)*
NAD^+	1	1.00	100
	3	3.08	103
	5	5.08	101
	7	7.25	104
	10	9.50	95
$NADP^+$	1	0.97	97
	3	2.83	95
	5	4.92	98
	7	6.92	99
	10	10.3	103

[a] NAD^+ and $NADP^+$ were usually determined by alcohol dehydrogenase and isocitrate dehydrogenase, respectively.

Recoveries of oxidized pyridine nucleotides

Table VI shows recovery data for NAD^+ and $NADP^+$ added to the medium containing yeast extract, casein hydrolysate, etc. These data show that recoveries of NAD^+ and $NADP^+$ were essentially complete.

Table VII shows that the recoveries of NAD^+ and $NADP^+$ were between 96 and 103 % when they were added to filtered broth.

From these data, the present method is believed to be more simple and convenient than the previously known methods for the separative determination of oxidized pyridine nucleotides in a large number of samples.

TABLE VII

RECOVERIES OF NAD^+ AND $NADP^+$ AFTER ADDITION TO THE CULTURE FILTRATE

Escherichia coli and *Saccharomyces cerevisiae* were grown in the medium described in Table VI. NAD^+ (0.190 μmole/ml) and $NADP^+$ (0.160 μmole/ml) were added to 50 μl of the culture filtrate and determined by the present method. NAD^+ was determined with alcohol dehydrogenase and $NADP^+$ with isocitrate dehydrogenase.

Strain cultured	*Amount of NAD^+ (μmole/ml)*		*Recovery (%)*	*Amount of $NADP^+$ (μmole/ml)*		*Recovery (%)*
	Added	*Found*		*Added*	*Found*	
E. coli	0.190	0.188	99	0.160	0.165	103
S. cerevisiae	0.190	0.190	100	0.160	0.154	96

ACKNOWLEDGEMENTS

We express our sincere thanks to Mr. T. TAKAYANAGI, Senior Managing Director of Research and Development, and Dr. K. FUJII, Director of Product Control Laboratory, for their encouragement during the course of this study, and grateful acknowledgement is made to Miss H. YAMAZATO for her technical assistance.

REFERENCES

1 E. RACKER, *J. Biol. Chem.*, 184 (1950) 313.
2 M. M. CIOTTI AND N. O. KAPLAN, *Methods Enzymol.*, 3 (1957) 890.
3 T. F. SLATER AND B. SAWYER, *Nature*, 193 (1962) 454.
4 J. S. NISSELBAUM AND S. GREEN, *Anal. Biochem.*, 27 (1969) 212.
5 D. KUPFER AND T. MUNSELL, *Anal. Biochem.*, 25 (1968) 10.
6 G. E. GLOCK AND P. MCLEAN, *Biochem. J.*, 61 (1955) 381.
7 O. H. LOWRY, J. V. PASSONNEAU, D. W. SCHULTZ AND M. K. ROCK, *J. Biol. Chem.*, 236 (1961) 2746.
8 J. W. HUFF, *J. Biol. Chem.*, 167 (1947) 151, 169.
9 K. J. CARPENTER AND E. KODICEK, *Biochem. J.*, 46 (1950) 421.
10 E. KODICEK AND K. K. REDDI, *Nature*, 168 (1951) 475.
11 S. TABUCHI, K. NISIDA AND Y. UEDA, *Vitamins (Japan)*, 27 (1963) 303.