The Institute of Biology's
Studies in Biology no. 28

The Biology of Respiration

Second Edition

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Readers' comments will be welcomed by the Education Officer of the Institute.

1979

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Preface

The first edition of this book was written because I felt that comparative biochemistry was a sadly neglected field. It is less neglected now and so I welcome the opportunity to revise a text nearly ten years old.

I have made many changes while retaining the same general approach. I have modernized the account of the role of membrane systems in energy transduction, and I have included a brief consideration of one of the world's most neglected biomes – the 'sulphide layer'. This occurs under surface sediments on the floors of all major bodies of water and provides a chemically reducing environment which harbours a host of primitive anaerobes. I have touched upon the evolutionary history of anaerobiosis and the origin of the eukaryotic cell. And finally, I have discussed a new idea that some parasites 'do without oxygen' in the midst of plenty and that it is other characteristics of their environments that enforce the anaerobic habit. All of this, I hope, combines to give a longer than usual view of the evolution of respiration.

Canberra, 1979

C. B.

Contents

1 Ecology and Respiration

The relationship between the environment of an animal and its respiratory metabolism is a very intimate one which has been often neglected by biochemists. An animal is fitted to its environment, and the fitness is manifested at the cellular level as well as at the level of the whole animal; unfortunately, the finer details tend to be overlooked. Considerable attention in the literature has been paid to physiological mechanisms underlying the adaptation of an animal to its ecological niche, but much less to the way in which biochemical mechanisms, in turn, provide the foundation which makes this adaptation possible.

It is important, when beginning the serious study of biology, to understand that all life does not conform to the patterns we learn from studies of the common laboratory animals and man. It is also necessary to be aware of the dangers of studying animals without taking into account the characteristics of the environments which produced them. Environment and organism comprise a unit which should ideally be studied as a whole. It is this concept that makes ecology the most important of today's biological sciences.

Environments which are different in many ways may yet have one or more important features in common which result in similar adaptations in the animals which occupy them. It is therefore necessary that great care should be taken to avoid the danger of postulating relationships between groups of animals on the basis of biochemical similarities. Such similarities may be the result of a common need, rather than a common ancestor. As an example, it proved to be erroneous to erect a hypothesis for the origin of vertebrates on a single biochemical system. It was suggested that echinoderm ancestors were related to ancestral vertebrates, because, amongst other things, both groups possessed creatine phosphate as an energy store. Invertebrates were thought to be the exclusive possessors of arginine phosphate. Unfortunately for this hypothesis, as larger numbers of groups were studied, echinoderms and invertebrates generally were found to possess both phosphagens. The distribution of phosphagens in the animal kingdom is based at least as much on the need for an energy store, whatever the components of the mechanism that produces it, as on an evolutionary relationship.

The bottom of a muddy pond is usually rich in organic matter and poor in O_2. Occupation of environments like this may result in the appearance in organisms of adaptations which resemble those found in organisms from other O_2-deficient environments. For example, there are common features in the cellular respiration of free-living flatworms from the pond, and the respiration of worms parasitic in the small intestine of the sheep.

They may indeed be due to a relationship, for the flatworm and the parasite are usually included in the same phylum, and the worms' respective successes in the ditch or in the gut may be because their ancestors possessed respiratory adaptations which permitted them to exploit O_2-poor environments. Or it may be that the animals are unrelated, and that they responded in a similar way to a similar challenge.

Another point to be considered is the conservative nature of life. Similar answers may be arrived at even where problems are only loosely connected. The occurrence of haemoglobin in such widely diverse forms as vertebrates, molluscs, insect larvae and intestinal parasites, merely reflects the usefulness of a sub-group of haemoproteins in transactions which involve the transport and storage of small molecules like O_2.

An important property of life is that it runs counter to the physical processes of the universe. The universe is running down, moving from a highly ordered state to a completely disordered one. On the other hand, life is characterized by its high degree of order which is maintained in the face of events which tend to break it down. The continued existence of life is therefore dependent on a continuous supply of energy to repair the depredations caused by the environment. Although its ultimate origin is solar radiation, in animals energy becomes available during the processes of respiration; it is not surprising that a system which fulfils such a basic requirement arose early in evolution and has been largely resistant to innovation. It must have arisen early and it must have been perfected early; subsequent modification has hardly been required.

