

Fluorimetric Technique for Monitoring Changes in the Level of Reduced Nicotinamide Nucleotides in Continuous Cultures of Microorganisms

D. E. F. HARRISON¹ AND BRITTON CHANCE

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received for publication 13 November 1969

A metabolite fluorimeter was modified to monitor nicotinamide nucleotides in cells growing in a chemostat culture. By illuminating and collecting fluorescence over a relatively large area of the side of the culture vessel, the noise level was kept low even in highly turbulent cultures. Calibration of the system was by enzymatic assay of samples. Changes in nicotinamide nucleotide of concentration less than 0.1 nmoles/ml could be detected [i.e., less than 10% aerobic/anaerobic response for a cell concentration of 5 mg (dry wt) per ml].

Nicotinamide nucleotides play a central role in the oxidative reactions of all organisms, and the intracellular redox potential of these compounds is an important parameter in the regulation of cell metabolism (9). There is, therefore, a great deal of information to be gained from following changes in the ratio of nicotinamide adenine dinucleotide (NAD⁺) to reduced nicotinamide dinucleotide (NADH) in response to changes in the extracellular environment. Such information may be obtained by chemical analytical techniques for measurement of the reduced and oxidized forms of nicotinamide nucleotide in samples (22), but the rapid and often complex changes in nicotinamide nucleotide that occur really necessitates a method for continuous determination.

The development of the very sensitive dual-beam spectrophotometric methods by Chance (3) provided a means for continuously monitoring the redox state of nicotinamide nucleotides inside cells by recording the absorption of light by NADH at 340 nm. By this technique, it was shown (5) that changes in the level of intracellular NADH correlated with the respiratory status of the cell and that a change in the extracellular environment from aerobic to anaerobic conditions caused a large increase in the level of NADH in resting-cell systems. Subsequently, the technique has been applied widely to studies of

regulatory systems in resting microorganisms (4, 17, 20).

Clearly, much useful information on the regulation of metabolism in growing cells should be obtained if the level of NADH could be monitored in continuous cultures of microorganisms. This may be achieved by spectrophotometric measurements of the culture circulated through a flow cell (Chance, *unpublished data*). However, any such circulation of cells entails a delay between the time a cell leaves the culture vessel and the measurement of the NADH content. The delay will be significant when compared with the rapid changes that can occur in the intracellular redox state. This difficulty may be overcome by the use of reflectance fluorimetry.

The fluorescent property of NADH and reduced nicotinamide adenine dinucleotide phosphate (NADPH) has been known for many years, and fluorimetry was applied to observations of enzyme preparations by Boyer and Theorell (2), by Duysens and Ames (11) to yeast cell suspensions, and by Chance and Balt-scheffsky (7) to mitochondrial suspensions. Estabrook (12) described the application of fluorimetric techniques for the measurement of metabolic intermediates. The adaptability of the technique is demonstrated by the variety of situations to which it has been applied, e.g., resting cell suspensions (1, 6), whole intact organs (10), and individual mammalian cells (18). Fluorimetry has recently been used to follow nicotinamide

¹ Present address: School of Chemical Engineering, University of Pennsylvania, Philadelphia, Pa.

nucleotide reduction in growing bacterial cultures by means of a flow cell (23). However, there appears to have been no attempt made to apply reflectance fluorimetry to continuous cultures of microorganisms, although it would seem that wealth of information may be gained by continuous monitoring of the level of an internal metabolic intermediate.

NAD⁺ and nicotinamide adenine dinucleotide phosphate (NADP⁺) have the property of accepting electrons and being converted to high-energy reduced forms (NADH and NADPH). In this form, both NADH and NADPH absorb light at 340 nm and emit light at 460 nm (7). This property of changing fluorescence at these wavelengths appears to be unique to NADH and NADPH with regard to the constituents of the living cell. The validity of the fluorimetric method for detecting changes in the level of NADH has been demonstrated in yeast cells by simultaneously monitoring the absorption changes at 340 nm (8).

