REVIEW ARTICLE

OXIDATIVE PHOSPHORYLATION

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It must be made clear that this review is in no sense comprehensive. The writer has made no attempt to cover the entire literature, even of the past few years. Rather, it is intended to give the general reader some idea of the present state of the problem, and will to that extent reflect the personal interests of the author.

Oxidative phosphorylation: P : O Ratios

The process of oxidative phosphorylation may be defined as the synthesis of adenosine triphosphate (ATP) from inorganic phosphate and adenosine diphosphate (ADP) during the passage of electrons along the respiratory chain of enzymes and coenzymes. It does not include glycolytic phosphorylations, such as the one observed in the oxidation of triosephosphate. Nevertheless, this phosphorylation serves as a very useful starting point for our discussion. In the oxidation of triosephosphate, 1 mol. of substrate is oxidised to 1 : 3-diphosphoglyceric acid, with the addition of 1 mol. of inorganic phosphate from the medium. Under the influence of the appropriate phosphokinase, this phosphate is transferred to ADP to make ATP. During the oxidation, 1 mol. of diphosphopyridine nucleotide (DPN) is reduced to DPNH. Thus 1 mol. of phosphate is transferred per mol. of hydrogen (or more exactly per pair of electrons) transferred. If now, the DPNH formed were to be oxidised by means of an artificial carrier system with the uptake of oxygen, it would be found that 1 atom of oxygen would be consumed per mol. of phosphate taken up, and the efficiency of the process could be expressed in this way, by taking the ratio of phosphate to oxygen, which in this instance would be 1·0.

If, however, one found that the ratio exceeded 1, when the oxidation was conducted by some system other than the artificial one, one would be forced to conclude that the oxidation of DPNH was itself yielding further phosphorylations, and in fact that a process of oxidative phosphorylation was taking place.

It is some time since Belitzer and Tsibakowa[1] and Ochoa[2], using systems oxidising substrates of the tricarboxylic acid cycle, were able to show that P : O ratios substantially above 1 were obtainable, and that participation of the electron transport chain in phosphorylation reactions was certain. Further studies have been concerned with the location of these phosphorylations along the chain, and to determining the number of phosphorylations which occur when a pair of electrons are transferred from pyridine nucleotide to oxygen.

Table I shows the transport chain together with the potentials of each couple, and may be used for reference. In Ochoa's early experiments, though he had been able to obtain P:O ratios above 1·0, it was found that the direct oxidation of reduced DPN would not itself give rise to phosphorylations, and this raised serious problems, especially since he also found that the oxidation of citrate, which is also coupled to a pyridine

TABLE I

ENERGY SCALE

	E_o m.volts
Pyridine nucleotide	−320
Flavoprotein	− 60
Cytochrome *b*	0
Cytochrome *c*	+260
Cytochrome *a*	+290
Oxygen	+815

nucleotide, in this case triphosphopyridine nucleotide (TPN), also failed to give rise to phosphorylations. It might therefore have been concluded that the phosphorylation did not occur during oxidation of the reduced coenzyme, but during the oxidation of the substrate by the coenzyme. Ogston and Smithies[3] examined this problem theoretically and pointed out that the potential of the DPN:DPNH couple would be adequate for this purpose only at ratios of DPN to DPNH so high that vanishingly small amounts of DPNH would be present and that oxygen uptake would be effectively zero, since the further oxidation of DPNH, catalysed by flavoprotein, requires a certain concentration of this substance. Clearly this result did not fit the observed facts, and subsequent investigation has shown why.

The work which is now to be considered began in 1947–48. At that time, Green and his co-workers[4,5] began to study the particulate "cyclophorase" system, obtained from a variety of tissues, and Lehninger[6] studied oxidations by particles derived from liver. At the same time, Hogeboom, Schneider and Pallade[7] devised methods for the isolation of cellular components, with the separation of intact mitochondria as probably their most significant contribution.

TABLE II

P:O RATIO OBTAINED WITH THREE SUBSTANCES DURING OXIDATION BY MITOCHONDRIA. RAT LIVER MITOCHONDRIA. BATH TEMPERATURE 15°

Substrate	O_2 uptake μ atoms	Phosphate uptake μ moles	P:O ratio
L-Glutamate	6·8	26	3·8
β(OH) butyrate	8·1	23·4	2·9
Succinate	12	21·9	1·8

All these preparations had the property in common that they were able to oxidise intermediates of the tricarboxylic acid cycle with the simultaneous esterification of inorganic phosphate, provided that Mg ions, ADP and inorganic phosphate were present. It was early demonstrated that the mitochondria were in fact the active units in the different preparations.

