ENERGY-TRANSDUCING REACTIONS IN BIOLOGICAL MEMBRANES

IV.* A MODEL OF OXIDATIVE PHOSPHORYLATION AND ION ACCUMULATION IN MITOCHONDRIA BASED ON A THIOACYLATION MECHANISM

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Summary

A hypothesis of oxidative phosphorylation, and ion transport, in mitochondria is proposed in which reduction and contraction of a respiratory electron-carrier complex is considered to result in mechanicochemical synthesis, within a membrane, of a thioester crosslink and release of a membrane weak base B:

$$R^1COOHB + R^2SH \rightleftharpoons R^1COSR^2 + B + H_2O$$

The base reacts with a proton from the medium and forms a complex with ADP and Mg^{2+}:

$$B + H^+ + ADPMg^- \rightleftharpoons BHADPMg$$

Oxidation of the electron-carrier complex expands the structure and the thioester crosslink breaks after reaction with water:

$$R^1COSR^2 + H_2O \rightleftharpoons R^1COOH + R^2SH$$

The liberated acidic poylmeric chain R^1COOH then adsorbs at a phosphorylating enzyme site and accepts hydroxyl ions arising from phosphorylation of ADP; Mg^{2+} act as an intermediary hydroxyl acceptor:

$$BHADPMg + P_iMg + R^1COOH \rightleftharpoons ATPMg_2 + R^1COOHB + H_2O$$

There is present also a K^+-complexing site X which competes with phosphorylation for reaction with R^1COOH:

$$R^1COOH + XK^+ \rightleftharpoons R^1COOKX + H^+$$

Thioacylation liberates alkalinity:

$$R^1COOKX + R^2SH \rightleftharpoons R^1COSR^2 + KOH + X$$

The alkalinity subsequently energizes substrate accumulation through a transporting system where a substrate anion, plus a proton from the medium, attaches to a weak-base carrier site; reaction with intramitochondrial alkali liberates the base and substrate is desorbed.

Ca^{2+}, Zn^{2+}, Cd^{2+}, uncoupling acids, gramicidin, and thyroxine are considered to uncouple phosphorylation by attachment to B. Valinomycin competes with X.

Lipid elasticity associated with electron and ion transport is postulated to influence these processes, and can be regulated by hormonal and some drug interactions.

I. Introduction

In the inner mitochondrial membrane there is located the respiratory carrier chain in which energy arising from the aerobic oxidation of NADH, or of succinate and some other acids, is utilized for the synthesis of ATP, for the translocation of

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ions, or for synthesis. Cations, particularly K^+ and Ca^{2+}, may be accumulated along with permeant anions, and in some cells this may be a specialized mitochondrial activity. The mitochondrial ion pump therefore differs from the sodium pump, which is a device for energizing essentially an *exchange* reaction between Na^+ and K^+ across a membrane.

This paper applies the concepts developed in earlier papers of this series (Weiss 1969*a*, 1969*b*, 1969*c*) to the accumulation of ions in mitochondria. Such a task necessitates first a detailed consideration of the process of oxidative phosphorylation, but only scant attention need be given to details of the complex processes associated with the respiratory carriers. Recent reviews by Ernster and Lee (1964), Griffiths (1965), Lehninger (1965), Green (1966), Mitchell (1966), Slater (1966*a*), Lehninger, Carafoli, and Rossi (1967), Pullman and Schatz (1967), Robertson (1967), and Slater (1967) cover in detail, and provide different emphasis on various aspects of, the mitochondrial energy-transducing reactions.

II. Current Hypotheses

In 1953, Slater first proposed the "chemical" hypothesis of oxidative phosphorylation in mitochondria. He, followed by others (see Slater 1966*a*, 1967), postulated the formation of high-energy intermediates of the respiratory carriers for coupling the synthesis of ATP with oxidation–reduction reactions in the respiratory train.

The difficulty of establishing unequivocally the presence of such intermediates has led some workers to question their existence and to propose alternative hypotheses. Studies of the accumulation of ions in biological membranes led Robertson, and others, to suggest (in 1948) that the mitochondrial membrane is impermeable to protons, and that the respiratory carriers induce translocation of ions by separating hydrogen and hydroxyl ions across the membrane (see Robertson 1967). In an extension of these ideas, Mitchell formulated in 1961 his "chemiosmotic" principle (see Mitchell 1966; Mitchell and Moyle 1969) in which ATP is considered to be synthesized in a vectorial process driven by the passage of protons across the membrane. Serious objections have been raised to this hypothesis (see Slater 1967) but it has stimulated a large amount of research. It was concluded by Slater, from a consideration of the data of Chance and Mela (1966) and others, that proton extrusion in mitochondria, which has been taken as evidence in favour of the chemiosmotic hypothesis, is the consequence of the energized uptake of permeant cations and is not involved in oxidative phosphorylation. This conclusion is supported also by later work (Addanki, Cahill, and Sotos 1968; Caswell 1968). These conclusions are still consistent with the proposal of Williams (1961, 1962, 1967) of a chemiosmotic-type reaction *within* a lipid membrane.

Yet another concept, a "conformational" hypothesis, was proposed by Boyer in 1964. From a consideration of muscle and of glyceraldehyde-3-phosphate dehydrogenase, he suggested that conformational changes, induced by the oxidation–reduction reactions of the respiratory carriers, form thioester linkages which subsequently energize the synthesis of ATP. This concept is related also to the mechanico-chemical ideas of Lehninger (1965), of Green (1966), and of Packer (see Utsumi and

Packer 1967). In the sense that a thioester contains a "high-energy bond", Boyer's hypothesis is related to the concept of the chemical hypothesis but eliminates the need for a chemical intermediate of the actual respiratory carriers. Falcone (1966) and Falcone and Hadler (1968) have also proposed oxidative phosphorylation mechanisms involving thioesters.

Following earlier studies, Harris *et al.* (1968) and Hackenbrock (1968) have shown that the mitochondrial inner membrane can assume a number of distinct morphological states under various well-defined conditions. They concluded also that a conformational change may be the means of coupling electron transport with oxidative phosphorylation.

In Part III of this series of papers (Weiss 1969*c*), the sodium pump has been considered as an ion-exchange system with variable crosslinking. ATP is considered to form thioester crosslinks under normal operating conditions and thereby change the ion selectivity. Under some other conditions, the energy of the ion concentration gradients across the membrane causes a flow of ions which reverses the pump and induces synthesis of ATP. This is accounted for by a contraction of the ion-exchange system of the pump induced by a reduction in the electrostatic repulsive forces between the cation-exchange sites in the presence of a high cation concentration. The contraction results in the mechanicochemical synthesis of crosslinks, whose subsequent hydrolysis energizes ATP synthesis. This mechanism is therefore related in principle to the proposed oxidative phosphorylation mechanism of Boyer since the ion-induced contraction of the ion-exchange sites of the sodium pump is replaced with a substrate-induced contraction of the respiratory carriers resulting from their reduction. Such a concept is consistent also with the model experiments of Kuhn, discussed in Part I (Weiss 1969*a*), who showed that mechanical extension of an electron-exchange polymer changes its oxidation–reduction potential. More recent oxygen-exchange studies of the mechanism of oxidative phosphorylation (Boyer 1967) show that it cannot be accounted for by formation of a phosphorylated intermediate, as has been proposed in the sodium-pump model.

This paper extends Boyer's concept and shows how respiratory energy, coupled with thioester membrane crosslinking, might drive oxidative phosphorylation or ion transport by mechanisms representing a synthesis of some of the key concepts of the chemical, conformational, and the chemiosmotic and Williams' hypotheses.