MATERIALS AND METHODS

The apparatus used was an adapted Johnson Foundation Metabolite Fluorimeter (21) built by D. Meyer. The light source and phototube holder were applied to the Pyrex surface of the vessel, a 1-liter, magnetically stirred chemostat, at an angle of approximately 60° to one another (Fig. 1). The vessel was carefully blacked out, and extraneous light was excluded from the phototube by surrounding the ends of the tube holders with black cardboard cups and covering the whole in black adhesive tape. The Pyrex ($\frac{3}{8}$ inch thick) absorbs little light at 360 nm.

The culture vessel was stirred magnetically; therefore, the phototube was shielded by surrounding it with a Nu-metal cylinder. This overcame most of the interference caused by the magnet, but the reading was susceptible to large changes in the rotation rate of the drive magnet, e.g., from 100 to 800 rev/min.

The culture used to test the apparatus was *Klebsiella aerogenes* (NCIB 8017) cultivated under the conditions described by Harrison and Pirt (16).

Calibration. Samples were withdrawn during the aerobic and anaerobic states and analyzed enzymatically. Care was taken to ensure that the time delay involved between the cells leaving the culture and mixing in the extracting solution was very short so that no significant changes would occur in the co-enzyme levels. This was achieved by using the rapid-sampling technique of Harrison and Maitra (15), which ensures that the cells are fixed within 0.2 sec of leaving the culture. The cells were extracted with 7% perchloric acid for analysis of NAD⁺ and NADP⁺, and with 3N KOH for analysis of NADH and NADPH. Analyses were carried out as described by Estabrook and Maitra (13) by using a metabolite fluorimeter (21) to follow the course of the enzyme-

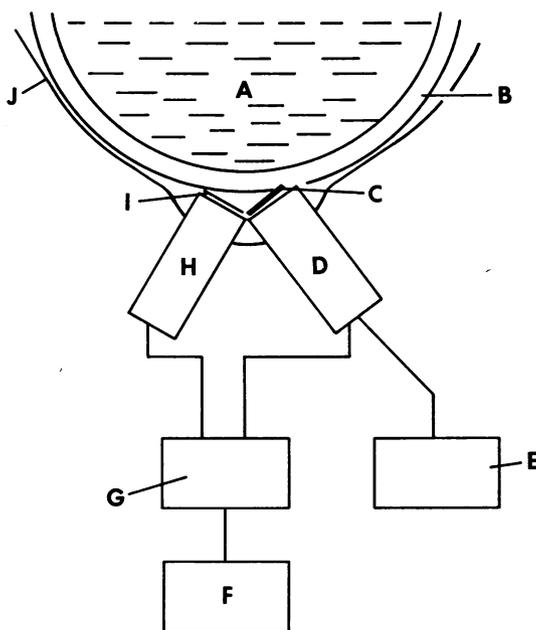


FIG. 1. Schematic diagram of apparatus used to monitor pyridine nucleotide fluorescence in a chemostat culture. Key: A, culture; B, Pyrex glass wall of chemostat vessel; C, optical filters (Kodak Wratten no. 32 + Corning no. 3389); D, photomultiplier (EMI 9524B); E, high-voltage supply (Kepco ABC 1500M); F, recorder; G, lamp supply and detector (Johnson Foundation); H, lamp (G.E. F4T4); I, optical filter (Corning no. 5840); J, black cardboard screening.

catalyzed reactions. NAD⁺ was determined by measuring the oxidation of ethyl alcohol by alcohol dehydrogenase (E.C.C. 1.1.1.1), NADP⁺ by measuring the oxidation of glucose-6-phosphate by Zwischenferment (1.1.1.49), NADH by measuring the oxidation of acetaldehyde by alcohol dehydrogenase (1.1.1.1.), and NADPH by measuring the oxidation of α -ketoglutarate by glutamic dehydrogenase (1.4.1.4).

