The Determination of Reduced Nicotinamide–Adenine Dinucleotide and Metabolic Intermediates in Picomole Amounts with Bacterial Luciferase

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Methods that use bacterial luciferase for the assay of NADH in the range from 1 pmol to 1 nmol are described. Optimal conditions for the assay of glycolytic intermediates, tricarboxylic acid-cycle intermediates and related amino acids from milligram amounts of tissue are presented. The whole spectrum of these intermediates can be determined on about 10 mg of liver tissue. The methods are simple, are suitable for routine use, and the instrumentation is inexpensive. The concentrations of glycolytic intermediates in rat livers were determined by conventional spectrometric methods and with luciferase, and the results found to be in good agreement.

In our work it became necessary to determine a large number of glycolytic and tricarboxylic acid-cycle intermediates in cytosol and mitochondria of hepatocytes incubated under various conditions. For conventional spectrophotometric assay, this requires 2–4 g of tissue, whereas we had only 50–100 mg of cells. Existing methods for the assay of metabolites in the picomole range are slow and require special skills. However, picomole or even femtomole amounts of ATP can be easily assayed by firefly luciferase luminescence. We thus explored the use of bacterial NADH-linked luciferase for our purpose. Bacterial luciferase catalyses the oxidation of several long-chain aldehydes to the corresponding acids in the presence of FMNH₂ and O₂ with the production of light. The bacteria also contain an NADH-linked FMN reductase, so that the overall reaction is:

Commercial extracts of luminescent bacteria contain both enzymes.

Bacterial chemiluminescence has been extensively studied [see review by Hastings (1978) and monograph edited by De Luca (1978)]. The application of bioluminescence for analysis has been reviewed by Campbell & Simpson (1979). However, the application of bacterial luciferase for the assay of metabolites has been limited. The assay of NADH

and NADPH and of several hexose 6-phosphates in the picomole range has been reported (Stanley, 1971, 1978; Cantarow & Stoller, 1976; Agren *et al.*, 1977). A liquid-scintillation counter or other specialized equipment was used for the measurement of the production of light. The scintillation counter, although a very sensitive light-detector, is a very expensive instrument and ill-suited for the determination of bacterial light-emission. In our hands, with the use of a simple photometer, the methods cited above were unsatisfactory. Moreover, optimal conditions for light-emission were not established. We now describe procedures for the assay of reduced nicotinamide nucleotides, and the enzymic assay of metabolites in the picomole range, that are accurate and suitable for routine work and that employ inexpensive instruments.

The standard enzymic procedures for the determination of metabolites by NADH-linked reactions were not always suitable for luciferase assays. When possible, we have selected procedures where NADH is produced rather than utilized, since the latter method depends on the difference between two separate determinations. In many standard enzymic methods the reactions are driven to completion by forming an adduct of the keto group with a compound such as hydrazine (for example, as in the assay of lactate or glutamate). This depresses the production of light, and alternative methods for assay were thus chosen. Bacterial luciferase reacts with NADPH and reduced 3-acetylpyridine–adenine dinucleotide, and these nucleotides were used in some assays.

Experimental

Instrument

A photometer with a rotating reaction chamber for 6 mm × 50 mm tubes in conjunction with an Integrator-Timer (Chem-Glow photometer and Integrator by Aminco, Silver Springs, MD, U.S.A.) was used. The disposable glass tubes that fit into the cuvette holder are made by Kimble (Toledo, OH, U.S.A.) (no. 73500-650).

Chemicals

FMN and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and dodecal (lauraldehyde) was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Luciferase was from *Photobacterium fischerii* and is a relatively crude preparation. In most of our work we have used type 1 from Sigma. However, the manufacturer discontinued this preparation, replacing it with type V. In later work we found a preparation (LS0004686) from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.) most satisfactory. It has properties similar to those of the Sigma type enzyme, but per unit weight emits about 50% more light. Other chemicals and enzymes were from Sigma or Calbiochem (San Diego, CA, U.S.A.).

