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Respiratory Chains and Sites of Coupled Phosphorylation

Studies in a bacterial system give further evidence of a basic biochemical unity between different forms.

Arnold F. Brodie and Joel Adelson

Oxidative phosphorylation, a vital cellular process for the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate was discovered during a period when many other biosynthetic processes were also being recognized. However, while the biosynthesis of many types of small molecules and even macromolecules has come to be understood in fairly close detail, the mechanism of generating the terminal high-energy phosphate of ATP during the respiratory process is still unclear. We understand rather well the biosynthesis of the adenine ring, and of the ribose moiety. But we do not understand in chemical terms how the stepwise downhill process of electron transport may be coupled to the production of "high-energy" phosphate bonds. Basically the reason for this gap in our knowledge is that structure at the intermacromolecular level is essential to the functioning of the complicated electron transport coupling process, and that most of our methods for studying biochemical processes depend on the resolution of the process into its component parts. When we apply the "gentlest" sorts of methods to fractionation of mitochondria we tend to lose the very reactions we wish to study.

The biochemical processes of bacteria and of mammalian tissues differ mainly in the pathways concerned with specialized activities. At a more basic biochemical level—such as glycolysis, the Krebs cycle, and protein synthesis—these widely diversified biological forms exhibit similar processes. Thus, bacterial systems have been investigated, not with the object of finding a radically new or different sort of coupling process, but with the hope of finding that at the mechanistic level the coupling process in bacteria is similar to that in mammalian mitochondria, yet sufficiently different in the fine details of structural organization to allow a meaningful and informative comparison between the two systems. As we discuss later, the chemical composition of the respiratory chains of *Mycobacterium phlei*, an organism capable of coupling phosphorylation to oxidation, as well

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as the electron transport sequence of the respiratory carriers of this microorganism, closely resembles analogous features of the mitochondrial system, while the physical organization does differ in an informative and useful way.

Respiratory-Chain Phosphorylation

Let us first examine the broad capabilities of extracts of the *Mycobacterium phlei* cell. The cell wall may be disrupted by ultrasonic vibration and centrifuged away at low speed. The resulting fluid is referred to as the crude extract. Biochemically, the coupled phosphorylation exhibited by the crude extract resembles that of mitochondria. All of the Krebs-cycle intermediates are oxidized, with concomitant production of ATP (*1*). Phosphorus-oxygen ratios greater than 1 were observed with a number of substrates. For example, with succinate as an electron donor the P/O ratio was 1.78. A wide variety of uncoupling agents were found to be effective with this bacterial system. The activity, like that exhibited by mitochondria, was labile to changes in tonicity, to freezing and thawing, and to further sonication. Other bacterial systems having similar properties have been described (*2–5*).

Examination of the crude extract revealed the presence of particles 70 to 180 millimicrons in diameter (*6*). High-speed centrifugation of the crude extract resulted in the separation of these particles from the supernatant fluid. Examination of the oxidative capabilities of the isolated particles revealed that none of the Krebs-cycle intermediates previously tested were appreciably oxidized, with the exception of succinate, which was oxidized slowly (*7*). The supernatant fraction had no oxidative capability. However, when the two fractions were recombined, both oxidation and phosphorylation occurred at the levels originally observed with the crude extract. The supernatant components were found to be protein in nature. Kinetic analysis revealed that the supernatant played a dual role and contained protein components necessary for oxidation and others required for phosphorylation (*7*). Thus, the *Mycobacterium phlei* system was shown to contain soluble coupling proteins necessary for ATP production (*8*). Similar coupling factors have been demonstrated in other systems of bacterial origin (*3–5, 9*). With mitochondria, on the other hand, more extensive treatment is necessary in the preparation of soluble coupling factors.

Bacterial systems vary in the number of soluble oxidative factors they contain, but all have soluble coupling factors. Of particular interest is the system from *Micrococcus lysodeikticus* of Ishikawa and Lehninger (*4*), which had no soluble oxidative factors but which contained soluble coupling factors. The *M. lysodeikticus* system was isolated by the "gentlest" technique available, lysozyme treatment followed by osmotic lysis of protoplasts. Thus, there is little reason to suspect that the soluble bacterial coupling factors are artifacts of isolation. There are, however, certain other findings associated with coupled phosphorylation in bacterial systems which make us alert to the possibility that the initial disruptive treatment used to isolate bacterial particles may have caused some damage to the structural integrity of the system. These findings are the low P/O ratios obtained with bacterial systems and the fact that these systems do not exhibit respiratory control. In a sense, the bacterial systems resemble some of the fragmented mitochondrial systems (*10*) which still have one or more intact phosphorylative sites.