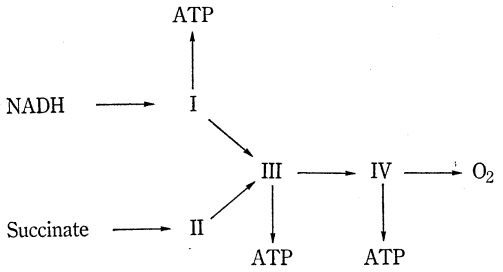
III. A Model of Oxidative Phosphorylation

(*a*) *Description of the Model*

A simplified model will be presented first to illustrate the proposed principle of oxidative phosphorylation under tightly coupled conditions in mitochondria.

ATP synthesis is coupled with three energy-conserving sites in the respiratory carrier chain within the inner mitochondrial membrane. As described by Green (1966), the system comprises four complexes, which will be designated "redox" complexes I, II, III, or IV respectively, between which electrons flow. Each complex contains an integrated assembly of respiratory carriers. There are present also "coupling factors", which are proteins whose combined presence is essential for

oxidative phosphorylation. The arrangement of the complexes is as follows, in a simplified representation of the system:



For the purpose of the model, the redox complexes need not necessarily have the detailed structure proposed by Green; it is sufficient for each to operate as a concerted oxidation–reduction assembly associated with a phospholipid membrane.

The model postulates that changes in the state of oxidation–reduction of the redox complexes induce conformational changes due to changes in charge, and electron-donating or -accepting properties of the electron-exchange sites. Such effects are observed in simple electron-exchange resins (see Part I).

"Coupling polymers", to which are attached the coupling factors, are considered to be bound within a lipoprotein membrane to the redox complexes by hydrophobic bonding, and by Mg^{2+}, which bridges weak-acid sites in the two systems. The expansion and contraction of the structure adjacent to the electron-exchange sites is thereby transmitted mechanically to the coupling polymer.

Each coupling polymer contains near the site of energy transduction an active carboxylic acid R^1COOH, a thiol R^2SH with a reactivity comparable with that of the active thiol in glyceraldehyde-3-phosphate dehydrogenase, and an associated catalytic histidine residue. Contraction brings the thiol, carboxylate, and histidine groups together in a favourable conformation where histidine catalyses the synthesis of a thioester crosslink in a mechanicochemical reaction; expansion tends to hydrolyse the crosslink and is associated with the synthesis of ATP. The histidine residue is postulated to catalyse the thioacylation reaction in either direction by the formation of an intermediate lactam, according to the mechanism elucidated by Bruice (1959). The thioacylation reaction is as follows, where B is a weak-base site within the membrane:

$$R^1COOHB+R^2SH \rightleftharpoons R^1COSR^2+B+H_2O \quad (1)$$

B thus liberated is removed by reaction with substrate ADP and Mg^{2+}, combined in a 1 : 1 complex, and involves a proton from the medium:

$$B+H^++ADPMg^- \rightleftharpoons BHADPMg \quad (2)$$

Subsequent hydrolysis of the thioester involves reaction with water and the creation of membrane acidity:

$$R^1COSR^2+H_2O \rightleftharpoons R^1COOH+R^2SH \quad (3)$$

Thioacylation is postulated to occur on the inner side of the membrane. Embedded at the outer surface, but adjacent to the inner thioacylation site, is a phosphorylation enzyme which is associated with coupling factors and is activated

by Mg^{2+} and ADP. R^1COOH is postulated to be attached to a polymer chain such that, when liberated by hydrolysis of the thioester crosslink, R^1COOH can either contact the outer surface of the lipoprotein membrane, or be adsorbed at the phosphorylating enzyme site, along with BHADPMg and P_iMg. The latter situation predominates when conditions favour oxidative phosphorylation. Phosphorylation of ADP by P_i results when the R^1COOH so bound accepts OH^- liberated from the following reaction:

$$BHADPMg + P_iMg + R^1COOH \rightleftharpoons ATPMg_2 + R^1COOHB + H_2O \quad (4)$$

It is postulated that Mg^{2+} acts as the initial acceptor of the OH^- liberated by the phosphorylation reaction. Such a mechanism is the converse of that proposed by Selwyn (1968) for the hydrolysis of ATP by Mg^{2+}. R^1COOH then reacts with the hydroxyl thus attached. A further requirement is that "effector ADP", distinct from "substrate ADP", must also bind at some additional site in the enzyme to induce a favourable conformation in polymeric components involved in phosphorylation.

When the redox polymer oxidizes, in the next step of the respiratory cycle, it expands, and the expansion, being mechanically transmitted to the coupling polymer, promotes hydrolysis of the thioester. As a consequence of the hydrolysis, the ratio $RCOOH : RCOO^-$ in the membrane rises and the resulting membrane acidity drives phosphorylation of ADP. The concept of membrane acidity driving phosphorylation has been proposed by Williams (1961, 1962, 1967) who suggested that protons reduce the activity of the water released during phosphorylation by forming H_3O^+, thereby assisting phosphorylation. Reaction of the water with membrane salt R^1COOHB could fulfil an analogous function. It is possible also that polymer chain R^1 becomes strained on forming the thioester crosslink, and that this strain remains after R^1COOH binds to the phosphorylation site. Relief of the strain by formation of R^1COOHB would then make an entropy contribution towards phosphorylation. Since none of the above mechanisms are mutually exclusive, removal of OH^- and water, and also a gain in entropy, may all cooperate in the mechanism which provides energy arising from the hydrolysis of the thioester crosslink for the phosphorylation of ADP without formation of an intermediate acylphosphate.

The net effect is that as a result of proton transfer between the phosphorylation and thioacylation reactions, ATP and water are synthesized in a mechanicochemical reaction involving ADP, P_i, Mg^{2+}, and a proton from the medium. Efficient proton transfer between phosphorylation and thioacylation would occur if BH^+ formed an ion pair with R^1COO^-; this would be facilitated if the reactions were to occur within a lipid environment, just as proton transfer between a carboxyl and weak-base ion-exchange resin is promoted by reducing polarity of the medium in the vicinity of the exchange sites (see Part I).

Because of participation of B in reaction (1), the pH of the inner solution does not change under tightly coupled conditions of oxidative phosphorylation, although the pH of the medium becomes more alkaline. Consequently a respiratory-derived proton gradient, as proposed in Mitchell's chemiosmotic concept, cannot drive this process of oxidative phosphorylation. This conclusion is consistent with experiment (see Slater 1967; Addanki, Cahill, and Sotos 1968; Caswell 1968; Mitchell and

Moyle 1969) since, as yet, there is no evidence that oxidative phosphorylation is associated with a respiratory-derived proton gradient.

The ability of ATP to reverse the system, and a mechanism for ion transport, will be discussed in later sections.

(*b*) *Evidence for a High-energy Intermediate*

The model proposes a thioester for intermediate energy conservation. Evidence that an energy conservation stage exists has been discussed by Pullman and Schatz (1967) and Slater (1966*a*).

The rapid formation of ATP ("ATP jump") observed when ADP is added to mitochondrial suspensions incubated in the presence of substrate and P_i supports the existence of stored energy in mitochondria (Eisenhardt and Rosenthal 1968). Further evidence comes from reversal of reactions in the respiratory chain in the absence of ADP [see Section V(*e*)] and the energized reduction of $NADP^+$ by NADH (Slater 1966*a*; Lee and Ernster 1968; see also Section IV).

Although such data prove that energy can accumulate in mitochondria under conditions where it cannot be converted into ATP, it has not been possible to choose between such energy being stored as a high-energy intermediate, as demanded by the chemical hypothesis, or as a membrane potential and pH gradient as proposed in Mitchell's chemiosmotic hypothesis.

