Oxidative Phosphorylation

Experiments with fragments of mitochondria offer new information about respiratory energy conversion.

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Oxidative phosphorylation is a vital process in the economy of aerobic cells, probably accounting for over 90 percent of all the adenosine triphosphate (ATP) generated from adenosine diphosphate (ADP) and phosphate at the expense of the energy liberated during catabolism of foodstuffs. Despite the great importance of this process and 20 years of intensive investigation, the enzymatic details of oxidative phosphorylation are still essentially unknown. It is certain, however, that during the exergonic passage of a pair of electrons from substrate to molecular oxygen, via the pyridine nucleotides, flavoprotein, and the cytochromes, 3 moles of ATP are generated in coupled reactions occurring at three points in the respiratory chain (*1–3*), presumably those shown in the upper portion of Fig. 1.

Both electron transport and oxidative phosphorylation take place in mitochondria, the structure of which must be relatively intact in order that oxidative phosphorylation may be observed in vitro. This finding has greatly limited the experimental approaches to the enzymatic mechanisms involved; it is largely for this reason that the mechanism of oxidative phosphorylation remains one of the most conspicuous mysteries in contemporary biochemistry.

In the last three years we have studied experimental procedures for making morphologically less organized preparations from rat liver mitochondria with which the enzymatic details of oxidative phosphorylation could be more directly studied, with the ultimate goal of resolving the mechanism of oxidative phosphorylation by enzyme separation and reconstruction approaches. We have found it possible to obtain fragments of the mitochondrial membrane which contain relatively intact respiratory chain assemblies, including the enzymes necessary for coupling phosphorylation to electron transport. This article summarizes the chemical and enzymatic properties of these membrane fragments, prepared by the action of digitonin on rat liver mitochondria, and the new information they have yielded concerning the mechanism of oxidative phosphorylation and its relation to other aspects of mitochondrial physiology.

Membrane Fragments

Preparation. Washed rat liver mitochondria are suspended in 0.05*M* sucrose and treated in the cold with an 0.8-percent solution of recrystallized, metal-free digitonin. The mitochondria undergo virtual dissolution, leaving a turbid brown solution which is centrifuged at 50,000 *g* for 25 minutes. The supernatant fluid, which is not clear but contains a gelatinous material, is then centrifuged at 100,000 *g* for 25 minutes to sediment the phosphorylating membrane fragments (*4, 5*). When suspensions of this preparation are incubated aerobically with D-β-hydroxybutyrate, ADP, and orthophosphate, oxygen is taken up, acetoacetate accumulates in equivalent amounts, and inorganic prosphate (P_i) disappears with equivalent formation of ATP. The observed P:2*e* ratios (moles of P_i taken up per pair of electrons transferred) have been as high as 2.8 [maximum expected, 3.0 (*2*)] but are more usually in the range 1.7 to 2.3. The addition of Mg^{++} to the test medium is not required for phosphorylation; however, the fragments contain a considerable amount of bound Mg^{++} (*4*).

Approximately 10 percent of the total nitrogen of the starting mitochondria is ordinarily recovered as the active digitonin preparation. Measurements of specific activity (micromoles of acetoacetate formed per milligram of N per minute) indicate that 40 to 60 percent of the total oxidative activity of the original mitochondria is recovered; the particles are thus from four to six times more active per milligram of N than the original mitochondria (*4, 5*).

Oxidative reactions in membrane fragments. These submitochondrial preparations do not catalyze the organized reactions of the Krebs tricarboxylic acid cycle or the fatty acid oxidation cycle. Only D-β-hydroxybutyrate and succinate, among a wide variety of substrates tested, yield significant rates of O_2 uptake. Fragmentation of the mitochondria with digitonin thus does not produce miniature replicas of intact mitochondria, but rather particles containing relatively intact assemblies of enzymes capable of phosphorylating electron transport, which are largely free of substrate-level enzymatic apparatus (*4*). The digitonin fragments are thus strikingly different from the phosphorylating submitochondrial preparations more recently described by Ziegler *et al.* (*6*) and Kielley and Bronk (*7*), which retain much more enzymatic organization on the substrate level.

On the other hand, the digitonin fragments do retain the characteristic organization of the respiratory chain of intact mitochondria. Difference spectra (*4, 5*) show the presence of cytochromes *a*, a_3, *b*, and *c* in the ratio 1.0:1:0:0.7:1.8, which is approximately that observed by Chance and Williams in intact liver mitochondria (*3*). Also present is an enzymatically reducible flavoprotein with the same absorption characteristics as that in intact mitochondria; its molar ratio to the cytochromes is slightly lower. Diphosphopyridine nucleotide (DPN) is present in a bound form which is highly reactive with the bound D-β-hydroxybutyric dehydrogenase but inactive with bound L-malic dehydrogenase, indicating the specificity of physical organization of the respiratory chain. Intact rat liver mitochondria contain some 30 to 40 moles of DPN per mole of cytochrome *a*; the membrane fragments contain less than 1 mole of DPN per mole of cytochrome *a*. Since the specific activity for oxidation of β-hydroxybutyr-