Table II shows the P:O ratios obtained with several substrates during oxidation by mitochondria. They are all high, are in substantial agreement with figures obtained by other workers, and show that electron transport chain participation must be extensive. Not only is the efficiency great, but the rates of oxidation are also rapid. The first significant step toward localisation in the transport chain was taken by Lehninger[8] who, repeating Ochoa's experiments with DPNH, was able to demonstrate a clear-cut phosphorylation associated with its oxidation.

TABLE III

SPAN DPNH TO OXYGEN

Substrate	DPNH disappearing μ moles	Phosphate uptake μ moles	P:O ratio
DPNH	3·68	3·98	1·08

The reason for this contrary finding lies in the mitochondrial structure. Lehninger correctly supposed that two pathways for DPNH oxidation must exist: one external, in which there was electron transport without phosphorylation, and the other internal, which was coupled to the phosphorylation mechanism. Hence the high P:O ratios with substrates of the cycle, and none with added DPNH. He surmounted the difficulty by exposing isolated mitochondria to hypotonic conditions, which increased their permeability to added DPNH. Table III shows that a well-marked phosphorylation resulted.

Now in the span from DPNH to oxygen, at least three phosphorylations can be accommodated, energetically speaking, and in this laboratory[9] we were able to show that at least one of these occurred during the oxidation of reduced cytochrome *c*. The P:O ratio for this step was about 1. Though at the time there was some doubt about the significance of these results, later work by Lehninger[10] and by Maley and Lardy[11] has extended and confirmed the earlier work (Table IV). Finally,

TABLE IV

PHOSPHORYLATION COUPLED TO OXIDATION OF REDUCED CYTOCHROME *C*

Oxygen uptake μ atoms	Phosphate uptake μ moles	P:O ratio
8·0	7·3	0·91
11·0	6·1	0·56

Lehninger[12] was able to demonstrate that two phosphorylations occurred in the span between DPNH and cytochrome *c*. (Table V).

Thus, a total of three phosphate molecules are esterified per pair of electrons passing between DPNH and oxygen, and the P:O ratio for substrates linked with pyridine nucleotide is therefore 3, since we have already seen that no phosphorylation is possible during the passage of electrons between substrate and nucleotide.

With one substrate, α-oxoglutarate, the ratio is 4, the reason for this being a substrate-linked phosphorylation, which occurs during the

oxidative decarboxylation, and which is entirely different from the electron chain phosphorylations. These high figures have given rise to much discussion about the overall efficiency of the whole process. It is clear that this calculation may only be made if one knows the potential span of the electron transport chain and the free energy of the terminal phosphate group of ATP. The former figure may be obtained readily from the

TABLE V

SPAN β(OH) BUTYRATE TO FERRICYTOCHROME

Substrate	½ Ferricytochrome *c* mμ moles	Phosphate uptake mμ moles	P:O ratio
β(OH) butyrate	13·2	21·2	1·61

published data, but it must be borne in mind that the significance of the value is in doubt until something is known of the relative concentrations of the reduced and oxidised form of each couple. Thus, we have already quoted figures of Ogston and Smithies[3] which show that phosphorylation could occur during electron transport between substrate and DPN, but only when the relative concentration of DPNH is so low that respiration could not take place at all. It is necessary, therefore, to determine whether the different components of the respiratory chain are in states which may be compared to those for which the data in the literature for their potentials were calculated; that is, for 50 per cent reduction, and a ratio of oxidised to reduced forms of 1·0.

The work of Chance[13] gives some solid ground for the determination of the necessary quantities. Using extremely sensitive methods of spectrophotometry, he has been able to observe the major components of the chain in isolated mitochondria under a variety of experimental conditions, and to determine their relative concentrations. It can be shown that in mitochondria catalysing active oxidative phosphorylation, the ratios of reduced to oxidised forms do not vary sufficiently from unity to make any correction necessary and that the standard values may be used without fear of too great error. This therefore gives us a total span of about 1,100 mv., and if it is taken as a rough rule that 250 mv. is equivalent to 12 kcal., then we get a figure of 54 kcal. for the whole span. The free energy change on hydrolysis of the terminal phosphate group of ATP must also be known in order to calculate the efficiency of the oxidative process, and here there has been much discussion over figures. Lipmann[14] in his now classical review, gave a calculated figure of 9 to 11 kcal. Since then, the figure has first risen to 12 kcal. and fallen to as low as 5·7 kcal. Burton[15] in a discussion of these figures gives a value of 8·4 kcal. for specified standard conditions, which when corrected for the conditions under which experimental determinations of oxidative phosphorylation are conducted, gives a value of about 12·5 kcal. With a P:O ratio of 3, this gives an efficiency of nearly 70 per cent. Whether these figures may be transferred to whole cells is a question which cannot be answered as yet.