Respiratory Chains

Although the structural organization of the bacterial system capable of coupling phosphorylation to oxidation differs from the organization of the mammalian mitochondrial system, the respiratory carriers of *Mycobacterium phlei* closely resemble those of the mammalian system in chemical composition. Chemical and spectrophotometric analysis of the carriers revealed the presence of bound nicotinamide adenine dinucleotide (NAD^+), flavins, a naphthoquinone, and cytochromes *b*, c_1, *c*, *a*, and a_3 (*11*). The only difference of particular interest was the observation that a naphthoquinone replaces the mammalian benzoquinone. The naphthoquinone was identified as vitamin K_9H (*12*).

The close resemblance beween the mitochondrial and bacterial systems became more apparent from studies of the participation of the respiratory carriers in electron transport under conditions of coupled phosphorylation. These studies involved both spectrophotometric techniques and the use of specific respiratory inhibitors. Reduction of cytochromes *b*, *c*, and *a* occurred with substrates representing different electron transport chains (*11*). The reduced steady-state levels of the cytochromes were obtained with succinate, β-hydroxybutyrate, and malate. The electron transport sequence of the terminal respiratory chain of this microorganism flows from cytochrome *b*, to *c*, to $a + a_3$, to oxygen. The cytochromes were found in the particulate fraction (*6, 11*).

The naphthoquinone (vitamin K_9H) plays an important role in the electron transport chain since it serves as a cofactor shuttling electrons between flavin adenine dinucleotide (FAD) and cytochrome *b* on both the NAD^+- and the malate-vitamin K reductase pathways. This carrier was found in large amounts in the particulate fraction and to a lesser extent in the supernatant fraction (*13*). The sensitivity of quinones to light in the near-ultraviolet region (360 mμ) permitted selective destruction of this cofactor *in situ* without damage to other cofactors or to structural integrity (*14*). Following irradiation (360 mμ), both oxidation and phosphorylation were lost with all substrates. Both activities were restored by the addition of the natural naphthoquinone or vitamin K_1 with malate or NAD^+-linked substrate. Succinoxidase activity was also destroyed by irradiation but was not restored by the addition of quinones (either benzo- or naphthoquinones or both in combination) or other cofactors (*14*).

The requirement for FAD for quinone reduction and the requirement for vitamin K, after irradiation of the system, for cytochrome *b* reduction indicate that the quinone functions between FAD and cytochrome *b* (*11*). Furthermore, the rate of reduction and oxidation of the reduced quinone was found to be consistent with the overall rate of oxidation of the electron transport chain. The reduced derivatives of vitamin K have been trapped with the "K-dependent" system and identified (*13*).

The electron transport pathways in *Mycobacterium phlei* are shown in Fig. 1. This figure may be used as a basis for comparing the mammalian and *M. phlei* electron transport chains. In Fig. 1, certain segments of the pathways are indicated as being localized within the particles, whereas others are found in the soluble fraction. The sites of interaction of the soluble oxidative factors are indicated as indentations in the particles (*7, 11*). The NAD^+- and succinate-linked chains converge at the cytochrome *b* level of oxidation (*11*).

Although the respiratory chains of *Mycobacterium phlei* are basically similar to those of mitochondria, the bacterial system was found to contain an additional respiratory chain, the first steps of which are soluble. The soluble enzyme which initiates malate oxidation has been purified and characterized. This enzyme, malate-vitamin K reductase, required the addition of FAD for oxidation of malate via the particulate chain. Evidence has been obtained that the pathway of electron flow proceeds from malate, through FAD, and enters the particles and converges with the NAD^+-linked chain at the naphthoquinone level (*11*). Another method of visualizing the reductase activity has been of great use in studying its properties, since the enzyme is entirely soluble. Under these conditions malate-vitamin K reductase required the addition of FAD, vitamin K_1, phospholipid, and a dye as a final acceptor. The product of malate oxidation was found to be oxalacetate. The two reactions may be illustrated as follows.

Interaction with particulate chain (1)

Soluble portion (1a)

$$\text{malate} \xrightarrow[\text{FAD}]{\text{MKR}} \text{oxalacetate} + FADH_2$$

Particulate portion (1b)

$$FADH_2 + \tfrac{1}{2}O_2 \xrightarrow{\text{particulate fraction}} H_2O + FAD$$

Soluble reaction (2)

$$\text{Malate} + \text{MTT (dye)} \xrightarrow[\text{FAD, MKR}]{K_1, \text{phospholipid}} \text{oxalacetate} + \text{formazan}$$

(MKR = malate-vitamin K reductase; MTT = thiazolyl blue tetrazolium.) With other electron transport enzymes, a requirement for phospholipid has been demonstrated (*15*). The phospholipid may serve as a vehicle for the interaction of lipid- and water-soluble cofactors. Alternatively, malate oxidation may proceed via the particulate NAD^+-linked chain. The enzyme which mediates this oxidation, malate dehydrogenase, is soluble and was separated from malate-vitamin K reductase.