The term respiration includes a variety of activities, from the metabolic pathways which enable the accomplishment of the capture of chemical energy, to the physiological and behavioural mechanisms which enable the organism to sample a portion of its environment, and extract from it O_2 which may enter solution in the body fluids and ultimately become available to the individual cells. It is at the latter levels that the greatest variability is encountered, because the body surface of the organism, whether an elephant or *Amoeba*, makes intimate contact with the outer world. External surfaces and forms are therefore the most plastic of structures. They interact with, and are moulded by, the environment, and their natures are dictated by the necessity for keeping the environment at bay. Elephant's hide and *Amoeba* membranes are two ways of achieving this. The surfaces are supportive, helping to maintain the integrity of the organisms, but they also form selective barriers which O_2 and other substances must somehow cross.

As environments are variable, it is not at all surprising that organisms have evolved many mechanisms for taking from them what they require. In *Amoeba*, diffusion is sufficient to transport O_2 from the environment to all parts of the organism. As it is an aquatic animal, it needs no elaborate devices for getting the O_2 into solution; O_2 arrives in that condition at the body surface, which is an advantage shared by most aquatic organisms, whether freshwater or marine.

As the size of the organism increases, skeletal and defensive requirements may result in most of the body becoming enclosed in armour of various sorts. Under these circumstances, a specialized respiratory surface develops, where the soft parts are directly exposed to the environment. Such a surface may be a vastly elaborated epithelium hidden under a carapace, as in the crayfish, or merely those parts which are exposed as they perform another function. An example is the tube foot of the sea-urchin. Increase in size also means that diffusion is no longer a suitable vehicle for the transport of O_2 and other substances throughout the animal. Some form of activated transport or circulatory system is necessary.

The need to maintain a relatively exposed surface for gaseous exchange renders the terrestrial organism more vulnerable than it might otherwise be. A moist membrane is required, so that O_2 arriving at its surface film can go into solution and pass across it; yet water loss must also be controlled. Considerable evaporation of water occurs through the tracheae of insects and lungs of vertebrates, and perpetuates their dependence on a source of external water, either free or with the diet. Many organisms living in deserts apparently survive on water generated during metabolic processes. It is clear that those functions which are at the mercy of the environment become as varied as external conditions.

Once inside the animal, however, it is a different story. All organisms possess the property of homoeostasis. In the face of a universe in which the degree of disorder is continually increasing, they seek to maintain themselves, to increase and grow. In the long run they lose the battle; probably all organisms are mortal. During their lifetimes, however, they endeavour to maintain internal conditions as steady as possible to provide the most favourable conditions for the continuance of life.

Over long periods there is evolutionary change. Evolution should not be thought of as a failure of homoeostasis, but rather as a homoeostatic compromise under new conditions of environment. The result is the innate conservativeness of the living organism, a resistance to the change which still occurs, slowly, but inexorably, over epochs; a resistance during which the basic cellular processes and the cellular environment change very little. The genetic machinery of an organism determines whether it will be a man or a mouse, but whether man or mouse, the principles upon which the machine works and the components which comprise it are similar. It is the mode of assembly which is different. The fundamental needs for survival of man or mouse are the same, and their metabolic machineries show even greater similarities because, in both animals, they do the same job.

This is a statement of the unifying principle of biochemistry. All organisms are alike, for there are only a limited number of ways of carrying out a given process efficiently, and the most efficient of these tends to be selected by all organisms. Animals resemble one another more nearly than they resemble plants, but a vistor to this planet would

not hesitate to classify plants and animals under the same heading. Apart from a few special functions, differences are confined to changes of fine detail in *general* processes. For example, the metabolic pathway for the fixation of CO_2 by green plants during photosynthesis shows resemblances to a pathway which occurs in mammalian liver. But plants convert CO_2 to sugars, whereas animals use the path in reverse for the metabolism of sugars to CO_2 and a number of other important metabolites. Bacteria show greater differences from animals and plants, but even in these organisms, metabolic processes are similar to those of higher organisms. They may have a few extra properties which animals, for example, do not possess, and the converse is equally true. However, even their genetic codes are similar, which is reflected in the fact that much research in this area is carried out on microorganisms.

