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Respiratory-Energy Transformation*

ALBERT L. LEHNINGER

Department of Physiological Chemistry, The Johns Hopkins School of Medicine, Baltimore 5, Maryland

INTRODUCTION

THIS article summarizes the broad outlines of current knowledge on some enzymatic and biophysical aspects of the conversion of respiratory energy into phosphate-bond energy. There are many problems for the biophysicist in this area which not only are pertinent to electron transport and respiratory-chain phosphorylation but also have wide applicability to more-general problems of ultrastructure and arrangement of enzymes in biologically organized "solid-state" arrays. More-detailed treatments of the subject can be found in review articles.[1–5]

ENERGY CYCLE IN AEROBIC CELLS

In all cells, the energy-requiring or *endergonic* functions are driven by chemical energy liberated during the energy-yielding or *exergonic* degradation of foodstuff molecules. The major energy-requiring functions or activities of cells include (a) *biosynthesis* of complex molecules from simple ones when such reactions proceed in an environment thermodynamically unfavorable for synthesis, as in the formation of glycogen from glucose in an aqueous system, (b) *mechanical work*, as in muscular contraction, (c) *active transport* or accumulation of substances against gradients of chemical potential, (d) *transmission* or conduction phenomena, and (e) *bioluminescence*. In aerobic cells, oxidative degradation of foodstuffs is the source of chemical energy. In strictly anaerobic cells, molecular oxygen does not intervene but internal oxido-reductions of fermentative or anaerobic metabolic cycles (such as alcoholic fermentation of glucose) are the primary energy source to drive endergonic functions.

The energy cycle in nature (as well as the carbon cycle) is then completed by the conversion of radiant energy of sunlight into chemical energy during photosynthesis, making possible reduction of carbon dioxide back to the oxidation level of carbohydrate, in reactions discussed in the companion paper by Calvin (p. 147). Respiratory-energy transformation and photosynthesis thus constitute the two complementary mainsprings of biological energy.

CONCEPT OF PHOSPHATE-BOND ENERGY

The general medium of energy exchange between certain exergonic reactions of oxidative catabolism and the endergonic functions is the so-called "high-energy phosphate bond" of ATP.

Phosphate esters of cells can be classed into two major groups on the basis of the magnitude of the standard free energy of hydrolysis of the phosphate bond.[6–8] Simple phosphate esters, such as 3-phosphoglyceric acid, glucose-6-phosphoric acid, and α-glycerophosphoric acid, etc., undergo hydrolysis enzymatically or by acid or base catalysis, often at widely different rates, with free energies of hydrolysis ($\Delta F°$) of -0.8–-3.0 kcal/mole at 25° and *p*H 7. These are the so-called "low-energy phosphate compounds." On the other hand, enol phosphate esters (phosphopyruvic acid), guanidine phosphates (phosphocreatine), acyl phosphates (1,3-diphosphoglyceric acid), and pyrophosphates (ATP) are in the high-energy class and have a $\Delta F°$ for hydrolysis of from 6 to 15 kcal/mole under standard conditions. This division is arbitrary and exact values of these esters are now in a state of flux because $\Delta F°$ values for hydrolysis of ATP are currently undergoing substantial revision.[9]

The *free energy of hydrolysis* represents the difference in energy content of reactants and hydrolysis products under standard conditions. It is *not* equivalent, of course, to the true bond energy or the zero-point energy; actually, input of energy is required to break chemical bonds. It must be made clear also that there is no particular biological magic involved in the difference between high- and low-energy compounds. Actually, the major reason for the relatively high free energy of hydrolysis of ATP and other high-energy compounds is that the immediate hydrolysis products of these compounds undergo secondary, spontaneous reactions with high equilibrium constants, leading to thermodynamically more-stable forms, either through resonance stabilization or tautomeric rearrangements. A second contribution to the high free energy of hydrolysis, in the case of ATP and phosphopyruvate, for example, is the separation and randomization of the neighboring like charges in these molecules at physiological *p*H. The structural, resonance, and charge contributions to the high-energy nature of these compounds have been discussed by Hill and Morales.[10] Classically, the free energy of hydrolysis of ATP has been assumed to have the value 12 kcal/mole, on the basis of a calorimetrically observed ΔH of approximately this figure and the assumption of only a small entropy change. However, recent work on the enzymatic equilibria of ATP and glutamate and the highly refined heat measurements by the important new method of Benzinger[11] have revealed that the standard free-energy change at

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1*M* reactants corrected for ionization changes at *p*H 7.5 is about −8.4 kcal, and that the true ΔH for hydrolysis is about −4.8 kcal (see Burton[9] for most recent evaluation). The value of the free energy of the hydrolysis of the ATP bond (−8.4 kcal) now seems to be a valid one; it is thus substantially lower than the classical value of 12 kcal. It must be pointed out, however, that, *under physiological conditions of* pH, *and intracellular concentration ranges of phosphate, ADP and ATP,* the recalculated $\Delta F'$ of ATP is about −12.5 kcal/mole or approximately the classical textbook value.[9] There is room for a great deal of careful work on the measurement of biochemically useful thermodynamic factors through refined heat measurements, enzymatic equilibria, and calculation of free energies of formation.[12,13] The values given above for ATP may be altered further when the hydrolysis is considered in a physiological milieu because of the lack of information on activity coefficients of the various ionic species of the reaction components—in particular, the Mg complexes of ATP and ADP in the presence of the high ionic strength background of intracellular fluid. Also, it must be pointed out that free-energy data for ATP obviously are reckoned on a macroscopic, statistical basis. In the intact cell, ATP-linked reactions occur with reactants bound to proteins (as in actomyosin) under nonequilibrium conditions where energy changes and transfers at the *microscopic* level dictate direction and rate of reactions.

There may be good reasons for the evolutionary choice of phosphoric acid, rather than sulfuric acid, HCl, or a carboxylic acid, as the vehicle for biological energy transfer through enzymatic group-transfer reactions. In the first place, phosphate anion has itself a rather high resonance energy; however, this property is not unique to phosphate. Perhaps it is more important that its anhydrides and amides are kinetically (as opposed to thermodynamically) much more stable than esters or amides of HCl, H_2SO_4, or carboxylic acids. Thus, acetic anhydride in H_2O at *p*H 7.0 decomposes rapidly, whereas the anhydride pyrophosphoric acid at the same *p*H has a much greater half-life despite the fact that the free energy of hydrolysis of the two compounds is of the same order of magnitude. Phosphate anhydrides and amides may have been selected biologically not only for their resonance characteristics but also for their property of remaining relatively unreactive in an aqueous solution in the absence of suitable directing enzymes; less-stable derivatives would tend to react spontaneously and thus not be subject to enzymatic control.

