The Estimation of the Oxidized and Reduced Forms of the Nicotinamide Nucleotides

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1. A method is described for the determination of the oxidized and reduced forms of the nicotinamide nucleotides by measuring the rate of the oxygen uptake with an oxygen electrode in a system in which the nucleotide acts as the rate-limiting carrier in a cyclic system. 2. The method permits the measurement of quantities as low as 0.02 $\mu\text{g.}$ of NAD^+ or NADH or 0.01 $\mu\text{g.}$ of NADP^+ or NADPH . 3. The method permits the measurement of the nucleotides in extracts that contain non-specific reducing substances, coloured compounds or fluorescent materials, e.g. green leaves. 4. The results obtained by the present method are compared with those reported in the literature.

It is now well established that the availability and the relative proportions of the oxidized and reduced forms of the nicotinamide nucleotide coenzymes play an important part in regulating the metabolic behaviour of cells (see Dickens, 1959). The increasing interest in this problem has led to a proliferation of methods available for measuring these nucleotides. The methods recently developed reflect two aspects of the problem: those in which the steady-state ratio of the nucleotides is the prime object (Chance, 1961; Hohorst, Kreutz & Reim, 1961) and those in which the main aim is to determine the concentrations and ratios at a fixed time in response to physiological change (Glock & McLean, 1955a; Jacobson & Astrachan, 1957; Bassham, Birt, Hems & Loening, 1959; Lowry, Passonneau, Schulz & Rock, 1961; Vilee, 1962; Pastan, Wills, Herring & Field, 1963). These latter methods may be divided into two broad groups: (a) those that employ a recycling technique, in which the nucleotide undergoes a cyclic oxidation and reduction as a component member of a series of coupled reactions, and the extent of the reaction is measured by the accumulation of an end product, i.e. the nucleotide acts in a catalytic manner; (b) those that involve a direct measurement in which the end product bears a 1:1 relationship with the nucleotide present, e.g. the sensitive fluorimetric system of Jacobson & Astrachan (1957), the radioactive assay of Pastan *et al.* (1963), or the more widely used, but less sensitive, determination depending on enzymic oxidation or reduction and measurement of the extinction at 340 $\text{m}\mu$.

Enzymic recycling systems offer many advantages, in particular the small amount of tissue required, and have been exploited with a variety of coupling systems. Pioneers of such methods were Haas, Harrer & Hogness (1942), who used NADPH -cytochrome *c* oxidoreductase and a manometric estimation of oxygen uptake for the assay of NADP . The method was extended by Glock & McLean (1955a), who employed the specific cytochrome *c* reductases to measure spectrophotometrically the oxidized and reduced forms of both NAD and NADP . The use of diaphorase and dichlorophenol-indophenol by Vilee (1962) obviated the necessity for employing enzymes that were relatively unstable and tedious to prepare. The observation of Dickens & McIlwain (1938) that phenazine methosulphate is a very effective electron acceptor, particularly for the hexose monophosphate pathway, paved the way for the replacement of cytochrome *c* reductase and diaphorase by this substance. Vilee (1962) proposed the use of this substance as a possible carrier, although he appeared to prefer diaphorase. Slater & Sawyer (1962), however, measured both NAD and NADP with phenazine methosulphate as carrier and dichlorophenol-indophenol as acceptor.

Most of these recycling methods share the common difficulty that many tissue extracts contain substances capable of producing reducing equivalents by chemical reaction (e.g. ascorbic acid and glutathione) without the intervention of specific enzymes and require either a system of blanks or a pretitration of the extract. Further, as most are

spectrophotometric methods, they require optically clear solutions, which necessitate either a high-speed centrifugation step or the use of very small volumes of the extract in large volumes of medium. Moreover, Bassham *et al.* (1959) have reported that during the alkaline extraction and on storage of the alkaline extract extensive reoxidation of the reduced nicotinamide nucleotides occurs and suggest that, in such extracts, both the oxidized and reduced forms of the nucleotides must be measured and it is the sum of these that corresponds to the original reduced form. This difficulty can be avoided by a cyclic estimation that would give the amount of reduced nicotinamide nucleotide by a single extraction. A new method has therefore been devised that counters all these difficulties. It uses either alcohol dehydrogenase (EC 1.1.1.1) or glucose 6-phosphate dehydrogenase (EC 1.1.1.49) as the specific reducing system and phenazine methosulphate as electron acceptor. The rate of hydrogen transfer is equivalent to the oxygen consumption as measured by an oxygen electrode coupled to a recorder. With this system quantities as low as 0.02 $\mu\text{g.}$ of NAD⁺ and 0.01 $\mu\text{g.}$ of NADP⁺ can be readily estimated in as little as 2–3 min.