It is clear, therefore, that where there are differences, they are the result of a different way of life, of the occupation of a different ecological niche. Obviously, an autotrophe like grass will need a different set of enzymes from the heterotrophic cow that feeds on it, but both grass and cow live in an environment rich in O_2. Organisms are metabolic opportunists and tend to use whatever is in plentiful supply, if it can be used. Cellular respiration in grass and cow converges at the point at which O_2 is essential.

Where differences occur in such a fundamental process as respiration, it is obviously important to try to find out why. In the following pages, the extent of the effect of the environment, as far as is known, will be examined, using examples drawn from the animal kingdom. Of special interest are those modifications which occur in animals occupying environments which are deficient in O_2, as do many intestinal parasites, or in animals which cut themselves off, either physically or physiologically, from the O_2 supply. It must be emphasized that it is an area of knowledge which is still imperfectly understood.

There are short-term solutions to the problems of survival without O_2. Even active mammals have to cope with a relative or effective shortage of O_2 for short periods. One example is a man sprinting a hundred yards; a more interesting example is the sounding of the whale, which may stay submerged for hours at a time. They both illustrate how the animal frees itself from the constraints imposed on it by the environment, and how a basic process has undergone modification in response to environmental demands. Such modifications contribute materially to the success of the organism by opening up to it hitherto unexploited evolutionary niches.

It is the purpose of this book to attempt to determine where differences occur in the fundamental biochemical process of respiration. If changes do occur in such a basically stable metabolic system, then it is important to try to find the cause of the departure from the very successful and hence very conservative pattern. It is important, not only from the point of view of the intrinsic interest of the exercise, but from the point of view of understanding the animal and its relationship to its environment.

2 Respiration and Electron Transport

2.1 Oxidation and electron transfer

Historically, the term 'oxidation' was first used to describe those processes in which a substance combined with O_2. Thus, the burning of carbon in air falls into this category quite readily:

$$C + O_2 \longrightarrow CO_2$$

The term 'reduction' was similarly reserved for combination with hydrogen. As an example, under certain rigidly defined conditions, nitrogen is reduced to ammonia:

$$N_2 + 3H_2 \longrightarrow 2NH_3$$

However, the oxidation of hydrogen to yield water creates a number of semantic problems, as the reaction can be regarded either as an oxidation or a reduction, depending on which element is taken as the referent:

$$2H_2 + O_2 \longrightarrow 2H_2O$$

Other definitions of oxidation were sought, and the best and most modern approach takes into account the transfer of electrons. Loss of electrons is oxidation; gain of electrons is reduction. Under this definition, the conversion of ferrous ion to ferric is oxidation:

$$\underset{\text{ferrous ion}}{Fe^{++}} \longrightarrow \underset{\text{ferric ion}}{Fe^{+++}} + \underset{\text{electron}}{e^-}$$

The above equation underlies one of the most fundamental processes in respiration. Certain molecules concerned with oxidation in cells contain iron. They are called cytochromes and their ability to undergo reversible oxidation–reduction reactions depends on the interconversion of ferrous to ferric ions and vice versa.

In cellular respiration, however, the problem assumes an even greater complexity because, in addition to oxidations mediated by electron transfer as indicated above, the oxidation of organic molecules will take place by dehydrogenation. Dehydrogenation is the removal of hydrogen from a molecule and is yet another way of looking at oxidation. It is mediated by specific catalysts or enzymes, and special electron acceptors. For example, succinic acid is oxidized to fumaric acid by an enzyme called succinic dehydrogenase:

$$\begin{array}{l} CH_2 \cdot COOH \\ | \\ CH_2 \cdot COOH \\ \text{succinic acid} \end{array} \longrightarrow \begin{array}{l} CH \cdot COOH \\ \| \\ CH \cdot COOH \\ \text{fumaric acid} \end{array} + 2H^+ \quad \text{protons} \quad + 2e^- \quad \text{electrons}$$

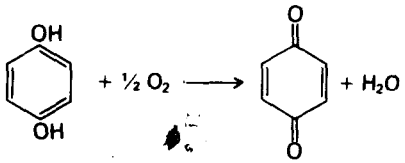
The enzyme has two distinct parts; a protein portion and a flavin portion. The former is the catalyst, and the latter accepts electrons and protons from succinic acid.