Regulatory Function of Oxidative Phosphorylation

During the discussion on the sites of phosphorylation, it was mentioned that isolated mitochondria will oxidise substrates rapidly in the presence of inorganic phosphate and ADP. It has long been known that the concentration of these substances is critical in determining the rate of respiration, and that when either falls to a low level respiration is reduced by a factor of about 10 at the limit.

Considering ADP first, Lardy and Wellman[16] were the first to show that as ADP was phosphorylated to ATP, so the rate of respiration declined to the limiting value, when nearly all the ADP had been converted. The addition of a system which withdraws the terminal phosphate group (for example, glucose and hexokinase; creatine and creatine phosphokinase) will cause an immediate acceleration of the respiratory rate. These results immediately suggest a mechanism whereby the rate of respiration may be regulated. Since the best figures for tissue analysis of adenine nucleotide suggest that ATP is the major, if not the only form present, it follows that mechanisms which permit loss of the terminal group will bring about acceleration of respiration. Thus hormonal regulation (for example, by thyroxine) may centre about this point.

Chance[13] has also studied this point in some detail. He was able to study the steady states of the respiratory carriers in isolated mitochondria under conditions in which active oxidative phosphorylation was taking place, with high levels of ADP present, and in others where ADP was almost exhausted, being converted to ATP. He found that in these two extreme conditions, the steady state levels of oxidised and reduced forms of the respiratory carriers showed large differences. Thus, when ADP was low, increasing reduction was observed throughout the chain, being maximal at the level of DPN, which was 90 per cent in the reduced form, and hardly noticeable at the level of cytochrome a_3 (cytochrome oxidase), the intermediate components fell into intermediate categories. Upon making ADP available, this state of affairs reversed itself, and increased oxidation was observed, the DPN now being about 50 per cent in the reduced form.

These observations are of the utmost importance, for they illustrate the phenomenon of coupling of respiration and phosphorylation in a most unambiguous way, in terms of the respiratory carriers. Incidentally, they also support the direct experiments on the localisation of phosphorylations, and show that one of these occurs at flavoprotein level, a fact which has so far escaped direct experimental proof. To return, however, to the coupling phenomenon, this interdependence of respiration and phosphorylation may only be observed in native mitochondrial preparations. Where, for example, the mitochondria have been damaged, as by exposure to detergents, or to hypotonic conditions, then the rate of respiration will be independent of nucleotide acceptor concentration and the phosphorylation mechanism itself may be destroyed. The mechanism by which this coupling occurs has been the subject of considerable discussion. Numerous schemes have been drawn up, the salient features of which are summarised below.

Mechanism and Coupling of Respiration and Phosphorylation

All the schemes have to account for the inhibition of respiratory rate when ADP becomes limiting, and therefore have to postulate a reaction during the process of dehydrogenation in which the respiratory carriers are phosphorylated or in which these carriers react with some other molecule which is thereby activated, and which may therefore react with inorganic phosphate in a phosphorolytic reaction. The subsequent step is transfer of phosphate to ADP to form ATP. It is easy to see that if the initial reaction, either of phosphate or some other molecule, is with reduced carrier, then the complex has to be split if further electron transport is to occur. And if we postulate that the transfer reaction to form ATP is rate-limiting, then the regulation of the rate of respiration by availability of ADP is readily explained.

Lipmann[17] formulated a scheme in which the respiratory carriers were phosphorylated, while Slater[18] suggested a reaction between the carriers and another compound which was then phosphorylated after dehydrogenation of the complex.

Chance[13] believes that the process is more complex. His views may be summarised as follows, where A and B are respiratory carriers and C and X are unknown compounds, C being an inhibitor of the respiratory chain, which is responsible for the slow rate of respiration in a coupled system when ADP concentration is limiting.

The oxidised form of B is in combination with C, thus

$$B + C \leftrightharpoons B.C$$

The reduced form of A, AH_2 then reacts with B.C

$$AH_2 + B\,C \leftrightharpoons A + BH_2{\sim}C$$

then

$$BH_2{\sim}C + X \leftrightharpoons BH_2 + X{\sim}C$$

and X∼C undergoes phosphorolysis and subsequent transfer of phosphate to ATP occurs,

$$X{\sim}C + ADP + \text{inorg. } P \leftrightharpoons X + C + ATP$$

It is plain that these are all paper schemes, but they do draw attention to two main points. Firstly, they can explain the existence of "uncoupled" respiration, for example in Chance's scheme, if the compound X∼C were able to react rapidly with water rather than inorganic phosphate, it is clear that the rate-limiting transfer reaction would be by-passed, that oxidation would become independent of ADP, and that no phosphorylation would be observed. This situation might be met where the mitochondria have been damaged. Secondly, they can be used to explain the mechanism of action of certain agents which are known to inhibit phosphorylation without affecting respiration.