METHODS

Animals. The animals used were either adult male or female albino rats or adult female hooded Norway rats. They were maintained on stock diet M.R.C. 41 B, with free access to food.

Materials. Alcohol dehydrogenase, glucose 6-phosphate dehydrogenase and glucose 6-phosphate were obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany; NAD⁺, NADH, NADP⁺ and NADPH were commercial preparations supplied by Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of tissue extracts. Rats were killed by cervical dislocation and the livers rapidly exposed. Two small pieces of liver, about 400–500 mg., were removed. One piece was used for an acid extraction and the other for an alkaline extraction (0.1 N-HCl and 0.1 N-NaOH respectively) by the procedure of Glock & McLean (1955a). After being cooled on ice the extracts were neutralized, the final volumes were noted and the extracts centrifuged to remove gross particulate matter. High-speed centrifugation is unnecessary. The centrifuged extracts were stored on ice and used for assay of the nicotinamide nucleotides.

In some experiments in which the effect of nicotinamide on the stability of the nucleotides was being examined, 0.01 ml. of 1.0 M-nicotinamide was added to the 10 ml. of either acid or alkali being used for the extraction procedure, which was otherwise carried out as above.

Assay of nicotinamide nucleotides. The oxygen electrode was of the Clark type (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.), which was inserted into the central chamber of a water-jacketed Perspex cell. The electrode was fitted with an 'O' ring to make an airtight fit except for a 1 mm. channel in the 'O' ring to permit the insertion of a syringe needle. The volume of the cell could

be decreased, if required, by the introduction of a Perspex sleeve to reduce the diameter. The smaller volume of medium then contained less dissolved oxygen so that the sensitivity of the system was correspondingly increased.

(a) Assay of the oxidized and reduced forms of NAD. A mixture (1.2 ml.), containing 250 μmoles of glycylglycine buffer, pH 9.0, 1 μmole of EDTA and 1.7 m-moles of ethanol, is placed in the cell of the oxygen electrode. Up to 0.7 ml. of the extract is then added, together with water to make up this volume to 0.7 ml., and finally 0.1 ml. of an aqueous solution of phenazine methosulphate (2 mg./ml.). When very small amounts of nucleotide are present (0.05–0.5 $\mu\text{g.}$) the sensitivity of the method can be improved up to three-fold by using 8 mg. of phenazine methosulphate/ml. After these additions the contents of the cell are stirred by a magnetic 'flea' and the electrode assembly is then carefully inserted and any trapped air bubbles are expelled. During the rest of the procedure the contents of the cell are stirred rapidly and continuously. After allowing 1–2 min. for temperature equilibration and for the completion of any purely chemical reduction, 0.05 ml. of alcohol dehydrogenase containing 0.3 mg. of enzyme protein is injected with a Hamilton micro-syringe. The increase in the rate of oxygen consumption is then recorded on a Kent Multilec mark III recorder (0–2 mv) for 2–3 min. A standard curve for NAD⁺ constructed over the range 0.5–5.0 $\mu\text{g.}$ is suitable for most purposes but can be extended in both directions, as shown below.

(b) Assay of the oxidized and reduced forms of NADP. The procedure for this nucleotide is identical with that used for NAD except that the reaction mixture (1.2 ml.) contains 100 μmoles of tris buffer, pH 8.0, 10 μmoles of glucose 6-phosphate and 1 μmole of EDTA. Phenazine methosulphate (2 mg./ml.) is added as before and the electrode assembled. The reaction is started by the addition of 0.05 ml. of glucose 6-phosphate dehydrogenase solution (containing 5 $\mu\text{g.}$ of enzyme protein in 0.1 M-tris buffer, pH 8.0), and the oxygen consumption recorded as before.