Malic acid is converted to oxaloacetic acid:

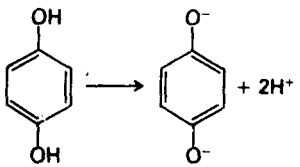
$$\begin{array}{l} CH(OH) \cdot COOH \\ | \\ CH_2 \cdot COOH \\ \text{malic acid} \end{array} + NAD^+ \longrightarrow \begin{array}{l} CO \cdot COOH \\ | \\ CH_2 \cdot COOH \\ \text{oxaloacetic acid} \end{array} + NADH + H^+$$

This reaction takes place in the presence of malic dehydrogenase. In this case, the acceptor of electrons and protons is not bound tightly to the enzyme, but it is still necessary for the reaction to occur. NAD^+ (nicotinamide adenine dinucleotide) is the oxidized form of the acceptor. In its reduced form, it is conventional to write it as $NADH + H^+$; it belongs to a group of substances which are ancillary to enzymes and are known as coenzymes or cofactors. The reduced form of NAD will itself become oxidized, at a later stage, by loss of electrons to the respiratory chain of mitochondria during aerobic respiration.

In order to make the processes of oxidation via electron transfer and proton (hydrogen ion) loss clearer, consider the oxidation of hydroquinone:

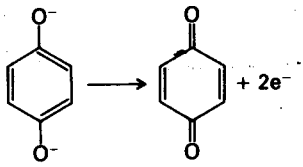


This reaction may be considered as a series of steps. The first is the dissociation of hydroquinone into its component ions:



In effect, by the loss of two protons, the parent molecule has become dehydrogenated.

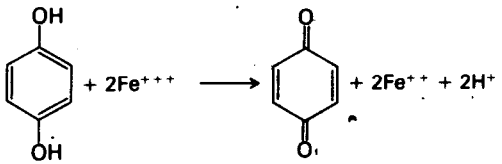
The second stage is the removal of electrons:



The electrons may then be accepted by another molecule. For example, if the ferric ion were the oxidant:

$$2e^- + 2Fe^{+++} \longrightarrow 2Fe^{++}$$

and the overall reaction becomes:



The hydroquinone reaction was deliberately chosen as an example because protons and electrons leave the parent molecule in well-defined stages. In other cases, especially in biological systems, protons and electrons depart simultaneously as intact hydrogen atoms or as hydride ions, which are merely hydrogen atoms carrying an additional electron apiece.

The earliest investigations into respiration were undertaken without any complicated equipment, although the experiments by which Lavoisier established that animals need O_2 would require a measure of ingenuity to duplicate even today. These and later studies all involved the enclosure of living material in air-tight vessels. Subsequent workers attached tubes called manometers, partially filled with liquid. Thus, if the volume of gas in the vessel containing the respiring organism changed, it would be reflected in a change in the level of liquid in the manometer. These devices were only possible if they possessed traps containing an alkali, which could absorb any CO_2 evolved. During aerobic respiration, the volume of CO_2 given out is close to the volume of O_2 utilized; when carbohydrates are being oxidized, the ratio is 1 : 1. In the absence of a trap, therefore, there will be no volume change.

