Mitochondrial Respiration

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Black-and-white
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<Opening titles>

<Thorn to camera>

It's a common experience that if we do some brisk muscular exercise, we soon find, among other things, that we begin to breathe more rapidly and deeply. And equally important that when we rest after a period of exercise, our breathing in a few minutes returns to its more normal rate. This sequence of responses to rate of changes in exercise is, of course, quite involuntary and it depends on a sequence of signals some of which you're, no doubt, already familiar with.

<Thorn narrates over flow chart>

The solid arrows represent chemical signals and the dotted arrows nervous signals. So, first of all, we have the nerve impulses prompting change in rate of muscular work. Then chemical changes which bring about a change in rate of oxidative metabolism to supply energy at an appropriate rate for the rate of muscular work. This metabolism in turn leads to changes in cellular chemistry which, by diffusion, are

transmitted to the blood, and this when circulated through the brain influences the respiratory centre, which in turn produces nerve impulses to alter the rate of ventilation. We, in this demonstration, are concerned with the early events in this chain of chemical signals: the way in which the rate of oxidative metabolism is matched to the rate of muscular work. A higher rate of metabolism for a high rate of muscular work; a lower rate of metabolism for a lower rate of muscular work.

<Thorn to camera>

We're going to demonstrate to you the polarographic oxygen electrode, the particular design of which is suitable for studying the respiration or oxygen consumption of a suspension of cells or of mitochondria prepared from living cells. Mitochondria, you remember, are the subcellular particles in which are embedded the respiratory enzyme systems. If you can get an experimental record of a series of experiments similar to those we're going to show you, you will be able to obtain, from these records, a number of qualitative and quantitative conclusions about the behaviour of the respiratory systems.

Well now, first of all then, let's see what an experimental record looks like coming out of the polarographic oxygen electrode apparatus. We have set up a simple experiment with yeast cells.

<Thorn narrates over film of polarographic apparatus readout>

The right-hand side trace represents the starting oxygen concentration. At the other end, it is anaerobic. And this section of the trace represents a slow respiration of yeast cells going on, and now we're going to add glucose. Is the glucose in? Oh, yes. That looks like an experimental artefact that we sometimes get. But, now you can see the recorder pen moving off much more rapidly down the chart. The rate of respiration has, in fact, increased on the addition of the substrate. We can interfere with respiration in several ways; you're all familiar with the respiratory inhibitor cyanide. And so now, let's see the effect of adding a small amount of cyanide. Can we have the cyanide? You can see the cyanide went in on that little blip of about 53

divisions. And after a small lag, you can see that the rate of respiration is diminishing. Diminished rate of respiration, of course, being represented by a flattening of the trace in respect of the time axis, which is represented, of course, by the direction of run of the chart paper.

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<Thorn to camera>

The next experiment will be done with a suspension of rat liver mitochondria. So, first of all, we have to wash out the apparatus and set it up again with a different lot of reagents. When it's been washed out, we'll have a quick look at it and see what it looks like. Meanwhile, some of you may be wondering why we start with a problem in muscle metabolism and then say we're going to do an experiment with rat liver mitochondria. Well, rat liver mitochondria and muscle mitochondria behave essentially the same for the purposes of this demonstration, and, in fact, liver mitochondria are a little simpler to prepare so that's why we're using them. I should also say that in a short demonstration of this kind, which presupposes some initial knowledge of mitochondria, it will be quite impossible, of course, to explain all the details and implications of what we're doing, so if any questions occur to you, please make a note of them and try and find out about them afterwards.

Well now, the apparatus has been washed out and we can now have a look at it.

<Thorn narrates over shots of polarographic apparatus>

Here is the reaction vessel. It's got water in it at the moment. And around it, you can see the water jacket through which water, at constant temperature, is circulating. The movement of the circulation you can see with the bubbles. When the apparatus is in use, the contents are stirred. You can see the magnetic stirrer now and its effect on the contents of the reaction vessel by the vortex running up the side. Also when it is in use, we close the reaction vessel, the reaction mixture, off from the atmosphere by means of a tightly fitting stopper. There is a rubber O-ring there, you can see sealing

the stopper closely to the walls of the reaction vessel. We seal it off from the atmosphere in this way because we start with a known volume of reaction medium containing a known concentration of oxygen and we don't want interference from atmospheric oxygen diffusing into the reaction mixture as time goes on. Nevertheless, there is a small hole, you saw there, in the middle of the stopper through which we pipette the reagents during the course of experimental runs.

