

Chapter 17

The Estimation of Small Quantities of Carbon-14 Labelled Adenine Nucleotides following their Separation by Ion Exchange Paper Chromatography

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The technique of polarography combined with the analysis of adenine nucleotides in a mitochondrial suspension provides a powerful analytical approach to certain aspects of oxidative phosphorylation. The use of the oxygen electrode alone is a well established technique. Its sensitivity, however, has tended to far outstrip the methods available for conveniently estimating the efficiency of the phosphorylation process. The present communication has the purpose of emphasizing the fact that the separation and estimation of the nucleotides derived from trace amounts of ADP is well within the scope of the teaching as well as the research laboratory.

A typical experiment for studying the efficiency of oxidative phosphorylation proceeds as follows. Samples to be analysed are withdrawn from the incubation medium containing the respiring mitochondria in volumes of 3 to 5 μl . The samples are then spotted on strips of DEAE 81 (OH form) ion exchange paper. For convenience, after being spotted the strips are cut from a previously ruled sheet. Ascending chromatography is then carried out using 20% formic acid at room temperature.

This type of chromatography results in a very tight binding of the nucleotides to the paper. The enzymatic reactions are immediately halted, thus doing away with the need for perchloric acid or TCA treatment. Following chromatography the strips are dried and cut into fragments about 1 cm square which are placed serially in separate vials containing the fluor solution. Immersion in this solution results in very little radioactivity leaving the paper.

The data obtained from the group vials give the total counts above background for the regions of the original chromatogram in which the ATP, ADP and AMP have become distributed. Each locus may have its activity spread over three to five vials depending on the concentration. This enables the activity due to each nucleotide to be calculated as a percentage of the total activity. The regions between the separated components have an activity which is the same as, or very close to, background.

Samples may be withdrawn at frequent intervals during an experiment. The open cell of the Gilson 'Oxygraph' lends itself to this procedure in that small samples may be obtained very conveniently and without disturbing the oxygen uptake recording. This procedure is illustrated in Fig. 1. The labelled compound is added through the same

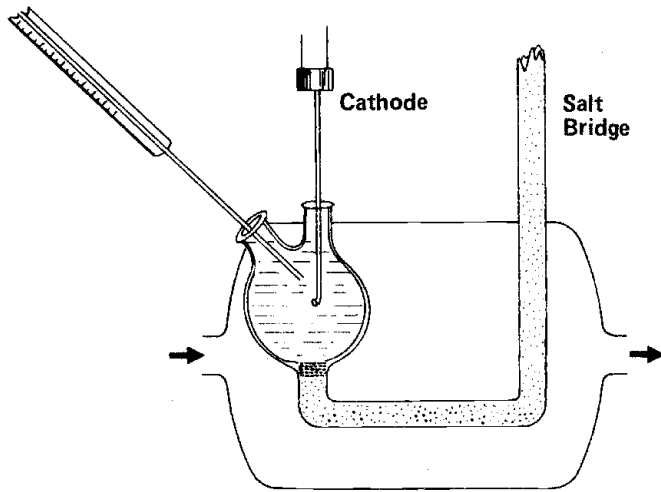


Fig. 1. The Gilson 'Oxygraph' cell showing the use of a Hamilton syringe for withdrawing small volumes. Large arrows indicate direction of flow in water jacket.

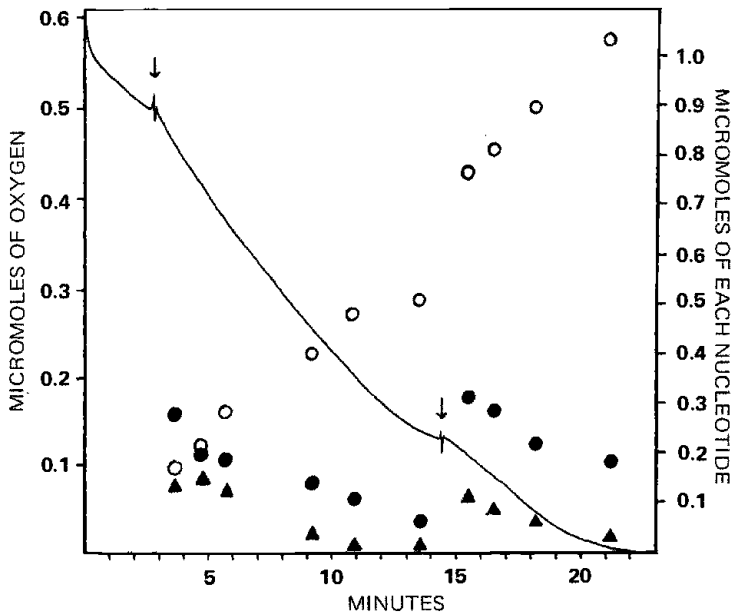


Fig. 2. The respiratory response to ADP in mitochondria isolated from the turtle liver. Arrows indicate times when ADP was added, $0.6 \mu\text{mol}$ in each case. \circ = ATP, \bullet = ADP and \blacktriangle = AMP. Total nucleotide concentration established at 260 nm. Substrate, succinate. Mitochondria present equivalent to 1 mg protein. (From experiment conducted with N. Grimes, University of Bowling Green, Ohio).

opening. Its volume is larger and the mixing procedure slightly deflects the oxygen recording. The combined data are shown in Fig. 2. In this case consecutive additions of ADP have been made. The reduced rate of oxygen uptake as the ADP is used up is clearly seen. Also shown is the renewed stimulation of respiration when fresh ADP was added.

Apart from the oxygen relationship, the study of the balance between the concentrations of the three nucleotides is a fertile field of research. Data quite similar to those in the present report (but obtained by means of the luciferase assay) have been published by Godinot *et al.*¹ A result which is sometimes surprising is the intense activity of myokinase and ATPase revealed in certain preparations, for example the embryo chick liver² and housefly sarcosomes.³

The use of the oxygen electrode in teaching the theory of oxidative phosphorylation in the laboratory has been adequately described.⁴ The combined approach presently described has also been very successful in practice. Students have been able to conduct oxidative phosphorylation experiments in a way which they have found to be particularly interesting. They have also obtained valuable experience in the technique of liquid scintillation counting and in the statistical handling of the data. The low activity of the isotopes made possible by this counting method is an additional asset in the teaching laboratory from the point of view of safety and economy.

REFERENCES

- 1 C. Godinot *et al.*, *Eur. J. Biochem.* 8, 385 (1969).
- 2 A. M. Holm, *Thesis*, University of Bowling Green, Ohio, U.S.A., 1968, p. 59.
- 3 G. C. Carney, *Life Sci.* 8, 435 (1969).
- 4 J. M. Foster, *Bioscience* 19, 541 (1969).

DISCUSSION

H. Dobbs: Comment: There is a one word answer to the questions raised in Dr. Carney's paper; it is 'combustion'. In our laboratories we have yet to find a sample which will not succumb to this technique. Quantitative measurements of the distribution of radioactivity on paper chromatograms can be made by cutting the chromatogram into strips and burning the individual pieces of paper. We have successfully applied combustion techniques to the assay of thin layer chromatograms. We scraped the areas of the plate (containing highly quenching materials adsorbed on alumina) into rice paper cachets. The alumina was mixed with powdered cellulose. Each cachet was then burned in the normal manner. After introduction of the liquid scintillator into the combustion flask the alumina was allowed to settle before an aliquot of the liquid scintillator was removed for counting.