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An Enzymic Method for the Assay of Pyridine Nucleotides in Extracts of Animal Tissues

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(Received 23 October 1961)

An enzymic assay of oxidized and reduced di- and tri-phosphopyridine nucleotides was described by Glock & McLean (1955). In their assay system the continuous reduction of DPN⁺ in the presence of an excess of ethanol and alcohol dehydrogenase was coupled with DPNH-cytochrome *c* reductase prepared from pig heart. In a similar fashion the continuous reduction of TPN⁺ in the presence of glucose 6-phosphate and glucose 6-phosphate dehydrogenase was coupled with TPNH-cytochrome *c* reductase prepared from brewer's yeast. The reduction of cytochrome *c* was followed spectrophotometrically at 550 m μ . The present report describes a modification of this assay.

Diaphorase and dichlorophenol-indophenol are used in place of the cytochrome *c* reductases and cytochrome *c*. This permits the assays to be carried out with enzyme preparations that are commercially available. The dehydrogenases are absolutely specific for their respective pyridine nucleotides; hence the additional specificity of the cytochrome *c*

reductases can be dispensed with. A preliminary account of this work has appeared previously (Vilee, 1961).

MATERIALS AND METHODS

Samples (about 200 mg.) of tissue are blotted free of blood and weighed rapidly on a torsion microbalance. Glass Potter-Elvehjem tissue homogenizers containing 5 ml. of 0.1N-HCl or 0.1N-NaOH are placed in a boiling-water bath. The tissues are added and 30 sec. later the homogenizers are removed from the boiling water. The tissues are homogenized for 90 sec. and then transferred to an ice bath. 0.2M-Tris buffer, pH 7.4 (1 ml.) is added to the content of each homogenizer and the solutions are neutralized by the dropwise addition, with constant stirring, of dilute acid or base. The neutralized extracts are then centrifuged at 18 800*g* at 0° for 30 min. The oxidized coenzymes are relatively stable in dilute alkali and the reduced coenzymes are relatively stable in dilute acid (Schlenk, 1951; Glock & McLean, 1955). Thus the acid extract contains DPNH and TPNH, whereas the alkaline extract contains DPN⁺ and TPN⁺. The usual volume of extract required for

DPN determinations is 0.05–0.1 ml., and for TPN is 0.05–2.0 ml. The total volume of the acid or alkaline extract is 20 ml. after acidification, neutralization and centrifuging.

Some tissues contain interfering substances that react spontaneously with the dichlorophenol-indophenol, changing it to the colourless form. To overcome this, small amounts of dye are added dropwise to the extracts after neutralization and centrifuging until a slight excess is present, as indicated by a pale blue colour which does not fade. Portions of the neutralized acid or alkaline extract are incubated with an excess of TPN-specific glucose 6-phosphate dehydrogenase (Sigma Chemical Co., type V) or with an excess of DPN-specific alcohol dehydrogenase (Sigma), together with an excess of diaphorase (Worthington Biochemical Corp.) and 2,6-dichlorophenol-indophenol. Preparations of glucose 6-phosphate dehydrogenase less pure than Sigma type V give some dye reduction in the control cuvette (Table 1, cuvette 3), and perhaps contain some diaphorase.

The diaphorase was prepared from *Clostridium kluyveri* (Mahler, Sarkar, Vernon & Alberty, 1952) and was free of pyridine nucleotides. Its specific activity was 50 units/mg. of protein.

Each cuvette contained 2.0 units of diaphorase and 0.1 μ mole of 2,6-dichlorophenol-indophenol. The reduction of the dye was followed in a Zeiss PMQ II spectrophotometer by measuring the decrease in absorption at 600 m μ . The assay system was set up so that the rate of reduction of the dye is limited only by the amount of pyridine nucleotide present. Diaphorase is present in tenfold excess over the highest rate of dichlorophenol-indophenol reduction measured. The details of the assay systems are given in Table 1.

The oxidation–reduction dye, phenazine methosulphate, at a concentration of 0.33 mM in the cuvette, can replace the diaphorase in mediating the transfer of hydrogen from reduced pyridine nucleotide to dichlorophenol-indophenol. With DPNH the rate of reduction of the dye is a linear function of the concentration of pyridine nucleotide over the range 0.5–3.0 μ m-moles of DPNH per cuvette. At any given concentration of DPNH the rate of dye reduction with phenazine methosulphate is approximately twice that with diaphorase as hydrogen-transfer agent. In assays of TPNH, the rate of dye reduction with phenazine methosulphate is less than half the rate with diaphorase for any given concentration of TPNH. The rate of dye reduction is a linear function of TPNH concentration over the range 1–5 μ m-moles per cuvette, but in the lower part of the range the rate of dye reduction is quite low and the values are subject to error.