This approach culminated in the development by Otto Warburg of the famous respirometer, which is surely to be found lurking in every biochemical laboratory in the world. The Warburg apparatus is a constant volume device; that is, it is used to determine differences in volumes by measuring the drop in pressure which occurs when O_2 is used up in an enclosed flask. If the pressure drops, the true volume of gas inside the flask is, when corrected to normal temperature and pressure, obviously smaller. It can also be used to measure gas output. A modern

Warburg apparatus possesses a mechanism for shaking the flasks in which the live material is placed, in order to ensure that the suspension medium remains fully saturated with respect to O_2. It has a thermostatically controlled bath to maintain the temperature of the experimental material, and a thermobarometer which allows the experimenter to compensate for minor changes in temperature and fluctuations in pressure which would otherwise introduce errors into measurements. The manometers are fixed and the flasks are removable, and there is provision for replacing the atmosphere in the flasks with other gases. The most modern versions of the apparatus give the corrected volume of the gas change on a digital readout. In the last few years, increasing use has been made of the oxygen electrode for measuring O_2 uptake. It is more sensitive than the manometric devices, and less easy to handle, but it is more accurate. An example of its use is given in Chapter 3.

The pioneer workers rightly considered that the interpretation of results obtained from the whole animal was too difficult to give a clear picture of the process involved in respiration. They therefore had recourse to the use of excised tissues (liver, kidney and minced pigeon breast muscle were favourites). The tissues were employed either whole, which was of limited usefulness because of the failure of O_2 to penetrate rapidly throughout, or they were broken up in various ways. The different types of preparation included slices cut very thin; breis, in which the tissue was chopped into very small pieces; minces; and homogenates.

A whole sub-science has built up around the preparation of homogenates and the separation of sub-cellular particles from them. In carefully prepared homogenates, the cell membrane is ruptured, spilling out the contents of the cell unharmed. As early as 1914 it was suspected that one of the cell inclusions, the mitochondrion, was somehow implicated in respiration. The first efforts to isolate mitochondria for further study were those of R. R. Bensley, in the 1930s, and although he only achieved a partial success, it represented a great advance over previous work. Bensley was inhibited by lack of both the proper suspension solution and an adequate centrifuge. It was not until 1948 that G. H. Hogeboom, W. C. Schneider and G. E. Palade reported on the excellence of sucrose solutions for the purpose of isolating mitochondria and the use of the technique of differential centrifugation for the preparation of intact mitochondria. Today, any manual of biochemistry will give the simple instructions which will enable anyone with access to a reasonable centrifuge to prepare a sample of mitochondria, suitable for all ordinary purposes, from rat liver.

In recent years, however, the need has arisen for absolutely pure preparations so that the worker can more easily distinguish between the respiratory and ancillary functions of the mitochondrion. This has led to a close scrutiny of all stages of preparation. The first step is the removal of the tissue to be investigated from the animal. Speed is essential as

irreversible changes occur very soon after death. Experienced workers can remove rat liver, for example, and cool it in the appropriate medium (such as isotonic sucrose) within 30 seconds of killing. It is of paramount importance that the tissue be cooled to between 0 and 4°C as quickly as possible, to slow down any enzyme reactions which may contribute to the breakdown of the system under investigation.

Having obtained the tissue and cooled and washed it in an appropriate medium, it is necessary next to homogenize it. A variety of tools have been used for this stage. One thing has to be borne in mind, however; the frictional heat generated by the homogenizer must not be allowed to warm the homogenate up, as damage may occur. Too vigorous homogenization may also denature the proteins. An efficient homogenizer is the ordinary kitchen blender, or modifications of it. It is very useful for large quantities of material, but types which can process less than 1 ml are also available. Blenders such as these, however, are often too rough, and the shear forces generated by the blades may rupture the sub-cellular particles as well as the cell envelope. More frequently used are the pestle homogenizers of various types which may be operated by hand or driven by a small electric motor. Other common methods include breaking up tissue by sonic disruption, by very rapid vibration in a vessel containing tiny glass beads, or by subjecting it to sudden, extreme changes of pressure.

Once the homogenate has been obtained in the correct suspension medium, the required particle must be separated. Separation processes have been simplified in recent years by the development of superior centrifuges, some of which are capable of achieving centrifugal forces in excess of 200 000 times the force of gravity (*g*). There are two fundamental techniques, either of which results in the production of a satisfactory preparation of mitochondria, and other sub-cellular fractions (Fig. 2–1).