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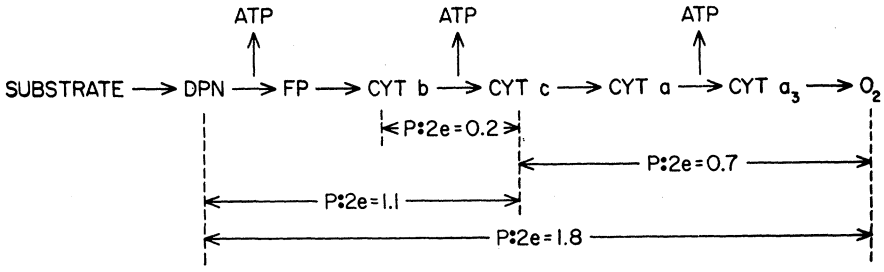

Fig. 1. Respiratory chain and phosphorylation sites. Probable sites of coupled phosphorylations along chain are shown (*2, 3*), as well as experimentally observed values for P:2*e* ratios of different portions of respiratory chain for digitonin particles.

ate in the fragment is fivefold greater than that of intact mitochondria, the bound DPN of the fragment is obviously far more active than the total DPN pool of intact mitochondria. The bound DPN of the membrane fragments is not discharged by phosphate, whereas most of the DPN of intact mitochondria is displaced on such treatment. These facts suggest that most of the DPN in intact mitochondria, although reducible by β-hydroxybutyrate, may be different in location and reactivity from that immediately associated with the respiratory chain. Observations on the kinetics of oxidation and reduction of the total pyridine nucleotide pool in intact mitochondria thus do not necessarily represent the behavior of the bound DPN which is immediately reactive with flavoprotein.

Oxidation of β-hydroxybutyrate in the membrane fragments may be completely inhibited by antimycin A, Amytal, and cyanide; spectrophotometric tests indicate that the pattern of inhibition is identical with that observed in intact liver mitochondria (*5*). However, *added* reduced DPN ($DPN_{red.}$) is oxidized by the preparations through a pathway largely insensitive to antimycin A; little or no phosphorylation is observed (*4*). Added reduced triphosphopyridine nucleotide ($TPN_{red.}$) is oxidized only if DPN is also provided; direct tests showed that the preparations contain a TPN-DPN transhydrogenase (*5*).

The preparations contain bound copper (*8*) and about six times as much iron as can be accounted for by the content of known cytochromes (*5*). Some of the noncytochrome iron may be associated with a pigment believed to be similar to the recently described "mitochrome" (*9*); it exhibits an absorption peak at 426 to 428 mμ in the presence of dithionite, but not with β-hydroxybutyrate. The reduced mitochrome peak of the digitonin particle can, however, be produced enzymatically by the combination of D-β-hydroxybutyrate *plus* succinate (*5*).

Phosphate acceptor specificity. Adenosine diphosphate is the specific phosphate acceptor in the coupled phosphorylations observed with the membrane fragments. The 5′-diphosphates of uridine, cytidine, guanosine, and inosine are completely inactive; they have no stimulatory or inhibitory effect on the action of ADP. Adenosine triphosphate was the sole product of phosphorylation of ADP observed by chromatographic analysis (*4*). The preparations contain little if any myokinase activity (*4, 10, 11*).

Phosphorylation sites. All three phosphorylation sites in the respiratory chain contribute to the over-all P:2*e* ratio during oxidtion of β-hydroxybutyrate by the digitonin fragments. The reduction of cytochrome *c* by β-hydroxybutyrate proceeds with a maximum P:2*e* ratio of about 1.1 (*12*) and the oxidation of reduced cytochrome *c* by oxygen with a maximum P:2*e* ratio of about 0.7 (*13*). When succinate is used to reduce cytochrome *c*, the maximum P:2*e* ratios observed are about 0.2 (*14*) (see Fig. 1). These findings suggest that in the digitonin fragments the phosphorylation site presumed to exist between DPN and flavoprotein is about 90 percent effective, that between cytochrome *b* and *c* about 20 percent, and that between cytochrome *c* and oxygen about 70 percent effective in the average digitonin preparation. These data indicate that there is some selectivity in inactivation of the three coupling mechanisms during preparation of the fragments.

Action of uncoupling agents. Oxidative phosphorylation in the membrane fragments is uncoupled by 2,4-dinitrophenol, Dicumarol, pentachlorophenol, gramicidin, arsenate, and methylene blue with about the same effectiveness shown by these agents in intact mitochondria (*4*). However, the very striking observation was made that two agents known to uncouple phosphorylation in intact mitochondria had absolutely no uncoupling action on the digitonin fragments, even in very high concentrations—namely, Ca^{++} and thyroxine and its analogs (*15*). Thus it appears that these agents have no direct action on the enzymes involved in oxidative phosphorylation, a finding which led us to examine other mechanisms by which thyroxine acts on respiration. Such experiments (*15–18*) have revealed that thyroxine greatly increases the rate of passive swelling of mitochondria by interfering with some aspect of the structure of the membrane. Dinitrophenol, on the other hand, inhibits mitochondrial swelling and antagonizes the effect of thyroxine, clearly demonstrating a fundamental difference in the action of these two agents on mitochondria. These findings have led to an interpretation of the mode of action of thyroxine which is rather different from a simple uncoupling action on oxidative phosphorylation such as is shown by dinitrophenol (see *17, 18*).