COMMON INTERMEDIATE PRINCIPLE IN UTILIZATION OF ATP ENERGY

Apart from certain photochemical and fluorescence phenomena, there is, in general, only one way in which energy can be transferred from one chemical reaction to another. The two reactions must be consecutive and *must have a common intermediate.*

The synthesis of sucrose provides a classical biochemical example.[14] The reaction

$$\text{glucose}+\text{fructose} \rightleftarrows \text{sucrose}+H_2O \quad (\Delta F=+5000) \qquad (1)$$

is strongly *endergonic.* Sugar-cane juice is 0.5*M* in sucrose. The concentrations of free glucose and fructose would have to be enormously greater than that of sucrose, and the water concentration low, in order for the reaction to proceed spontaneously in the sugar cane. This is not the case and sucrose is not made in this way. Enzyme studies show that it is formed at the expense of the hydrolysis of ATP in a coupled reaction. The driving reaction is the strongly *exergonic* hydrolysis of ATP:

$$\text{ATP}+H_2O \rightarrow \text{ADP}+\text{P} \quad (\Delta F=-12\,000). \qquad (2)$$

Reactions (1) and (2) are the partial thermodynamic reactions in sucrose synthesis. The actual enzymatic mechanism is the following:

$$\text{ATP}+\text{glucose} \text{ - - } \rightarrow \text{glucose}-1-P+\text{ADP} \quad (\Delta F=-7000 \text{ cal}) \qquad (3)$$

$$\text{glucose}-1-\text{P}+\text{fructose} \rightarrow \text{sucrose}+H_3PO_4 \quad (\Delta F=\sim 0 \text{ cal}). \qquad (4)$$

The over-all ΔF is −7000 cal and synthesis of sucrose proceeds spontaneously in the presence of the enzymes. In this sequence, glucose has been raised to the energy level of a glycoside by phosphorylation; glucose−1−P is now the common intermediate of two consecutive reactions which together have a negative net free-energy change, but in which a product of the first, *exergonic,* reaction has sufficient "group potential" in the form of the glycosyl phosphate to drive the second, *endergonic,* reaction to form sucrose.

In principle, this pattern underlies all endergonic biosynthetic reactions, such as formation of glycogen from glucose, proteins from amino acids, and complex lipids from fatty acids. ATP and an intervening phosphate-group transfer reaction represent the mode of energy transfer.

COMMON INTERMEDIATE PRINCIPLE IN BIOLOGICAL CONVERSION OF OXIDATIVE ENERGY INTO PHOSPHATE-BOND ENERGY

From the foregoing, the breakdown of ATP to ADP and P is necessary to drive endergonic functions. The ATP then is regenerated continuously from ADP and P at the expense of catabolic reactions, primarily those of oxidation-reduction reactions, in order to complete the energy cycle.

The regeneration of ATP from ADP and P is very largely a matter of the transfer of the energy liberated in certain highly exergonic oxidations of metabolism to a reaction resynthesizing ATP from ADP and P. Such

respiratory energy conversion also occurs by the principle of the common intermediate.

The best-understood example for this discussion is the enzymatic oxidation of glyceraldehyde−3−phosphate to 3−phosphoglyceric acid by DPN which occurs in the anaerobic breakdown of glucose to lactate, to which is coupled the synthesis of ATP from ADP and phosphate. The over-all reaction first can be broken down into two partial thermodynamic reactions. The first, the oxidation of an aldehyde to a carboxylic acid, is highly exergonic, and the second, formation of ATP, highly endergonic.

$$R-\underset{\underset{O}{\|}}{CH}+DPN_{ox} \rightleftarrows R-\underset{\underset{O}{\|}}{C}-O^- + DPN_{red} + H^+$$

$$(\Delta F + -16 \text{ kcal}) \quad (5)$$

$$P + ADP \rightarrow ATP + H_2O \ (\Delta F = +12 \text{ kcal}). \quad (6)$$

The energy liberated in the oxidation of an aldehyde to a carboxylic acid is recovered as ATP, with energy left to spare, the net driving force being some 4 kcal, as is shown in the following over-all reaction:

$$\text{glyceraldehyde-3-phosphate} + P_i + DPN_{ox} \rightleftarrows \text{1,3-diphosphoglycerate} + DPN_{red} + H^+, \quad (7)$$

$$\text{1,3-diphosphoglycerate} + ADP \rightleftarrows \text{3-phosphoglycerate} + ATP. \quad (8)$$

It is seen that the oxido-reduction of reaction (7) results in formation of 1,3-diphosphoglycerate, a high-energy mixed anhydride of a carboxylic acid and phosphoric acid. This is the intermediate which is common in the two reactions and acts as a high-energy phosphate donor in the formation of ATP [reaction (8)]. Although this reaction is well understood, it accounts for only a very small fraction of ATP synthesis in aerobic cells, but it is an extremely important model of how the great bulk of ATP may be formed during respiration.

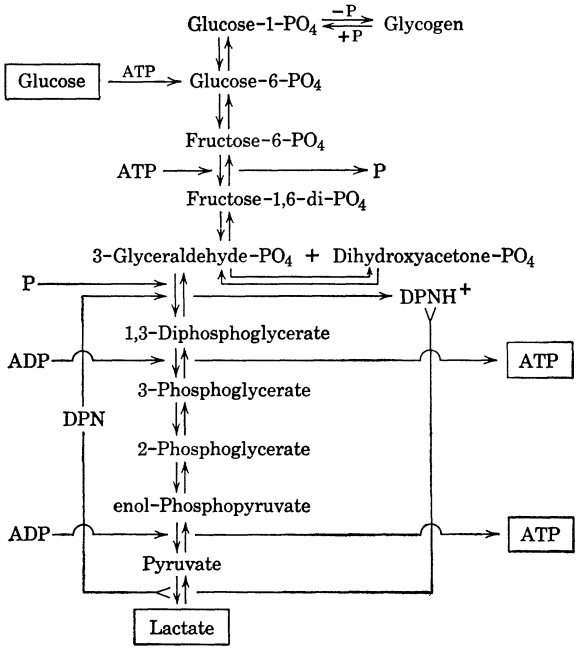


FIG. 1. The mechanism of anaerobic glycolysis.

RELATIVE CONTRIBUTION OF FERMENTATIVE AND OXIDATIVE REACTIONS TO ATP SYNTHESIS

Figures 1 and 2 show the outlines of the mechanism of anaerobic glycolysis and the Krebs citric-acid cycle, the main aerobic phase of carbohydrate oxidation. However, the amount of energy liberated from a glucose molecule during glycolysis to lactate ($\Delta F = -49\,700$ cal) represents a few percent only of the total energy liberated by complete oxidation of glucose to carbon dioxide and water ($\Delta F = -686\,000$ cal/mole). However, this small yield of energy in the anaerobic phase is quite efficiently recovered by the two phosphorylations occurring in the cycle, and from the following equations it is seen that some 50% of the energy liberated when 2 moles of lactate are formed from 1 of glucose is recovered in the form of the phosphate-bond energy of two moles of ATP.