(*c*) *Phosphorylation Reaction*

ADP is the first demonstrable nucleotide acceptor for a phosphoryl group in oxidative phosphorylation (Hill and Boyer 1967; Colli and Pullman 1969). There is also evidence that the phosphorylation reaction, and the production of water associated with it, occurs at sites other than those of the oxidation–reduction reactions (Boyer *et al.* 1966). Provided ADP is present, arsenate stimulates respiration in the absence of P_i (Estabrook 1961; Hinkle, Penefsky and Racker, 1967; Ter Welle and Slater, 1967). Oxygen-exchange reactions, to be discussed in Section VII, are consistent with water formation from an oxygen of P_i by a concerted reaction involving ADP, P_i, and Mg^{2+} ions in energized ATP formation (see Boyer 1967). Such observations are consistent with the model.

IV. Energy-linked Hydrogen Transfer Reactions and Oxidation of NADH

(*a*) *NADH Oxidation and Pyridine Nucleotide Transhydrogenation*

The thioester associated with redox complex I in the model, where NADH is the substrate, is postulated to be reduced by NADH according to the following reactions:

$$R^1COSR^2 + NADH + H^+ \rightleftharpoons R^1{-}\overset{\displaystyle H}{\underset{\displaystyle OH}{C}}{-}SR^2 + NAD^+ \qquad (5)$$

$$R^1{-}\overset{\displaystyle H}{\underset{\displaystyle OH}{C}}{-}SR^2 \rightleftharpoons R^1CHO + R^2SH \qquad (6)$$

The reaction begins with the formation of a charge-transfer complex between NAD^+ and the thiolhemiacetal, and has properties expected of a high-energy inter-

mediate of NAD^+. The formation of an aldehyde in reaction (6) involves rupture of the crosslink. Reactions (5) and (6) resemble those proposed for coenzyme A-linked aldehyde dehydrogenase (Rudolph, Purich, and Fromm 1968). The aldehyde is then postulated to be oxidized to R^1COOH by the flavoprotein in the redox complex I. The acidity thus liberated drives phosphorylation, and the thioester reforms when the reduced flavoprotein in turn reduces another component in the redox complex which induces contraction.

When $NADP^+$ is present, the type of equilibrium represented by reaction (5) can involve either NAD^+ or $NADP^+$, but to different extents. The following exchange reaction thereby occurs:

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH \qquad (7)$$

Such an exchange reaction depends therefore on the presence of a thioester whose production requires the expenditure of energy either from respiration or from ATP [see Section V(*e*)].

An hydrogen exchange with the above features is well known in mitochondria (see Ernster and Lee 1964; Lee and Ernster 1968; Papa *et al.* 1968). "High-energy" derivatives of NAD^+ have been observed which may be charge-transfer complexes with the postulated thioester (see Griffiths 1965). Charge-transfer complexes have been observed when NADH reduces glyceraldehyde-3-phosphate dehydrogenase; NAD^+ forms a charge-transfer complex with the donating thiolhemiacetal group thus formed (Smith 1966). It is known also that the NADH–oxidase activity of submitochondrial particles is abolished by reagents which are known inhibitors of vicinal dithiols (Fluharty and Sanadi 1960). The similarity between the respiratory chain iron metalloproteins, and related metalloproteins such as aldehyde oxidase, has been noted by Handler, Rajagopalan, and Aleman (1964). Jacoby (1958) has concluded that a vicinal dithiol plays a role in the latter enzyme.

There is therefore substantial support for the proposed interaction between NADH and the thioester of the model.

(*b*) *Reaction with Formaldehyde*

Related to reactions (5) and (6) is the following postulated reaction of the model with formaldehyde:

$$R^2SH + HCHO \rightleftharpoons R^2S\overset{\displaystyle H}{\underset{\displaystyle OH}{C}}H \qquad (8)$$

$$R^2S\overset{\displaystyle H}{\underset{\displaystyle OH}{C}}H \xrightarrow{-2H} R^2S\overset{\displaystyle \|}{\underset{\displaystyle O}{C}}H \xrightarrow{H_2O} R^2SH + HCOOH \qquad (9)$$

In such a reaction a thiolhemiacetal is formed, which is then oxidized and subsequently released as formate by a dehydrogenase [reaction (9)]. The overall effect is therefore the oxidation of formaldehyde to formic acid without necessarily the consumption of oxygen, and inhibition of the phosphorylating site associated with oxidative phosphorylation.

Such a reaction is consistent with the findings of Buskirk and Frisell (1967), and of Tyler (1969), who established that the site of formaldehyde inhibition is in the NAD–flavin region of the respiratory chain. Reaction (8) is analogous to that which occurs in glyceraldehyde-3-phosphate dehydrogenase (Colowick, Eys, and Park 1966).

V. The Translocation of Ions

(*a*) *Energy Distribution*

There are three major energy-transducing units in the model: (1) the redox complexes, (2) the coupling polymers, and (3) an ion pump, associated with thioacylation, which energizes transport of cations across the membrane. The resistances to change in each of the three units determines the division of energy between the different systems (see Rottenberg and Caplan 1967). There will therefore be no strict stoicheiometry between the number of cations transported for the passage of each pair of electrons across each energy-conserving site (accumulation ratio). Accumulation ratios ranging from a small fraction of unity to values in excess of 7 ("super-stoicheiometry") have been reported (Carafoli *et al.* 1967; Rossi and Azzone 1968).

The energy-transducing processes are reversible. ATP can reverse the state of oxidation of the respiratory carriers, or energize ion movements; "downhill" movements of ions can, under some conditions, synthesize ATP (Cockrell, Harris, and Pressman 1967; Caswell 1968).

(*b*) K^+ *Transport*

It is postulated that there is present in the membrane a chelating structure X which can form ion–dipole complexes XK^+ (see Part I) with K^+. Reaction of XK^+ with R^1COOH releases a proton to the medium:

$$XK^+ + R^1COOH \rightleftharpoons R^1COOKX + H^+ \qquad (10)$$

This reaction with R^1COOH is competitive with its forming a complex with BHADPMg and MgP_i, and is favoured by increasing pH of the medium, which reduces protonation of B and of R^1COO^-, and by absence of $ADPMg^-$. Subsequent thioacylation releases potassium hydroxide in the inner solution and liberates X:

$$R^1COOKX + R^2SH \rightleftharpoons R^1COSR^2 + KOH + X \qquad (11)$$

The net result of such energized cation transport is the development of acidity in the medium and of alkalinity in the intramitochondrial solution.

The mechanism is consistent with observations that ion transport varies inversely with oxidative phosphorylation (Lynn and Brown 1966), and is competitively inhibited by ADP (Papa *et al.* 1969). When unaccompanied by permeant anions, K^+ accumulation involves a 1 : 1 exchange of K^+ for H^+, and does not require an inward shift of anions or a K^+–Na^+ exchange (Christie *et al.* 1965; see also Lehninger, Carafoli, and Rossi 1967; Harris and Pressman 1969). Cation transport results in acidification of the medium and alkalinization of the inner solution (Addanki, Cahill,

and Sotos 1968; Caswell 1968). Monovalent ion transport is stimulated by EDTA (Packer, Utsumi, and Mustafer 1966; Settlemire, Hunter, and Brierley 1968). This suggests that by removing Mg^{2+} by reaction with EDTA, adsorption of ADP by B is prevented so that formation of R^1COOKX is favoured as a result of release of R^1COOH [see Section V(*h*)].