Chemical and physical nature of membrane fragments. The digitonin preparations contain about 65 percent protein, about 28 percent phospholipid, and 1 to 2 percent cholesterol. The preparations also contain about 5 percent bound digitonin (*5*).

Ultracentrifugal analysis shows the particles to have an average sedimentation constant of about $S_{w,\ 20°} = 200$. The particles are obviously polydisperse since the sedimenting boundaries spread rapidly. While the material is too crude for exact measurements, an average particle weight of 40 million is suggested as a very gross approximation. When the preparation is subfractionated by differential ultracentrifugation and the separate fractions are tested for activity, all fractions are found to be approximately equal in specific oxidative activity per milligram of N, and the P:2*e* ratio is essentially constant, indicating that the digitonin particles are enzymatically homogeneous but differ in size (*5, 19*). Preliminary electron microscope observations suggest that the digitonin particles are fragments of the mitochondrial membrane, a view confirmed by Siekevitz and Watson (*20*). These considerations suggest that the respiratory enzyme assemblies are more or less evenly distributed throughout the mitochondrial membranes, particularly in those making up the cristae. Treatment with digitonin is believed to disrupt the membranes along fragile lines of cleavage so that polydisperse frag-

ments result which are made up of multiples of a single structural unit, each of which may contain only a single respiratory enzyme assembly (*5, 19, 21*) (see Fig. 2). Calculations based on the known content of some respiratory carrier proteins in the liver mitochondria suggest that as much as 10 to 20 percent of the substance of the mitochondrial membrane is made up of the catalytically active proteins of the respiratory assemblies.

When the digitonin fragments are exposed to sonic vibration at 9 kcy/sec, there is a great decrease in the average sedimentation constant of the fragments without any change in the respiratory or phosphorylating activity. These findings suggest that the sonic vibration produces particles which approach in size and composition the basic recurring unit which, it has been suggested, make up the mitochondrial membrane, as is shown in Fig. 2.

The molecular and geometrical configuration of the mitochondrial membrane is a function of the oxidation-reduction state of the respiratory carriers; recent experiments have demonstrated that when the electron carriers of intact mitochondria are fully reduced the mitochondria are not susceptible to swelling in the presence of thyroxine and other agents (*22*). When the carriers are fully oxidized, the mitochondria are maximally responsive to the action of swelling agents.

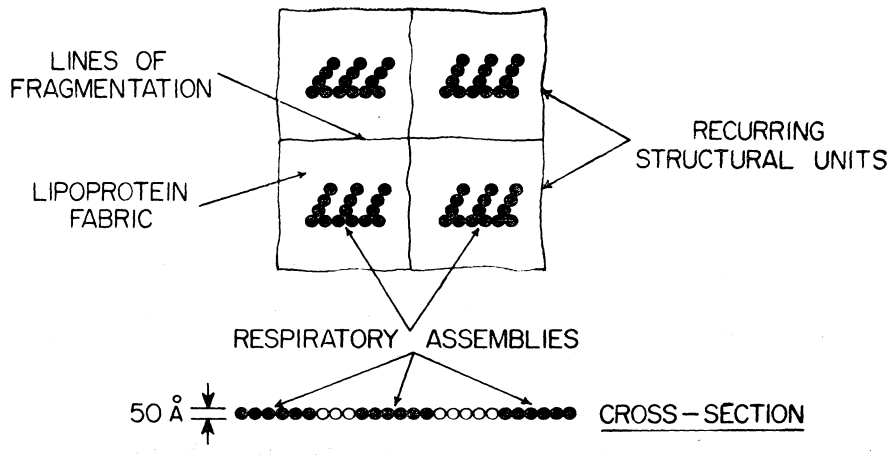

Fig. 2. Molecular representation of mitochondrial membrane. Each respiratory assembly is assumed to consist of six carrier proteins and three ancillary enzyme molecules at each coupling site to yield structure of molecular weight 1.5 million, assuming molecular weight of 100,000 for each protein. Since assembly molecules make up as much as 20 percent of the membrane, the simplest recurring unit may have a particle weight of some 7.5 million. The measured thickness of membrane suggests a monolayer of protein molecules.

Coupling Mechanism

Experimental approaches. It has been proposed by a number of investigators (see, for instance, *2, 3, 11, 23*) that at each of the three phosphorylation sites in the respiratory chain coupled electron transfer results in the formation of a "high-energy" derivative of an electron carrier, either with phosphate or with some other compound. This "high-energy" intermediate may thus be viewed as the chemical form in which the energy liberated in electron transfer has been conserved. It is then proposed that this intermediate can donate its "high-energy" phosphate group to ADP to form ATP, either directly or following intervening group-transfer reactions. Such a formulation (*2, 11*) is given in skeleton form in the following equations:

$$\text{Carrier} + X \xrightarrow{\text{electron transfer}} \text{Carrier} \sim X \quad (1)$$

$$\text{Carrier} \sim X + P_i + \text{ADP} \rightleftarrows \text{Carrier} + \text{ATP} + X \quad (2)$$

in which X is the hypothetical coupling molecule (possibly an enzyme) and $\sim$ denotes a "high-energy" bond; in all probability, X is a group-transferring enzyme.