RESULTS

Figure 2 shows a typical fluorescence trace obtained from a chemostat culture of *K. aerogenes* in response to reversible changes from aerobic to anaerobic conditions upon interrupting the aeration of the culture for 4 min. As the dissolved oxygen tension fell, there was initially a small, slow increase in NADH level followed by a rapid increase when the oxygen tension reading had fallen below 10 mm of Hg. The speed of response of the oxygen electrode was very much slower than that of the fluorimeter, requiring 1 min for 90% response compared with 1 sec; thus, the actual oxygen tension at which the fast in-

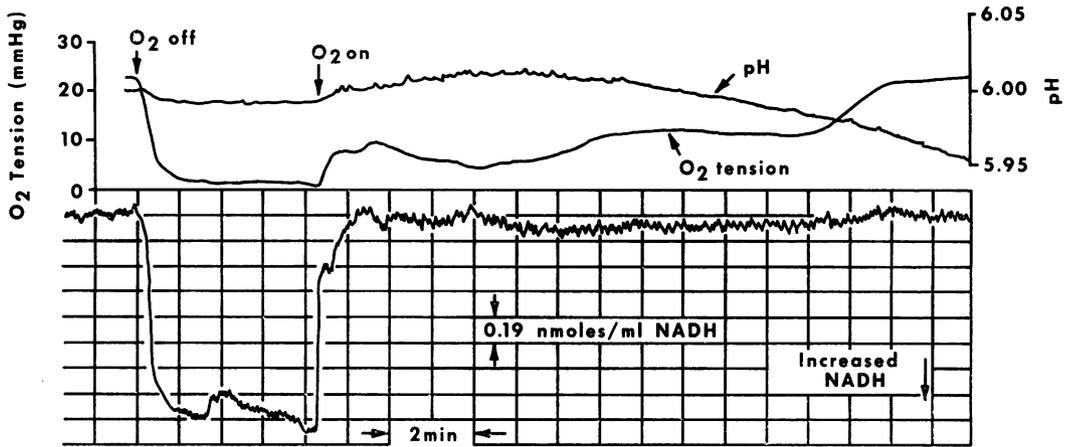


Fig. 2. Effect of an aerobic/anaerobic change on the nicotinamide nucleotide fluorescence in a chemostat culture of *K. aerogenes* [5 mg (dry weight) per ml]. The culture was glucose-limited at a growth rate of 0.2 hr^{-1} . The 90% response time of the oxygen electrode was approximately 1 min, compared with 1 sec for the fluorimeter.

crease in NADH occurred was much lower than 10 mm of Hg. After an initial small oscillation, the fluorescence assumed a new steady anaerobic level. Upon reoxygenating the culture, the rise in dissolved oxygen tension was accompanied by a rapid reoxidation of NADH until it reached three-quarters of the previous aerobic steady-state level and eventually reached the aerobic steady-state level. The biphasic nature of the oxygen-tension response to reoxygenation has been studied and discussed previously (15). This large change in fluorescence of NADH on an aerobic to anaerobic transition is in agreement with the type of response that would be expected from studies with nongrowing systems (1, 6).

Calibration of the system by the addition of free NADH would not be meaningful, as it has been shown by Estabrook (12) (and substantiated by the author) who found that NADH dissolved in the extracellular fluid has a much lower fluorescence yield than that inside the intact cell (which is probably mostly in a bound state). Thus, enzymatic analysis of samples is the only method available for calibrating the apparatus. To this end, samples were taken and analyzed, as described above, with the culture in the aerobic and anaerobic steady states. Results of such a calibration, shown in Table 1, are in good agreement with similar results for *Escherichia coli* (19). It can be seen that even in the anaerobically grown cells most of the nicotinamide nucleotide is present in the oxidized form, although the triphosphate which constitutes only about 25% of the total nicotinamide nucleotide remains largely in the reduced state. The difference between the measured change in NAD^+ and NADH is prob-

TABLE 1. Typical results of chemical calibration of a fluorimeter

Sample ^a	Concn ^b			
	NAD^+	NADH	NADP^+	NADPH
Aerobic	13.8 14.1	2.1 1.8	0.57	4.55
Anaerobic	12.9 12.4	3.5 3.5	0.50	4.45
Mean change	-1.3	1.6	-0.07	-0.05

^a Glucose-limited, chemostat culture of *K. aerogenes* was used [4.5 mg/ml (dry weight)]. Samples were taken from the culture under aerobic and anaerobic conditions, rapidly extracted, and analyzed for nicotinamide nucleotides.