(*c*) *Substrate Transport*

The inner mitochondrial membrane contains a number of specific substrate-transporting systems across which substrate anions and P_i must pass for subsequent reaction with the respiratory carriers (see Chappell 1968). Anions can accumulate in the inner solution at concentrations in excess of those in the surrounding medium, and the process seems to be associated with the accumulation of K^+ (see Kraayenhof, Tsou, and Van Dam 1969). It is proposed that the transporters contain weak-base sites to which the protonated form of the substrate acids bind; in the case of multivalent anions, only one of their groups would be required for attachment to the carrier. Substrate thus bound is considered to induce a conformational change which brings it in contact with the inner solution. As a result of alkalinization of this solution through cation transport [reaction (11)], substrate acid desorbs as reaction of the alkali with the carrier reforms the free base. Such "permeant" anions therefore promote cation transport by reducing inner solution alkalinity which tends to inhibit thioacylation by reversal of reaction (11). Accumulation of substrate and K^+ stimulates respiration and induces swelling through osmosis.

Because multivalent anions can be transported by a reaction which need involve only one proton and hydroxyl ion, the remaining substrate anions could be accompanied by cations to an extent determined by the pH of the membrane. Consequently more than one cation could be transported for the expenditure of each high-energy bond. For every K^+ accumulated, a proton will be released to the medium, but this will tend to be compensated by the loss of a proton for binding substrate; hence medium pH should be higher when K^+ accumulation occurs in the presence of permeant anions than in their absence.

There is substantial support for such a mechanism which conforms with the concept of Harris, Höfer, and Pressman (1967*a*) of a respiratory-energized ion pump coupled with the transport of substrate anions or P_i. K^+ seems to be essential for mitochondrial functioning (Pressman and Lardy 1955; but see Smith and Beyer 1967). K^+ transport stimulates respiration (Rossi, Scarpa, and Azzone 1967), and is accompanied by Kreb's cycle anions (Gamble 1965). Addition of permeant anions reduces the internal alkalinization (Addanki, Cahill, and Sotos 1968) and raises the external pH; with increasing pH of the medium, succinate uptake and mitochondrial swelling are reduced, but the ratio of protons rejected to K^+ ions accumulated increases and approaches unity (Rossi, Scarpa, and Azzone 1967).

(*d*) *Stimulation of K^+ Transport by Zn^{2+}*

In the presence of a permeant anion such as acetate, and P_i or arsenate, K^+ transport is stimulated by the addition of Zn^{2+} (Brierley and Settlemire 1967), which releases endogenous Mg^{2+} (Brierley, Knight, and Settlemire 1968). If a monovalent

complex formed between Zn^{2+}, P_i, and the acetate it might be adsorbed at BH^+ and displace $ADPMg^-$. This would uncouple phosphorylation and stimulate cation transport by releasing R^1COOH from R^1COOHB [see Section V(*f*)].

(*e*) *Influence of Ionophorous Antibiotics*

A variety of polypeptide antibiotics form specific ion complexes which can induce ion permeability changes in membranes (see Part I). These have been studied extensively, particularly by Pressman and Chappell and their respective colleagues, and can be classified into various categories (see Pressman 1968; Henderson, McGiven, and Chappell 1969).

(i) *Valinomycin-type Antibiotics*

These antibiotics induce K^+–K^+ exchange across lipid membranes. When added to mitochondria in the presence of small amounts of K^+, a marked stimulation of cation transport, swelling, and respiration occurs, although higher K^+ concentrations inhibit phosphorylation but not respiration. Under respiratory-energized conditions, strong uptake of K^+ occurs in the presence of valinomycin if ADP is absent; addition of ADP results in phosphorylation of ADP but ion transfer resumes once phosphorylation is almost completed (Hofer and Pressman 1966; Harris, Catlin, and Pressman 1967; Harris, Hofer, and Pressman 1967; Harris and Pressman 1969). Mitochondria containing valinomycin continue to respire rapidly even after net uptake of K^+ ceases, but K^+ infiux remains at a high level provided energy is available (Harris, Catlin, and Pressman 1967; Caswell 1968). When oxidative phosphorylation is inhibited by oligomycin, one proton is rejected for each K^+ transported in a valinomycin-stimulated reaction; a short pulse of acid immediately reduces K^+ uptake and respiration (Mitchell and Moyle 1969).

These observations have been interpreted in two ways. One is based on Mitchell's chemiosmotic hypothesis (see Henderson, McGiven, and Chappell 1969; Mitchell and Moyle 1969). Alternatively, Pressman and his colleagues consider that increased cation permeability due to the antibiotic diverts energy at the expense of oxidative phosphorylation to drive a cation pump; passive substrate anion accumulation follows which in turn stimulates respiration (see Harris and Pressman 1969). An interpretation of the latter type follows logically from the ion-pump model discussed above.

It is proposed that because of the highly stable complex formed between K^+ and valinomycin, and its lipophilic character, valinomycin complexes K^+ in preference to X and the complex forms a particularly stable ion pair with R^1COO^- by displacing a proton from R^1COOH. These reactions uncouple phosphorylation. Subsequent thioacylation releases potassium hydroxide which drives substrate accumulation and thereby stimulates respiration. If only small amounts of K^+ are present, the extracellular K^+ concentration decreases as the accumulation proceeds and this factor, coupled with stimulation of respiration, leads to enhanced phosphorylation. At higher K^+ concentrations, the uncoupling effect of K^+–valinomycin is no longer diminished sufficiently to be compensated by the enhanced respiration so that inhibition of phosphorylation, but not respiration, sets in.

Once a steady-state K^+ gradient has been established, a K^+–K^+ exchange reaction occurs across the membrane in the presence of valinomycin, which maintains a high influx of K^+ and a high level of respiration. This is possible because K^+–valinomycin, formed from inner K^+, can react with R^1COOH liberated immediately after thioacylation and can participate in further thioacylation or exchange across the membrane; such a reaction is more difficult in the absence of the antibiotic since it is postulated that aqueous K^+ ions from the inner solution cannot easily contact R^1COOH and X within the lipid membrane. Addition of pulses of acid, or alkali, influence the steady state, and therefore the membrane potential, by changing the degree of ionization of R^1COO^-.

If rotenone be added to block respiratory energy for ATP synthesis, such synthesis can nevertheless occur in mitochondria with a strong, outwardly directed K^+ gradient on addition of valinomycin, ADP, and P_i (Cockrell, Harris, and Pressman 1967); this behaviour is consistent with reversal of the proposed ion pump.

(ii) *Gramicidin*

This antibiotic, unlike valinomycin, has an inherent ability to uncouple phosphorylation analogous to that of classical uncoupling agents. Like dinitrophenol (DNP) and Ca^{2+}, gramicidin in the presence of Na^+ or K^+ and permeant anions inhibits phosphorylation but stimulates respiration. Unlike valinomycin, gramicidin inhibits $P_i \rightleftharpoons$ ATP exchange (Harris, Hofer, and Pressman 1967). The mitochondrial membrane permeability pattern in the presence of gramicidin is not characteristic of gramicidin (Falcone and Hadler 1968).

Such behaviour suggests that gramicidin may compete with ADPHMg for adsorption at B. Liberation of R^1COOH stimulates ion transport, substrate accumulation, and respiration. An adsorption process might account also for the activity of gramicidin being dependent on the presence of an hydroxyl group (Falcone and Hadler 1968) if the latter were involved in its binding to the adsorption site at B. With such an interpretation it is not clear why *N*-ethylmaleimide does not inhibit gramicidin-induced swelling (Weinstein, Scott, and Hunter 1964), although *p*-mercuribenzoic acid does so inhibit (Falcone and Hadler 1968). Perhaps the antibiotic alters accessibility to the thiol; Hubbell and McConnell (1968) observed that gramicidin reduces the extent of fluid hydrophobic regions in lipid membranes.