It is evident from these reactions that two general approaches to study of the coupling mechanism are possible. The first approach, seemingly the more direct one, is to search for and identify chemically the primary "high-energy" form of the three specific respiratory carriers which engage in the energy coupling. However, although the major electron carriers in the respiratory chain are known, there is considerable uncertainty about the participation of additional carrier molecules or enzymes beyond those shown in Fig. 1. For instance, much recent activity in a number of laboratories suggests the participation of substances such as vitamin K_1, α-tocopherol, an unidentified quinone, copper (and also an additional cytochrome (c_1), but the sequence and precise role of these elements is not known, and therefore the precise location of the three phosphorylation sites is still somewhat uncertain.

On the other hand, a second approach to the coupling mechanism is implicit in the scheme of reactions 1 and 2—namely, the identification of the terminal reaction(s) leading to formation of ATP through a transphosphorylation to ADP. By working back in reaction 2 from ATP, it is evident that the mechanism of the participation of phosphate and ADP may be elucidated, in principle, before the specific electron carriers involved in coupling are identified. This is the "back-door" approach to oxidative phosphorylation, and in the following paragraphs it is shown that this approach has yielded significant new information.

The membrane fragments bring about three characteristic transformations of ATP in the absence of *net* transfer of electrons, and each of these is greatly modified by the classical uncoupling agent dinitrophenol. These are (i) adenosine triphosphatase activity (*10*), (ii) the ATP-P_i^{32} exchange reaction (*11*), and (iii) the ATP-ADP exchange reaction (*11, 24, 25*). These reactions and their significance are described in the following paragraphs.

Adenosine triphosphatase activity. The phosphorylating digitonin fragments are capable of hydrolyzing ATP to ADP and P_i (*10*). This ATP-ase activity is believed to be related to the coupling mechanism since the addition of the uncoupling agent dinitrophenol greatly accelerates the hydrolysis of ATP; in this respect the ATP-ase activity of the fragments resembles that in intact mitochondria. The ATP-ase activity is completely specific for ATP; the fragments do not act on the triphosphate of inosine, guanosine, uridine, or cytidine; this is in agreement with the complete specificity for ADP shown by oxidative phosphorylation. There is no ADP-ase activity, nor are the diphosphates of the other nucleosides attacked. The ATP-ase activity of the digitonin fragments is great enough to account for at least some of the deficit in the P:O ratios observed. Appropriate tracer experiments have revealed that the ATP-ase activity is not "turned off" during oxidative phosphorylation (*10*).

The hypothetical coupling mechanism shown in reactions 1 and 2 could account for the DNP-stimulated ATP-ase. It may be postulated that ATP is split by reversal of reaction 2 followed by hydrolytic decomposition of carrier $\sim X$; DNP is generally believed to cause rapid decomposition of an energy-rich intermediate (such as carrier $\sim X$) by a displacement reaction, leading to net hydrolysis of ATP.

Exchange Reactions

The ATP-P_i^{32} exchange reaction. When the digitonin fragments are incubated aerobically with ATP and inorganic phosphate labeled with P^{32}, in the *absence* of oxidizable substrate or net electron transport, P_i^{32} is incorporated into the terminal phosphate of ATP in a reaction which is inhibited by dinitrophenol. No other nucleoside 5′-tri- or -diphosphate will replace ATP (*11*). This exchange reaction is thus similar to that occurring in intact mitochondria, and it is believed to represent the action of a portion of the coupling mechanism (*11, 25*). The exchange reaction is inhibited by those agents which uncouple phosphorylation, but not by Ca^{++} or thyroxine. The exchange activity disappears on aging of the digitonin fragments at the same initial rate as that of the loss of activity in oxidative phosphorylation and the dinitrophenol-stimulation of ATP-ase. This property clearly indicates that a labile common factor is shared in all three reactions.

The rate of the ATP-P_i^{32} exchange reaction is modified by the presence of ADP in a manner suggesting that ATP, ADP, and P_i participate in an equilibrium (*11*). When ATP concentration is reduced to 1 mM or lower and the phosphate concentration is high (about 10 mM), then the presence of ADP becomes absolutely necessary for the incorporation of P_i in ATP. Adenosine diphosphate is thus an obligatory component of the ATP-P_i^{32} exchange reaction.

Participation of ATP, ADP, and P_i in an equilibrium could be expected by the simple hypothesis stated in reactions 1 and 2. As will be seen below, however, reaction 2 can be dissociated into two consecutive reaction steps.