Solutions

Luciferase (1 mg/ml) was dissolved in a buffer solution [final concns. 0.1 M-potassium phosphate buffer, pH 7.0, 1 mM-EDTA, 10% (v/v) glycerol, 1 mM-dithiothreitol, and 0.1% defatted bovine albumin]. The solution was divided into small portions and kept frozen at -20°C. It was stable for at least 2 months.

FMN

FMN was dissolved in water (2.0 mg/ml), and diluted to 2.5 µg/ml in 0.1 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA. It was kept in a dark bottle and out of the light, and the solution was made up fresh daily.

Dodecal

A 5 µl sample was dissolved in 15 ml of methanol and made up fresh daily.

NADH standard

A stock solution of about 0.1 mM-NADH in 0.1 M-triethanolamine/HCl buffer, pH 9.6, was standardized daily in the spectrophotometer. It was diluted as required in the above buffer. Very dilute solutions of NADH are unstable, and we found that a 5 µM solution decayed by as much as 25% in 4 h at room temperature at neutral pH. Hence dilutions were made shortly before use. All solutions were filtered before use.

General procedure for NADH assay

To a sample in a 6 mm × 50 mm tube were added rapidly, in sequence, buffer, FMN, dodecal and luciferase. The tube was vortex-mixed vigorously and promptly put into the chamber. The integrator settings were adjusted to read from 200 to 2000 (arbitrary units) in 30 s. The first readings were disregarded because of transient spurious light from FMN. In most conditions (see below) the second 30 s reading was the value used. A blank with no NADH was included. The solutions were at room temperature ($22 \pm 2^\circ\text{C}$). High-precision dispensers are important for accurate and rapid delivery of the small volumes. With some experience duplicate samples agree within 2–3%.

Conditions of assay

For the range 1–10 pmol, the mixture contained 0.20 ml (0.5 µg) of FMN, 0.01 ml (2.5 µg) of dodecal and 0.05 ml (50 µg) of luciferase in a final volume of 0.31 ml. For the range 10–50 pmol, the mixture contained 0.25 ml (0.625 µg) of FMN, 0.01 ml (2.5 µg) of dodecal and 0.025 ml (25 µg) of luciferase in a final volume of 0.335 ml. For the range 50–100 pmol, the mixture contained 0.5 ml (1.25 µg) of FMN, 0.01 ml (2.5 µg) of dodecal and 0.025 ml (25 µg) of luciferase in a final volume of 0.585 ml.

Metabolites

Extracts of liver tissue or cells, about 50 mg wet wt./ml of neutralized HClO_4 , were prepared. In general, a medium of buffer, nucleotide and enzymes at twice the concentration listed in Table 1 was prepared and added to an equal volume of metabolite in small tubes. The volume usually was 0.2 ml. At the end of the reaction time, samples were taken for luciferase assay. The procedures may be classified into (a) reactions forming NADH, (b) reactions utilizing NADH and (c) reactions involving other nucleotides. The conditions are described in Table 1.

Formation of NADH

Whenever possible, this assay was chosen. Most conditions shown in Table 1 are minor modifications of standard methods described in Bergmeyer & Gawehn (1974), except that the reaction time is longer with the low substrate concentrations.

NADH utilization

This is determined as a difference between two samples, one with and one without the substrate. The approximate concentration of the compound was estimated by a preliminary determination in order to employ a concentration of NADH about twice that of the substrate. The enzymic reactions proceeded at pH 7.4–7.6. To avoid NADH loss, which occurs in dilute neutral solution, an equal volume of 0.1 M-

Table 1. *Determination of metabolites in liver*

A 50mg sample of liver in about 1ml of neutralized HClO_4 extract was used. Key for symbols and abbreviations: Fru-6-P, fructose 6-phosphate; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Glc-6-P, glucose 6-phosphate; Grn-P, dihydroxyacetone phosphate; Gro-3-P, glycerol 3-phosphate; AcPyAD, 3-acetylpyridine-adenine dinucleotide; ALD, aldolase; CS, citrate synthase; CL, citrate lyase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDH, glycerol 3-phosphate dehydrogenase; GLDH, glutamate dehydrogenase; GOT, glutamate-oxalacetate transaminase; G6PDH, glucose 6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; TIM, triose phosphate isomerase; UDPGDH, UDP-glucose dehydrogenase.