(iii) *Nigicerin-type Antibiotics*

The nigicerin-type antibiotics consist of linear chains of oxygen-containing heterocyclic rings, with terminal carboxyl and hydroxyl groups at each end of the chain. When the carboxyl group is ionized, hydrogen bonding between the carboxylate and hydroxyl groups, in which two water molecules also serve as bridges, converts the linear chain into a ring which chelates alkali-metal cations by ion–dipole interactions with the oxygen atoms (see Pressman 1968). Consequently such antibiotics can carry either protons or K^+ ions, but not both simultaneously, across membranes. Addition of nigicerin to a mitochondrial membrane, where establishment of a cation gradient has also established a pH gradient, therefore results in dissipation of both gradients (see Cockrell, Harris, and Pressman 1967; Harris and Pressman 1969; Henderson, McGiven, and Chappell 1969).

(*f*) *Respiratory-energized Ca^{2+} Transport*

Ca^{2+} is accumulated in mitochondria with particular ease and is an effective uncoupler of oxidative phosphorylation (see Lehninger, Carafoli, and Rossi 1967).

Ca^{2+} accumulation involves a small number of sites having an exceptionally high affinity for Ca^{2+}, and also for Mn^{2+}, Sr^{2+}, and Zn^{2+}. K^+ and Mg^{2+} are much less readily adsorbed and cannot displace the former ions easily. Decreasing pH decreases binding of Ca^{2+} at such sites. In respiring mitochondria, binding of Ca^{2+} displaces protons, but if the respiratory carrier system is inhibited no such proton displacement occurs. Ca^{2+} binding is inhibited by DNP, which inhibits Ca^{2+} accumulation driven by respiration, but is not inhibited by gramicidin, valinomycin, or oligomycin (Reynafarje and Lehninger 1969).

Addition of Ca^{2+}, in the absence of ATP and permeant anions, results initially in respiratory-energized transfer of a limited amount of Ca^{2+} across the membrane after which respiration is inhibited. The process is facilitated by increasing the pH of the medium. DNP relieves such respiratory inhibition. There is no fixed stoicheiometry because of the inhibition of respiration, and high accumulation ratios for the transfer (super-stoicheiometry) have been observed (Addanki, Cahill, and Sotos 1968; Rossi and Azzone 1968). These phenomena provide strong support for the existence of the postulated membrane base B, which will be identified as the high-affinity Ca^{2+} sites. Strong chelation of Ca^{2+} by B enables it to competitively displace ADPHMg. R^1COOH then reacts with the chelated Ca^{2+} to form an ion pair with release of a proton:

$$BCa^{2+}+R^1COOH \rightleftharpoons R^1COOCa^+B+H^+ \quad (12)$$

Respiratory-energized thioacylation releases Ca^{2+} plus one OH^- and B:

$$R^1COOCa^+B+R^2SH \rightleftharpoons R^1COSR^2+Ca^{2+}+OH^-+B \quad (13)$$

Ca^{2+} release from B following thioacylation occurs possibly because of the cross-linking inducing a conformational change which alters the affinity of B, or because of the presence of substrate anions capable of chelating Ca^{2+}. Inhibition of respiration arises from the imbalance of charge associated with reactions (12) and (13) and respiration ceases once a double layer establishes itself. The process is facilitated by increasing pH of the medium which reduces protonation of B and thereby favours chelation with Ca^{2+}. The ratio of protons rejected to the medium to Ca^{2+} accumulated shows no fixed stoicheiometry (Rossi, Azzone, and Azzi 1967). Under some conditions of the medium the imbalance of charge can be relieved by inner protons attaching to weak-base substrate carrier sites and moving out across the membrane to be neutralized by excess anions, such as succinate, remaining in the medium as a result of reaction (12). The H^+/Ca^{2+} ratio can approach 2 to 1 under favourable circumstances. DNP inhibits Ca^{2+} accumulation by protonation and strong adsorption at B [see Section VI(*a*)]. When the respiratory carriers are blocked so that hydrolysis of the thioester is inhibited, Ca^{2+} can still bind at B but no protons are released owing to the absence of R^1COOH. The observation that gramicidin does not competitively displace Ca^{2+}, despite the fact that its adsorption at B is postulated to account for its activity, would require that its binding affinity for B is much less than that of Ca^{2+}.

Addition of P_i stimulates respiratory-energized Ca^{2+} accumulation; two Ca^{2+} plus one P_i are transferred for each high-energy bond expended (Addanki, Cahill, and Sotos 1968; see also Lehninger, Carafoli, and Rossi 1967). If CaP_i were to bind at the site normally occupied by MgP_i, it may form a neutral complex with R^1COOCa^+B.

$$CaP_i \rightleftharpoons CaP_i^- + H^+ \tag{14}$$

$$R^1COOCa^+B + CaP_i^- \rightleftharpoons R^1COOCa_2P_iB \tag{15}$$

Thioacylation would then deposit two Ca^{2+} plus one P_i on the inner side of the membrane. The reaction would be favoured by subsequent chelation of Ca^{2+} by substrate anions capable of forming a stronger Ca^{2+} complex than that with P_i.

(*g*) *Reversed Operations with ATP*

Reactions in the respiratory chain can be reversed by ATP (Chance and Hollinger 1961; see Pullman and Schatz 1967). ATP can also energize cation transport (see Lehninger, Carafoli, and Rossi 1967). Because oligomycin inhibits ATP-energized, but not respiratory-energized cation accumulation (Pressman 1964; Christie *et al.* 1965), and Mg^{2+} influences reversed and forward operations in the presence of oligomycin to different extents (see Lee and Ernster 1968), reversed operations with ATP would seem to involve a different reaction mechanism than that in oxidative phosphorylation.

Such a conclusion is consistent with the model since if ATP resulted in desorption of effector ADP, the conformation of the phosphorylating enzyme would change and might be such as to favour phosphorylation of R^1COO^- by ATP (cf. the sodium pump, Part III). Acyl phosphate could then energize thioacylation and would result in phosphate, plus its associated cations, being transferred across the membrane:

$$R^1COOP^{2-} + R^2SH \rightleftharpoons R^1COSR^2 + P_i^{2-} \tag{16}$$

The contraction resulting from thioester crosslinking would change the oxidation–reduction potential of the associated redox complex.

In ATP-energized Ca^{2+} accumulation, the expenditure of one molecule of ATP results in the transport of two Ca^{2+} and one P_i across the membrane; the process is inhibited by DNP (see Lehninger, Carafoli, and Rossi 1967; Addanki, Cahill, and Sotos 1968) and also by Zn^{2+} or Cd^{2+} (Brierley 1967). ATP-energized transport of oxaloacetate depends on energized accumulation of Ca^{2+}, and the stoicheiometry is similar to that for accumulation of Ca^{2+} and P_i (Haslam and Griffiths 1968). These observations suggest the following mechanism.

One ATP molecule containing Ca^{2+} may initially complex with R^1COOCa^+B leading to formation of a joint R^1COOP_iCa–CaB complex. Thioacylation, energized by the acyl phosphate [reaction (16)], might then release two Ca^{2+} plus P_i. Reaction of Ca^{2+} with oxaloacetate, if the complex were more stable than hydroxyapatite, would facilitate transfer and provide a driving force for passive accumulation of oxaloacetate via its transporting system. Evidence will be cited later [Section VI(*a*)] suggesting that DNP has a high affinity for B. Inhibition of Ca^{2+} transfer by DNP, and also by Cd^{2+} and Zn^{2+}, can be accounted for by competitive displacement of Ca^{2+} from B.