In the base of the reaction vessel, there is an oxygen-sensing device. You can't see it at the moment, but we'll show you in a diagram. But, meanwhile, you can see the electric motor with the magnet on the rotating spindle that activates the stirrer. The oxygen-sensing device is fed with a small direct current supplied from the control box, which you can now see in the left-hand corner. And the signals from the oxygen-sensing device are fed back through the control box into the recorder that you can now see standing there.

In the background, you can just see the outlines of the constant-temperature water bath, which serves two purposes, one as a supplier of constant-temperature water for circulating through the jacket of the apparatus, and secondly, in that water bath, we keep our reagents equilibrated to the correct temperature and also aerated with air to make sure that the oxygen concentration in them is at equilibrium with oxygen in the atmosphere.

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<Thorn narrates over diagram of polarographic apparatus, using indicator>

Here, you will be able to identify the parts that you've already seen: reaction vessel, water jacket, stirrer, stopper and the Perspex base over a motor-driven magnet. In this area here, we have the oxygen-sensing device. In the centre, there is a platinum cathode and surrounding it, a circular silver/silver chloride anode. Electrical contact between those two electrodes is made by means of a few drops of dilute KCl solution and the electrodes are covered over with a very fine Teflon membrane, which is

firmly clamped at the edges with a rubber band so that the electrode compartment and the reaction compartment are isolated from one another by the membrane.

From the control box, a small DC potential of 0.6 volts is applied across the electrodes. This is too small to cause the electrolytic decomposition of water. And the high electrical resistance means that only a very small current can normally flow. This is augmented, however, by the fact that dissolved oxygen molecules coming in contact with the cathode polarised at -0.6 volts can pick up electrons from it, thereby being reduced, probably to water. Oxygen molecules which disappear by this chemical reaction are replaced by others diffusing into the vicinity of the cathode. The amount of extra current depends on the rate at which this diffusion of oxygen molecules can take place. And this, in turn, is proportional to their concentration. Since the membrane is permeable to oxygen, the main source of dissolved oxygen which can diffuse to the cathode is, of course, the stirred reaction medium.

The design of the apparatus, its geometry and stirring arrangement, is an attempt to ensure that changes in oxygen concentration in the reaction medium are accurately reflected in changes in the electrode current due to diffusion of oxygen. And it is these changes which are manipulated by means of the control box into changes in position of the recorder such as you have seen.

Well, we're nearly ready to start an experiment with rat liver mitochondria, but before we do, let's just refresh our memories about the structurally organised enzyme system we're dealing with.

<Thorn narrates over diagram>

This region here represents the inner, or crystal, membrane of the mitochondria, completely surrounding the matrix. In this membrane are associated the enzymes and carriers of the respiratory chain and of the phosphorylation system. Most of them have been omitted for the sake of clarity, but notice particularly a dehydrogenase and cytochrome oxidase. The dehydrogenase catalyses the oxidation of its substrate, accepting two hydrogen atoms which, by oxidation-reduction transfers, are passed

along the respiratory chain. Under normal circumstances of mitochondrial metabolism, the bulk of that so-called substrate is, of course, the reduced form of the cofactor NAD, the reduced form being continually regenerated by the action of other dehydrogenases on their substrates in the mitochondrial matrix.

In our experiments, we're going to use succinate which has to pass through the membrane before it can reach its dehydrogenase on the inside of the membrane. Although normally, of course, succinate would be formed in the Krebs cycle in the matrix space. You may be able later to think of other substrates that we could have added. The other enzyme, cytochrome oxidase, is responsible for the final reduction of oxygen to water using reducing power coming out of the other end of the respiratory chain. Notice that the stoichiometry of this is effectively one atom of oxygen to one molecule of substrate.