The first technique is that of differential centrifugation, in which use is made of the fact that particles in the different fractions are of different sizes. Mitchondria are larger and heavier than microsomes, for example, and will sediment at a greater rate in a constant centrifugal field. Thus, a preliminary centrifugation at low speeds is employed to remove the large chunks of cell debris. The material which has remained in suspension after this treatment consists entirely of particles which are smaller than whole cells, and is called the supernatant. In the next run, the supernatant is centrifuged at about 10 000 × *g*, whereupon the mitochondria sediment under the increased centrifugal force. The supernatant from this spin will yield microsomes and other particles if desired. The mitochondrial pellet is usually washed several times by repeating the spin at 10 000 × *g* before use.

An alternative technique is that of density gradient centrifugation. In this case, the homogenate is layered carefully on the top of the suspension medium in the centrifuge tube. The concentration of the medium in the

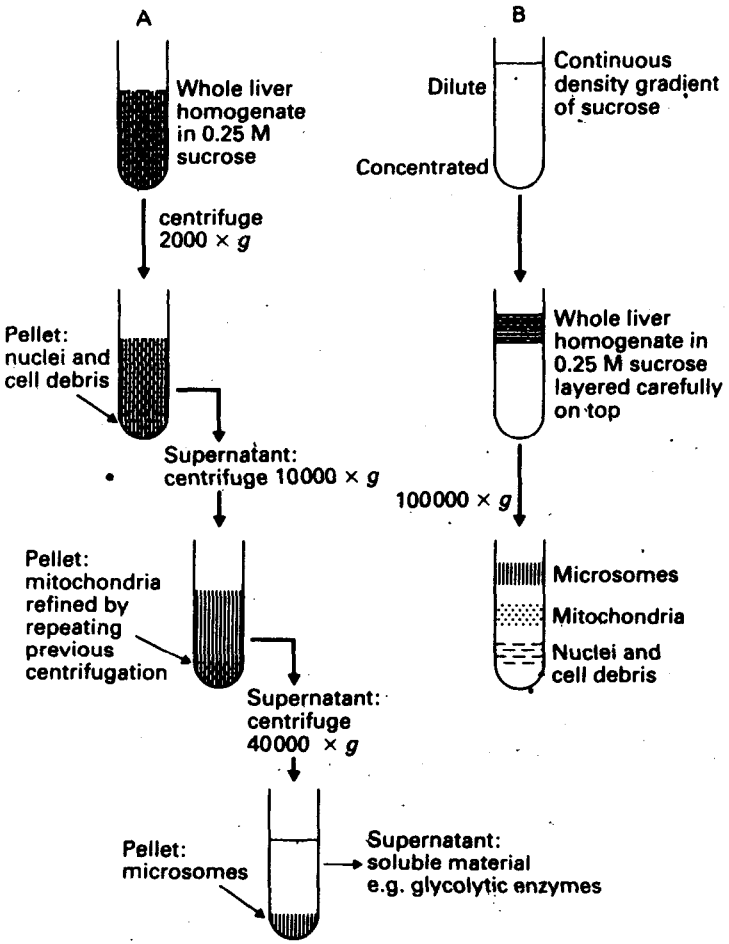


Fig. 2–1 Diagram illustrating the differences between differential (A) and density gradient centrifugation (B).

tube has been carefully adjusted so that it forms a gradient in which it is highly concentrated at the bottom, and relatively dilute at the top. When it is subjected to high centrifugal force, the density gradient does not alter, but the elements from the homogenate will travel down the gradient until they reach the levels at which their own densities are equal to that of the medium. There will then be no further progress. When the centrifuge is stopped, the tube is carefully removed and the appropriate layer withdrawn by means of a hypodermic syringe.