RESULTS AND DISCUSSION

Extraction procedure

A number of methods have been reported for the differential extraction of the oxidized and reduced forms of the nicotinamide nucleotides that are based on the relative stability of these two forms in acid and alkali (see Schlenk, 1951). In general they all give comparable results and equivalent recoveries of NAD⁺, NADH, NADP⁺ and NADPH when these nucleotides are added during the extraction procedure. In the present work 0.1 N-hydrochloric acid and 0.1 N-sodium hydroxide (Glock & McLean, 1955a) have been used since these appear to be best documented and in our hands yield satisfactory recoveries. We have, in addition, tested the effect of adding nicotinamide to the extraction mixture in the hope that this might stabilize the extracted nucleotides against the action of heat-stable nucleosidases (Jacobson & Kaplan, 1957) (Fig. 1). The nucleotides were extracted in either 0.1 N-hydrochloric acid or 0.1 N-sodium hydroxide in either the

Fig. 1. Stability of the nicotinamide nucleotides in neutralized liver extracts. The oxidized forms were extracted in 0.1N-HCl and the reduced forms in 0.1N-NaOH, as described in the Methods section, and then neutralized. Samples of the extracts were estimated immediately and the remainder stored at -15° until used. $\circ$ and $\bullet$, NAD $^{+}$; $\triangle$ and $\blacktriangle$, NADH; ∇ and $\blacktriangledown$, NADP $^{+}$; $\square$ and $\blacksquare$, NADPH. $\circ$, $\triangle$, ∇ and $\square$, in the absence of added nicotinamide; $\bullet$, $\blacktriangle$, $\blacktriangledown$ and $\blacksquare$, in the presence of 1 mM-nicotinamide.

presence or absence of 1 mM-nicotinamide and were estimated immediately. Several samples of each extract were frozen and stored at -15° . At 24 and 48 hr. samples were thawed and estimated again. Although NADP $^{+}$ remained stable over this period, all the others showed some decline (16, 26 and 27% for NADH, NADPH and NAD $^{+}$ respectively), the greater part of which occurs in the period 24–48 hr. Nicotinamide partially prevents this loss but in no case entirely eliminates it. Since there is virtually no loss of any of the four nucleotides over the first 24 hr., a finding in agreement with the results of Bassham *et al.* (1959), all estimations were carried out on the same day as the extractions.

Effects of varying the concentrations of components used in the assay procedure

Alcohol dehydrogenase. The optimum amount of enzyme for the assay was determined by estimating 1 μ g. and 3 μ g. of NAD $^{+}$ in the presence of the standard reaction mixture (see the Methods section) with increasing concentrations of alcohol dehydrogenase, from 0.03 mg. to 0.6 mg. in the 2 ml. reaction volume. The rate of oxygen consumption increased with increasing concentration of enzyme up to 0.15 mg. of enzyme and then remained constant (Fig. 2). Therefore 0.3 mg. of alcohol dehydrogenase was used as a routine in the assay of both NAD $^{+}$ and NADH. The volume of ethanol added to the reac-

Fig. 2. Effect of the concentration of enzyme (alcohol dehydrogenase for NAD $^{+}$; glucose 6-phosphate dehydrogenase for NADP $^{+}$) on the oxygen uptake due to 1.0 μ g. of NAD $^{+}$ (a) or NADP $^{+}$ (b). The conditions of the assay were as described in the Methods section, except for the different amounts of enzyme.

tion mixture appears to be sufficient to saturate the enzyme without being inhibitory.

Glucose 6-phosphate dehydrogenase. The commercially available glucose 6-phosphate dehydrogenase is said by the makers to be contaminated by NADPH-glutathione oxidoreductase. The presence of this enzyme would provide an alternative route for oxidation of NADPH if oxidized glutathione were present in the extracts. Therefore glucose 6-phosphate dehydrogenase preparations were always checked for the presence of glutathione reductase by measuring the effect of the addition of both oxidized and reduced forms of glutathione (8–32 μ g./2 ml. of reaction mixture) on the oxygen consumption due to a standard amount (1 or 3 μ g.) of NADP $^{+}$ under otherwise standard conditions. Of a number of batches so tested only one showed an appreciable contamination with NADPH-glutathione oxidoreductase and this was discarded. In general the commercial enzyme seemed free of this contaminant and was used for the assay procedure without further purification. The optimum amount of enzyme required for the assay was determined as for the NAD $^{+}$ assay except that the assay mixture