Substrate	Method	Final concentration per ml in reaction				Time of reaction (min)
		Buffer	Enzyme (units)		Nucleotide	
		Formation of NADH				
Glc-6- <i>P</i>	Lang & Michal (1974)	75 mM-Tris/HCl, pH 7.8, 10 mM-MgCl ₂	G6PDH*	0.5	0.5 mM-NAD ⁺	10
Glc-6- <i>P</i> ± Fru-6- <i>P</i>	Same as for Glc-6- <i>P</i>	Same as for Glc-6- <i>P</i>	G6PDH*	0.5	0.5 mM-NAD ⁺	20
Grn- <i>P</i>	Schmidt <i>et al.</i> (1970)	20 mM-Triethanolamine/HCl pH 9.0, 2 mM-Pi, 30 mM-sodium arsenate, 0.75 mM-dithiothreitol	PGI PGK GAPDH TIM	4 5 5 5	3 mM-NAD ⁺	30
Fru-1,6- <i>P</i> ₂	Schmidt <i>et al.</i> (1970)	Same as for Grn- <i>P</i>	PGK GAPDH ALD	5 5 0.5	3 mM-NAD ⁺	90
Gro-3- <i>P</i> †	Schmidt <i>et al.</i> (1970)	Same as for Grn- <i>P</i>	PGK GAPDH GDH TIM	5 5 7.5 5	3 mM-NAD ⁺	90
Malate	Schoner (1974)	12.5 mM-Triethanolamine/HCl, pH 9.6, 0.125 mM-Acetyl-CoA	MDH CS	5 1	2 mM-NAD ⁺	30
UDP-glucose†	Keppler & Decker (1974)	280 mM-Glycine/NaOH, pH 8.7, 4.5 mM-EDTA	UDPGDH	0.04	2 mM-NAD ⁺	30
Disappearance of NADH						
Pyruvate	Czok & Lamprecht (1974)	37.5 mM-Triethanolamine/HCl, pH 7.6, 0.375 mM-EDTA	LDH	1.25	10 μM-NADH	30
Aspartate	Bergmeyer <i>et al.</i> (1974)	30 mM-Triethanolamine/HCl, pH 7.4, 1.5 mM-2-oxoglutarate	GOT MDH	0.05 1.25	10 μM-NADH	30
Citrate	Dagley (1974)	10 mM-Triethanolamine/HCl, pH 7.4, 20 μM-ZnSO ₄	MDH CL‡	5 0.2	10 μM-NADH	30
Glutamate	Witt (1974)	22 mM-P _i , pH 8.2	3-Acetylpyridine-adenine dinucleotide GLDH	13	0.2 mM-AcPyAD ⁺	90

* NAD-specific, from *L. mesenteroides*.

† 2 mol of NADH formed/mol.

‡ Made fresh.

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triethanolamine/HCl, pH 9.6, was added at the end of the reaction period.

Other nucleotides

For glutamate determination, 3-acetylpyridine-adenine dinucleotide was used to drive the reaction to completion. Bacterial luciferase reacts with NADPH and reduced 3-acetylpyridine-adenine dinucleotide, but the light-intensity is less than that with an equal amount of NADH, and higher concentrations of luciferase are employed. 3-Acetylpyridine-adenine dinucleotide was also used in the

determination of lactate, but we were not successful in driving the reaction to completion, and the amount of required nucleotide gave very high blanks. Lactate was, however, present in cells in concentrations sufficient for spectrophotometric assay.