Phosphorylative Sites in Mycobacterium phlei

Bacterial systems which couple phosphorylation to oxidation lack respiratory control. This fact precludes the use of "crossover" techniques of the type used by Chance and Williams (*16*) as a means of identifying phosphorylative sites. Instead, respiratory-chain segments, created by fractionation or by the use of artificial "electron sinks" which tap the respiratory chain at different levels of oxidation (*17*), have permitted analysis of the different respiratory areas to which phosphorylation is coupled in *M. phlei*.

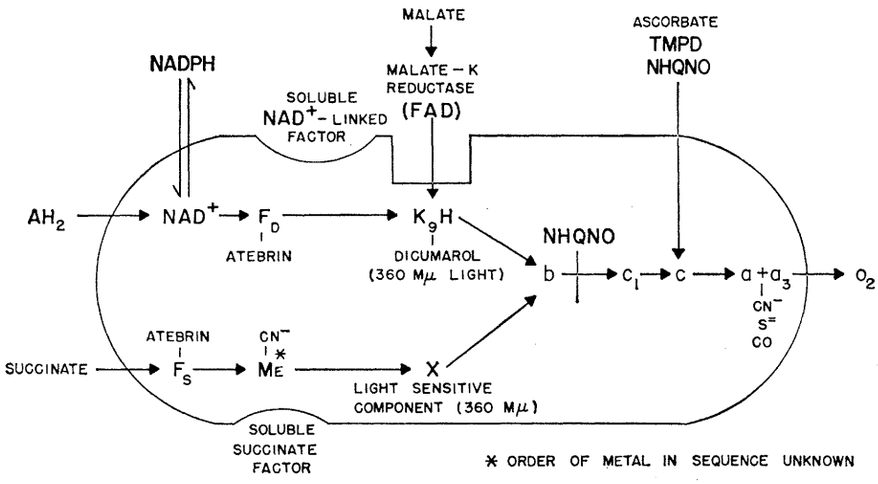

Fig. 1. Respiratory chain of *Mycobacterium phlei* particles. [Modified from Asano and Brodie (*11*)]

Studies of phosphorylative sites require a system whose respiratory chain is known in detail and choice of a substrate which undergoes only a one-step oxidation (*17*). The product of oxidation must not be further metabolized by the system under study. Since both these requirements were fulfilled by the fractionated *M. phlei* system, a study to localize the phosphorylative sites was undertaken. One-step oxidation was obtained with substrates representing each of the three distinct pathways: the succinate chain, the malate-vitamin K reductase chain, and the NAD^+-linked chain. The three chains couple phosphorylation to oxidation. The P/O ratios for the succinate and malate-vitamin K reductase chains were similar and ranged between 0.4 and 0.8, whereas P/O ratios greater than 1 were observed with NAD^+-linked substrates such as β-hydroxybutyrate and ethanol. These studies were carried out with the fractionated system in which the terminal phosphorylative site (between cyto-

Table 1. Summary of phosphorylative sites in *Mycobacterium phlei*.

Pathway	Localization	Evidence	Remarks
NAD^+-linked chain	NADH-flavoprotein level	Certain analogs of K_1 fail to restore phosphorylation with added NADH but exhibit phosphorylation with particle-bound NAD^+ (*18*).	Oxidation of coupled pathway sensitive to amytal.
NAD^+-linked chain	Quinone-cytochrome *b* level	Competitive inhibition of phosphorylation by antagonists of vitamin K (*19*).	Competitive inhibition by dicumarol and lapachol.
		Lack of phosphorylation but not of oxidation on substitution of slightly modified quinones for vitamin K_1 (*14, 19*).	Dihydrophytyl vitamin K_1 and lapachol restore oxidation by the same electron transport pathway as vitamin K_1.
		Rapid loss of phosphorylation with brief periods of irradiation (360 mμ).	Oxidation remains sensitive to respiratory inhibitors.
Succinate-, NAD^+-, and malate-vitamin K reductase–linked pathways	Cytochrome *c* to oxygen	Phosphorylation exhibit with ascorbate and TMPD* as electron donors to reduce cytochrome *c*. Endogenous oxidation blocked at cytochrome *b* level with inhibitor.	Phosphorylation requires specific coupling proteins. Phosphorylation sensitive to DNP.
Malate-vitamin K reductase pathway	FAD or naphthoquinone level	Synthesis of ATP (P^{32}) on oxidation of malate by soluble malate-vitamin K reductase. Activity dependent on FAD, K_1, phospholipid, and coupling proteins (*20*).	Sensitive to uncoupling agents and respiratory inhibitors which inhibit coupled activity with malate in *M. phlei*.