^b Values are expressed as nanomoles per milliliter of sample.

ably within the experimental error of the methods. In these cells, changes in NADPH are small and would contribute little to the fluorescence changes, so that it may be assumed that observed changes in fluorescence during the transition from aerobic to anaerobic states are due to changes in NADH level. Such analyses were used to calibrate the type of transitions shown in Fig. 2.

The response of nicotinamide nucleotide fluorescence to intermediate conditions between the fully aerobic and anaerobic states is shown in Table 2. There was no measurable change in fluorescence until the oxygen tension was lowered to 3 mm of Hg at which time a small increase was observed. With subsequent decreases in

oxygen supply, however, there were corresponding increases in fluorescence. The greatest increases in fluorescence occurred at oxygen tensions which were below the minimal sensitive range of the electrode, but intermediate states in the reduction of nicotinamide nucleotide may exist in the growing cell as oxygen becomes limiting.

Effect of organism concentration. It was found that the change in the concentration of the bacterial population had a profound effect on the sensitivity of the apparatus. This is to be expected as cells will in fact receive less light, the light penetrates further into the culture, and the light intensity falls as the square root of distance. There should be no upper limit to the cell concentration that may be used. Ideally, only the surface of the culture should be illuminated, as the longer the light path within the culture the greater the possibility of reabsorption and scattering of emitted light by cells. Table 3 shows the effect of cell (dry weight) concentration on the noise level of the fluorescence reading. It can be seen that at a cell concentration of 5 mg/ml the apparatus has a low noise level but the sensitivity decreases considerably at a cell concentration of 0.5 mg/ml. Thus, a cell concentration of at least 3 mg/ml is required for acceptable sensitivity.

Stability. For periods of 1 hr or less, the stability of the fluorimeter reading was generally extremely good, with no detectable drift. However, the drift in reading over a 24-hr period

TABLE 2. Changes in the nicotinamide nucleotide fluorescence with changes in culture oxygen tension^a

Oxygen partial pressure in gas phase (mm of Hg)	Dissolved oxygen tension (mm of Hg)	Fluorimeter reading (mv)
154	115	0
77	38	0
54	15	0
40	3	+0.20
35	<1.0	+0.60
31	<1.0	+1.58
0	0.0	+1.98

^a Dissolved oxygen tension was altered by changing the partial pressure of oxygen in the gas phase with mixtures of air and nitrogen. The oxygen electrode used was insensitive to oxygen tensions below 1.0 mm of Hg, and values lower than this could not be recorded. The fluorimeter reading is expressed in millivolts relative to the reading with air in the gas phase which was set arbitrarily at zero. The culture was a glucose-limited chemostat culture of *K. aerogenes* maintained at a growth rate of 0.2 hr⁻¹.

TABLE 3. Effect of cell concentration on the noise level of the fluorescence trace^a

Cell concn ^b	Per cent noise level ^c
0.5	15
3.0	7
5.0	2.6

^a Results are for glucose-limited cultures of *K. aerogenes*.

^b Expressed as milligrams (dry weight) per milliliter of sample.

^c Calculated from: [(anaerobic reading - aerobic reading)/amplitude of noise] × 100.

of constant monitoring was found to vary up to 100% of the aerobic/anaerobic response. When the temperature of the lamp was maintained at 30 C by means of a water-jacket, the drift was reduced to less than 20% of the aerobic/anaerobic response. Slow long-term drift is probably caused by changes in physical properties of the culture rather than by the instrument. Changes in cell concentration, size and number of the bubbles, growth of organisms on the glass walls of the culture vessel, etc. would be expected to alter the absolute reading of the fluorimeter.

