METHODS IN MICROBIOLOGY

VOLUME 6B

METHODS in MICROBIOLOGY

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PREFACE

The main theme in Volume 6 of "Methods in Microbiology" is the application of biochemical techniques to the study of micro-organisms. The topics covered include the use of biochemical and enzymic tests to characterize microbial types, the quantitative separation and analysis of fermentation products produced by micro-organisms, a critical appraisal of methods available to elucidate metabolic pathways and the control of enzyme synthesis, the assay of selected enzymes in crude extracts of cells as indicators of metabolic pathways, the use of antimetabolites to study biosynthesis and electron transport. The applications of radiotracer techniques are described in detail in five chapters: respiratory measurements with dyes and with electrodes for oxygen and carbon dioxide are included while manometric methods, which are so well described elsewhere (Umbreit, Burris and Stauffer, 1964) have been excluded from this series; articles on nitrogen fixation, calorimetry, electrochemical measurements and methods for studying sporulation and germination are also provided.

The detailed choice of the contents of the various contributions has been left largely to the individual authors. We have, as in the past, edited only to conserve consistency, bridge the gaps and avoid, where possible, overlaps between the articles.

Volume 6, like Volumes 3 and 5, has been divided into two parts. The division of the contributions, although somewhat arbitrary, was made by grouping the more chemical and enzymological topics into Volume 6A and allocating the more physical techniques of isotopes, electrodes, electrometry and calorimetry to Volume 6B.

We are grateful for the pleasant way in which our contributors have co-operated with us during the last three or four years. We must particularly thank those authors who have had the patience to wait for this publication. Some completed manuscripts were received three years ago, and many were subsequently revised by their authors.

J. R. NORRIS

D. W. RIBBONS

September, 1971

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CHAPTER I

The Use of Electron Acceptors, Donors and Carriers

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I. INTRODUCTION

In the investigation of electron (hydrogen) transfer chains, it is common practice to introduce an artificial electron donor or acceptor into the experimental system. Usually, this is a substance whose oxidation or reduction is easily measured and it is added to the system in place of one of the natural substrates. It may react at either end of the catalytic chain or at an intermediate point. Substances which react at more than one point may also be used catalytically to by-pass portions of the chain. By a suitable choice of such reagents, different assays may be devised for the functional separation of the chain into its component parts. A few natural electron carriers, notably NAD, are also readily available for use in such studies.

This section deals with the use of natural or artificial reagents as donors, acceptors or carriers of electrons. When necessary, the term "electron carrier" will be used collectively to include all such substances; it will be obvious from the context when the more limited reference to substances acting catalytically is intended. With micro-organisms, the use of these reagents has not been limited to the study of aerobic species and the scope of this contribution will be taken to include fermentative as well as respiratory systems. Published information on the use of electron carriers is both widely scattered and deeply buried, so that important practical details are easily overlooked. In consequence, the topics given detailed treatment in this contribution reflect the writer's interests and experiences and some topics have been treated in review form.

II. TECHNIQUES

A. Measurement of the reaction

1. *Spectral methods*

At the present time, the vast majority of oxidation–reduction reactions are followed by making use of changes in the absorption spectrum of the electron donor or acceptor. These methods have the advantage that observations can usually be made without disturbing the reaction mixture or consuming portions of it. Tests of this kind were first made by visual observations of colour changes and were put on a quantitative basis by measuring the time taken for complete decolorization of dyes. This gives only a crude indication of the reaction rate since the progress of the reaction is rarely, if ever, linear over the full period of the reaction. The successive introduction of colorimeters and spectrophotometers and subsequent improvements in their performance and availability have enabled more refined measurements to be made with ease, so that the precise and rapid or continuous measurement of optical density over a narrow waveband is now a common place laboratory operation. Provided that the Beer–Lambert Law is obeyed by the chromophore being monitored, such measurements are proportional to concentration and give a direct indication of the progress of the reaction. The spectrophotometer has thus become the apparatus of choice for following oxidation–reduction reactions. Measurement of decolorization time however, is still useful as a simple means of dealing with turbid preparations that avoids the need for more sophisticated instruments.

Details of the construction and operation of individual colorimeters and spectrophotometers are obtainable from manufacturers' literature and will not be given here. Remarks will be confined to two practical matters concerning the reaction vessels. First, when making kinetic experiments it is desirable to start the reaction by adding the final component and to mix quickly and at a precise time. A convenient method applicable to standard cuvettes is to use a thin glass or plastic rod, the lower end of which is flanged or shaped into a cup. The required component, in a small volume, is pipetted onto this end of the rod to form a drop. The addition and mixing are then accomplished rapidly by lowering the end of the rod into the cuvette and agitating.