The assay is based on the principle that the concentrations of reduced pyridine nucleotides are very much smaller (0.5–4.0 μ mM) than the K_m for diaphorase (20 μ mM) and that the rate of diaphorase reaction is therefore proportional to the concentration of pyridine nucleotide. The linear relationship between concentration of pyridine nucleotide and the rate of dye reduction begins to break down at a concentration of 5 μ mM. At a concentration of 7 μ mM-TPN the linear relationship between dye reduction and pyridine nucleotide concentration can be restored by increasing the amount of diaphorase in the cuvette threefold. The usual assay reactions are completed in 3 min. and in this time there is no interference due to the reoxidation of dichlorophenol-indophenol by air.

RESULTS

The diaphorase from *Clostridium kluyveri* will react with either DPNH or TPNH. In measuring the amount of reduced pyridine nucleotide in a tissue extract by this system any DPNH or TPNH present will quickly be oxidized by the great excess of diaphorase and dye. However, no further reduction of the dye occurs unless the pyridine nucleotides are reduced by their specific dehydrogenases. In the presence of an excess of DPN-specific alcohol dehydrogenase and ethanol, the rate of subsequent reduction of the dye is a linear function of the amount of DPNH present over the range 0.5–4.0 μ m-moles of DPNH per cuvette. In the presence of an excess of TPN-specific glucose 6-phosphate dehydrogenase and glucose 6-phosphate, the rate of subsequent reduction of the dye is a linear function of the amount of TPNH present over the range 0.5–4.0 μ m-moles of TPNH per cuvette. Suitable control experiments showed that the addition of DPN did not interfere with the assay for TPN nor did the addition of TPN interfere with the assay for DPN. Tissue extracts treated with both acid and alkali gave no reduction of the dye when tested in the assays for either DPN or TPN. The results are corrected for the small separately-determined rate of non-enzymic reduction of the dye by the tissue extract alone (Table 1, cuvette 3) and by a combination of diaphorase, dehydrogenase, and substrate in the absence of the tissue extract (Table 1, cuvette 1).

Analyses of rat liver, kidney and muscle gave values which agreed well with those obtained by Glock & McLean (1955) (Table 2). In all of these tissues DPN is present largely in the oxidized form whereas TPN is present mainly in the reduced form. There is no measurable breakdown of either DPN or TPN by the diaphorase preparation. This was determined by incubating diaphorase in buffer at room temperature with DPN. A control vessel was set up without DPN. After 30 min. DPN was added to the control vessel, and ethanol, alcohol dehydrogenase and dye were added to both. The rate of dye reduction measured spectrophotometrically was similar in both cuvettes. An analogous experiment was carried out with TPN, with glucose 6-phosphate, glucose 6-phosphate dehydrogenase and dye added after 30 min. In both experiments the rates of dye reduction were similar in the two cuvettes.

Analyses of standard solutions of DPN and TPN established the linear relation between pyridine nucleotide concentration and the rate of reduction of dichlorophenol-indophenol. Standard solutions of four concentrations of DPN and four of TPN were analysed each day along with the tissue extracts to ensure the linearity of the assays.

Table 1. *Assay systems for pyridine nucleotides*

Substrate, enzyme, tissue extract (tris buffer, pH 7.4, in cuvette 1), 2,6-dichlorophenol-indophenol and tris buffer, pH 7.4, are mixed (total vol. 2.9 ml.) and the extinctions at 600 m μ measured. The extinction of cuvette 1 is set at 0.600, and diaphorase (tris buffer, pH 7.4, in cuvette 3) added. The contents of the cuvettes are mixed, and the extinction of each measured at intervals of 30 sec. Each cuvette contained a final total vol. of 3.0 ml., and a final total of 60 μ moles of tris.

							Vol. (ml.)		
Cuvettes	...	...	...	...	...	...	1	2	3
(a) Assay for DPNH									
Ethanol (95%)							0.1	0.1	0.1
Alcohol dehydrogenase*							0.15	0.15	0.15
Tissue extract							0	+	+
2,6-Dichlorophenol-indophenol (0.35 mg./ml.)							0.1	0.1	0.1
Diaphorase							0.1	0.1	0
							Vol. (ml.)		
Cuvettes	...	...	...	...	...	...	1	2	3
(b) Assay for TPNH									
Glucose 6-phosphate (3.3 mg./ml.)							0.3	0.3	0.3
Glucose 6-phosphate dehydrogenase (0.4 Kornberg unit/0.01 ml.)							0.01	0.01	0.01
Tissue extract							0	+	+
2,6-Dichlorophenol-indophenol (0.35 mg./ml.)							0.1	0.1	0.1
Diaphorase							0.1	0.1	0

* Alcohol dehydrogenase is the Sigma preparation of crystalline enzyme diluted 1:30 with distilled water.

Table 2. *Concentrations of pyridine nucleotides in rat tissues*

Results are given as μ moles/g. of tissue.

Tissue	DPN ⁺	DPNH	TPN ⁺	TPNH
Liver	0.39	0.17	0.015	0.35
Kidney	0.72	0.40	0.036	0.22
Muscle	0.51	0.064	<0.005	0.025

Table 3. *Recovery of pyridine nucleotides added to tissue slices*

The amount of each nucleotide added was 0.1 μ mole.