(*h*) *Energy-linked Transport of* Mg^{2+}

Respiratory-energized uptake of Mg^{2+} alone is not facile, and differs from that observed with Ca^{2+}, Mn^{2+}, or Zn^{2+}; it does not stimulate oxygen consumption. Accumulation of Mg^{2+} occurs in beef heart mitochondria in the presence of substrate and P_i; Mg^{2+} then accumulates as $Mg_3(PO_4)_2$. Such accumulation is inhibited by addition of Ca^{2+} or ADP (see Lehninger, Carafoli, and Rossi 1967). Addition of Mg^{2+}-chelating agents, such as EDTA, stimulates K^+ uptake; Mg^{2+} inhibits the swelling and respiration induced by EDTA (Azzi, Rossi, and Azzone, 1966). Addition of Zn^{2+} stimulates Mg^{2+} accumulation (Brierley and Knight 1967). Addition of parathyroid hormone stimulates respiration in the presence of Mg^{2+}, particularly if acetate is present (Rasmussen and Ogata 1966).

These observations suggest that Mg^{2+} may compete with K^+ for reaction with X, and that $Mg^{2+}X$ then reacts with R^1COOH; the imbalance of charge then resembles that associated with BCa^{2+} and can be relieved by addition of phosphate to form a neutral complex. Addition of parathyroid hormone forms a more stable Mg^{2+} complex than that with X, but facile accumulation requires acetate to form a neutral complex. The inhibitory effect of Mg^{2+} on thyroxine-stimulated ion uptake may be due to the formation of R^1COOMg^+X which would inhibit cation uptake. Addition of guanidines, which might react with R^1COO^-, leads to increased binding of P_i (Pressman 1963). X may therefore be the binding site for MgP_i in the phosphorylating enzyme complex.

VI. Some Inhibitors of Oxidative Phosphorylation

(*a*) *Uncoupling Acids*

The classical uncoupling agents are lipid-soluble acids, such as DNP, whose activity is related to their acidic strength, lipid solubility, and ability to bind to proteins (see Slater 1966*a*; Weinbach and Garbus 1969). Uncoupling activity of DNP, and its derivatives, depends on the presence of appropriate *ortho* and *para* negative substituents and a free hydroxyl group (Shaw, Lannon, and Tapley 1959). Weinbach and Garbus (1969) suggest that by binding to ϵ-amino groups in the phosphorylating enzyme, they induce a conformational change which is adverse for phosphorylation. High activity is associated with the presence of π-electron systems with strong electron-withdrawing properties, which suggests that binding may involve formation of a charge-transfer complex with electron-donating basic sites.

Mitochondria accumulate anions, probably in association with energized cation accumulation (see Section V), and anions of uncoupling acids compete in the process. Respiratory inhibition induced by the accumulation of such uncoupling anions can be relieved by raising the substrate concentration (Van Dam 1967; Caswell and Pressman 1968; Kraayenhof and Van Dam 1969). Uncoupling acids exhibit other effects which cannot be attributed to their influence on substrate transport.

Addition of acidic uncouplers inhibits phosphorylation but stimulates respiration and mitochondrial swelling; at higher concentrations they inhibit all these processes. In the absence of ADP, the maximum respiratory stimulation does not exceed that obtained by ADP alone; at uncoupling concentrations below that corresponding to maximum stimulation, addition of ADP enhances respiration (Van Dam 1967). Addition of small amounts of hydrochloric acid, when the respiratory chain is reduced

and inhibited by lack of ADP, stimulates respiration and oxidation of DNPH, but higher concentrations inhibit these reactions (Lowenstein and Chance 1968). DNP inhibits K^+–K^+ exchange and K^+ transport (see Lehninger, Carafoli, and Rossi 1967), the $P_i \rightleftharpoons ATP$ and $ATP \rightleftharpoons H_2{}^{18}O$ exchange reactions (see Boyer 1967), and the release of protons when Ca^{2+} is added (Gear *et al.* 1967).

Van Dam and Tsou (1969) concluded that such behaviour indicates that ATP synthesis and uncoupling acids compete for a common high-energy intermediate or state with a higher affinity for the former process. The above results can be interpreted as follows in a manner consistent with such a conclusion.

DNP, and other uncoupling acids, are postulated to have a high affinity for the basic site B, with which they form a charge-transfer complex. Such adsorption inhibits phosphorylation by competitive displacement of ADPHMg, and releases R^1COOH. Participation of the latter in energized thioacylation and cation transport results in respiratory stimulation as a consequence of the associated accumulation of substrate. Small additions of hydrochloric acid sufficient to combine with B, but not to a significant extent with R^1COO^-, stimulate these reactions analogously. Higher amounts of uncoupling, or mineral, acids inhibit cation transport, and K^+–K^+ exchange, by protonation of R^1COO^-, and thereby inhibit reaction of R^1COOH with K^+ [reaction (10)].

Small amounts of DNP stimulate, but higher amounts inhibit ATPase activity in the absence of ADP; inhibition is competitive with ATP and is stronger at low ATP concentrations (Kraayenhof and Van Dam 1969). ATPase activity requires the presence of monovalent cations (Papa *et al.* 1969), and is accompanied by release of protons (see Lee in Mitchell and Moyle 1967). ATPase activity is inhibited when electron transfer is inhibited with amytal or actinomycin or both (Hemker 1964). Such observations suggest the following mechanism.

Small additions of uncoupling acid displace $ADPMg^-$ from B and release R^1COOH which reacts with medium alkalinity to form R^1COOK. These reactions stimulate hydrolysis of ATP:

$$ATPMg_2 + R^1COO^- + H_2O \rightleftharpoons R^1COOH + ADPMg^- + MgP_i \qquad (17)$$

Once excess uncoupler is added over that required for reaction with B, inhibition of reaction (17) occurs through protonation of R^1COO^-. ATPase activity can be inhibited also by blocking electron transfer so that hydrolysis of thioester, and release of R^1COOH, is prevented.

(*b*) *Thyroxine*

Like the acidic uncoupling acids, low concentrations of thyroxine uncouple phosphorylation and induce mitochondrial swelling. Activity in thyroxine and its derivatives requires the presence of the iodo-substituted diphenyl ether and is independent of the structure of the side chain. Methylation of the phenolic hydroxyl group does not alter activity *in vitro* (Shaw, Lannon, and Tapley 1959). DNP is a poor competitor with thyroxine for adsorption in the membrane but is an effective inhibitor of the swelling induced by thyroxine (Tapley and Basso 1959). K^+ stimulates the ability of thyroxine to induce swelling which depends on electron transport, and the generation of a high-energy intermediate, but is not associated with any

particular positions of the electron-transport chain. Unlike DNP, thyroxine does not inhibit swelling at higher concentrations. The behaviour of thyroxine and gramicidin are strikingly similar (Scott and Hunter 1966). Thyroxine-induced swelling is markedly enhanced by Ca^{2+} or Zn^{2+}, but is inhibited by Mg^{2+} and Sr^{2+}, and there are similarities between the swelling behaviour of thyroxine and Ca^{2+} or Zn^{2+}. Thyroxine chelates Ca^{2+}, Zn^{2+}, and Mg^{2+} (Cash *et al.* 1966). These observations suggest the following interpretation.