was that described in the Methods section for NADP⁺. The range of the enzyme concentrations used was 0.5–10 $\mu\text{g.}/2\text{ml.}$ of reaction mixture. The rate of oxygen consumption increased rapidly up to 2.5 $\mu\text{g.}$ and then slowly up to 5 $\mu\text{g.}$, after which the addition of further enzyme had little effect on the rate (Fig. 2). Therefore 5 $\mu\text{g.}$ of enzyme was chosen as the amount used for routine assays. In practice the commercial preparation was diluted 1:50 with 0.1 M-tris buffer, pH 8.0, shortly before use and 50 $\mu\text{l.}$ used for each reaction. The enzyme is sufficiently stable to give consistent results for several hours.

Phenazine methosulphate. The effect of increasing amounts of phenazine methosulphate in assay mixtures containing either NAD⁺ or NADP⁺ is shown in Fig. 3. Increasing the concentration of the carrier increases the rate of oxygen consumption, and similar results were obtained at all of the concentrations of nucleotide tested. The stimulation of oxygen consumption was most marked at the lower concentrations of phenazine methosulphate and fell off more rapidly for NADP⁺ than for NAD⁺. Although at any particular concentration of phenazine methosulphate there was still a linear relationship between the amount of nucleotide present and the oxygen consumption, the slope of this curve was dependent on the concentration of carrier. It is therefore obvious that by varying the concentration of the phenazine methosulphate it is possible to increase or decrease the sensitivity of the assay. With concentrations of the nucleotides in the range 0.5–10 $\mu\text{g.}$ in the sample volume the most convenient concentration of phenazine methosulphate is 0.1 ml. of a solution of 2 mg./ml. In the range 0.05–1.0 $\mu\text{g.}$ a more convenient concentration would be 0.1 ml. of 8 mg./ml. Solutions where the nucleotide content is too high to fall within these ranges can either be

diluted, or estimated with a phenazine methosulphate solution of lower concentration, say 0.1 ml. of 0.2 mg./ml. In each case a standard curve with the appropriate concentration of phenazine methosulphate is required. By varying the concentration of phenazine methosulphate the range of the method can be extended to cover the range 0.02–50 $\mu\text{g.}$ of NAD⁺ or NADP⁺. Thus the method can be readily used to estimate as little as 0.04 μmole of nucleotide. This compares well with the sensitivity of other procedures, e.g. that of Glock & McLean (1955a), 1 μmole , that of Vilee (1962), 0.5 μmole , the fluorimetric procedures of Jacobson & Astrachan (1957), 0.01 μmole , of Bassham *et al.* (1959), 0.1 μmole , of Slater, Sawyer & Sträuli (1964), 0.2 μmole , and the micro-modification of this method described by Slater, Heath & Graymore (1962), 0.02 μmole . The method of Lowry *et al.* (1961) combines an enzymic recycling procedure for 30 min. with a final fluorimetric assay and can measure 0.001 μmole . The method of Pastan *et al.* (1963), which uses radioactive 6-phosphogluconate, can estimate 1 μmole of NADP and is, presumably,

Fig. 3. Effect of the concentration of phenazine methosulphate on the oxygen uptake due to 1.0 $\mu\text{g.}$ of either NAD⁺ (●) or NADP⁺ (▲). The conditions of the assay were as described in the Methods section, except for the different amounts of phenazine methosulphate.

Fig. 4. Calibration curves for the estimation of NAD⁺ (a) and NADP⁺ (b) in the presence of 0.32 mM- (▲) or 0.64 mM- (●) phenazine methosulphate. The conditions of the assay were as described in the Methods section.

capable of even greater sensitivity if transformed into an enzymic recycling procedure.

The choice of a level of 0.1 ml. of a 2 mg./ml. solution of phenazine methosulphate for routine assay was conditioned by the necessity to make the method as sensitive as possible without introducing the need for blanks. Phenazine methosulphate can undergo photochemical oxidation (Ramage & Landquist, 1959) and be a potential donor of electrons. At concentrations of dye up to 4 mg./ml. this is not detectable under our conditions, but at 8 mg./ml. a slight oxygen consumption can be measured in the absence of any nucleotide, so that at these high concentrations a blank run in the absence of added nucleotide is essential. The extent of this reaction can be limited by decreasing the amount of direct light falling on the cell, or by painting the exterior of the water jacket black. Photochemical oxidation under these conditions is minimal and is of much less significance than in optical methods for assaying the nicotinamide nucleotides with this carrier.