Over-all reaction:

$$\text{glucose} + 2ADP + 2P \rightarrow 2 \text{ lactate} + 2ATP \quad (\Delta F = -25\,700 \text{ cal}).$$

Partial reactions:

(a) glucose → 2 lactate ($\Delta F = -49\,700$ cal)

(b) $2ADP + 2P \rightarrow 2ATP$ ($\Delta F = +24\,000$ cal).

However, the real energetic mainspring of aerobic cells, regenerating 90% or more of the ATP required, is the oxidative phase of catabolism.

Despite the absence of phosphorylated intermediates in the Krebs cycle as it is depicted in Fig. 2, it is known now that a very large number of phosphorylations of ADP accompany such aerobic oxidations, approximately 36 per mole of glucose oxidized. In addition, the efficiency of energy recovery during this aerobic phase is even higher than in the anaerobic phase, approaching some 70%.

OXIDATIVE PHOSPHORYLATION (SYNONYMS: AEROBIC PHOSPHORYLATION, RESPIRATORY-CHAIN PHOSPHORYLATION)

This term denotes the coupled phosphorylations of ADP which occur simultaneously with certain organized oxido-reduction reactions in the aerobic phase of metabolism. The biological significance of phosphorylations causally coupled to oxidations with molecular oxygen first was recognized by Kalckar in 1937, but the quantitative importance of these in cellular metabolism was not appreciated fully until the penetrating studies of Belitser in Russia in 1939–1941, which were

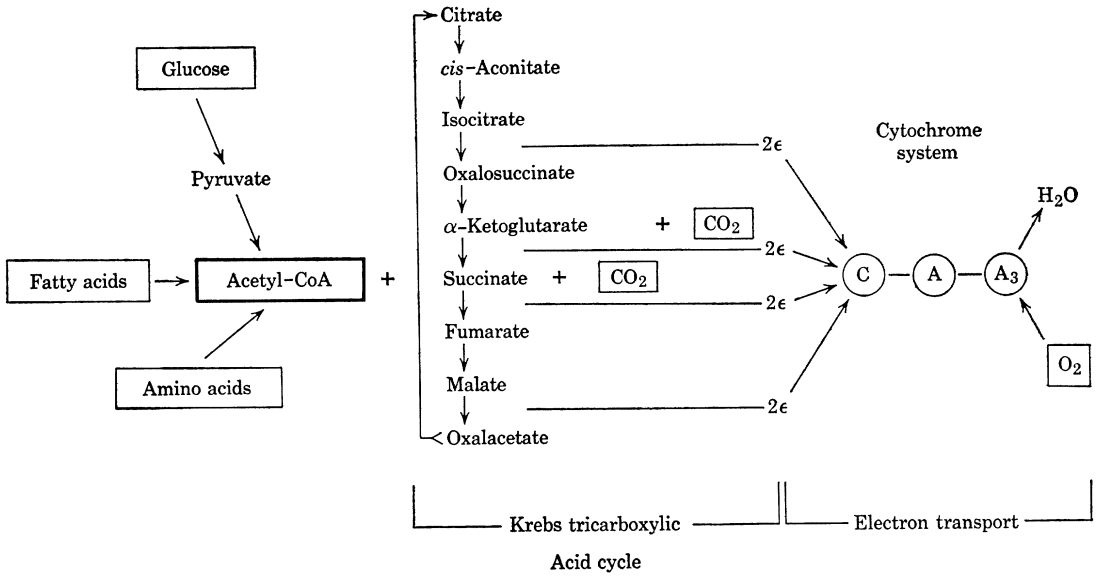


FIG. 2. The Krebs tricarboxylic-acid cycle.

followed during and after World War II by a number of independent and confirmatory studies by Ochoa, Cori, Hunter, and others (cf. references 4, 2, 3). These studies clearly demonstrated that, for the passage of every pair of electrons from a substrate of the Krebs citric-acid cycle to molecular oxygen during aerobic metabolism, an average of three moles of ATP were synthesized from ADP and P_i; i.e., the P:O ratio (moles P_1 taken up per atom oxygen used) was 3.0. Thus, the over-all equation for the oxidation of pyruvate to carbon dioxide and water as it occurs in the Krebs cycle was found to be

$$CH_3COCOOH+15P+15ADP+5O \rightarrow 3CO_2+15ATP+2H_2O. \quad (9)$$

This reaction may be broken down as follows:

$$CH_3COCOOH+5O \rightarrow 3CO_2+2H_2O \quad (\Delta F=-273 \text{ kcal}), \quad (10)$$

$$15P+15ADP \rightarrow 15ATP \quad (\Delta F=12\times 15) \quad (\Delta F=+12\times 15=180 \text{ kcal}), \quad (11)$$

$$\text{efficiency}=180\times 100/273=67\%.$$

GENERAL PROPERTIES OF OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation classically has been recognized as an unstable and evanescent phenomenon which occurs only with relatively "native" and unfractionated extracts or homogenates of tissues. Aging of such extracts, or application of some of the common procedures for separation or purification of enzymes, quickly inactivated the coupled phosphorylation of ADP, whereas the purely oxidative activity of Krebs-cycle reactions remained rather stable. The findings thus suggested that coupled phosphorylation of ADP is not an obligatory accompaniment of oxidation, that it may be inactivated or uncoupled. The great lability of the coupling mechanisms to even relatively mild separation procedures has been a major technical obstacle to a fuller understanding of the mechanism of oxidative phosphorylation.

The great lability just described is explained in part by the fact that oxidative phosphorylation, along with the organized Krebs-cycle reactions and fatty-acid oxidation cycle, has been found to occur in rather highly organized subcellular structures, *the mitochondria*, as was first shown by Kennedy and Lehninger,[15] and independently by Schneider (cf. Ernster and Lindberg[16]), a finding which very quickly was confirmed and extended in many other laboratories.[17] Mitochondria isolated by the well-known sucrose procedure have become the standard study object in this field. It is important that mitochondria from many different cell types—plant, animal and microbial—show the respiratory-chain phosphorylation, thus providing a sound enzymatic basis for the suggestion by Claude that mitochondria are the power plants of the cell.