The ATP-ADP exchange reaction. Direct participation of ADP in an exchange reaction with ATP was then demonstrated conclusively by incubating ADP labeled in the terminal phosphate with P^{32} together with unlabeled ATP and the membrane fragments. The ATP was then isolated by chromatography of the medium in Dowex-1 columns and was found to have become labeled (*11*). Fortunately the digitonin fragments are essentially free of adenylate kinase, an enzyme which is present in considerable amounts in intact mitochondria and which causes conversion of labeled ADP to ATP, a reaction which could "mask" and interfere seriously with the study of the ATP-ADP exchange.

On closer study, by using C^{14}-labeled ADP and paper chromatography for separation of the nucleotides, it has been found that the incorporation of ADP into ATP is in fact a very fast reaction and quickly proceeds to complete isotopic equilibrium between the ATP and ADP present (*24*).

The ATP-ADP exchange has been found to share a common denominator with the ATP-ase activity and the ATP-P_i^{32} exchange: it is inhibited (Table 1) by the uncoupling agents dinitrophenol, Dicumarol, and gramicidin in fresh preparations of digitonin fragments (*24*). It was a very important finding, the significance of which is pointed out below, that the rate of the ATP-ADP exchange reaction is not modified by the presence or the concentration of inorganic phosphate, as is seen in Table 1 (*24*).

Significance of exchange reactions of ATP. Information on these DNP-sensitive reactions of ATP obtained from the digitonin fragments is far more useful than that derived from study of intact mitochondria, in which other exchange reactions of ATP occur which are essentially extraneous to oxidative phosphorylation, such as the adenylate kinase reaction. Furthermore, intact mitochondria show morphological "compartmentation" of their "pools" of DPN, H_2O, phosphate, and nucleotides (*26, 27*), leading to great complexities in interpretation of the kinetics of these exchange reactions.

The importance of the exchange reactions of ATP in making the "back-door" approach to the mechanism of oxidative phosphorylation may now be shown. It will be seen that close study of these reactions has permitted (i) determination of the sequence and general character of the terminal reactions of oxidative phosphorylation, (ii) separation of the last enzyme in the sequence in soluble form, free of interfering reactions, and (iii) experimental approaches to the function of the respiratory carriers in energy coupling.

Sequence of interaction of phosphate and ADP. The properties and requirements of the ATP-P_i^{32} and ATP-ADP exchange reactions make it possible to establish the sequence of the terminal reactions of oxidative phosphorylation with some certainty. The over-all general equation (Eq. 2) for interaction of ADP and P_i already given, namely

$$\text{Carrier} \sim X + P_i + \text{ADP} \rightleftarrows \text{ATP} + \text{carrier} + X \quad (2)$$

can be visualized as a sequence of two consecutive reaction steps, in which the entry of phosphate and ADP occurs in separate reactions.

Here two possibilities are open, as follows:

Sequence I

$$\text{Carrier} \sim X + P_i \rightleftarrows \text{Carrier} + P \sim X \quad (3)$$

$$P \sim X + \text{ADP} \rightleftarrows X + \text{ATP} \quad (4)$$

Sequence II

$$\text{Carrier} \sim X + \text{ADP} \rightleftarrows \text{ADP} \sim X + \text{carrier} \quad (5)$$

$$\text{ADP} \sim X + P_i \rightleftarrows X + \text{ATP} \quad (6)$$

It is seen that both sequences permit P_i^{32} to be incorporated into ATP and labeled ADP into ATP, without net electron transfer. However, in sequence I, incorporation of P_i^{32} into ATP cannot occur in the absence of ADP, but incorporation of ADP into ATP would not require the presence of P_i. Sequence II demands a converse dependence of the exchange rates.

It has already been pointed out that

Table 1. Characteristics of ATP exchange reactions in digitonin fragments. The basic test system contained 0.006M ATP and 100 μg of enzyme N in 1.0 ml (pH 6.5). The test for ADP exchange contained 0.003M ADP-C^{14}; the test for P_i^{32} exchange contained from 0.0002 to 0.01M P_i^{32}. Incubation period 20 minutes, at 25°C.

Test	ATP-ADP (Δmμmole of ATP-C^{14})	ATP-P_i (Δmμmole of ATP^{32})
I. Effect of uncouplers		
None	397	68
$5 \times 10^{-5}M$ dinitrophenol	100	0
$5 \times 10^{-5}M$ Dicumarol	140	0
0.001M sodium azide	395	3
0.001M p-chloromercuribenzoate	0	0
II. Effect of phosphate additions		
None added	397	68
Plus 0.005M P_i	394	141
Plus 0.02M P_i	398	162
III. Effect of aging at 2°C		
Fresh	412	157
24 hours	421	74
48 hours	397	2.3
72 hours	380	0