Second, the standard optical cuvette of 1 cm light path and 3 ml operating volume is designed primarily for the precise measurement of optical density. As a reaction vessel it is inferior to the test tube in cost, robustness and ease of handling and cleaning. In some instances, and especially where high precision is not called for, or large numbers of tests have to be made, it is often more convenient to use tubes. These may be used down to about 300 nm where glass ceases to be transparent. Suitable adaptors for this purpose are available with some instruments, or can be readily made. Tubes with similar optical properties may be made from selected lengths of glass tubing or from precision bore tubing. Alternatively, batches of ordinary tubes may be screened to select those with similar optical properties within certain limits of tolerance; this may be done by determining the extinctions of each tube empty and when filled with a standard solution of a suitably coloured substance. As the optical paths of such tubes usually differ across different diameters, the tubes should be marked so that the same face may always be presented to the light path.

2. *Other methods*

Because spectral measurements conveniently satisfy the majority of experimental requirements and especially those for routine enzymic assays, other methods of following the reaction of electron carriers have received comparatively little attention. Further, many artificial carriers in common use have been selected with a view to spectrophotometric use. The useful range of concentration over which they can be employed is determined by their extinction coefficients and other methods of following reactions are mainly used when it is necessary to work outside this concentration range. This may arise for example, when Michaelis constants are being determined, when high concentrations are required to permit the determination of other reactants or products, or when concentration influences the specificity of action of the electron carrier.

As instruments suitable for the continuous measurement at biological temperatures of other physical properties such as optical rotation become more readily available it may be expected that they will be applied to the measurement of electron carriers and that future electron carriers will be selected with the appropriate properties in mind. At present, it seems unlikely that newer physical methods will oust spectrophotometry for general use because of the resources of practical knowledge and equipment which have accumulated in relation to the latter. Moreover, spectral methods utilizing electron carriers which give a visible colour change have the advantage that qualitative preliminary experiments are easily done visually.

Mention will be made here of three alternative modes of following oxidation–reduction reactions which have been used to follow the reactions of electron carriers and which appear capable of wider application.

(a) *Fluorescence measurements*. Fluorescence methods have been used for the measurement of NAD and NADP and are described by Udenfriend (1962). The method is much more sensitive than absorption spectrophotometry but is more susceptible to interference and requires more careful use. To date, the method has been mainly used for measuring the NAD and NADP contents of tissues and following changes in their intracellular concentration.

(b) *Potentiometric measurements*. Measurements of electrode potential may be used to follow the reaction of some electron carriers. Whittaker and Redfearn (1967) have used this method to investigate the reduction of ferricyanide by succinate in presence of heart muscle preparations. The potential difference between a platinum electrode and a standard calomel electrode inserted into the reaction vessel was recorded against time. Potential differences were converted to ferricyanide concentrations using a calibration curve obtained by measuring the potential difference with known ferricyanide–ferrocyanide mixtures in buffer containing the heart muscle preparation. With this method it was possible to study the enzyme system over a wider range of ferricyanide concentration than was possible by either spectrophotometric or manometric means. The apparatus is described in more detail by Spikes *et al.* (1954).

The potentiometric method was used as early as 1929 to follow the reduction of dichlorophenolindophenol (Lehmann, 1929) and would appear to be capable of wider application, especially with electron carriers whose spectral properties are unsuited to spectrophotometry.

(c) *Measurements of gas exchange*. Many reactions involving artificial electron carriers can be made to produce an output or uptake of gas. This may be followed manometrically using the standard Warburg techniques (e.g. Umbreit *et al.*, 1964), or in the case of oxygen and carbon dioxide, by electrode methods. Suitable apparatus and procedure for the polarographic measurement of oxygen has been described by Bellamy and Bartley (1960), Stickland (1960), Peel (1963) and by Beechey and Ribbons (this Series). The use of carbon dioxide electrodes is described by Nicholls *et al.* (1967) and by Garland and Nicholls (this Series).

For convenient measurement, the manometric method requires gas exchanges of several μmoles and these changes are frequently 10–100 greater than those suitable for spectrophotometric observations. Because the manometric method measures exchanges rather than the amount of gas present at any one time, rates may be measured with large concentrations of reactants. The electrode methods, especially those for oxygen, are much more sensitive and typical ranges of concentration are up to 0·25 mM. The electrode methods also lend themselves readily to continuous automatic recording, a further advantage over the manometric methods. The manometric and electrode methods are unaffected by turbidity and so can be easily used with cell suspensions and insoluble enzyme preparations, reactants or products.