The mitochondria are almost completely self-contained units capable of carrying out both respiration and phosphorylation. It is necessary only to supplement a suspension of mitochondria with a substrate such as pyruvate, a trace amount of a 4-carbon catalyst such as fumarate or oxalacetate as substrates or fuel for the cycle, inorganic phosphate and ADP, and also Mg^{++}, which help to preserve mitochondrial structure. All of the necessary coenzymes and other co-factors are contained in the mitochondrial structure in such an organized manner that the complex cycle oxidations are smooth and complete with no obvious or conspicuous bottle-

necks, indicating the high degree of enzymatic and morphological organization in the mitochondria.

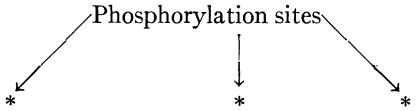
The studies on oxidative phosphorylation have revealed, however, that relatively intact mitochondrial structure is necessary; simple procedures, such as freezing and thawing, treatment with detergents, exposure to hypotonic or strongly hypertonic agents, or solution, all cause uncoupling of the phosphorylation.[4]

Another important property of oxidative phosphorylation is that certain inhibitors in relatively minute concentrations are capable of uncoupling phosphorylation; that is, in their presence, no phosphorylation of ADP takes place, but oxidation proceeds nearly normally. These uncoupling agents include 2,4-dinitrophenol, as well as other nitro- and halo-phenols, the antibiotic gramicidin, and the anticoagulant Dicumarol, in addition to a number of oxidation-reduction dyes such as methylene blue. These agents have become extremely important tools for the elucidation of the character and sequence of the intermediate reactions of oxidative phosphorylation.[18]

LOCALIZATION OF PHOSPHORYLATION SITES IN RESPIRATORY CHAIN[1-4]

To explain the occurrence of these oxidative phosphorylations, it was suggested at first that the intermediates of the Krebs cycle actually might occur in the form of phosphorylated derivatives rather than the free acids, analogs to the glycolytic reactions. Such phosphorylated intermediates never have been found. In 1940, however, Belitser postulated that the aerobic phosphorylations did not occur actually in the Krebs-cycle reactions proper, but rather during the phenomenon of electron transport from primary dehydrogenase to molecular oxygen via the known electron carriers making up the respiratory chain, such as diphosphopyridine nucleotide, flavoprotein, and the cytochromes. Belitser pointed out that the ΔF for the transfer of a pair of electrons from reduced diphosphopyridine nucleotide to molecular oxygen is approximately $-55\,000$ cal/mole. Since formation of one mole of ATP requires the input of 12 000 cal, it is evident that rather more than 4 moles of ATP theoretically could be generated during transport of each pair of electrons to oxygen, providing suitable enzymatic coupling mechanisms existed. As logical as the Belitser hypothesis appeared, experimental proof for this pattern was difficult to muster, and it was not until 1949–1951 that direct proof was adduced.[19,20] Highly purified, chemically reduced diphosphopyridine nucleotide was used as the actual substrate for the oxidation, in suspension of rat-liver mitochondria which had been pretreated in a hypotonic medium to produce sufficient alteration in permeability of the mitochondrial membrane so as to admit the reduced pyridine nucleotide to the oxidative centers within the mitochondria. Oxidation of reduced DPN via the known components of the respiratory chain, including the flavoprotein and cytochromes, culminated in oxygen uptake and coupled uptake of inorganic phosphate and the formation of ATP, with P:O ratios approaching the value 3.0. Oxidative phosphorylation thus occurs along the respiratory chain and may proceed in the absence of Krebs-cycle intermediates.

Similar experiments with pure cytochrome-*c*, used as either electron donor or acceptor, make possible further localization of the phosphorylations along the respiratory chain. Thus, passage of electrons from reduced DPN to cytochrome-*c* caused the formation of two moles of ATP.[4] Also, despite earlier experiments of Slater to the contrary, it finally was possible to demonstrate conclusively that the passage of a pair of electrons from cytochrome-*c* to oxygen yields a single phosphorylation.[21] More-recent spectroscopic investigations of Chance have confirmed these gross locations of phosphorylation sites.[1] Thermodynamic considerations of the known oxidation-reduction potentials of the carrier (Fig. 3), together with enzymatic and spectroscopic methods, suggest that one of the phosphorylations occurs between DPN and flavoprotein, the second between cytochrome-*b* and cytochrome-*c*. The site of the third phosphorylation is less certain: Chance suggests it lies between cytochrome-*c* and -*a*,[1] but other considerations indicate that it occurs between cytochrome-*a* and -a_3. These findings are summarized as follows:



DPN→flavoprotein→cyt *b*→cyt *c*→cyt *a*→cyt a_3→O

The over-all reaction of the respiratory chain is thus

$$DPN_{red}+O+3P+3ADP \rightarrow DPN_{ox}+H_2O+3ATP, \quad (12)$$

and may be considered as the partial reaction

$$DPN_{red}+O \rightarrow DPN_{ox}+H_2O \quad (\Delta F=-55\,000 \text{ cal}) \quad (13)$$

$$3P+3ADP \rightarrow 3ATP+H_2O \quad (\Delta F=+36\,000 \text{ cal}). \quad (14)$$

From these results, it can be seen at once that the multimembered respiratory chain must represent a device for breaking up the large chunks of oxidative energy into smaller, biologically useful packets of approximately 12 kcal to accommodate the dimensions of the energetic currency of the cell—namely, the free energy of formation of ATP from ADP and phosphate. If all 55 kcal were released in one chemical-reaction step, even if coupled to a phosphorylation mechanism, only one ATP could be generated in this fashion.

MECHANISM OF RESPIRATORY-CHAIN PHOSPHORYLATION

The mechanism of oxidative phosphorylation, in enzymatic and chemical terms, remains as one of the

