OXIDATIVE PHOSPHORYLATION

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A brief review is given of the history of the experimental demonstration of oxidative phosphorylation. The properties of the system which can be measured using mitochondria have been described and these have been brought together in a diagrammatic scheme which illustrates the dissection of the process into various parts by the use of toxic substances.

In this lecture my aim is to tell you mainly about the practical aspects of the problem and to illustrate the points discussed with examples using the preparation of mitochondria we use at Carshalton. Since the properties of mitochondria may vary with the method of preparation I think it will be useful to consider as many aspects as possible on one preparation. The problem of oxidative phosphorylation and its reaction mechanism is in a state of flux at the moment. I shall try to show the extent of our knowledge.

The importance of adenosine triphosphate (ATP) to the economy of biological organisms hardly needs to be emphasized. It is a key substance and is the means whereby the energy liberated by the oxidation of substances (particularly carbohydrates) may be trapped, stored and utilised for the synthesis of large molecular weight substances of the body. It is well known that ATP is synthesised during anaerobic glycolysis. Two molecules of ATP are used to produce hexose 1,6 diphosphate from glucose. After splitting the molecule into triose phosphate, each half yields two molecules of ATP, one at the phosphoglycerolaldehyde and the other from phospho-enolpyruvate stages. Therefore four molecules of ATP are formed and two used giving an overall yield of two molecules of ATP from each glucose converted to lactate. The free energy loss for glucose to two moles lactate is \(-57\) K. cal/mole whereas from two moles lactate to carbon dioxide the loss in free energy is \(-629\) K. cal/mole. By anaerobic glycolysis only a small proportion of the available energy is trapped.

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The early observations (1937-40) of Kalcher (1, 2) and more particularly Belitzer (3, 4) first demonstrated that during the oxidation of three carbon substrates, inorganic phosphate disappeared from the medium and was recovered as phospho creatine, creatine acting as the phosphate acceptor. These processes are prevented by cyanide or under anaerobic conditions and were therefore definitely related to oxidative processes. The ratio of phosphate removed to oxygen absorbed (P/O ratio = \( \mu \) mole phosphate removed / \( \mu \) atom oxygen absorbed) was approximately 2.0. Belitzer pointed out that if the intermediates of the Krebs tricarboxylic acid cycle were all phosphorylated and the phosphate transferred to ADP to form ATP then a P/O ratio of not more than 1.0 could be obtained. It was therefore suggested that the energy from electron transport was being utilised to form ATP. Unlike anaerobic glycolysis the oxidation of pyruvate by the tricarboxylic acid cycle does not involve phosphorylated substrates in the formation of ATP.

The measurement of accurate yields of oxidative phosphorylation are technically difficult. The early work was carried out upon minces of tissue where the breakdown of ATP formed was rather high. The demonstration (5, 6) that isolated mitochondria carry out oxidative phosphorylation was a major step forward. However, the system whereby electron transport is coupled so that ATP is formed is a very labile one and damage to mitochondria will cause large losses of ATP due to hydrolysis. In addition disrupted mitochondria such as Kirlin-Hartree preparations carry out oxidation of succinate but no phosphorylation is associated with it.

It is only since the demonstration that mitochondria carry out oxidative phosphorylation (5, 6) that the determination of the yield of oxidative phosphorylation may be attempted. Unlike tissue homogenates slices and minces, mitochondrial preparations contain little endogenous substrate. Since the preparation of mitochondria was first placed on a practical working basis (7, 8) the P/O ratios have steadily risen so that now values of 2.7 may be obtained for pyruvate oxidation even when determined at 37°. With early preparations and also still with mitochondria from some tissues it was essential to work at lower temperatures to ensure reasonable stability of the preparation. It seems therefore that the P/O ratio for pyruvate oxidation should be 3.0. We have recently carried out some experiments with a preparation of stable liver mitochondria. When hexokinase and glucose is added to these mitochondria oxidising pyruvate a stimulation of the oxygen uptake of about four times is obtained. This continues while inorganic phosphate is being incorporated through ATP into glucose to form glucose 6-phosphate until all the inorganic phosphate has been removed from the medium. When this occurs the rate of oxygen uptake falls to exactly the same rate as without hexokinase. The unstimulated oxygen uptake may therefore be considered separately and even if phosphorylation is in-
Oxidative phosphorylation measured using minimal amounts of hexokinase.

Liver mitochondria  Pyruvate 37°
Hexokinase 16 units/flask
For other conditions see (21).