It should also be noted that the reaction mechanism involves several steps, the minimum being two, first a phosphorolysis, and second a transfer. The phosphorolysis may itself proceed in two stages. All these steps are presumably catalysed by enzymes, but it is not impossible that the phosphorolytic step is spontaneous and non-enzymatic. For example, Stadtman and White[19] have shown that *N*-acetyl imidazole will

react reversibly with inorganic phosphate to give acetyl phosphate, and it might well be that some similar mechanism is involved in the early stages of the oxidative phosphorylation reaction.

We have up to now talked of the coupling phenomenon as though the dependence of respiration on ADP were absolute, and it might be assumed from this that the rate of respiration is stoichiometrically related to the concentration of the nucleotide. This is not the case, for Chance has shown that respiration may proceed linearly until the ADP is nearly exhausted, and that the rate is essentially the same within a 10,000 fold variation in the ratio of ATP to ADP. Nevertheless, this should not be taken to mean that the respiratory rate is not governed by acceptor availability *in vivo*, for as we have already noted, it is probable that in whole cells, the major part of the adenine nucleotide is present as ATP.

The dependence of respiration on the presence of a sufficiency of inorganic phosphate has been demonstrated many times[9], and it is clear that its mechanism may be treated in the same way that has been applied to ADP. Thus in the reaction scheme which was given above, if there were inadequate inorganic phosphate present, the phosphorolysis of X~C would be reduced in rate, and hence the electron transport would also be slowed. That these schemes may have basis in fact is demonstrated by their affording satisfactory explanations for the phenomena of uncoupling by chemical reagents, which will now be considered.

Action of Uncouplers of Oxidative Phosphorylation

In 1948, Loomis and Lipmann[20] showed that low concentrations of 2:4-dinitrophenol completely inhibited the phosphate uptake of a mitochondrial preparation while leaving its respiration unaltered. Since that time, a vast amount of work has been done on the subject[5,8,9,21] and numerous other compounds have been found to do the same thing.

However, dinitrophenol is an excellent example to consider here, and the discussion will centre around this substance. Loomis and Lipmann[20] were also able to show that when their enzyme preparations were incubated in a medium deficient in inorganic phosphate, the respiration rate fell to low levels, and that uncoupling concentrations of dinitrophenol stimulated this rate to normal figures (Table VI). They concluded that dinitrophenol might "replace" inorganic phosphate in their system, perhaps in a similar way to arsenate, which actually does replace orthophosphate in the glycolytic phosphorylation.

That this is not the case is shown clearly by the experiments of Judah[9] who demonstrated that the "replacement" only took place when small amounts of inorganic phosphate were present in the medium. This implied that dinitrophenol was actually sparing inorganic phosphate by preventing its uptake into ATP. Judah and Williams-Ashman[21] also made similar observations with respect to adeninenucleotide (Table VI). They found that when ADP was omitted from the medium the respiration rate was low and that addition of dinitrophenol greatly stimulated the rate of respiration under these conditions. However, if the mitochondrial preparations were well washed, then dinitrophenol was no longer effective,

suggesting that as with inorganic phosphate, a certain small amount of nucleotide is required, and that once again this is a sparing effect of dinitrophenol. Now if this is so, then something is clearly wrong. For in our consideration of the mechanism by which inorganic phosphate is esterified it became apparent that a phosphorolysis followed by a transfer reaction must occur. Dinitrophenol is presumably inhibiting one or both of these steps, and if so should make the system independent of

TABLE VI

"REPLACEMENT" EFFECT OF DINITROPHENOL (DNP). RAT LIVER MITOCHONDRIA OXIDISING GLUTAMATE

	μl. O_2 respiration	
	No DNP	+DNP
Complete system	255	258
No phosphate	80	288
No nucleotide	99	180

either inorganic phosphate, adenine nucleotide or both. The fact that it does not do any of these things is a puzzle which is largely ignored by those considering the mechanism by which it works. It may be that there is some other reason for the failure of dinitrophenol to replace adenine nucleotide. For example, the mitochondrial system may require small amounts of adenine nucleotide not solely for the respiratory function, but also for maintenance of structure, in which case it might well be that it would be difficult to demonstrate complete independence of the former.