* Tetramethyl-*p*-phenylenediamine.

chrome *c* and oxygen) was missing. These results indicated the presence of phosphorylative sites in broad areas of the respiratory chain.

An attempt to define more precisely the location of the phosphorylative sites was made in studies in which smaller segments of the respiratory chain were utilized. By selection of appropriate electron donors and acceptors the spans from substrate to cytochrome *c*, from malate to the naphthoquinone on the malate-vitamin K reductase pathway, from NAD^+ to flavoprotein, and from cytochrome *c* to oxygen were examined for their ability to couple phosphorylation to oxidation. The results are summarized in Table 1.

Analogs which transfer electrons by the pathway utilized by vitamin K, but which do not participate in phosphorylation have been useful in demonstrating a phosphorylative site at the NAD^+-level (*18*). For example, substitution of lapachol for vitamin K_1 resulted in restoration only of oxidation with added NADH (the reduced form of NAD^+) as an electron donor. However, phosphorylation was observed with this analog when electron transport proceeded via bound NAD^+, with P/O ratios between 0.3 and 0.5. In contrast to the oxidation with added NADH, the oxidation with lapachol and the particulate-bound NAD^+ was found to be sensitive to amytal. Thus, the phosphorylation observed with the analog appears to be associated wtih the NAD^+-flavin region of the chain.

Several aspects of the bacterial system presented in Table 1 differ markedly from findings for the mammalian system studied so far and thus are informative and useful. Studies with the quinone-dependent system (after irradiation) have suggested a phosphorylative site at the quinone-cytochrome *b* level of oxidation. The fact that certain quinones, lapachol and dihydrophytyl vitamin K_1, restore oxidation by the same pathway as that by which vitamin K_1 restores it but do not restore phosphorylation suggests the direct participation of the quinone in the phosphorylative events (*19*). The naphthoquinones which restored oxidative phosphorylation were found to contain a methyl group in the two-position and at least a 5-carbon (β, γ-unsaturated) side chain in the three-position of the naphthoquinone nucleus. In addition, known competitive antagonists of vitamin K—dicumarol or lapachol—were found to be competitive inhibitors of phosphorylation (*19*).

Phosphorylation associated with the electron transport span from cytochrome *c* to oxygen has been demonstrated in the *Mycobacterium phlei* system by the method of Lehninger (*17*). Phosphorylation at this region was demonstrated with the crude extract from *M. phlei* but not with the particulate and fractionated supernatant system. Coupling proteins for this site have been isolated from the crude supernatant material. When these coupling proteins were added to particles they restored phosphorylation in the region from cytochrome *c* to oxygen.

The other site of phosphorylation presented in Table 1 was catalyzed by the soluble fraction which contained malate-vitamin K reductase and coupling proteins (*20*). Net synthesis of ATP occurred with this fraction and was found to be dependent on malate oxidation and on the addition of FAD, vitamin K_1, phospholipid, dye, ADP, and inorganic phosphate (P_i).

$$\text{Malate} + \text{ADP} + P_i + \text{MTT} \xrightarrow[\text{phospholipid}]{\text{FAD, } K_1} \text{oxalacetate} + \text{ATP} + \text{formazan} \quad (3)$$

The ATP formed was measured, after chromatographic separation of the nucleotides, as ATP (P^{32}) or glucose-6-phosphate (P^{32}). The P/2*e* ratio (moles of P_i taken up per pair of electrons transferred) for this reaction was found to be less than 1 and ranged between 0.3 and 0.6. Neither dye reduction nor ATP formation occurred on substitution of oxalacetate, pyruvate, or NADH for malate. Formation of ATP was inhibited by respiratory inhibitors which affect malate oxidation and was uncoupled by *m*-Cl-CCP (*m*-chlorocarbonylcyanide phenylhydrazone) and hydroxylamine in low concentrations. Although phosphorylation occurs with malate-vitamin K reductase, further evidence is necessary to establish the relationship of this phosphorylation to that observed with malate via the malate-vitamin K reductase chain illustrated in reaction 1.