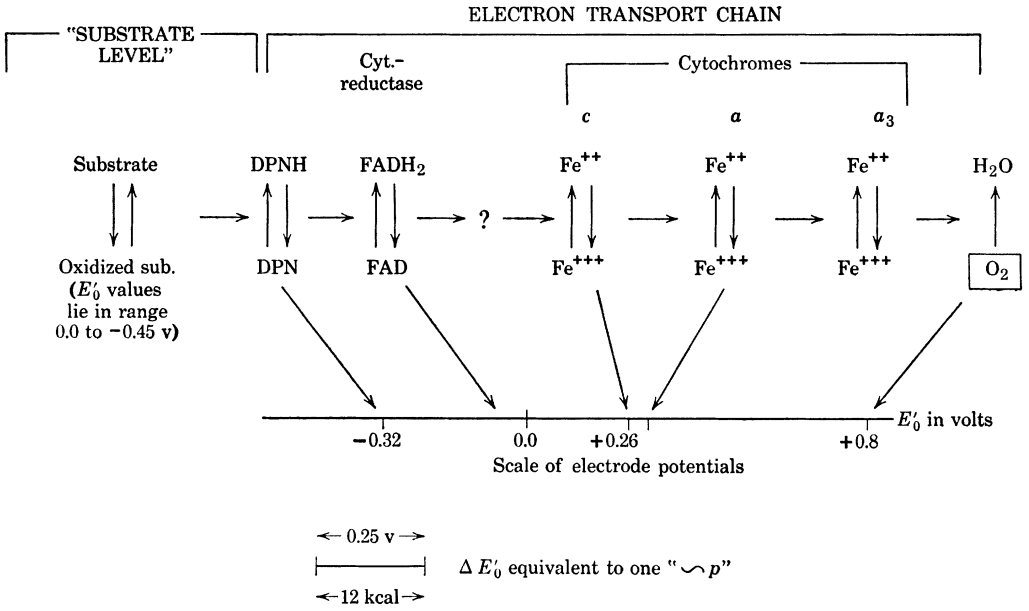


FIG. 3. The thermodynamic relationships in the respiratory chain. Electrons normally flow from substrates at the left to oxygen at the right. The approximate oxidation-reduction potentials (pH = 7.0) of the carriers are shown on the scale. The free energy of hydrolysis of ATP of about 12 kcal is equivalent to the maximum free-energy change as a pair of electrons flow over a 0.25-v span, as is shown in the yardstick, from the relation $F = nf\Delta E$ [from A. L. Lehninger in *Harvey Lectures* (Academic Press, Inc., New York, 1953–1954), Vol. IL, p. 176].

most conspicuous and challenging unsolved problems of contemporary biochemistry. The immediate situation is that the classical approach of separation and isolation of enzymes making up a complex metabolic sequence, followed by *in vitro* reconstruction of the enzyme system, simply has not been possible to date, not only owing to the fact that the process is very labile but also because there is a requirement for geometrical organization of the many enzymes concerned in the characteristic morphology of the mitochondria. In addition, there is the fundamental dilemma of how a set of respiratory carriers of even moderate substrate specificity can catalyze both nonphosphorylating and phosphorylating electron transport, particularly in view of the fact that highly purified respiratory carriers, such as cytochrome-*c*, DPN, cytochrome-*c* reductase, and other respiratory carriers, show absolutely no ability to phosphorylate when tested in highly purified form in the test tube [cf. reference 3].

To facilitate discussion of the problem of the mechanism of oxidative phosphorylation and some recent successes in unraveling part of the coupling mechanism, it is best to consider first the general mechanisms which have been proposed to account for energy coupling in the respiratory chain, which again make use of the principle of the common intermediate described below.

The following mechanism is one we have proposed and for which considerable evidence has accumulated.[3,22,5] It incorporates ideas originally proposed by Lipmann.[8] Similar mechanisms have been discussed since by Lardy, Hunter, Slater, Chance, and others (see references 2, 1, 23). In skeleton form, it is

$$\text{carrier}_{red} + X + \text{oxidant} \rightarrow \text{carrier}_{ox} \sim X + \text{reductant}, \quad (15)$$

$$\text{carrier}_{ox} \sim X + P_i \rightleftarrows \text{carrier} + P \sim X, \quad (16)$$

$$P \sim X + ADP \rightleftarrows ATP + X. \quad (17)$$

In this scheme, the substance X is the vehicle of energy coupling and forms a high-energy compound with the carrier during the course of transfer of electrons from the reduced carrier to the next in the chain (designated as "oxidant"). $\text{Carrier}_{ox} \sim X$ then undergoes phosphorolysis to form $P \sim X$ (a high-energy compound, presumably a phosphoenzyme) which in turn can donate its phosphate to ADP. Thus, carrier $\sim X$ is a common intermediate shared by the exergonic oxido-reduction of reaction (1) and by the endergonic reaction leading to formation of ATP in reactions (2) and (3). The energy liberated in the oxido-reduction thus is utilized to cause the formation of ATP. It is possible that phosphate interacts with the carrier directly to form a high-energy carrier$\sim$phosphate compound; however, for a number of reasons, it appears more likely that some compound other than phosphate is the first reactant with the carrier in the coupling mechanism. This hypothesis is written in skeleton form to indicate in the simplest way the principle of the common intermediate. It is possible and likely, however, that there are additional intermediate reactions between reactions (1) and (2) and also between (2) and (3).

This sequence now may be visualized as occurring

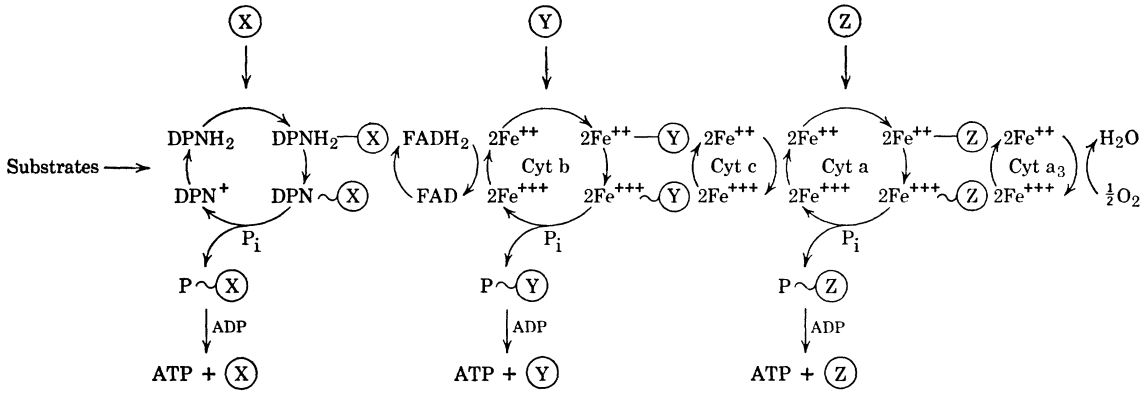


FIG. 4. A diagrammatic conception of the phosphorylation-coupled respiratory chain. This diagram is based on the coupling mechanism described in the text and assumes that the coupled carriers are DPN, cytochrome-*b*, and cytochrome-*a*. [from A. L. Lehninger, C. L. Wadkins, C. Cooper, T. M. Devlin, and J. L. Gamble, Jr., Science **128**, 450 (1958)].

with three different carrier pairs in the respiratory chain, as is shown in the diagram (Fig. 4) to account for the three different phosphorylations occurring along the chain.