Standards

For reactions forming NADH, a solution of the metabolite to be analysed was used as standard. For NADH-utilizing reactions, the standard was a solution of NADH. We prepared concentrated stock

solutions of the metabolites or of NADH, assayed them spectrophotometrically and made appropriate dilutions. Blanks without substrate were run with every determination.

Results and Discussion

Kinetics of light-emission

The light-intensity and the kinetics of emission are affected by all the components of the medium. The emission of light is proportional to the square of the enzyme concentration. Thus doubling the concentration of the crude enzyme quadruples light intensity. The reason is that the preparation contains a mixture of two enzymes, an FMN dehydrogenase with NADH as the hydrogen donor, and luciferase

proper, which oxidizes the aldehyde. Production of light is proportional to the product of the two enzymes. The effect of enzyme concentration is shown in Fig. 1. The sensitivity of the reaction is thus greatly dependent on enzyme concentration.

In Fig. 2 the kinetics of light-emission and the effects of FMN and dodecal are shown. Maximal light-emission occurs by 1 min, with the intensity decaying for the next 3 min. Higher amounts of FMN are inhibitory. Moreover, with excess FMN the reaction mixture is light-sensitive, with spurious emission in the absence of luciferase, so that the analysis must be conducted in a darkened room. Excess dodecal is also inhibitory.

Thiol compounds have been commonly used in the luciferase assays. Fig. 3 shows the marked effect of thiol compounds on the light-intensity and kinetics. Dithiothreitol greatly stimulated light-intensity. When mercaptoethanol rather than dithiothreitol is used, light-intensity is much diminished, and the kinetics are strikingly affected. Peak intensity is attained in 2–3 min, and the curves are quite flat and decay occurs relatively slowly. When 1 mM or 2 mM-dithiothreitol is used, light-intensity is nearly tripled, but the peak occurs early and the decay is rapid. Higher concentrations of dithiothreitol gave higher yields of light on the day of preparation, but were inhibitory when stored overnight. When the enzyme solution was prepared in 5 mM-dithiothreitol (rather than 1 mM), it lost nearly all activity overnight (Fig. 3). The other additions, EDTA, glycerol and albumin, have a smaller stimulatory effect and were not studied in detail.

The concentration of luciferase was varied according to the quantity of the substrate. Higher enzyme concentration was attained by either increasing enzyme amounts or decreasing volumes. Under optimal conditions for different ranges, NADH in amounts from 1 to 1000 pmol may be assayed. The assay is linear, as shown in Fig. 4. At the highest luciferase concentration the light-emission of the blank is about 40% of that with 1 pmol of NADH. The lower limit of detection of metabolite depends on the value of the blank. Commercial enzyme preparations differ in their blank readings. This may be due to the presence of absorbed NADH on the enzyme proteins.

Our results show that light-emission by the luciferase preparations from *Photobacterium fischerii* is a complex process and that the kinetics depend greatly on conditions. Our concern here is with practical application to metabolite assays. The variability in kinetics permits great flexibility in adapting the methods to a wide range of metabolite concentrations and to instruments differing greatly in their sensitivity. Thus with the Aminco photometer we chose conditions for high light-intensity and a

Fig. 1. Effect of enzyme concentration on light-emission. The volume was 0.6 ml, with three concentrations of type 1 (Sigma) luciferase: —, 50 µg; ····, 25 µg; ----, 12.5 µg. Experimental details are indicated in the text.

Fig. 2. Effect of FMN and dodecal on light-emission
The tubes contained 200pmol of NADH per 0.59ml, with various amounts of FMN as shown. The amounts of dodecal were (per tube): —, 2.5 µg, ····, 5 µg; ----, 10 µg. Experimental details are indicated in the text.