DISCUSSION

The main difficulties connected with optical measurements in a stirred bacterial suspension are light penetration, interference due to air bubbles, and turbulence. In fluorimetric techniques, light penetration poses no problem because incident light needs to penetrate only a short distance into the culture. In fact, the higher the cell concentration and the lower the light penetration, the greater is the sensitivity of the fluorimeter, since light scattering and reabsorption of emitted light are diminished. In practice, a cell (dry weight) concentration of at least 3 mg/ml was found to be desirable. Interference due to bubbles is avoided in this system by illuminating and collecting over a relatively large area (approximately 10 cm²) rather than by focusing the light. This has the effect of integrating the fluorescence over that area and minimizing the effect of small bubbles. The response time of the amplifier was set at 0.5 sec so that occasional large bubbles or bubble clusters passing rapidly through the field of illumination would not cause a large deflection in reading.

The sensitivity of the fluorimetric method applied to the chemostat compares well with that of fluorescent studies in resting-cell suspensions provided a cell concentration of 5 mg/ml or more is used. The fluorescence recordings

obtained during aerobic/anaerobic transitions in the chemostat culture were similar to those obtained in resting-cell suspensions (6, 13), in which a rapid reduction of nicotinamide nucleotide takes place as the culture becomes devoid of oxygen. Thus, this technique offers the possibility of studying changes in the intracellular redox potential in response to the extracellular environment in growing cells and should reveal information on the metabolic control systems operating in dividing cells. Some studies have already been carried out on steady-state chemostat cultures by using continuous fluorimetric measurement of NADH. They have revealed new oscillating phenomena in dividing cells (14; Harrison, J. Cell Physiol., *in press*).

Some of the most interesting and profound changes in the metabolism of facultative organisms in response to oxygen concentration occur at oxygen levels which are below the sensitive range of membrane oxygen electrodes (14). The studies reported here show that the NADH fluorescence shows the greatest sensitivity to oxygen availability at this range of oxygen concentrations. Thus, nicotinamide nucleotide reduction provides a possible alternative monitor for cultures under limited-oxygen conditions. This method is more sensitive than the measurement of oxygen tension and probably more meaningful than extracellular redox potential. The control of metabolic pathways which are sensitive to low oxygen tensions or redox potentials may possibly be correlated with the NAD⁺/NADH ratio, as this represents the intracellular redox potential. In the case of fermentation products, the production of which depends on maintaining a certain low oxygen level or redox potential, NADH fluorescence may provide the best monitor or control parameter for the process.

No doubt this technique can be developed into a quantitative, stable metering system which could find widespread applicability to studies on cultures of microorganisms. Combined with a system for controlling oxygen tension in a culture, the measurement of reduced pyridine nucleotide levels is a very useful tool, enabling the observation of changes of the redox state inside the cell in response to extracellular stimuli.

ACKNOWLEDGMENT

We are grateful to D. Meyer for his expert advice on instrumentation.