<table>
<thead>
<tr>
<th></th>
<th>Expt. (1)</th>
<th>Expt. (2)</th>
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\[ \text{O}_2 \text{ uptake, with hexokinase,} \]
\[ (\mu\text{g atom/mg. protein/hr.}) \]
\[ 9.49 \pm 0.26 \]
\[ 10.45 \pm 0.23 \]

\[ \text{O}_2 \text{ uptake, unstimulated.} \]
\[ (\mu\text{g atom/mg. protein/hr.}) \]
\[ 2.96 \pm 0.11 \]
\[ 2.89 \pm 0.18 \]

\[ \text{Difference} \]
\[ 7.23 \pm 0.28 \]
\[ 7.56 \pm 0.28 \]

\[ \text{P. uptake} \]
\[ (\mu\text{mole/mg. protein/hr.}) \]
\[ 21.95 \pm 1.04 \]
\[ 22.35 \pm 0.65 \]

\[ \text{P/O ratio} \]
\[ 3.04 \pm 0.19 \]
\[ 2.96 \pm 0.14 \]
With three phosphate molecules incorporated for every atom of oxygen absorbed it seems very probable that there should be three sites of phosphorylation in the electron transport chain. The experimental demonstration that there are three sites has been made, but involved many technical difficulties, the main one being that added coenzymes do not readily penetrate into intact mitochondria. For instance, although $\beta$-hydroxybutyrate is readily oxidised by intact mitochondria without added cytochrome C, added reduced coenzyme I is not oxidised unless cytochrome C is added (9). Phosphate is esterified with $\beta$-hydroxybutyrate as substrate but not with reduced coenzyme I with cytochrome C present. The latter oxidation is not inhibited by Antimycin A whereas the former is very sensitive (10). Lehninger showed that pretreatment of the mitochondria with hypotonic solutions allowed reduced coenzyme I to be oxidised without added cytochrome C; this oxidation is sensitive to Antimycin A and phosphate is esterified. These findings are explained by postulating that the reduced coenzyme I cannot penetrate unless the permeability of the mitochondria is modified by pretreatment with hypotonic solutions for a short time. Experiments using these techniques produced the first direct proof that phosphorylation occurs in the electron transport chain between reduced coenzyme I and oxygen with $^{15}O$ ratios of more than 2.0 (9).

Phosphorylation between cytochrome C and oxygen has also been demonstrated (11, 12). Ascorbic acid was used to reduce cytochrome C non enzymatically and the mitochondria were pretreated with hypotonic solutions to allow penetration of reduced cytochrome C. More recently a direct measurement of phosphorylation associated with the oxidation of reduced cytochrome C by oxygen has been made (13), $^{18}O$ ratios of less than 1.0 being obtained. By similar techniques phosphorylation associated with the oxidation of $\beta$-hydroxybutyrate by cytochrome C (14) has been measured, the $^{18}O$ ratios being more than 1.5 but less than 2.0.

There are therefore three phosphorylation steps associated with the passage of electrons up the respiratory chain. One step is associated with the oxidation of reduced cytochrome C and the other two probably with the oxidation of reduced coenzyme I by flavo protein and of reduced flavo protein by cytochrome C. Three phosphorylation steps in the respiratory chain are also in good agreement with thermodynamic data upon the free energy change of the reactions. The free energy change from coenzyme I to oxygen is 50-51 K.cals/mole (15) and that due to the formation of three moles ATP is 36 K.cals. (16), an efficiency of utilisation of available energy of around 70%.

All the available experimental evidence points to the oxidation of one mole of succinate being associated with the formation of two moles of ATP; this is also in agreement with the free energy data available (15, 16) with an efficiency of utilisation of energy of 60-70%.