Thyroxine forms a much stronger charge-transfer complex with B than does DNP. Since methylation of hydroquinone does not interfere with its ability to form charge-transfer complexes with benzoquinone (Michaelis and Granick 1944), it is reasonable to propose that methylation of thyroxine does not inhibit complex formation with B. Displacement of R^1COOH, as with DNP, through adsorption of thyroxine at B stimulates ion transport and induces swelling. Because DNP cannot readily displace adsorbed thyroxine its addition inhibits ion transport, and swelling, by protonation of R^1COO^-. Potentiation of swelling by Ca^{2+} or Zn^{2+} may be the result of their chelation by thyroxine and formation of a neutral complex between R^1COOCa^+B and thyroxine. Mg^{2+}, which is only weakly bound by B [see Section V(*f*)], possibly inhibits by complexing with thyroxine alone but prevents formation of a strong membrane complex. Since Sr^{2+} binds tightly to B, and presumably chelates also with thyroxine, it is not clear why it should inhibit. Also it is not clear why excess thyroxine does not inhibit cation transport since it is a reasonably strong acid. Perhaps excess thyroxine binds elsewhere in the membrane, or chelates with ions which destroy its acidity.

(*c*) *Thiol Inhibitors*

Oxidative phosphorylation is inhibited by a variety of thiol inhibitors which also induce ATPase activity. The respiratory burst due to addition of Ca^{2+}, and respiratory response to ADP, or arsenate, is inhibited by *p*-mercuribenzoate, *N*-ethylmaleimide, and 5,5′-dithio-bis(2-nitrobenzoic acid) (Boyer 1964; Falcone 1966; Fonyo and Bessman 1966; Haugaard *et al.* 1969). Thiol inhibitors also induce K^+–K^+ exchange across the membrane (see Lehninger, Carafoli, and Rossi 1967). Small amounts of DNP, in the presence of thiol inhibitors, stimulate discharge of a K^+ gradient across the membrane, but larger amounts inhibit (see Brierley, Knight, and Settlemire 1968). A thiol is present in coupling factor "B" which combines with an appropriate submitochondrial particle to confer ability to participate in oxidative phosphorylation, ATP-driven NAD^+ reduction, ATP-driven transhydrogenase activity, and ATP–P_i exchange (Sanadi, Lam, and Ramakrishna Kurup 1968).

These observations suggest that combination of a thiol inhibitor with R^2SH liberates R^1COOH for participation in K^+–K^+ exchange across the membrane or in ATPase activity [reaction (17)]. Small amounts of DNP stimulate discharge of K^+, in the presence of thiol inhibitors, by liberating R^1COOH from B, but larger amounts inhibit this reaction by protonation of R^1COO^-. The behaviour of factor "B" is consistent also with the postulated role of a thioester in oxidative phosphorylation and the pyridine nucleotide transhydrogenation reactions.

Inhibition of oxidative phosphorylation by anionic mercurials, but not by *N*-ethylmaleimide, is preceded by stimulation of cation transport and respiration

in ATP-energized systems, but not under respiratory-energized systems requiring P_i; addition of P_i inhibits such stimulation (Brierley, Knight, and Settlemire 1968). Anionic mercurials inhibit entry of P_i through its membrane transporting system (Haugaard *et al*, 1969; Tyler 1969). These observations suggest that the anionic mercurials compete with P_i for basic sites in its transporting system. Since P_i is not required in ATP-energized ion transport, entry of the anionic mercurials function as permeant anions which stimulate thioacylation, and respiration, by removing the alkalinization associated with cation transport [see Section V(*c*)].

(*d*) *Oligomycin*

In intact mitochondria, oligomycin inhibits oxidative phosphorylation, the $P_i \rightleftharpoons ATP$ and $P_i \rightleftharpoons H_2{}^{18}O$ exchange reactions (see Section VII), uncoupling by arsenate, ATPase activity, and ATP-energized, but not respiratory-energized, ion-transport. Respiratory inhibition, under tight oxidative phosphorylation conditions, can be relieved by the addition of DNP; oligomycin stimulates the pyridine transhydrogenation reactions (see Fonyo and Bessman 1966; Lee and Ernster 1968). It does not inhibit binding of Ca^{2+} to its high-affinity sites (Reynafarje and Lehninger 1969).

This behaviour suggests that oligomycin might interfere with hydroxyl-ion transfer from adsorbed P_iMg to R^1COOH at the phosphorylation site and with phosphorylation of P^1COOH by ATP. From studies with EDTA submitochondrial particles, Lee and Ernster (1968) concluded that small amounts of oligomycin inhibit hydrolysis of a high-energy intermediate which, in their reaction path, corresponds with the postulated thioester. Bruice (1959) observed the tendency of P_i to hydrolyse thioesters in his studies of imidazole thioester catalysis. Perhaps P_i has a similar tendency in mitochondrial particles and the presence of adsorbed oligomycin stabilizes the thioester by preventing reaction with P_i.

(*e*) *Fatty Acids*

The uncoupling effect of fatty acids (see Lehninger 1965) may be accounted for by their competing with R^1COOH.

(*f*) *Guanidines*

The uncoupling ability of alkyl guanidines and biguanidines (Schafer 1964), their ability to inhibit the $ADP \rightleftharpoons ATP$ exchange reaction (Guillary and Slater 1965), and the observation that their binding parallels that of P_i (Pressman 1963) suggests competition with BH^+ by such lipid-soluble bases for R^1COO^-.

VII. Exchange Reactions

An unique feature of oxidative phosphorylation systems is their ability to catalyse an $ATP \rightleftharpoons H_2{}^{18}O$ exchange reaction; $ATP \rightleftharpoons P_i$ and $P_i \rightleftharpoons H_2{}^{18}O$ exchange reactions are observed also (see Boyer 1967).

The $ATP \rightleftharpoons H_2{}^{18}O$ and $ATP \rightleftharpoons P_i$ exchanges are accounted for by reaction (4) which depends on effector ADP; removal of ADP therefore inhibits such exchange (see Boyer 1967). The acids DNP and *p*-mercuribenzoate might inhibit the exchange

(see Boyer 1967) through protonation of R^1COO^-. Reaction (4) might account also for the $P_i \rightleftharpoons H_2{}^{18}O$ exchange reaction, which requires ATP, but not ADP, and requires less Mg^{2+} than does oxidative phosphorylation (see Boyer 1967), if hydroxyl-ion transfer between ATP and Mg^{2+} and Mg^{2+} and P_i is not dependent of effector ADP, but the latter may regulate OH^- transfer to RCOOH. Uncoupling acids, in excess of uncoupling concentrations, might block such an exchange by reducing the steady-state concentration of Mg^+OH in the enzyme.

From the above postulated mechanisms of the exchange reactions, of ion transport, and of the behaviour of inhibitors and uncoupling agents, a consistent pattern emerges. Ca^{2+}, Cd^{2+}, Zn^{2+}, uncoupling acids like DNP and thyroxine, and gramicidin compete with protons plus $ADPMg^{2-}$ for adsorption at B and uncouple phosphorylation; further competitive studies between these agents should be made. When electrically neutral complexes form, R^1COOH is liberated and ion transport or ATPase activity is stimulated. Valinomycin, and related antibiotics, compete with X. Reaction of thiol inhibitors likewise liberates R^1COOH with stimulation of K^+–K^+ exchange and ATPase activity. Fatty acids compete with R^1COOH, and lipid-soluble bases compete with B. The postulated mechansim for oxidative phosphorylation and ion transport involves carboxyl, basic, and K^+-chelation structures; inhibitors are known with each type of structure. Thiol inhibitors, and the pyridine nucleotide transhydrogenation reactions and other observations, provide evidence for the participation of a thiol and a thioester. The unifying concept that emerges from the basic postulates provides strong support for the validity of the concept.