LITERATURE CITED

1. Betz, A., and B. Chance. 1965. Influence of inhibitors and temperature on the oscillation of reduced pyridine nucleotides in yeast cells. *Arch. Biochem. Biophys.* **109**:579-584
2. Boyer, P. D., and H. Theorell. 1956. The changes in reduced diphosphopyridine nucleotide (DPNH) fluorescence upon combination with liver alcohol dehydrogenase (ADH). *Acta Chem. Scand.* **10**:447-450.
3. Chance, B. 1952. Spectra and reaction kinetics of respiratory pigments of homogenised and intact cells. *Nature (London)* **169**:215-221.
4. Chance, B. 1954. Spectrophotometry of intracellular respiratory pigments. *Science* **120**:767-775.
5. Chance, B. 1954. Enzymes in action in living cells: the steady state of reduced pyridine nucleotide. *Harvey Lect.* **40**:145-175.
6. Chance, B. 1964. Feedback control of metabolism in *Ascites* tumor cells. *Acta Unio Int. Contra Cancrum* **20**:1028.
7. Chance, B., and H. Baltscheffsky. 1956. Respiratory enzymes in oxidative phosphorylation. VII. Binding of intramitochondrial reduced pyridine nucleotide. *J. Biol. Chem.* **233**:736-739.
8. Chance, B., R. W. Estabrook, and A. Ghosh. 1964. Damped sinusoidal oscillations of cytoplasmic reduced pyridine nucleotide in yeast cells. *Proc. Nat. Acad. Sci. U.S.A.* **51**:1244-1251.
9. Chance, B., and G. R. Williams. 1956. The respiratory chain and oxidative phosphorylation. *Advan. Enzymol.* **17**:65-134.
10. Chance, B., J. R. Williamson, D. Jamieson, and B. Schoener. 1965. Properties and kinetics of reduced pyridine nucleotide fluorescence of isolated *in vivo* rat heart. *Biochem. Z.* **341**:357-377.
11. Duysens, L. N. M., and J. Ames. 1956. Fluorescence and spectrophotometry of reduced diphosphopyridine nucleotide in intact cells in the near-ultraviolet and visible region. *Biochem. Biophys. Acta* **24**:19-26.
12. Estabrook, R. W. 1962. Fluorimetric measurement of reduced pyridine nucleotide in cellular and subcellular particles. *Anal. Biochem.* **4**:231-245.
13. Estabrook, R. W., and P. K. Maitra. 1962. A fluorimetric method for quantitative micro-analysis of adenine and pyridine nucleotides. *Anal. Biochem.* **3**:367-382.
14. Harrison, D. E. F., D. J. MacLennan, and S. J. Pirt. 1968. Responses of bacteria to dissolved oxygen tension, p. 117-144. *In* D. Perlman (ed.), *Fermentation advances*. Academic Press, Inc., New York.
15. Harrison, D. E. F., and P. K. Maitra. 1969. Control of respiration and metabolism in growing *Klebsiella aerogenes*: role of adenine nucleotides. *Biochem. J.* **112**:647-656.
16. Harrison, D. E. F., and S. J. Pirt. 1967. The influence of dissolved oxygen concentration on the respiration and glucose metabolism of *Klebsiella aerogenes* during growth. *J. Gen. Microbiol.* **46**:193-211.
17. Hempfling, W. P., M. Höfer, E. J. Harris, and B. C. Pressman. 1967. Correlation between changes in metabolite concentrations and rate of ion transport following glucose addition to *Escherichia coli* B. *Biochem. Biophys. Acta* **141**:391-400.
18. Kohen, E. 1964. Oxidation-reduction kinetics of pyridine nucleotide: a micro-fluorimetric study in insect spermatids and ascites cells. *Exp. Cell Res.* **35**:26-37.
19. London, J., and M. Knight. 1966. Concentrations of nicotinamide nucleotides coenzymes in microorganisms. *J. Gen. Microbiol.* **44**:241-254.
20. Maitra, P. K., R. W. Estabrook, and B. Chance. 1963. Yeast as a model for the study of the regulation of cellular metabolism, p. 491-506. *In* J. C. Senez (ed.), *Mécanismes de régulation des activités cellulaires chez les microorganismes*. Colloques Internationaux du Centre National de la Recherche Scientifique, Paris.
21. Meyer, D. H., J. R. Williamson, and V. Legallais. 1969. A sensitive filter fluorimeter for metabolic assays. *Chem. Instrumentation* **1**:383-389.
22. Purvis, J. L. 1958. Forms of pyridine nucleotide in rat-liver mitochondria. *Nature (London)* **182**:711-712.
23. Schön, G., and G. Drews. 1968. The influence of pO₂ on the redox balance of NAD(P) and of cytochromes b and c₁ in some Athiorhodaceae. *Arch. Mikrobiol.* **61**:317-326.