Before concluding, we should briefly mention work done on potential intermediates in oxidative phosphorylation. One of these, histidine phosphate, was formed by the supernatant fraction. Formation of this compound occurred with the particles alone, but to a lesser extent than with the supernatant fraction. Although both fractions are required for oxidative phosphorylation, recombination of the two fractions failed to stimulate formation of this compound. Photooxidation of the coupling factors of the supernatant with methylene blue and visible light resulted in the loss of 97 percent of the ability to form phosphohistidine without loss in the oxidative phosphorylation capacity of the preparation (*21*). Further, treatment of the particles and supernatant fraction with light (360 $m\mu$) to destroy the ability to couple phosphorylation to oxidation had no effect on the level of phosphohistidine formed by this system. Thus, in agreement with the results of Pressman (*22*) for the mammalian system, we can conclude, at the present level of resolution of the process into its components, that histidine phosphate is not an intermediate in oxidative phosphorylation. An alternative possibility, suggested by Boyer and his associates (*23*), is that histidine phosphate is formed during catalysis at the substrate level by succinyl thiokinase, an enzyme present in the *Microbacterium phlei* supernatant. Formation of ATP has also been demonstrated with the *M. phlei* system on oxidation of synthetic 6-chromanyl phosphate of vitamin K_1 (*24*); however, the nature of the reduced derivative of the endogenous quinone has not been elucidated.

Conclusion

Studies with bacterial systems capable of oxidative phosphorylation have further established the concept of the existence of a basic biochemical unity between widely different biological forms. In broad outline, it may be readily seen that a good deal of the internal structure of both mammalian and bacterial cells is devoted to providing a framework for ATP synthesis. Both systems contain structurally based electron transports chains, and in both the electron transport sequence is coupled to the production of ATP. The coupled activity exhibited by bacteria appears to be similar to that exhibited by mammalian mitochondria. Agents which disrupt electron flow or which act to uncouple are effective in both systems. In fine detail, however, there are differences in structural organization which are reflected in the electron transport chains and phosphorylative coupling factors. These differences yield information regarding the mechanism of phosphate-bond energy generation. Of particular interest is the soluble malate-vitamin K reductase pathway which yields ATP on oxidation of malate. This sort of soluble activity has not been seen in other systems which cou-

ple phosphorylation to oxidation. The requirement for a phospholipid for this activity may represent an intermediate step between reactions mediated by soluble enzymes and those mediated by, and dependent on, highly organized structures like the mitochondrion. Further study with the "soluble system" may provide an understanding of the mechanism of oxidative phosphorylation and of the broader question of enzyme interaction in complex structures.

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Social Facilitation

A solution is suggested for an old unresolved social psychological problem.

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Most textbook definitions of social psychology involve considerations about the influence of man upon man, or, more generally, of individual upon individual. And most of them, explicitly or implicity, commit the main efforts of social psychology to the problem of how and why the *behavior* of one individual affects the behavior of another. The influences of individuals on each others' behavior which are of interest to social psychologists today take on very complex forms. Often they involve vast networks of interindividual effects, such as one finds in studying the process of group decision-making, competition, or conformity to a group norm. But the fundamental forms of interindividual influence are represented by the oldest experimental paradigm of social psychology: social facilitation. This paradigm, dating back to Triplett's original experiments on pacing and competition, carried out in 1897 (*1*), examines the consequences upon behavior which derive from the sheer presence of other individuals.

Until the late 1930's, interest in social facilitation was quite active, but with the outbreak of World War II it suddenly died. And it is truly regrettable that it died, because the basic questions about social facilitation—its dynamics and its causes—which are in effect the basic questions of social psychology, were never solved. It is with these questions that this article is concerned. I first examine past results in this nearly completely abandoned area of research and then suggest a general hypothesis which might explain them.

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Research in the area of social facilitation may be classified in terms of two experimental paradigms: audience effects and co-action effects. The first experimental paradigm involves the observation of behavior when it occurs in the presence of passive spectators. The second examines behavior when it occurs in the presence of other individuals also engaged in the same activity. We shall consider past literature in these two areas separately.

Audience Effects

Simple motor responses are particularly sensitive to social facilitation effects. In 1925 Travis (*2*) obtained such effects in a study in which he used the pursuit-rotor task. In this task the subject is required to follow a small revolving target by means of a stylus which he holds in his hand. If the stylus is even momentarily off target during a revolution, the revolution counts as an error. First each subject was trained for several consecutive days until his performance reached a stable level. One day after the conclusion of the training the subject was called to the laboratory, given five trials alone, and then ten trials in the presence of from four to eight upperclassmen and graduate students. They had been asked by the experimenter to watch the subject quietly and attentively. Travis found a clear improvement in performance when his subjects were confronted with an audience. Their accuracy on the ten trials before an au-