Earlier it was pointed out that oxidative phosphorylation is particularly sensitive to damage of the mitochondria and until recently all
attempts have failed to fractionate the mitochondria into smaller particles and still obtain oxidative phosphorylation. However, Lehninger and co-workers have succeeded in disrupting liver mitochondria by treatment with diethyl and have isolated submitochondrial particles which will carry out oxidative phosphorylation with \( \beta \)-hydroxybutyrate as substrate (17). This preparation is proving extremely valuable in the study of mechanisms of oxidative phosphorylations; I shall be describing some of this work later. However, these submitochondrial particles only oxidise \( \beta \)-hydroxybutyrate and succinate. At Carshalton we were interested in the metabolism of whole intact mitochondria and in particular wished to use results obtained on the action of trialkylammoniums on mitochondria in vitro to explain results obtained in vivo. Briefly, using a special homogeniser and 0.3 M sucrose for isolation and a medium with the ratio of the concentrations of potassium, magnesium and phosphate roughly as is found in the intracellular fluid of the liver, a reliable preparation of liver mitochondria has been obtained (18). Oxidation is stable for up to 3 hr. at 37°C in an electrolyte medium and upon the addition of enzymes utilising ATP such as hexokinase and glucose (see Fig. 1) or potato amyrase a 3-4 fold stimulation of oxygen uptake is obtained. No coenzymes need be added. Their stability is indicated by the results of an experiment illustrated in Fig. 2. When amyrase is added at zero true oxidation is stimulated 4-fold. Unstimulated oxidation by anaerobic rate in 2 hr. If amyrase is then tipped in again a 4-fold stimulation is obtained. This indicates that the unstimulated mitochondria which have been metabolising pyruvate for 2 hr. at 37°C have still sufficient coenzymes to give the maximum rate of oxidation uptake upon the addition of amyrase. A similar stimulation is obtained upon adding 2:4-dinitrophenol. The importance of the large stimulation by amyrase or hexokinase is that we know that \( 70-80\% \) of oxygen uptake is associated with phosphorylation. For studies of oxidative phosphorylation this is extremely valuable. It also follows from such a stimulation that just as phosphorylation is dependent upon oxygen uptake so also is oxygen uptake dependent upon phosphorylation of ADP. This means that the inhibition of oxygen uptake will be obtained by inhibiting any enzyme in the electron transport chain or in the energy transferring chain between electron transport and ATP. This has important consequences for the interpretation of results showing that respiration is inhibited.

I now wish to consider what measurements may be made with these mitochondria. The \( P/O \) ratio may be easily measured. A lowering indicates uncoupling of oxidative phosphorylation. Much work has been carried out with 2:4-dinitrophenol which will lower inorganic phosphate uptake without lowering oxygen uptake (19). This is also true of several other phenols (20). When oxygen uptake is lowered as well the \( P/O \) ratio it is essential to show that with the particular preparation of mitochondria that a true respiratory inhibition does not also lower
the P/O ratio due to secondary disruptive processes (21). It is curious how few papers contain this essential control experiment. Another point is that there is a difference between uncoupling and inhibiting oxidative phosphorylation. If phosphate uptake is lowered with no lowering of oxygen uptake then uncoupling has been demonstrated. However when oxygen uptake is also inhibited it is much more difficult to decide at what stage oxygen uptake is being prevented.

The inorganic phosphate – ATP exchange reaction is another property which may be readily measured. If liver mitochondria are placed in a medium containing ATP but no substrate and radioactive inorganic phosphate is added there is a rapid exchange between the $P_{32}$ and the terminal phosphorus atoms of ATP (22, 23, 24). With liver mitochondria both adenosine diphosphate (ADP) and ATP become labelled but not the monophosphate (AMP). The labelling of the diphosphate may be due to the presence of myokinase in liver mitochondria (25). In Table 2 are the results of an experiment showing the incorporation of labelled inorganic phosphate into ADP and ATP and the influence of the
Table 2.
Effect of inorganic phosphate concentrations upon the ATP–P³² exchange

For basic medium see (21)
pH 6.7–6.8, Temp. 37°

<table>
<thead>
<tr>
<th>Inorganic P concentration (μmolc/ml)</th>
<th>P exchanged (μmole/mg. protein/hr.)</th>
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<tr>
<td></td>
<td>After 10 min.</td>
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<tr>
<td>0.35</td>
<td>1.11</td>
</tr>
<tr>
<td>0.50</td>
<td>1.17</td>
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<tr>
<td>1.30</td>
<td>2.39</td>
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<tr>
<td>4.21</td>
<td>4.13</td>
</tr>
<tr>
<td>15.1</td>
<td>5.75</td>
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concentration of inorganic phosphate upon this labelling. This process is rapid for, in 10 min., the ATP is labelled halfway toward the equilibrium position. Both ADP and ATP are labelled but AMP is not. As the concentration of inorganic phosphate is raised the amount of phosphate incorporated into the nucleotides is increased. Fig. 3 shows that, as would be expected, the increase is exponentially related to the concentration of inorganic phosphate. This is a useful finding for it means that when determinations of this exchange reaction are made in the presence of substances which cause breakdown of ATP and the liberation of inorganic phosphate the rate of the exchange may be corrected for this rise in inorganic phosphate concentration. No exchange occurs between P³² and any other nucleotides such as those of guanosine, cytidine or inosine triphosphate (24).