A clue to the nature of dinitrophenol action was provided by the observation of Lardy and Wellman[22] that it greatly increases the ATP-ase activity of intact mitochondria. However, this cannot be the sole explanation for its uncoupling action because (1) Slater[23,24] has shown that the ATP-ase action may be anulled by increasing the activity of the hexokinase normally added to the reaction mixture, which thus successfully competes for ATP. (2) While dinitrophenol reduces the phosphorylation coupled to DPN-linked substrate oxidation, it leaves untouched the esterification of inorganic phosphate which is coupled to the anaerobic dismutation of α-oxoglutarate with ammonia[9]. (3) Reduction of incubation temperature reduces the activity of the ATP-ase, but has no effect on the dinitrophenol inhibition of oxidative phosphorylation. Lardy and Wellman[22] suggest that this apparent ATP-ase activity is in fact due to interaction of dinitrophenol with an intermediate in the phosphorylation process (see the schemes suggested by various authors and described earlier). In other words, the ATP-ase activity is due to a reversal of the oxidative phosphorylation, and inorganic phosphate is liberated in the presence of dinitrophenol because of the hydrolysis of an unknown phosphorylated intermediate. Thus,

$$\text{ATP} + \text{X} \leftrightharpoons \text{ADP} + \text{X}\sim\text{P}$$
$$\text{X}\sim\text{P} + \text{DNP} \leftrightharpoons \text{X} + \text{iP}$$

or

$$\text{X}\sim\text{P} + \text{DNP} \leftrightharpoons \text{DNP}\sim\text{P}$$
$$\text{DNP}\sim\text{P} \rightarrow \text{DNP} + \text{iP}$$

Other suggestions including that of Chance[13] have been put forward. The latter authors suggest that dinitrophenol interacts with X∼C in their scheme, rather than with a phosphorylated intermediate. A compound dinitrophenol∼C is then formed, and this accounts for the acceleration of respiration by dinitrophenol in systems which are limited by ADP concentration, since C is inhibitory to the respiratory chain and the formation of the compound with dinitrophenol lifts this inhibition. These ingenious ideas explain the findings very well, and are supported by the experiments of Slater and Lewis[25], who found that insect sarcosomes oxidised α-oxoglutarate with a P:O ratio of around 1·0. They were also able to show that addition of dinitrophenol accelerated the respiration by a factor of 3, despite the fact that hexokinase was present and that ADP concentration could not be limiting; not only this, but there was also an *increase* in the amount of phosphate esterified, a most unusual finding. It is suggested that the insect system possesses a very slow transfer reaction from intermediate to ADP, compared with the usual mammalian systems, and that the effect of dintrophenol may be explained by its interaction with an intermediate, as shown above. However, there is one fact which cannot be explained by these theories, and it is that low bath temperatures will prevent the loss of inorganic phosphate from ATP in the presence of dinitrophenol while the effect of the phenol does not vary. It seems hard to get round this point.

Though a great deal of work has been done on dinitrophenol, other substances which uncouple phosphorylation from respiration are also known[8,21], though not so much attention has been paid to them. A few words concerning some of these substances would not be out of place here. Azide is best known for its inhibitory effect on cytochrome oxidase, and it is not generally appreciated that in low concentrations it is a powerful inhibitor of oxidative phosphorylation[9,26]. Experiments based on its function as a cytochrome oxidase inhibitor are liable to be complicated by this other property. Azide evidently acts differently from dinitrophenol. For instance, it will not accelerate the respiration of phosphate-deficient mitochondrial systems, but little else is known about it.

Dicoumarol, the antagonist of vitamin K, has been shown by Martius and Nitz-Litzow[27,28] to be a powerful uncoupler of oxidative phosphorylation. They suggested that this inhibition was related to the antagonism to vitamin K and that naphthoquinones were playing a part in electron transport and phosphorylation. In support of this view, they were able to show that mitochondria isolated from vitamin K deficient birds gave low P:O ratios, which were elevated by addition of vitamin K. Recently, Lehninger [29–31] has been able to show that dicoumarol inhibits phosphorylation at every step in the electron transport chain and it therefore seems that naphthoquinones must either operate at every step in the process as a component of the phosphorylation system, or that dicoumarol has another action separate from its antagonism to vitamin K. In any event, the hypothesis put forward by Martius to explain his results is no longer tenable. He suggested that vitamin K was actually part of the electron-transport chain, acting between DPN and cytochrome *c*, and

that dicoumarol caused a diversion of electron flow to a non-phosphorylating path. These experiments are all extremely interesting and thought-provoking and further developments are to be hoped for.