The postulated skeleton-reaction mechanism indicates two possible approaches to unraveling the mechanism of energy coupling in oxidative phosphorylation. The first and seemingly more direct approach is to carry out investigations to isolate and to identify the molecular species of the respiratory carrier which exists in the energy-charged form—in short, the structure and enzymatic reactions of carrier ~X or its analogs. However, not all of the known respiratory carriers have been obtained so far in purified soluble form suitable for examination of possible complexes of this kind and those which have been isolated to date have shown no propensity to phosphorylate in the test tube in simple systems. Furthermore, there now is increasing evidence that additional factors, such as vitamin K_1, α-tocopherol, quinones, copper, nonheme iron, and other substances capable of undergoing reversible oxidation-reduction changes, may be members of the respiratory chain in addition to the classically accepted flavoprotein and cytochromes, but the site of these in the chain is still quite uncertain. Direct identification of the coupled carriers as a first step to determining the identity of X also is made difficult by the probability that a coupled form such as carrier ~X reasonably could be expected to be rather labile. However, the spectroscopic studies of the kinetics of interaction of the carriers in the chain carried out by Chance in intact mitochondria provide some important landmarks for such efforts.[1]

There is another approach to the mechanism of oxidative phosphorylation, which sometimes is called the "back-door approach," and which we have chosen to take in our recent investigations.[5,18,24] The back-door approach starts with the end product of oxidative phosphorylation, namely ATP, and works back through the partial reactions to the carrier level. Our recent approaches to the mechanism of oxidative phosphorylation have been made possible by two developments. The first was our finding, in 1955, that phosphorylating submitochondrial fragments can be prepared from rat-liver mitochondria by the action of digitonin. These fragments are very much smaller than mitochondria but still retain phosphorylative activity. They are not simply miniature mitochondria since they no longer are capable of the Krebs-cycle reactions or of fatty-acid oxidation. However, these fragments, which are believed to be derived from the mitochondrial membrane, contain more or less intact respiratory chains together with the enzymatic factors responsible for energy coupling. Study of coupling in these so-called "digitonin fragments" is not complicated by the morphological compartmentation and permeability effects seen in intact mitochondria, which also catalyze many enzymatic reactions which are extraneous to oxidative phosphorylation.[25–31,22,5]

The second development is the finding that these particles catalyze two different isotopic exchange reactions of ATP which occur in the absence of electron transfers. The first is the ATP-P_i^{32} exchange, in which labeled P_i^{32} is rapidly incorporated into the terminal phosphate group of ATP.[22] The second is the so-called ATP-ADP exchange, in which P^{32}- or C^{14}-labeled ADP is incorporated bodily into ATP.[18] Both of the reactions in fresh fragments are completely inhibited by the classical uncoupling agent DNP, indicating their relationship to oxidative phosphorylation and distinguishing them from many other similar exchange reactions of ATP which have nothing to do with oxidative phosphorylation.

Close study of the interrelationship of these exchange reactions has revealed that the ATP-ADP exchange reaction is in reality the terminal reaction of oxidative phosphorylation

$$P\sim X + ADP \rightleftarrows ATP + X,$$

and is itself an intermediate step in the ATP-P_i^{32} exchange. Since phosphate is not involved in the ADP

exchange, but ADP is an obligatory component of the P_i^{32} exchange, the sequence of the two terminal reactions of oxidative phosphorylation is established as

$$R\sim X+P_i \rightleftarrows P\sim X+R$$
$$P\sim X+ADP \rightleftarrows ATP+X$$

where R may be an electron-carrier molecule. Further, it has been found that the terminal reaction is not itself inherently sensitive to DNP but that sensitivity to DNP is conferred on it because it is in equilibrium with the preceding reaction, which is DNP-sensitive.[5,18]

These experiments have led very recently to the separation of the enzyme catalyzing the ATP-ADP exchange in soluble form from the mitochondrial fragments. It has been purified by chromatography on cellulose derivatives.[5] The nature of the active site participating in formation of the phospho-enzyme intermediate is now under investigation.

With this terminal enzyme in hand as a foundation for reconstruction approaches, we now propose to work nearer the carrier level and hope to soon obtain definitive information on the oxidation-reduction state of the energy-rich form of the carriers[1,5,32] which drives the phosphorylation, as well as the identification of the three coupled carriers in the chain.

RESPIRATORY-ENZYME ASSEMBLIES AND THE STRUCTURE OF THE MITOCHONDRION

Consider now oxidative phosphorylation in the context of its natural habitat—namely, the mitochondrion.

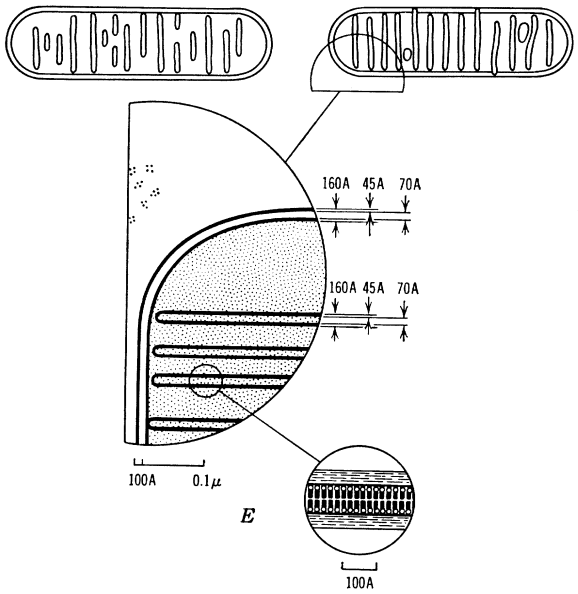


FIG. 5. Diagrammatic representation of the structure of mitochondrion according to Sjöstrand.[33] Note the double-layer character of outer membrane and the transverse "cristae." The magnified cross-section of the membrane suggests two protein monolayers separated by an oriented double layer of lipid molecules [from F. S. Sjöstrand in *Fine Structure of Cells—Symposium, Eighth Congress on Cellular Biology, Leyden, 1954* (Interscience Publishers, Inc., New York, 1955), pp. 16, 222].

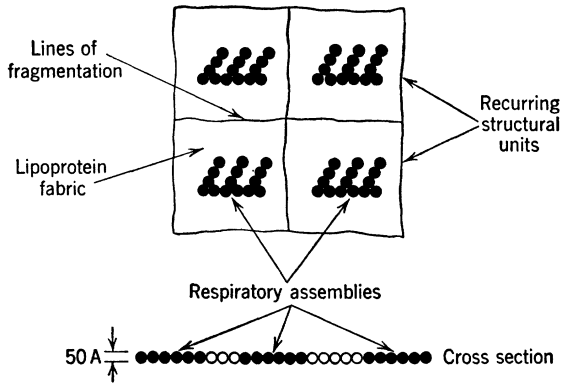


FIG. 6. Respiratory-enzyme assemblies in recurring units of mitochondrial membrane. The assembly consists of 6 electron-carrier protein molecules, supplemented by three sets of coupling enzymes, each consisting of three proteins as an approximation. This is a simplified representation since there may be several flavoprotein molecules per mole of cytochrome-*a* and several DPN-dehydrogenase molecules[1] [from A. L. Lehninger, C. L. Wadkins, C. Cooper, T. M. Devlin, and J. L. Gamble, Jr., Science **128**, 450 (1958)].