A recent addition to the exchange reactions is the incorporation of labelled ADP into ATP (94, 96). This has been demonstrated with ADP labelled with P³² or C¹⁴ but has only been carried out with submitochondrial particles and not with intact mitochondria. Many other enzymes can catalyse such an exchange and it is essential to have a preparation free from such enzymes. The ADP–ATP exchange is a fast reaction and is not influenced by the concentration of inorganic phosphate (27). Although this exchange is prevented by 2:4-dinitrophenol, after ageing the particles the reaction becomes insensitive to 2:4-dinitrophenol as the P³² – ATP exchange reaction disappears (27). It is considered that the ADP–ATP exchange reaction is a property of the terminal step in oxidative phosphorylation process.

Finally there is oxygen uptake, and adenosine triphosphatase (ATPase) stimulated by 2:4-dinitrophenol. We have been using 2:4-dinitrophenol to dissect the energy transferring chain and have been examining the effect of drugs upon these two properties (21). The justification of the use of 2:4-dinitrophenol in this way will be discussed later. In Table 3 is a list of the properties and their values which we have obtained using as far as possible strictly comparable conditions.
Fig. 3. Effect of the concentration of inorganic phosphate on the ATP-P\(^{32}\) exchange reaction. Conditions as in Table 2.

Table 3.

Rates of processes carried out by liver mitochondria

<table>
<thead>
<tr>
<th>Process</th>
<th>Units</th>
<th>Value 1 ± Standard Error (Number of Experiments)</th>
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<tbody>
<tr>
<td>Oxygen uptake</td>
<td>μlO₂/mg protein/hr</td>
<td>24.1 ± 3.5 (11)</td>
</tr>
<tr>
<td>Unstimulated</td>
<td></td>
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<tr>
<td>With aprylase</td>
<td></td>
<td>94.2 ± 9.4 (11)</td>
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<tr>
<td>With 2 : 4 Dinitrophenol (1.5 - 3 × 10⁻⁵M)</td>
<td></td>
<td>93.4 ± 7.8 (7)</td>
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<tr>
<td>With hexokinase</td>
<td></td>
<td>99.4 ± 14.9 (27)</td>
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<tr>
<td>Phosphate metabolism</td>
<td>μmole P/mg protein/hr</td>
<td></td>
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<tr>
<td>ATPase</td>
<td></td>
<td>0.39 ± 0.20 (9)</td>
</tr>
<tr>
<td>ATPase + 2 : 4 Dinitrophenol (3 × 10⁻⁵M)</td>
<td></td>
<td>13.1 ± 0.83 (6)</td>
</tr>
<tr>
<td>Oxidative phosphorylation (P/O ratio)</td>
<td></td>
<td>2.61 ± 0.18 (27)</td>
</tr>
<tr>
<td>Oxidative phosphorylation (phosphate uptake)</td>
<td></td>
<td>952 ± 4.1</td>
</tr>
<tr>
<td>ATP - P(^{32}) exchange</td>
<td></td>
<td>7.2</td>
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</table>
The oxidative rate in the presence of hexokinase and glucose, apyrase or 2:4-dinitrophenol is the same with pyruvate as substrate. The adenosine triphosphatase activity of the mitochondria is very low but is increased 35 times by the addition of $3 \times 10^{-4}$ M. 2:4-dinitrophenol. Phosphorylation associated with pyruvate oxidation proceeds at a rate of 20-97 μ mole P/mg protein/hr. 2:4-dinitrophenol activated ATPase at a little more than half and the $P^{32}$-ATP exchange reaction at less than one third of this rate. Whether these results indicate real differences in mechanisms or whether they are due to the experimental conditions cannot be decided at present.

I would like to consider now how all these properties of oxidation and phosphorylation are fitted together to give our present hypotheses about the processes of oxidative phosphorylation. 2:4-Dinitrophenol is being used confirmally as a tool to study oxidative phosphorylation. What is the evidence that its action is due to a modification of the normal processes of oxidative phosphorylation? 2:4-Dinitrophenol prevents phosphate uptake without affecting oxygen uptake (19, 20) and also stimulates the hydrolysis of adenosine triphosphate by mitochondria (29). The original suggestion that this stimulation is due to a modification of the energy transferring chain is due to Hunter (29) but has now been generally accepted (30, 31, 32, 33). The concentration which stimulates ATPase activity is the same as that which prevents phosphate uptake during oxidation (29, 30, 31, 20). Studies on submitochondrial particles produced by sonic disintegration (34) or treatment with digitonin (17) have shown that ATPase stimulated by 2:4 dinitrophenol and oxidative phosphorylation have the same specificity for nucleotides of adenine and the same pH activity range. Strong evidence that both ATPase and oxygen uptake induced by 2:4-dinitrophenol are modifications of the normal oxidative phosphorylation processes may be derived from studies of a range of nitro and halo phenols (20) and the trialkyltin (21). The concentrations of the phenols which stimulate oxygen uptake uncouple oxidative phosphorylation and stimulate ATPase are identical (20). Each of these systems are inhibited to the same degree by each of six trialkyltin tested (21).