Many of the techniques and pieces of apparatus mentioned in this section are exceedingly complex. However, even with the limited facilities available to them, the early workers provided a large amount of important data. Until the end of the 1930s, all this information was derived from nothing more refined than a crude homogenate. Especially important were the remarkable studies of Otto Warburg, but another worker who contributed much in this period was T. L. Thunberg. As an example of the sort of information that could be gained using quite unsophisticated apparatus, we can consider some of the experiments in which 'redox' dyes were used. The term 'redox' is a convenient contraction of 'reduction–oxidation'. In this case, it refers to the property of some compounds which enables them to undergo simple, reversible reduction reactions, accompanied by colour changes. Methylene blue is a good 'redox' dye (Fig. 2–2). It will accept a pair of electrons from, say, a

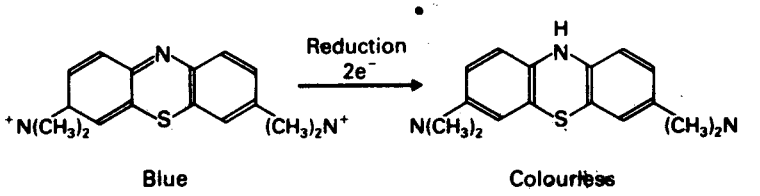


Fig. 2–2 The reduction of methylene blue to the colourless, leuco-form.

respiring mitochondrial system and be converted to the colourless leuco-form. If O_2 is present in the reaction vessel, the dye will immediately give up these electrons to O_2, reverting to the blue, oxidized, form. The O_2 is reduced to water.

Thunberg utilized this respiration 'indicator' in many experiments to study the nature of biological oxidation. No complex apparatus was required, just the glassware illustrated in Fig. 2–3. When substrate, tissue preparation and methylene blue were mixed in the absence of O_2, respiration commenced. The rate of respiration could be determined by measuring the time taken for the methylene blue to decolourize. Results obtained by the use of such simple equipment were later confirmed and extended.

When the problems associated with preparation of pure, active mitochondrial fractions were solved, it offered substantial encouragement to the development of the technical means for their study. Greatly improved techniques of spectroscopy, centrifugation and the introduction of the oxygen electrode led to an explosion of knowledge. The way in which these instruments contributed to our understanding of the processes of respiration by mitochondria will be outlined in the remainder of this chapter and the next.

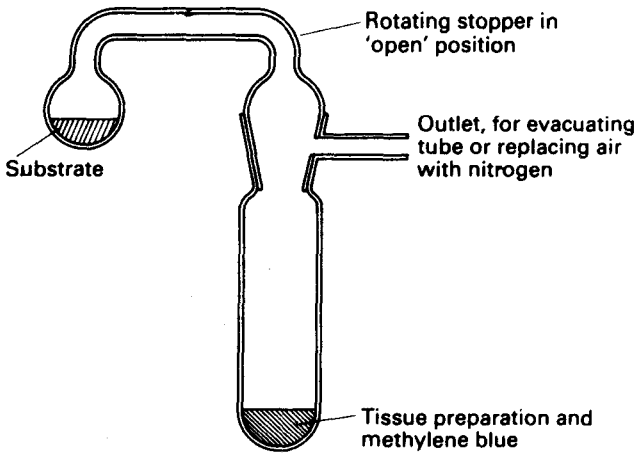


Fig. 2–3 Illustration of a simple experiment which may be performed with a Thunberg tube. When the substrate, tissue homogenate and methylene blue are mixed in an O_2-free system, the dye rapidly decolourizes.

2.2 The mitochondrion

Mitochondria occur in most cells, and are the major sites of respiratory activity within the cells. Understanding the structure of the mitochondrion provides a key to understanding its function. Before the advent of the electron microscope, mitochondria were considered to be structureless particles, perhaps vesicles, which were in some way implicated in oxidative processes. Their most spectacular property was the capacity to take up a vital dye, Janus Green B. The term 'vital' refers to its ability to stain only living tissue.

When the high resolution of the electron microscope became available, it was immediately found that the mitochondrion had an elaborate architecture (Fig. 2–4) formed from an inner and an outer membrane. The surface of the inner membrane was much expanded, thrown into numerous folds or cristae, which served to increase the area. The cristae penetrated the whole of the interior of the mitochondrion. Between the cristae, and between the inner and outer membranes was a structureless material which was termed the matrix. Until recently, this was considered to be the extent of the structure. However, a new technique for the preparation of sections for examination by electron microscopy was applied to preparations of mitochondria.

The standard technique of staining for electron microscopy involves the deposition of heavy metals (which are electron-dense) on the membranes of the mitochondria themselves. H. Fernández-Morán and