The last compound which is to be considered here is the hormone, thyroxine. In view of the similarities in effect on animals (increased basal metabolic rate with wasting), produced by dinitrophenol and thyroxine, and the fact that both are substituted phenols, it is not surprising that many workers have tried to show similar actions of the two substances *in vitro*, The early work was hampered by an inability of the thyroxine to reach the active centres in the mitrochondria, but Martius and Hess[32] found that pre-incubation of the particles with low concentrations of thyroxine produced typical uncoupling effects. Hoch and Lipmann[33] made the fascinating observation that mitochondria from hamster liver are normally more permeable than those from rat liver, and that on these former, thyroxine worked readily without pre-incubation to give low P:O ratios without significant effect on respiration. Unfortunately, the interpretation of these results is placed in doubt by the recent work of Lehninger[34] who has shown that a particle derived from mitochondria (which will be referred to later) is capable of a brisk oxidative phosphorylation which is uncoupled by dinitrophenol, but which is quite insensitive to thyroxine. He has also shown that the action of thyroxine on intact mitochondria is probably due to the swelling brought about in these particles when they are incubated with the hormone. The question, of course, is whether this action may in any way be related to the physiological effect of the hormone. At the present time the writer is quite unable to see any answer.

Exchange Reactions and Oxidative Phosphorylation

In the foregoing sections, we have dealt with phenomena which are undoubtedly coupled to electron flow, but here it is intended to deal with exchange reactions, in which by the use of a label, either ^{32}P or ^{18}O, it is possible to show that phosphate groups or the oxygen of phosphate are transferred, in some cases in the absence of a net oxygen uptake.

Cohn[35] was the first to show that ^{18}O-labelled phosphate exchanged its oxygen with water during the process of oxidative phosphorylation. The most significant part of her work lay in the demonstration that some 6 cycles of oxygen exchange took place for each mol. of inorganic phosphate esterified during the passage of electrons along the respiratory chain, and she was also able to show that dinitrophenol inhibited the exchange phenomenon.

It is clear that a P–O link is being repeatedly formed and broken, and the inference is that some compound is being formed with phosphate which can bring its oxygen into equilibrium with the oxygen of water. Boyer, Falcone and Harrison[36] have also observed that this oxygen exchange may occur in the absence of a net oxygen uptake. Using mitochondrial preparations, they found that providing ATP were present, a rapid exchange took place between inorganic P and water and that this exchange was independent of substrate and took place in anaerobic

conditions. If ^{32}P were the label, then a group transfer was found, and the label appeared in ATP. The rate of the phosphate exchange was about a seventh of the oxygen exchange. Dinitrophenol and azide block these exchanges. If ADP and not ATP is present, the exchange, at least of ^{32}P is negligible. Boyer and his colleagues suggest that these reactions represent the reversal of oxidative phosphorylation. Since the electron transport chain in these mitochondria will be in a variety of oxidation states, representing the steady state for an inactive system, that is, with DPN almost completely reduced, and the intermediate components between DPNH and oxygen being in intermediate conditions, it is not hard to see that electron flux might well take place and might cause the observed reactions. It might also be that these reactions depend on the existence of intermediates of oxidative phosphorylation. Thus if an active intermediate existed, and this has been postulated in the scheme, it is not hard to see how it could be in equilibrium with both inorganic phosphate and the terminal phosphate of ATP. Such an intermediate could also react with water to give the oxygen exchange.

The available evidence does not give much support to either theory at the moment. Thus it can be shown that antimycin A, which is an inhibitor of the electron transport chain which acts between cytochrome *b* and cytochrome *c*, will not affect the ^{32}P exchange. This might mean either that the bulk of the electron flux occurs between DPNH and flavoprotein, or that the electron flux is of no importance in the exchange reaction. Quite recently, Cohn and Drysdale[37] reported that during oxidative phosphorylation, not only was there a marked oxygen exchange between inorganic phosphate and water, but that a further exchange took place before the phosphate was taken up into ATP. This could only mean that an intermediate existed which was itself rapidly exchanging oxygen with water, and was the first evidence of such a compound. Unfortunately, Boyer and his colleagues[38] have shown that the concentration of intermediate, calculated from the figures of Cohn and Drysdale, would have to be very large indeed to satisfy the theory of an intermediate, and suggest an alternate mechanism for the findings. This is that the substrate, β-hydroxybutyrate, was being converted to the coenzyme A derivative. As the similar acetate activation process is accompanied by oxygen transfer from acetate carboxyl to the phosphate of AMP, derived from ATP, it seems plausible that the exchange observed by Cohn is in fact not related to oxidative phosphorylation.

We are therefore left with these interesting observations. They may prove valuable in the elucidation of the problems of oxidative phosphorylation, but at the moment there would appear to be no certainty as to interpretation.