By similar experiments it is considered that the ATP-P$_{32}$ and the ADP-ATP exchange reactions are also part of the normal oxidative phosphorylation processes. All have the same pH optimum (84) the same nucleotide specificity (27), and are inhibited by 2:4-dinitrophenol in fresh preparations of submitochondrial particles (27). Recent observations have, however, shown that the trialkyltin inhibit the ATP-P$_{32}$ exchange (35) but not the ADP-ATP exchange (36).

It is therefore the current view that all of these processes are properties of the normal oxidative phosphorylation processes.

In Fig. 4 the position is presented diagrammatically. The respiratory chain given is that accepted as the present position though there is still some doubt about the positions of cytochromes b and c (43). It has been
presumed that the initial steps of the energy transferring chains from the three sites must involve different reactions. The three sites of oxidative phosphorylation are all completely inhibited by $10^{-4}$ M 2:4-dinitrophenol (38, 39) and oxidation phosphorylation associated with pyruvate and succinate oxidation are inhibited by trialkyltins (21). It has however been suggested that the ATPase in liver mitochondria with different pH optima may be associated with the different sites of oxidative phosphorylation (30, 37).

However, the relation of these measurements to those described above is not understood for only one of the ATPases (pH optimum 6.3) is

![Diagram of oxidative phosphorylation](image)

*Fig. 4. Diagrammatic representation of the processes of oxidative phosphorylation.*
activated by $10^{-5}$ M 2 : 4-dinitrophenol, the other two (pH optima at 8.5 and 7.4) requiring higher concentrations (33). The simplest hypothesis is that the energy transferring chains from the point where 2 : 4-dinitrophenol acts to ATP formation are similar, if not identical, and have accordingly been shown as a single process in Fig. 4.

The point where 2 : 4-dinitrophenol is shown to act is quite arbitrary but the other observations are shown relative to it. Without substrate present ATP is hydrolysed. When substrate is being oxidised the energy of some intermediate is diverted and not used to form ATP. The stimulated ATPase is rather a symptom of the primary disorder produced by 2 : 4-dinitrophenol which may be detected when the mitochondria are examined without substrate. Since oxidative phosphorylation, oxidation and ATPase stimulated by 2 : 4-dinitrophenol are all equally sensitive to trialkyltns, it seems probable they act at the same site though they must have different mechanisms of action (21).

The ADP-ATP exchange is not sensitive to 2 : 4-dinitrophenol when it is dissociated from the ATP-P$_{58}$ exchange (27). It is considered to be the last stage in the oxidative phosphorylation. The position of the ATP-P$_{58}$ exchange is much more uncertain. It is inhibited by trialkyltns (33) and therefore, since trialkyltns and 2 : 4-dinitrophenol act at the same site, an intermediate containing phosphate should be involved. However, on other grounds other workers have reached the opposite conclusion (34) and it has been suggested that the ATP-P$_{58}$ exchange is only concerned with one of the sites of oxidative phosphorylation (44). Therefore although in Fig. 4 the ATP-P$_{58}$ exchange is shown to extend further towards electron transport than the 2 : 4-dinitrophenol stimulated ATPase this is not yet certain.

Finally it has been shown by several workers that barbiturates do not inhibit oxidative phosphorylation with succinate as substrate (40, 41, 42) and this has been confirmed in our laboratory (45). It must be concluded on the basis of the current views of the respiratory chain that barbiturates act only on oxidative phosphorylation associated with the dehydrogenase using coenzyme I. In addition barbiturates do not inhibit ATPase induced by 2 : 4-dinitrophenol.

It should be emphasized that this is only a working hypothesis and in many ways my personal one. There is no doubt that it will be drastically modified as more experimental results are obtained.

I think you will have been interested to hear of the part played by toxic substances in reaching our current views on oxidative phosphorylation. It is our view at Carshalton that they will play an ever increasing part in the intracellular dissection of biochemical processes.
REFERENCES


Sadržaj

OKSIDATIVNA FOSFORILACIJA

Kratki prikaz razvoja eksperimentalnog istraživanja na području oksidativne fosforilacije. Opisana je upotreba mitokondrija u svrhu ispitivanja svojstava aktčna oksidativne fosforilacije, a hematski prikaz ilustrira pojedine stepene procesa, koje se moglo dokazati upotrebom toksičkih supstancija.

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