At intermediate points along the respiratory chain, simplified in the diagram to just one point, we have the so-called coupling system, here shown rather noncommittally as a circle of arrowheads. And it's by means of this coupling system that the chemical potential of oxidation of substrate by oxygen, a very non-reversible type of reaction, can be applied to the ADP/ATP system in such a way as to drive it in the relatively unfavourable direction of combination of ADP with inorganic phosphate to form a new pyrophosphate bond in ATP. We believe the important enzyme in this reaction, and it's an enzyme that has been isolated from the mitochondria, is this one which we call an ATPase. And it's called an ATPase because when isolated it catalyses the reverse reaction to the one shown there, namely the hydrolysis of ATP rather than its synthesis.

So, remember then when we come to do experiments with mitochondria that the reaction medium contains, among other things, substrate, oxygen, ADP and inorganic phosphate. And in addition to these, an inert substance, sucrose, which is added to the medium to make it approximately isosmotic with the mitochondria and therefore to preserve the properties of the intact, enclosing membrane. We shall start the experiments by finally adding mitochondria, although it is, of course, possible to add the reagents in different orders.

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<Thorn narrates over film of polarographic apparatus readout, interspersed with narration over slides>

Well, now we can start and watch for the effect of the stirring on the ropy mitochondrial suspension as it comes in. And the medium is now uniformly opaque. Now let's see what the recorder is doing. And they're off. And you can see that a fairly rapid rate of respiration is now taking place; and these mitochondria with succinate as the substrate. You may be able to tell that it now looks as if there is a deceleration of reaction rate happening, just about at that point. You can see now that there is now a second, definitely lower rate happening, and we have to ask ourselves, what is limiting at this point? Well, it's not oxygen, it's not substrate or inorganic phosphate because we added those in excess and, in fact, ADP was the reagent added in smallest quantity, so can we add a little more ADP? The ADP has gone in and now you can see that the trace has accelerated again. In fact, as the trace now goes on, we intend to continue to add small amounts of ADP and repeat this cycle of events as long as it will.

This important ability of mitochondrial respiration to slow up when ADP is limiting is called the respiratory control. And it appears to be such an important property that we define a measure of its effectiveness <*slide*> and call it the respiratory control ratio, which is the rate of oxygen consumption in the presence of ADP divided by oxygen consumption after ADP has been phosphorylated. <*Return to film of readout*> If you remember at the beginning, we said: increased rate of oxygen consumption for higher work performance, decreased rate for lower work performance. And you can see the parallel now. It looks as if addition of ADP corresponds to a higher work performance, depletion of ADP lower work performance. And if you recall that for most cellular work performances, ADP is formed from ATP during the work performance, you can see that ADP is not only a substrate for oxidated phosphorylation but also seems to act as a signal for an increase in cellular, that is mitochondrial, respiration when work is being done.

Apart from this calculation of being a respiratory work ratio, we can also, making due assumptions, calculate how much ATP is formed during the consumption of a given amount of oxygen. This is known as the P/O ratio <*slide*>, and this was effectively the *n* in the equations in the previous diagram, if you remember seeing *n*s in the ADP/ATP system equation. So, P/O ratio: moles of ADP phosphorylated to ATP divided by moles of oxygen atoms consumed.

<*Return to film of readout*> The number of pen divisions in a fast phase of oxygen consumption compared with the total pen divisions traversed from the start of the experiment to the point of going anaerobic gives the fraction of the known starting amount of oxygen in the reaction medium used. And given the amount of ADP we added, we can calculate a P/O ratio for the substrate used.

Now, we'd like to do two more experiments in which rat liver mitochondria will be subjected to different treatments. This run, of course, is now tailing off as the reaction medium goes anaerobic; you can see the trace flattening there on the time axis approaching nought divisions on the scale.

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<Thorn narrates over slide>

As I said, we'll do two more experiments and these will be concerning interference treatments for this complex, membrane-bound enzyme system. You've already seen the effect of cyanide on yeast respiration. The cyanide, as you know, inhibits the cytochrome oxidase of the respiratory chain. The compound that we're now going to add is dinitrophenol. This compound is described as an uncoupler because its effect is to dissociate the ATPase system from the respiratory chain. We can show you the effect on respiration because this is what we are measuring with this apparatus, but you might like to think about what the effect might also be on the ATPase system. Suppose you were to take mitochondria mixed with the dinitrophenol and then add to them a mixture of equal parts of ADP and ATP, and by chemical analysis find out

what happens to the chemical composition of that mixture. Would it stay the same or would it change? And if it changed, in what way would it change?