Mitochondrial Preparations: Fractionation of the Phosphorylation System

It seems worth while to consider the variety of preparations which are available for the study of oxidative phosphorylation. Numerous attempts have been made to break the mitochondrial system down to simpler

units, and in addition a variety of tissues have been used. A summary of the present state is therefore attempted here.

Mitochondria

The classical preparation is a modification of that of Hogeboom, Schneider and Pallade[7], who used 0·88 M sucrose for homogenising rat liver, and isolated intracellular particles by differential centrifugation at low temperatures. The modification consists in using 0·25M sucrose, and is due to Schneider[39]. Better activities are obtained with the lower concentration of sucrose and lower centrifugal speeds are required, though the mitochondria tend to lose their normal elongated shape. Rat liver has been the material of choice for most studies, but Slater[23] has used particles isolated from heart muscle. These preparations have yielded much information in Slater's hands, but they have the drawback that they are relatively difficult to set free from the muscle cells and that yield is limited. Furthermore, the heart contains large amounts of calcium,

TABLE VII

COBALT EFFECT ON BRAIN MITOCHONDRIA PRE-TREATED WITH 1:10 PHENANTHROLINE

	No cobalt	Cobalt $3{\cdot}3 \times 10^{-6}$M
Respiration μ atoms O	8·7	11·2
Phosphate uptake μ moles ..	5·2	14·0
P:O ratio	0·6	1·3

which during the isolation procedure becomes attached to the mitochondria, and which acts as a powerful inhibitor of oxidative phosphorylation. In order to obviate this, the homogenate must be made in relatively high concentrations of ethylenediamine tetra-acetic acid (edetic acid) in order to bind the calcium. The effect of calcium has itself been used to study the phosphorylation system and will be considered later. Mitochondria have also been isolated from brain[40]. These particles are very similar to those from liver. They need edetic acid, however, in much the same way that heart sarcosomes do, and also show a peculiar inability to oxidise added citrate, despite the fact that added pyruvate is oxidised rapidly without accumulation of citrate in the flasks[40]. These workers also found that incubation of brain mitochondria with a variety of chelating agents resulted in powerful inhibition of respiration. For example, *o*-phenanthroline when added to mitochondria and then removed by washing on the centrifuge, resulted in preparations which rapidly lost their respiratory activity. At the same time, the P:O ratio fell to low figures. Reversal could be brought about by cobaltous salts in low concentration; nickel salts also had the same effect, and it is probable that there is no specificity in this result, despite the fact that a range of other ions had no effect (Table VII).

The whole problem of metal requirements for oxidative phosphorylation is exceedingly complicated. Thus, Lindberg and Ernster[41,42] have shown that calcium ions when added to rat liver mitochondria bring about a

requirement for manganese. If the particles are pre-incubated with calcium, oxidative phosphorylation falls to low levels. The addition of Mn will bring about some reversal when added later, and will prevent the inhibition if added at the same time. The presence of ATP together with Mn gives the best protection. Cleland and Slater[43] have shown that calcium ions disrupt the structure of the mitochondria, and this finding, together with those of Christie and colleagues[40] make one wonder whether these metal effects do not represent some structural effect on the particles rather than a fundamental one on the phosphorylating system? The position of magnesium in this matter was at one time unassailable. It is required for phosphorylation by all mitochondrial systems, and its known function in other phosphokinase systems (for example, hexokinase) made it seem all the more likely that this was a true requirement. The work of Lehninger now to be considered casts doubt on this belief.

Mitochondrial Fractionation

The breakdown of the mitochondrial structure invariably results in almost total loss of activity, with the exception of one process. Thus, the suspension of the particles in water which brings about gross swelling and the escape of soluble protein, nucleotides, and other substances, reduces the P:O ratio to small figures, increases the ATP-ase activity to maximum and damages the oxidative mechanism. The addition of surface active agents has similar effects. The exposure of the mitochondria to solvents such as ethanol or acetone in any great concentration destroys them utterly. In face of these facts, it is not surprising that attempts to reduce the particle system to simpler units have had little success. Cooper and Lehninger[29] have, however, succeeded in obtaining minute particles from rat-liver mitochondria which will oxidise β-hydroxybutrate and succinate with a coupled phosphorylation. They used digitonin to split the mitochondria, and followed this with differential centrifugation at high speed. The final centrifugation at $105{,}000 \times g$ gave a small pellet of active particles, the particle weight of which is roughly calculated at about 1/3000th that of the parent mitochondrion.