ADP is a necessary reactant for incorporation of P_i^{32} into ATP, as required for sequence I. Furthermore, the data in Table 1 show that phosphate concentration does not affect the rate of the ATP-ADP exchange, suggesting that phosphate is not a necessary participant in the ATP-ADP exchange reaction. This was conclusively proved by demonstrating that the ATP-ADP exchange can occur at maximum rates in the absence of incorporation of labeled inorganic phosphate into ATP. The data in Table 1 show that the uncoupling agent sodium azide does not affect the incorporation of ADP into ATP but that it does inhibit the ATP-P_i^{32} exchange completely within experimental error (*24*). Furthermore, it has been found that aging of the digitonin enzyme preparation at 2°C causes complete loss of the ability to incorporate P_i^{32} into ATP, while the activity in incorporating ADP into ATP is almost completely retained (Table 1). These findings thus demonstrate clearly that phosphate and ADP are taken up in two separate reactions during the energy coupling. The data also exclude sequence II and are compatible with the formulation of sequence I as representing in principle the two terminal reactions of oxidative phosphorylation. This conclusion is also supported by recent experiments of Boyer with O^{18} as tracer (*28*); they eliminate an ADP ~ enzyme compound as an intermediate. These experiments are compatible with, but do not yet prove, the hypothesis that a covalently-bonded phosphoenzyme is the chemical form of the phosphate donor for ADP as formulated; however, current developments in the study of phosphate-transferring enzymes makes this formulation most probable.

Properties and separation of terminal phosphotransferase. The above findings indicate that the terminal reaction of oxidative phosphorylation can be designated as

$$P \sim X + ADP \rightleftarrows X + ATP \quad (4)$$

where X is an enzyme capable of undergoing reversible phosphorylation by ATP. It has been found that the enzyme can be assayed quantitatively under appropriate conditions by measuring the initial rate of the incorporation of labeled ADP into ATP. The reaction is heat-labile and has an optimum *p*H at 6.5. It is inhibited by *p*-chloromercuribenzoate, and the inhibition can be reversed by cysteine. In fresh preparations of the mitochondrial fragments, the ADP incorporation is inhibited by dinitrophenol, gramicidin, and Dicumarol, but not by azide. It is very striking, however, that when the preparations are aged at 2°C, very little of the exchange activity is lost. However, the ATP-ADP exchange gradually loses its sensitivity to inhibition by dinitrophenol on aging and ultimately becomes completely insensitive. This finding thus demonstrates that the DNP-sensitivity of the ATP-ADP exchange in fresh mitochondrial fragments is conferred on it, probably because it shares a common intermediate with a preceding reaction which is sensitive to dinitrophenol (*24*). Thus it may be postulated that neither azide nor dinitrophenol acts on the terminal reaction (Eq. 4) but on the preceding reaction tentatively designated as

$$\text{Carrier} \sim X + P_i \rightleftarrows \text{Carrier} + P \sim X \quad (5)$$

More recent experiments suggest even closer localization of the action of these uncoupling agents (*24*).

The relatively great stability of the enzyme catalyzing the terminal ATP-ADP exchange reaction is in direct contrast to the generally labile nature of the coupling mechanism and suggested the possibility that the enzyme could be separated in soluble form from the digitonin fragments. This has now been accomplished (*24*): aqueous extracts of acetone-dried mitochondrial fragments have been found to contain 90 percent of the exchange activity in soluble form. A tenfold purification of the terminal phosphotransferase activity has been effected.

The purified soluble enzyme preparation contains only traces of adenylate kinase activity, requires no dialyzable cofactors other than Mg^{++} or Mn^{++}, and has no ATP-ase activity, nor does it catalyze the ATP-P_i^{32} exchange reaction (*29*).

The importance of the separation of the terminal enzyme of oxidative phosphorylation cannot be overemphasized; having the purified enzyme at hand now makes it possible to use it as a known base to identify and reconstruct experimentally the immediately preceding reaction, and thus, by working backward, to elucidate the separate reaction steps of the coupling mechanism.

Oxidation-reduction state of electron carriers and the coupling mechanism. Study of the exchange reactions of ATP also makes possible experimental approaches to the nature of the reaction by which oxidoreduction of the respiratory carrier leads to generation of the primary high-energy compound which ultimately becomes the phosphate donor.

The formulation of reaction 1 for the participation of the carrier is noncommittal even with regard to the gross aspects of the mechanism:

$$\text{Carrier} + X \xrightarrow{\text{electron transfer}} \text{Carrier} \sim X \quad (1)$$

However, in the light of current knowledge of energy-conserving mechanisms in oxidoreductions, more specific mechanisms may be written. Actually two major reaction types are possible and have been proposed for the mechanism of oxidative phosphorylation. The first implies that the high-energy complex of a specific carrier is formed when a specific carrier is *reduced* (*3*); generation of ATP is then caused by phosphorolysis of the reduced carrier complex:

Mechanism I

$$\text{Carrier} + X + 2e \rightarrow \text{Carrier}_{red.} \sim X \quad (7)$$

$$\text{Carrier}_{red.} \sim X + P_i \rightleftarrows \text{Carrier}_{red.} + P \sim X \quad (8)$$

$$P \sim X + ADP \rightleftarrows ATP + X \quad (9)$$

On the other hand, an alternative mechanism may be written, in which the high-energy form of the carrier with X is generated by the act of oxidation or removal of electrons from a reduced carrier, analogous to the known mechanisms of energy conservation in the case of triose phosphate dehydrogenase and α-keto acid oxidases. Such a mechanism follows:

Mechanism II

$$\text{Carrier}_{red.} + X \rightarrow \text{Carrier}_{ox.} \sim X + 2e \quad (10)$$

$$\text{Carrier}_{ox.} \sim X + P_i \rightleftarrows \text{Carrier}_{ox.} + P \sim X \quad (11)$$

$$P \sim X + ADP \rightleftarrows ATP + X \quad (12)$$

These two alternative formulations thus suggest that the rate of the ATP-P_i^{32} exchange and the ATP-ase activity may be a function of the oxidation-reduction state of the respiratory carriers in the chain. If this can be shown to be true, it would constitute very important evidence for a coupling mechanism of this general type. Furthermore, such experiments could be of assistance in deciding which of the two mechanisms depicted is correct. For example, if the respiratory chain were kept in the fully reduced state, but with no net flow of electrons, the rate of the ATP-P_i^{32} exchange in mechanism I could be expected to be maximal, but in mechanism

II could be expected to approach zero. Conversely, the fully oxidized state should produce complete inhibition of the exchange in mechanism I, but a maximal rate if mechanism II is correct.

The effect of the oxidation-reduction state of the respiratory carriers on the ATP-P_i^{32} exchange as it occurs in the digitonin membrane fragments has been examined (*30, 31*). Following institution of the two extreme oxidoreduction states, which were monitored by measurement of difference spectra, it was found that the rate of the exchange reaction was maximal when the carriers were fully oxidized; when the carriers were in the reduced state, the rate of the exchange was greatly and sometimes completely inhibited. Similar results were observed in intact mitochondria when appropriate precautions were taken to measure initial rates of the exchange following full reduction of the carriers. Intermediate oxidation-reduction states yielded intermediate rates of exchange.

Because the ATP-P_i^{32} exchange reaction may proceed to isotopic equilibrium between the P_i and the terminal P of ATP rather quickly, it is evident that this exchange must occur through the intervention of a reaction intermediate *which has approximately the same energy of hydrolysis as the terminal phosphate bond of ATP*. This thermodynamic consideration makes it necessary to conclude that the *oxidized* state, which yields maximum rates of exchange, is the "energy-rich" or "charged" form of the carriers or is the primary determinant of an energy-rich intermediate ultimately responsible for the formation of ATP. This picture is thus analogous to other enzymatic oxidoreductions in which coupled synthesis of a new "high-energy" linkage occurs, as in the case of triose phosphate dehydrogenase. In both these reactions it is the *oxidative partial reaction* which is the energy-conserving phase of the over-all oxidoreduction.

These findings are not consistent with an alternative hypothesis (*3, 32*)—namely, that the *reduced* form of the carriers is the energy-rich form, which was based primarily on the finding that, in the absence of ADP, respiration of intact mitochondria ceases with the carriers largely in the reduced state (that is, combined with an inhibitor "I"). Addition of ADP caused the restoration of respiration with a shift toward the more oxidized form of the carriers in the steady state. Such properties are not dissimilar to those of the digitonin fragments, but they do not necessarily indicate the energy content of the so-called "inhibited" form of the respiratory carriers.

It is necessary to point out that there are some restrictions on conclusions drawn from experiments on the rate of the ATP-P_i^{32} exchange reaction. There are three phosphorylation sites in the respiratory chain. It is probable but not proved that all three participate in the observed ATP-P_i^{32} exchange reaction. The individual contribution of each phosphorylation site to the measured total ATP-P_i^{32} exchange rate is, however, still unknown, and it is conceivable that one of the three sites may contribute only a very small part of the observed exchange, of the order of a few percent. Obviously the dependence of this site on the oxidation-reduction state could not be determined satisfactorily by the experiments described. Furthermore, it is conceivable that the energy-rich or charged form of the carrier may be the oxidized state for only one or two sites and the reduced state for the other(s). From the statistical point of view, however, we can conclude that the oxidized state yields maximum rates of the ATP-P_i^{32} exchange and also ATP-ase activity.

With the availability of the purified terminal phosphotransferase enzyme, it is now feasible to approach in a direct manner the reconstruction of ATP-ase and ATP-P_i^{32} exchange activity. This approach is now being taken in current investigations in this laboratory and is expected to yield definite answers not only on the participation of the carriers in these reactions but on the important question of which oxidation state of the carrier is the energy-rich one.

A pattern of phosphorylating electron transport. The information obtained from study of the ATP-ADP exchange reaction and the oxidation-reduction dependence of the ATP-P_i^{32} exchange may now be formulated diagrammatically into a working hypothesis of the mechanism of phosphorylating electron transport, as shown in Figure 3, which illustrates the simplest possible form of a mechanism accounting for all the facts at hand.