<Thorn narrates over film of polarographic apparatus readout, interspersed with narration over slides>

Well now, we've taken the same amount of mitochondria and we've started this experiment in exactly the same way as the previous one; remember just succinate, oxygen, ADP and inorganic phosphate to start with, and so we expect to see a repeat performance of what the mitochondria were doing last time. And you can see that they're coming under control and we have the beginnings of the slower phase of oxygen consumption now that ADP is exhausted. Now we're going to add dinitrophenol. Can we have the dinitrophenol? Well, you can see that the effect of the dinitrophenol comes evident at round about 80 divisions there. We have a rapid rate of respiration, the pen is running away down to the bottom of the chart in an unrestrained manner. Let's add a little more ADP, just to see whether ADP has a further accelerating effect. The ADP went in at about the point there and, in fact, I think you can tell by looking that the rate is unaffected by this so that the rate of oxygen consumption with the dinitrophenol is not susceptible to alteration of the addition of ADP anymore.

<*Slide*> Well, you've seen the effect of dinitrophenol in producing uncontrolled respiration; did you work out what the effect might be on the ATPase system? Well, of course, it would be to bring about catalysis in the natural direction of that reaction, namely the direction of hydrolysis.

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Finally, we have set up an experiment, the second kind of treatment which we were going to show with mitochondria which have been subjected to hypotonic conditions at room temperature for about 45 minutes, instead of being kept in sucrose at nought <*film of readout*>. They are, in fact, respiring as you can see. Perhaps, you think that they're not going as fast the previous lot but we've certainly put in as many

mitochondria, the same amount of mitochondria as there have been in the other experiments. Well now, round about here on the previous occasions, we've expected to see the beginning of a deceleration due to respiratory control and the depletion of ADP, but it hasn't taken place there so it looks as if something has happened. Now, you may agree that somewhere about this point, it does look as if some kind of control, some kind of deceleration, is taking place, but it's taken place later than usual and the deceleration is not very convincing.

Well, let's add some dinitrophenol which will give us some indication of whether the mitochondria are totally uncoupled. Can we have the dinitrophenol? That's it. Well, you can see an unmistakable acceleration in the presence of dinitrophenol, so it looks as if the mitochondria are not totally uncoupled, but judging from the criteria of respiratory control and P/O ratio, they certainly have been the coupling system – it looks as if it's been damaged.

<Thorn to camera>

Well, what you have seen is one method, a quick and convenient one for examining the properties of the respiration system of mitochondria. If you have access to a record of a series of experiments similar to these, here is what you can do.

<Thorn narrates over paper readout displayed on wall, using pen as indicator>

Here is an experimental record. Starting oxygen concentration down to anaerobic. And this point represents the addition of a small amount of ADP. Now, for determining respiratory control ratio, all you have to do is to line the ruler up carefully on the ADP stimulated rate and extrapolate the line as far as the chart will allow, and then do the same thing for the rate after ADP is exhausted and draw a line as far as the chart will allow. And the ratio of the fast rate to the slow rate gives you the respiratory control ratio.

If you want to work out the P/O ratio, what you need to know is the number of divisions between the point where ADP was added and brought about the stimulation

and this point here where ADP was exhausted. In order to do that, you may need to make another little construction line to find out the exact point of intersection of this slow rate here with the fast rate after ADP was added. Then if you compare that number of divisions with the total number of scale divisions traversed in the experiment, you can work out how much oxygen was used. And given the amount of ADP added, you can work out the P/O ratio.

<Thorn narrates over slide>

Well now, probably, the most important single thing that emerges from experiments of this sort is that the rate of respiration appears to be limited by the availability of ADP and that the amount of ADP present in its turn is limited by the rate at which the cell is required to perform work.

<Thorn to camera>

This cellular control mechanism is important in explaining the chain of circumstances involved in the control of ventilation, the problem we started with. But, it has more far-reaching significance than this because the ratio of the concentrations of ADP and ATP which the mitochondrial respiration system seems to be geared to maintain has far-reaching consequences. Clearly, if the ATP concentration falls drastically and is not restored, the whole of the cell's energy-requiring mechanisms are put in jeopardy. But, smaller changes in the ADP/ATP ratio can also have far-reaching effects in influencing other pathways of metabolism in the cell, but this is another story.

<End credits>