These submitochondrial particles require only substrate, inorganic orthophosphate and ADP for their activity. Magnesium is not required, and is indeed somewhat inhibitory in the usual concentration. Calcium has no effect on the phosphorylation. We must therefore bear in mind the possibility that the effect of both these ions on intact mitochondria is related to their action on mitochondrial structure. That this is probably the case with calcium has already been mentioned, but the observations of Lehninger are the first good evidence that Mg requirement is in the same general category, and fits in with the remarks made about Mn and Co earlier in this review.

Lehninger and his colleagues have studied this preparation in some detail. Amongst their important findings is that ADP is the sole acceptor of phosphate. Other nucleoside diphosphates are inactive in this system. This point could never be settled with intact mitochondria, for it is known that they contain a variety of nucleotides, and while ADP has been used

constantly with such systems, there was always the chance that the initial phosphorylation involved some other compound (for example, inosine diphosphate, which is known to be a primary acceptor in the substrate-linked phosphorylation coupled to the oxidation of α-ketoglutarate to succinate). They have also shown that phosphorylation occurs in the span substrate to cytochrome *c* and in the span from reduced cytochrome *c* to oxygen. Thus the whole chain is complete and present. Their observations on dicumarol have already been referred to.

Since the publication of Lehninger's work, Hatefi and Lester[44] have described successful fractionation of beef-heart mitochondria. The investigation of their particles has not been reported with the same detail as has Lehninger's, and further work is awaited. At least one major difference is known, however. Whereas the digitonin preparation gives particles which will oxidise only succinate and β-hydroxybutyrate, the beef-heart particles appear to contain all the enzymes of the tricarboxylic acid cycle, together with the phosphorylating enzymes (see Table VIII for summary).

TABLE VIII

ATTEMPTS AT FRACTIONATION

	Lehninger preparation	Hatefi and Lester preparation
Method	1 per cent digitonin	15 per cent ethanol
Source	Rat liver mitochondria	Beef heat mitochondria
Yield	0·5 per cent	7·5 per cent
Substrate specificity	β(OH) butyrate and succinate (low)	All T.C.A. intermediates
Activity	Same as starting material or better	Variable
Stability	Unstable	Stable to freezing

The last type of preparation which is to be considered is of some interest. Hunter[45] found that when isolated mitochondria were incubated with 0·02M inorganic orthophosphate for 10 minutes at 30°, they were unable to oxidise β-hydroxybutyrate. The addition of DPN to such preparations stimulated the oxygen uptake, and also resulted in a coupled phosphorylation. It appears that the treatment renders the particles DPN-less, without interfering with the phosphorylation system. This is in marked contrast to other situations where DPN is required by mitochondria. For example, ageing of the particles causes loss of nucleotides into the medium, and a strong DPN requirement is then observed, but there is no coupled phosphorylation. Christie and Judah[46], investigating the action of carbon tetrachloride on the liver, found that mitochondria isolated from such livers required the addition of DPN for respiration, and that this respiration was associated with normal P:O ratios. The same result can be obtained by soaking isolated mitochondria in CCl_4 *in vitro*, but the degradative process is not readily controlled and may proceed too far.

Finally, a word or two about respiratory pathways in mitochondria. It would appear that there are at least two pathways for electron transport, one "internal" and coupled to the phosphorylating system, the other

"external" and non-phosphorylating. Thus, we have seen that in the case of DPNH, the reduced nucleotide is rapidly oxidised by intact mitochondria without a coupled phosphorylation unless access is assured to the internal pathway by hypotonic treatment. But the problem is not so simple as that. Thus, in Lehninger's submitochondrial particles, β-hydroxybutyrate is oxidised with a coupled phosphorylation, whereas added DPNH is oxidised without phosphorylation, as in the case of intact mitochondria. These particles do not, however, have the same permeability characteristics as intact mitochondria. They are immune to hypotonic conditions, they do not respond to calcium or to Hunter's phosphate treatment. Why, therefore, do they not show a coupled phosphorylation with added DPNH?

Lehninger[29] thinks that electron-transport in this case is probably mediated by a DPN-cytochrome *c* reductase which is not coupled to a phosphorylating system, and which is also insensitive to antimycin A, and which is similar to that described by Vernon, Mahler and Sarkar[47].

It follows that the mitochondrial system is a sort of maze of pathways, that aside from the two well-defined "internal" and "external" ones, there may be systems which normally do not function in electron transport, but which may come into action upon disturbance of the mitochondrial structure.

Finally, it must be emphasised that all attempts to fractionate or degrade the mitochondrial system have only succeeded in producing smaller structures. In every case there is a considerable organisation left intact. No one has yet succeeded in obtaining a preparation in which the oxidative phosphorylation system can be reconstructed from any of its parts. It is either complete or it does not appear to exist.

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