The structural details of mitochondria have been visualized by electron microscopy in thin sections of intact tissues in the laboratories of Sjöstrand, Palade, and others.[33] Although there are some differences in interpretation of electron micrographs of these organelles, the diagrammatic representation of Sjöstrand (Fig. 5) shows that the mitochondrion is surrounded by an outer double-layered membrane and also contains double-layered lamellar structures across the lumen called the "cristae" by Palade. These membranes, which are easily separable into fragmented form, are rich in lipid, containing some 30 to 40% which is mostly phospholipid. Sjöstrand has pointed out, as shown in his diagram, that the double-layered membranes correspond approximately to two monolayers of protein molecules separated by a double layer of oriented lipid molecules in the fashion suggested, and a similar arrangement in the cristae. These dimensions are constant in the mitochondria of all cell types examined.[33] The inner matrix of the mitochondrion contains soluble proteins and enzymes, electrolytes, and nucleotides which are released rather easily into the medium by mechanical or by osmotic rupture of the membranes. The results of many investigators suggest that most of the substrate-level enzymes of the Krebs cycle and the fatty-acid oxidation cycle are present either in the lumen or in the intercristal space. They are released easily in soluble form after damage of the membrane. On the other hand, it has been a universal finding that the enzymes involved in electron transport and coupled oxidative phosphorylation are present entirely in the insoluble membranes in organized "solid-state" systems; the separate enzymes of this system are not dissociated easily in truly soluble form.[5,16,34–36]

In phosphorylating subfragments of the mitochondrial membranes, the several electron carriers making

up the respiratory chain occur in approximately equimolar ratios,[29] suggesting that each respiratory "assembly" consists of the six (or more) carrier molecules arranged in contiguous manner, presumably adjacent to each other. These are supplemented at three points along the chain by three sets of two or three (or more) auxiliary enzyme proteins which are necessary for the energy-coupling process, as in the coupling mechanism postulated. Such an arrangement thus would form a complete respiratory-phosphorylating multienzyme assembly, as is shown in Fig. 6.

The question arises as to the distribution of such respiratory assemblies along the membrane. We have subjected sonically-prepared fragments of the mitochondrial membrane to differential ultracentrifugation and have obtained a wide spectrum of particle sizes. The relative distribution of respiratory carriers in these particles of widely different sedimentation rate has been examined with the finding that, no matter what the size of the fragment, the ratio of the respiratory carriers to total protein was always constant, and the ratio of carriers to each other was relatively constant also. Phosphorylation activity is preserved in all fractions.[30] This finding indicates that the respiratory carriers are distributed more or less evenly along the membrane, and that fragmentation of the membrane produces pieces of different size made up of multiples of a basically recurring unit, as is indicated in the diagram, where each recurring unit contains a complete and organized respiratory assembly.[5]

Calculations involving the known extinction coefficients of some of the respiratory carriers such as cytochrome-*c* indicate that, in the membranes of a single rat-liver mitochondrion, there may be several thousand such respiratory assemblies. Still further analyses of this kind make it possible to estimate the total fraction of the mitochondrial membrane substance contributed by catalytically active proteins of these assemblies (assuming twelve protein molecules per assembly, each having a molecular weight of 10^6) to be as high as 20% and possibly higher. From such considerations, it can be calculated that the average particle weight of a single respiratory assembly, together with its "filler" protein and lipid, may be 7×10^{-7} approximately.[37]

From such considerations, it is clear that the mitochondrial membrane is not simply a "dead wall," but rather a highly complex fabric in which are embedded the respiratory assemblies in a highly oriented and purposeful manner. These respiratory assemblies thus represent the very warp and woof of the membrane fabric.

MECHANICAL PROPERTIES OF MITOCHONDRIAL MEMBRANE

The mitochondrial membrane is not only the site of the highly organized respiratory assemblies, but it is also a structure capable of characteristic and selective permeability and active-transport phenomena. By optical measurements, it has been observed that the permeability may be altered by the ambient concentrations of ATP, magnesium, sulfhydryl reagents, and inorganic phosphate. In addition, we have found that the permeability of the mitochondrial membrane may be changed very drastically by addition of minute concentrations of the thyroid hormone, which causes rapid swelling and increased permeability to sucrose and other small molecules.[38–40] The mitochondrial membrane is capable of quite drastic extension and swelling under certain circumstances; it may increase in volume as much as several fold without actual rupture of the membranes (cf. Werkheiser and Bartley[41]). Phase contrast films of mitochondria in an intact cell show that they have remarkable plasticity of shape, and undergo rhythmic swelling and contraction as they wander through the canaliculi of the cytoplasm. The contractile properties of the mitochondrial membrane also can be observed *in vitro* when adenosine triphosphate is added to suspensions of heart mitochondria or when phosphorylating respiration is instituted. These agencies cause a rapid contraction of the mitochondrial membrane with the extrusion of water, and it has been postulated that the mitochondria, at least in specialized tissue such as the kidney, are active in water transport.[16,42] The contractility of the mitochondrial membrane thus may resemble the configurational changes in the myosin molecule similarly coupled to ATP energy. The number of water molecules extruded from mitochondria is out of all proportion to the number of molecules of ATP formed or utilized.

Very recently, we found that the permeability and contractility of the mitochondrial membrane are conditioned by the oxidation state of the respiratory carrier enzymes present in the membrane. Thyroxine and inorganic phosphate, which are potent swelling agents of mitochondria, increasing the permeability of the membrane, have this action only when the respiratory carriers are in the *oxidized* state. The action of these compounds results in a relaxation of the membrane and in increased permeability to certain test substances such as sucrose. On the other hand, when the respiratory carriers are maintained in the fully *reduced* state, the permeability of the mitochondrial membrane cannot be changed by addition of phosphate or thyroid hormone.[39,40]

The mitochondrial respiratory assemblies thus not only are the site of phosphorylating oxidation and major building blocks of the membrane, but also are controlling factors of rather dramatic mechanical and configurational changes which govern the rate of entry of essential metabolites such as substrates and inorganic phosphate, as well as the exit of respiration products such as H_2O, ATP, carbon dioxide, etc. The membrane-bound respiratory assembly thus becomes an example of a "mechanoenzyme" system, to use

Engelhardt's term for myosin. Indeed, the mitochondrial membrane has many characteristics resembling those of myosin, such as ATP-ase activity, DNP-stimulation, striking ionic strength relationships, as well as the configurational changes.