The phosphorylation sites shown are those generally accepted (*2, 3*), although the evidence is as yet indirect and inferential. However, if the sites are correct in location and if no additional respiratory carriers participate in phosphorylating respiration beyond those shown, then it is interesting to note that the three coupled carrier pairs are separated from each other by single, noncoupled "connecting" carriers in a symmetrical manner. A "phosphate-collecting" enzyme could be added to this formulation to provide a common terminal reaction for formation of ATP from $X \sim P$, $Y \sim P$ and $Z \sim P$. This diagrammatic formulation is considered to be more realistic and informative than diagrams of respiratory carrier sequences without some mechanistic provision for the coupled phosphorylations which, after all, represent the whole purpose of the respiratory chain (*1, 2*). Furthermore, such a formulation demonstrates the thermodynamic necessity for having a multimembered respiratory chain, in order to quantize the liberation of respiratory energy efficiently into packets equivalent to the energy of hydrolysis of ATP.

Significance of digitonin fragments in active transport. Intact mitochondria are capable of accumulating electrolytes such as K^+, Na^+, H^+, and phosphate when oxidation and phosphorylation take place (see *33*). The digitonin fragments of the mitochondrial membrane have been found to contain a considerable amount of bound K^+, approximately 100 to 400 mμmole/mg of N. The behavior of this bound potassium has been investigated in this laboratory (*34*). It has been found that the bound K^+ is rapidly lost to the medium on aging the digitonin fragments. However the loss can be prevented if the fragments are actively oxi-

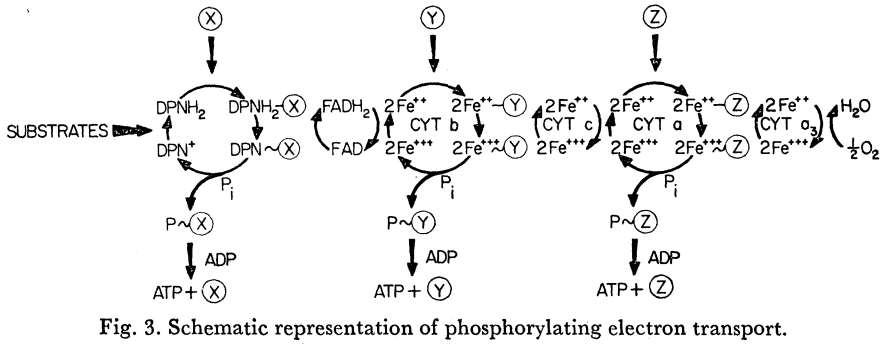

Fig. 3. Schematic representation of phosphorylating electron transport.

dizing β-hydroxybutyrate. Furthermore, active exchange of the K^+ bound to the mitochondrial membrane fragments coupled to respiration has been demonstrated with K^{42} as a tracer. It is inhibited by cyanide or dinitrophenol, demonstrating that the binding of the potassium to the digitonin fragments is linked to electron transport and phosphorylation mechanisms. However, ATP cannot substitute for respiration in causing binding of radioactive K^+. Since the preparations have relatively insignificant capacity to bind Na^+ under any conditions, this active potassium-binding mechanism of the mitochondrial membrane appears to be selective.

These experiments demonstrate that the digitonin fragments may provide an important lead for investigation of the molecular mechanisms involved in active transport; further work on identification of the binding sites for K^+ is in progress.

Conclusion

Although the membrane fragments are still highly organized, they are considerably less complex than intact mitochondria and thus relatively free of extraneous side reactions not relevant to oxidative phosphorylation and of structural "compartmentation" of pools of intermediates. With the information gained on these preparations, still less organized portions of the complex enzymatic machinery may soon be dissociated and studied separately, toward the ultimate goal of demonstrating the mechanism of oxidative phosphorylation.

These investigations are being carried out with full appreciation of the eventuality that such classical approaches to reconstruction applied to the highly integrated and structure-dependent respiratory chain and its energy-coupling mechanisms may never succeed to the same extent or in the same way as they have for other metabolic cycles which occur by interaction of essentially soluble enzymes, because of a possible necessity for special polymolecular arrangements to direct protein-protein collisions. For this reason, the problem of electron transport and oxidative phosphorylation represents a great challenge in the large and relatively uncharted area of "solid-state" enzymology.

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Retention of Radioactive Bone-Seekers

Calculations based on the power function cast doubt on the present concept of biological half-times.

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To calculate quantities pertaining to or derived from the biological retention patterns of radioactive isotopes, it is desirable to have direct measurements of retention over the entire period of interest. However, in the case of radioelements which show an affinity for bone, it is well known that the period during which retention is biologically important is usually very long and may cover the entire life span of man. The consequent paucity of data covering such extended periods of time requires that current predictions of retention and related quantities depend upon extrapolations based upon data taken over relatively short intervals.

Earlier attempts at such extrapolations were based upon the assumption that the retentions of bone-seeking radioelements decreased as first-order exponentials. Thus, a derived constant, the "biological half-life," is often used as a fundamental parameter of such retention phenomena, especially in calculations of permissible body burdens and permissible daily intakes of radioactive isotopes (*1, 2*).

Over the past several years evidence has accumulated to show that the concept of an exponential decrease in retention is altogether untenable when applied to bone-seekers. More specifically, these data show that the *over-all* retention patterns of many bone-seeking elements, when administered as soluble

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