Table 2. Assay of liver by spectrophotometric (A) and luciferase (B) methods
Experimental details are indicated in the text. Key for symbols: Fru-6-P, fructose 6-phosphate; Fru-1,6-P₂, fructose 1,6-bisphosphate; Glc-6-P, glucose 6-phosphate; Grn-P, dihydroxyacetone phosphate; Gro-3-P, glycerol 3-phosphate.

		Content (nmol/g wet wt.)							
Condition of rat	Methods	Fed		Starved		Diabetic fed		Diabetic starved	
		A	B	A	B	A	B	A	B
	Glc-6-P	296	280	64	52	408	420	476	512
	Glc-6-P + Fru-6-P	352	360	84	60	472	472	552	580
	Grn-P			28	28	104	108	44	46
	Fru-1,6-P ₂			5	11	60	84	36	32
	Gro-3-P	724	724	928	916	3316	3848	1440	1472
	Malate	676	712	848	912	900	844	1584	1500
	Aspartate	644	772	432	524	680	872	1820	2096
	Citrate	196	204	300	312	100	88	21	60
	Glutamate	2680	2880	2608	2868	2464	2560	2204	2472

rapid approach to peak emission such as obtained with dithiothreitol. On the other hand, when a liquid-scintillation counter is used, which is over

100-fold more sensitive, a lower light-emission and a broader plateau, such as is obtained with mercaptoethanol, may be more desirable.

Fig. 3. *Effect of thiol compounds and other additions on light-emission*

The tubes contained, in a volume of 0.59 ml, 250 pmol of NADH and 25 μ g of enzyme and the following additions: ●, albumin (0.1%), glycerol (10%, v/v), EDTA (1 mM) and dithiothreitol (1 mM); ○, only dithiothreitol (1 mM); ▲, albumin (0.1%), glycerol (10%, v/v), EDTA (1 mM) and mercaptoethanol (1 mM); △, mercaptoethanol only (1 mM); ◆, no additions; ◇, enzyme made up in 5 mM-dithiothreitol, and stored overnight in the cold. Experimental details are indicated in the text.

Assay of liver extracts

HClO₄ extracts of freeze-clamped liver (250 mg of liver/ml of neutralized extract) were prepared from fed, starved, diabetic fed and diabetic starved rats. In Table 2 we compare the results of analysis of liver

Fig. 4. *Standard curves for NADH from 1 to 1000 pmol*
For conditions see the text.

extracts by standard spectrophotometric methods and with luciferase. In most cases agreement was satisfactory, although values with luciferase tended to be slightly higher. When the concentrations were very low (fructose 1,6-bisphosphate in starved animals and citrate in diabetic animals), the value estimated by the use of luciferase was considerably higher. It is probable that at these concentrations the assay with luciferase is more reliable.

Determination of other compounds

3-Phosphoglycerate and phosphoenolpyruvate are not described in Table 1, but were determined by us satisfactorily in liver extracts by the utilization of NADH in an assay coupled with pyruvate kinase and lactate dehydrogenase. A similar assay can be used to measure ADP. We have assayed also 2-oxoglutarate and glutamine, and it is probable that most spectrophotometric assays based on NADH or NADPH can be adapted for bacterial luciferase. One phosphate ester for which no convenient NADH-based assay is available is 1,3-bisphosphoglycerate, which is present in very low concentrations. Probably this is best determined by the production of ATP with firefly luciferase. The determination requires prior removal of endogenous ATP by adsorption on charcoal.

Our methods were designed for extracts from 50–100 mg of liver, but can be readily adapted to the determination of a large metabolic spectrum in less than 10 mg of tissue. One advantage is the cheapness of the instruments. The photometer and integrator used by us retail currently in the U.S.A. for about \$3000. Several instruments in this price range are available from American and European manufacturers. The performance of several photometers has been compared (Picciolo *et al.*, 1978). The cost of enzymes and chemicals is very small. The luciferase assay is in our experience more reproducible than fluorimetric methods. It is probable that bacterial luciferase offers many other potential applications for the assay of metabolites in very small amounts of tissues.

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