SOME BIOPHYSICAL PROBLEMS ASSOCIATED WITH RESPIRATORY-ENERGY CONVERSIONS AND "SOLID-STATE ENZYMOLOGY"

Most well-known enzyme systems, such as the citric-acid cycle and glycolytic cycle, for example, involve low molecular-weight, rapidly diffusible intermediate molecules like di- and tri-carboxylic acids or phosphorylated sugars, as shuttles between relatively slowly diffusing enzyme proteins in an alternating manner. The respiratory-chain system, however, differs strikingly; it involves protein-protein interactions exclusively without low molecular-weight intermediates (Fig. 7). Since the rate of diffusion of protein molecules is extremely low as compared with that of simple sugars, amino acids, etc., a multienzyme sequence of a dozen consecutive reactions, each of which is brought about through protein-protein collisions alone, can be expected to occur at very low rates only if there were no means to direct collisions. In fact, it can be calculated readily that the known high rates of respiration of cells could not occur if all of the enzymes involved were distributed equally in free solution within the volume of the cell.[43] The assembly of the individual proteins making up this system into a solid-state array, as in the mitochondrial membrane, provides a molecular adaptation to overcome this kinetic problem. A variety of questions arises, however.

For one thing, do ordinary mass-law and ideal-collision considerations govern the behavior of protein molecules in such a "solid-state" array? The question is important and underlies all attempts to analyze reaction kinetics and reaction mechanisms in solid-state enzyme systems by the application of Michaelis-Menten principles and other kinetic parameters. Chance has commented on this problem.[1]

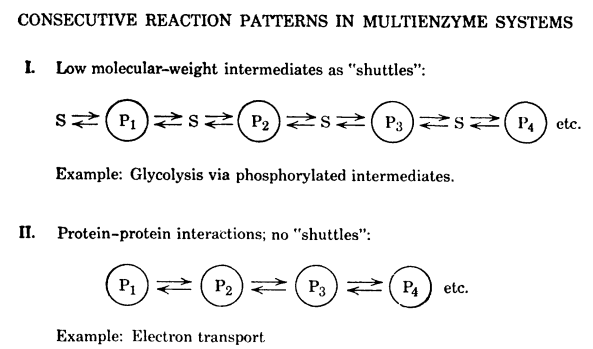


FIG. 7. Diffusion rates as factors in molecular interactions in multienzyme systems. Low molecular-weight substrate (product) molecules S, S_2, etc., have high-diffusion coefficients and may "shuttle" between high molecular-weight enzyme proteins; in the respiratory chain, slow-moving proteins must interact.

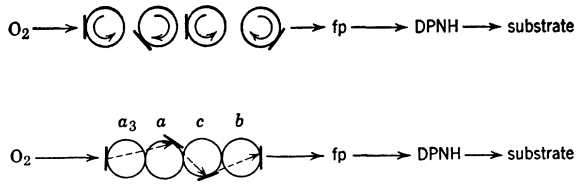


FIG. 8. Modes of electron transfer along chain of fixed electron-carrier molecules (after Chance and Williams[1]).

New methods thus will be required to examine the interaction of the individual catalyst molecules in such solid-state arrays, and to determine the translational, rotational, and vibrational degrees of freedom involved in these protein-protein interactions. Possibly, useful information will come from refined application of both electron-paramagnetic and nuclear-magnetic resonance spectroscopy, to supplement the already-extensive light spectroscopic studies of Chance and his colleagues.[1] Another question arises. How can the mechanism of electron transfer along the respiratory chain be accounted for, in view of the known dimensions of some of these proteins and their prosthetic groups, even assuming close juxtaposition of carriers? Chance has shown[1] two alternatives for the mechanism of electron transfer from one protein carrier to another along the chain (Fig. 8). In one, restricted rotation is postulated to permit collision of the prosthetic groups. In the second, the molecules are fixed, and electrons then must pass through the protein moieties to the prosthetic groups, possibly by π-electron interactions as in the so-called "charge-transfer" complexes. The passage of electrons or energy through the protein moiety of heme proteins has been considered frequently, since the classical experiments of Bücher on the constant quantum efficiency of 1.0 in the photodissociation of carbon monoxide myoglobin at all wavelengths tested.[44] Recently, Shore and Pardee have considered the physics of such energy transfers, which may take place through fluorescence and sensitization phenomena.[45,46] All of the respiratory carriers have quite characteristic fluorescence spectra. Szent-Györgyi, Weber, and others have suggested that fluorescence phenomena may conceivably play a physiological role in energy transfers along the carrier chain.[47]

The occurrence of such solid-state arrays of catalytically active proteins, the individual members of which are so difficult and as yet impossible to separate from each other with preservation of activity, presents a whole new area of protein chemistry and techniques. To date, it has been sufficiently difficult to examine the physical parameters of proteins in more or less ideal solutions; the interaction of two proteins with each other in dilute solution actually has been just barely touched upon in recent years. However, these far more complex solid-state arrays described above obviously present difficulties of a much greater order of magnitude. Similarly, the separation and identification of

such proteins making up solid-state protein arrays will involve a whole new category of experimental approaches, as students of this area of enzyme chemistry already are painfully aware. If new techniques finally will permit separation of all of the carrier enzymes in soluble form, then reconstruction of these systems *in vitro* also can be expected to present great difficulty, because of the problem of restoring the spatial relationships among the members of such a complex in the test tube. Perhaps full *in vitro* reconstruction to yield soluble systems of high-catalytic activity never really will be attained.

These are but a few of the many biophysical and molecular problems posed by the solid-state character of the respiratory-energy transformers of mitochondria.

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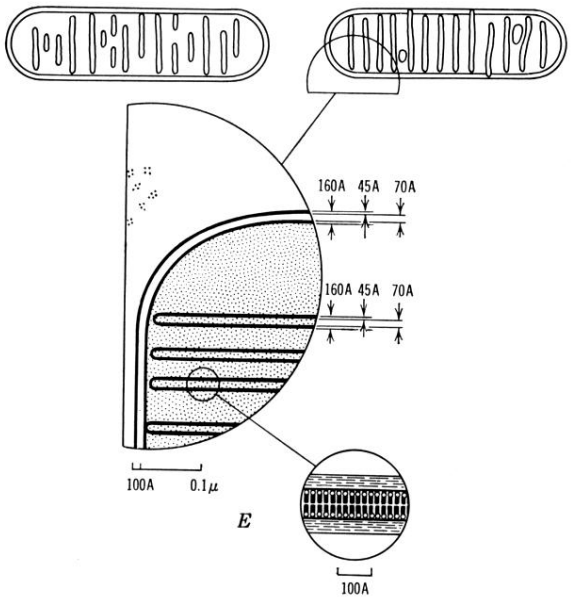

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