Measurements of oxygen uptake may be used to assay many dehydrogenases by adding autoxidizable electron carriers, singly or in combination, to substitute for natural carriers transferring electrons to oxygen. One much used example is the assay of succinic dehydrogenase with phenazine methosulphate (Singer and Kearney, 1957). The suitability of particular carriers depends upon the specificity of their action with natural electron carriers and dehydrogenases. Thus methylene blue reacts directly with several dehydrogenases but not with NAD. The method may be extended to NAD-linked dehydrogenases by adding a diaphorase (e.g. lipoic dehydrogenase) to bridge the electron transfer gap between NAD and methylene blue.

Oxygen may also be used in the absence of added electron carriers as an "unphysiological" oxidant with preparations from strictly anaerobic bacteria. This method was used by Stadtman and Barker (1949) to study the oxidation of ethanol and butyrate by dried cells of *Clostridium kluyveri* and by Nisman (1954) to study the oxidation of amino acids by washed cells of *Clostridium sporogenes*.

Measurements of carbon dioxide exchange in bicarbonate buffer may be used to follow reactions which are accompanied by the gain or loss of protons. The ferricyanide–bicarbonate system was first introduced by Quastel and Wheatley (1938) for succinic dehydrogenase. When ferricyanide is reduced by succinate, the transfer of each electron results in the release of one molecule of carbon dioxide and the system is thus four times as sensitive as when oxygen is the terminal oxidant. Hochster and Quastel (1952) later used manganese dioxide as the oxidant with a bicarbonate buffer. In that case, the transfer of a pair of electrons to the manganese dioxide results in the uptake of one molecule of carbon dioxide. These bicarbonate buffer systems are used with carbon dioxide/nitrogen mixtures as the gas phase and are thus particularly useful with enzyme preparations which are sensitive to oxygen.

Measurements of hydrogen exchange have been used in conjunction with substrate amounts of artificial electron carriers for the manometric assay of hydrogenase in either direction in several bacterial species (Peck and Gest, 1956). Dithionite was used as the reductant when assaying hydrogenase by release of hydrogen and methylene blue was used as the oxidant when measuring the enzyme by hydrogen uptake.

B. Anaerobic techniques

1. *Introduction*

For various reasons it is frequently necessary to make measurements under anaerobic conditions. First, the electron acceptor may be autoxidized at a sufficient rate to interfere unduly with measurements of its reduction. Second, the reduced form of the electron acceptor may be subject to enzymic, as opposed to non-enzymic, oxidation when oxygen is present. This occurs commonly with NADH and NADPH and, perhaps unexpectedly, crude extracts of many strictly anaerobic bacteria possess powerful NADH oxidase activity. With *Peptostreptococcus elsdenii* for example, a typical activity for a crude extract is 10 μmoles NADH oxidized/h/mg protein at 37°, and this completely prevents the spectrophotometric detection of several dehydrogenases reducing NAD, unless strictly anaerobic conditions are used (Somerville, 1965). Third, the enzyme preparations or their substrates may be sensitive to oxygen. Fourth, the substrate of the reaction under investigation may be a gas, as in the case of hydrogenase. To meet these requirements special reaction vessels and methods of purifying gases are used.

2. *Anaerobic reaction vessels*

Manometric apparatus may be used anaerobically without modification. For spectral measurements, the use of evacuated reaction tubes was first introduced by Thunberg (1918). A single evacuation with a water pump is often adequate; if not, the tube is refilled with oxygen-free gas and evacuated again, repeating the process until a sufficiently low oxygen tension is reached. The original pattern of tube used by Thunberg had no second compartment for separating the substrate (or other component) from the reaction mixture until the evacuation was complete. Quastel and Whetham (1924) met this requirement by using an evacuated U-tube; Keilin (1929) introduced tubes with a curved hollow stopper which have come to be regarded as the standard form of the Thunberg tube. With tubes made to fit colorimeters, or those spectrophotometers suitable for round tubes, the progress of the reaction may be followed quantitatively. With many spectrophotometers, however, and for precision work, measurements must be made in an optical cuvette of square cross section and this has resulted in modified Thunberg tubes terminating in a cuvette (Fig. 1a). A variation which abandons the hollow stopper and uses a side arm is illustrated in Fig. 1b. Although this pattern is longer it occupies less room laterally and is particularly useful when a version with two side arms is needed.

A disadvantage of the evacuated tube is that, because of the unavoidable boiling which occurs on evacuation, a minimum length of free tube is required between the surface of the liquid and the side arm or hollow stopper. This results in tubes which cannot be accommodated within the confines of the cell compartment of most spectrophotometers, although the difficulty may often be overcome by substituting an inverted box for the normal lid of the compartment. The pattern of cuvette illustrated in Fig. 1c is much