Amounts recovered (μ mole)			
DPN ⁺	DPNH	TPN ⁺	TPNH
0.075	0.130	0.098	0.083
0.096	0.057	0.107	0.059
0.109	0.074	0.104	0.092
0.101	0.098	0.111	0.102
0.092	0.078	0.110	0.119
0.103	0.107	0.094	0.132
0.096	0.091	0.104	0.098
$\pm$ 0.005	$\pm$ 0.010	$\pm$ 0.003	$\pm$ 0.010

In three experiments, each carried out in duplicate, 0.1 μ mole of DPN and 0.1 μ mole of TPN were added to homogenizers containing slices of rat liver and extracts were prepared in the usual fashion. Control slices, to which no nucleotides were added, were extracted separately. From the

differences between the analyses of control and experimental tissues it was calculated that 96 % of the added DPN⁺ and 104 % of the added TPN⁺ were recovered (Table 3). The recovery of added DPNH was 91 % and that of added TPNH was 98 %.

Direct measurements of the concentrations of oxidized and reduced DPN and TPN in human term placenta (Table 4) showed that, as in rat liver, DPN is present largely in its oxidized form and TPN is present largely in its reduced form. The concentration of oxidized TPN in term placenta is quite low. Independent estimates of the concentrations of oxidized pyridine nucleotides were made by the fluorimetric method of Jacobson & Astrachan (1957). This measures the fluorescence of oxidized pyridine nucleotides in 5N-NaOH at 460 m μ with an excitation beam of 370 m μ . The results of the enzymic assays and fluorimetric assays are in good agreement (Table 4).

The concentrations of oxidized and reduced pyridine nucleotides in a number of human foetal tissues have been assayed by this method (Table 5). These measurements indicate that in foetal tissues as in adult tissues the DPN is present predominantly in its oxidized form and TPN predominantly in its reduced form. The levels of TPN⁺ and TPNH found were quite low. The low level of TPNH in these tissues was an unexpected finding in view of the important role of TPNH in biosynthetic reactions.

Table 4. *Concentration of oxidized and reduced pyridine nucleotides in human term placenta*

The values are means $\pm$ s.e. of 8 determinations of eight term placentas, expressed as μ moles/g. of fresh tissue.

Nucleotide	Enzymic assays	Fluorimetric assays
DPN ⁺	0.224 $\pm$ 0.026	0.236 $\pm$ 0.022
DPNH	0.055 $\pm$ 0.011	—
TPN ⁺	0.0056 $\pm$ 0.0008	0.004 $\pm$ 0.0007
TPNH	0.0095 $\pm$ 0.0016	—

Table 5. *Concentrations of pyridine nucleotides in human foetal tissues*

Results are given as μ moles/g. of tissue. Each figure represents an analysis of a separate tissue sample.

Tissue	DPN ⁺	DPNH	TPN ⁺	TPNH
Liver	0.410	0.049	0.014	0.027
	0.412	0.059	0.032	0.047
	0.652	0.063	0.018	0.068
	0.460	0.054	0.016	0.053
Lung	0.194	0.041	<0.0039	0.0059
Heart	0.396	0.118	<0.010	0.016
	0.589	0.128	<0.017	0.017
Brain	0.198	0.022	<0.0054	0.0059
	0.159	0.023	<0.0082	0.011
Kidney	0.267	0.038	<0.0083	0.013

DISCUSSION

This modification of the Glock & McLean (1955) method takes advantage of the complete specificity of the glucose 6-phosphate dehydrogenase for TPN⁺ as hydrogen acceptor and the complete specificity of alcohol dehydrogenase for DPN⁺ as hydrogen acceptor. The addition of DPN did not interfere with the standard TPN assay nor did the addition of TPN interfere with the standard DPN assay. This specificity has also been shown by Passonneau & Lowry (1961) who used it to measure, by a system utilizing enzymic amplification, the very small amounts of pyridine nucleotides present

in single cells. The present assay provides a convenient means for measuring oxidized and reduced di- and tri-phosphopyridine nucleotides by using commercially available enzyme preparations. Phenazine methosulphate at a concentration in the cuvettes of 0.33 mM can replace the diaphorase in mediating the transfer of hydrogen from reduced pyridine nucleotides to dichlorophenol-indophenol. The specificities of the assays, which result from the specificity of the dehydrogenases used, are not altered by substituting the phenazine methosulphate for diaphorase.

SUMMARY

1. The concentrations of oxidized and reduced pyridine nucleotides in tissue extracts can be estimated by an enzyme assay which couples the specific dehydrogenases and a non-specific diaphorase and measures the rate of reduction of 2,6-dichlorophenol-indophenol.

2. The results of these assays agree well with those obtained by a fluorimetric assay and with the assays of Glock & McLean (1955) who used cytochrome *c* reductases and cytochrome *c*.

I wish to express my thanks to Miss Joyce Spencer for her careful and expert technical assistance in developing this method. The work was aided by grants from the Charles A. and Marjorie King Fund, from the Association for the Aid of Crippled Children, and Grant C-2400, U.S. Public Health Service.

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