Influence of other factors

Effect of temperature. The assay is usually carried out at 25°. The rate can be increased by raising the temperature, with a corresponding increase in sensitivity. However, at temperatures above 30° the formation of air bubbles in the closed cell becomes troublesome.

Effect of pH. The pH selected for the reaction mixture is, in each case, the optimum pH of the enzyme being used. The response of the electrode is not affected by variations of pH (Laitinen & Kolthoff, 1941).

Effect of other reducing substances. Tissue extracts, especially those from some specialized organs such as the adrenals, may contain substances such as ascorbic acid or glutathione that could effect a non-enzymic reduction of phenazine methosulphate. Such a reduction can be followed visually on the recording and the addition of the specific dehydrogenase delayed until this non-specific reduction is seen to be complete. The period before the addition of the specific enzyme automatically provides a blank for each assay.

Calibration curves. Typical calibration curves for NAD⁺ (or NADH) and NADP⁺ (or NADPH) in the presence of two concentrations of phenazine methosulphate are shown in Fig. 4. Both nucleotides show linear calibration curves over both of the concentration ranges.

Concentration of nicotinamide nucleotides in different tissues

A comparison of the results obtained by the present method with those given in the literature is given in Table 1; the method gives values that are in good agreement with the values previously pub-

Table 1. Concentrations of the nicotinamide nucleotides in various tissues

References: 1, Glock & McLean (1955b); 2, Holzer, Busch, & Kröger (1958); 3, Lowry *et al.* (1961); 4, Bassham *et al.* (1959); 5, Caiger, Morton, Filsell & Jarrett (1962); 6, Pastan *et al.* (1963); 7, McLean (1958); 8, Briggs (1960); 9, Glock & McLean (1957); 10, Nides & Reid (1964); 11, Anderson & Vennesland (1954); 12, this study.

Tissue	Concn. of nucleotide (μg./g./wet wt. of tissue)				Reference
	NAD ⁺	NADH	NADP ⁺	NADPH	
Liver	425	116	25	231	1
	505	192	6	227	2
	414	168	85	374	3
	320	101	19	186	4
	280	71	21	281	5
	—	—	66	243	6
Adipose tissue	531 ± 42 (10)*	146 ± 17 (10)*	28 ± 3 (10)*	183 ± 14 (10)*	12
	—	—	0.7	5.3–22.8	6
	41.2 ± 3.7 (6)*	5.3 ± 0.4 (6)*	3.1 ± 0.1 (6)*	5.8 ± 0.3 (6)*	12
Mammary gland†	1760	350	18	330	7
Hepatomas	1070 (4)*	348 (4)*	54 (4)*	138 (4)*	12
	176	—	24	—	8
	255	25	< 3	28	9
	180‡	—	76‡	44‡	10
	57 ± 12 (6)*§	24 ± 6 (6)*§	7 ± 1 (6)*§	19 ± 5 (6)*§	12
Leaves of green peas	8.5	—	7.5	—	11
	11.7	1.9	7.0	0.7	12

* Number of animals given in parentheses.
† Nicotinamide nucleotide content calculated as μg./total abdominal glands (range of gland weights, 5.3–8.8 g.).
‡ Spontaneous mouse hepatomas.
§ Azo-dye hepatomas from rats given the dye for 9 months.

lished. Of special interest are the values obtained for the nucleotides of the green leaves of peas. The estimation of the nucleotides in higher plants has always presented considerable difficulties owing to the presence of coloured and fluorescent materials in the extracts. In the present method plant materials pose no special difficulty and may readily be estimated by normal procedures. The values obtained are in good agreement with those of Anderson & Vennesland (1954). This tissue is exceptional in having NADP⁺ concentration higher than that of NADPH, a fact presumably associated with photosynthetic activity. The method has also permitted, for the first time, an estimation of all four nicotinamide nucleotides in adipose tissue. The values for NADP⁺ and NADPH obtained in this study show excellent correspondence with those obtained by Pastan *et al.* (1963), and are noteworthy in that this tissue shows an unusually low NADPH/NADP⁺ ratio that could well be related to the specialized function of this organ as a site of lipogenesis.

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