VIII. Conformational Changes in Mitochondrial Membranes

It is a fundamental feature of the model that a swelling–contraction cycle is involved in mitochondrial energy transduction. Thermodynamic calculations suggest the feasibility of a mechanicochemical concept (Veeger, in Slater 1966*b*). There are experimental data in support of such a concept.

Optical rotatory dispersion studies suggest that in the presence of 3M guanidine hydrochloride, a minimum of 15 amino acid residues out of a total of 104 shift into another formation when ferricytochrome *c* is reduced, and there is evidence for a similar transition for the lipid-bound cytochrome *c* (Urry and Doty 1965). The effect is more than a simple swelling phenomenon induced by a change in charge and probably reflects a change in the coordinating ability of the haem iron in the different oxidation states. Most likely reduction, which enhances the electron-donating ability of the iron, results in attachment of the imidazole group of a histidine residue as a ligand. Recent hydrogen–deuterium exchange studies also provide evidence for a marked conformational difference between reduced and oxidized cytochrome *c* (Kägi and Ulmer 1968). Fluorescence probe studies indicate structural changes of the mitochondria which are associated with energy conservation (Azzi *et al.* 1969; Packer, Donovan, and Wrigglesworth 1969). Their time course studies show that they precede the utilization of intermediates of energy conservation, as indicated by configurational changes associated with ion transport (Packer *et al.* 1969).

Detailed electron microscope studies by Harris *et al.* (1968), and Hackenbrock (1968), have provided direct evidence for conformational changes in mitochondrial

membranes. These papers also note earlier related evidence by Lehninger (1965), Packer, and others (see Packer, Utsumi, and Mustafa 1966). The energy-producing reactions of mitochondria occur within the membrane of the christae attached to the inner membrane. According to conditions, Harris *et al.* (1968) and Hackenbrock (1968) have observed that the christae may exist in orthodox, aggregated (ag), or comminuted (com) modes which are a reflection of the conformation of the repeating units of which they are composed. The latter may exist in a non-energized (NE), energized (E), or energized–twisted (ET) state. Experimental conditions have been established for the interconversion of the different states. Comparisons of the effect of a given respiratory transition in different mitochondria show consistent changes in the extent of membrane folding, the volume of the inner compartment, and the density of the matrix. It has been claimed that the rate of conformational change is of the right order of magnitude for it to be associated with oxidative phosphorylation and the transport of ions (see Harris *et al.* 1968). These observations strongly support a mechanicochemical process and may be interpreted in terms of the model as follows.

The addition of oxygen increases the state of oxidation of the respiratory carriers which swell, and thereby account for the energized state (E_{ag}). Inhibition of electron transport by the addition of actinomycin or rotenone, or of the transport of substrate to its reaction sites by the addition of uncoupling acids, converts E_{ag} into the non-energized state (NE_{ag}). ATP can induce electron transport via thioacylation contraction–expansion cycles, and so, like substrate, energizes the respiratory carriers into the E_{ag} conformation. If, in the presence of oxygen and substrate, P_i only is added, it stimulates ion transport and the polymers expand into the ET_{com} state through osmosis. Addition of ADP uncouples ion transport by stimulating phosphorylation and the swelling is reduced.

IX. The Role of Lipid

A curious, and characteristic feature of many biological membranes which reaches a supreme state of complexity in mitochondria, is the manner in which they can be fractionated into subunits by treatment with detergents and subsequently recombined (see Harris *et al.* 1968). There is little doubt that the subunits are united in the intact membrane as a result of interactions between the hydrocarbon chains of adjacent particles and of alkyl side chains of proteins; most likely bilayers are formed. If the role of such lipid interactions were purely structural, is it not likely that during the process of evolution more stable, covalently crosslinked structures would have evolved? The lipid bilayer rubber hypothesis, proposed in Part I, suggests that the lipids in such structures may function as elastic structures which oppose dilatory processes occurring within the subunits as they extend the hydrocarbon chains. The hypothesis suggests also that such elastic structures stabilize the structure and provide control points for hormone interactions with steroids, and the like, which regulate energy-transducing processes by controlling membrane elasticity. There are some observations supporting such a concept in mitochondrial oxidative phosphorylation.

In Part I evidence has been cited showing the presence and importance of unsaturated and *cis,cis* lipid hydrocarbon conformations in mitochondrial membranes

and the stabilizing effect of vitamin A which, like the ubiquinones, has a carotene side chain. Such structures are consistent with the postulated rubber-like properties of such hydrocarbon chains.

According to the hypothesesis, changing the elasticity of redox complex lipid should change its oxidation–reduction potential and such a change should follow from addition of anaesthetics, which are swelling solvents for hydrocarbons. Amytal, which in peripheral nerve behaves as an anaesthetic, also inhibits electron transfer (see Pumphrey and Redfearn 1963), but is antagonized by vitamin K_3 (Toth *et al.* 1965). Several site-specific inhibitory effects are induced by progesterone and deoxycorticosterone (Vallejos and Stoppani 1967). NADH dehydrogenase is inhibited by diethyl ether (Kaniuga, Gardus, and Jakubiak 1968).

As it has been proposed that a strained conformation of R^1 makes an entropy contribution towards phosphorylation, the presence of swelling agents that can interact with R^1 might be expected to release the strain and uncouple phosphorylation. There is some support for such a thesis.

Since the distribution of energy between oxidative phosphorylation and ion transport is related to the osmotic equilibrium that can be established by ion transport across the membrane, changing the elasticity of the containing walls of the intramitochondrial spaces, in which the ions accumulate, as a result of interactions with lipid swelling agents should alter the osmotic equilibrium, as occurs in changing the crosslinking of an ion-exchange resin, and the magnitude of ion accumulation, and so should influence ion transport and oxidative phosphorylation. Halothane, carbon tetrachloride, chloroform, benzene, and diethyl ether uncouple oxidative phosphorylation and cause mitochondrial swelling; their activity correlates with their relative oil solubilities. Halothane uncouples at all the phosphorylating sites at positions which are most probably between the respiratory chain and the phosphorylation sites (Snodgrass and Piras 1966). Vitamin A induces mitochondrial swelling, and uncouples phosphorylation (Etingof and Shukolyukov 1963); vitamin D opposes such swelling (Billitteri and Raoul 1965). Swelling induced by Ca^{2+}, potassium phosphate, or thyroxine, is reduced by vitamin D (Billitteri and Raoul 1965).

These effects are therefore consistent with related observations of the effects of analogous agents, and of the phenomenon of drug antagonism, in nerve (see Part II) and in the sodium pump (see Part III).

X. Discussion

The model provides a detailed molecular mechanism for a chemiosmotic process for the synthesis of ATP but, since it is confined to within the membrane, it is more in accord with the general concept proposed by Williams (1961, 1962, 1967) than that of Mitchell (1966), whose first chemiosmotic concept first appeared also in 1961. The hypothesis of this paper accounts for the pH gradient associated with ion transport and for alkalinization of the medium during phosphorylation. Robertson's earlier concept (see Robertson 1967) of a respiratory-driven proton gradient driving ion transport could be applied to the proposed ion-pump mechanism, but in so doing an alternative mechanism would be required to account for substrate accumulation, and for the proton uptake from the medium which is observed along with substrate

accumulation, since this mechanism is postulated to be driven by mitochondrial alkalinization.

The proposed model suggests a number of control possibilities. Adsorption interactions between effector ADP, ATP, and AMP might regulate ATP levels. Thyroxine and fatty acids may regulate uncoupling of oxidative phosphorylation, perhaps with the objective of converting respiratory energy into heat for temperature control. K^+ and Ca^{2+} levels may regulate substrate concentrations within mitochondria and thereby influence respiratory rate. Some steroids, and possibly vitamins A and D, may also influence respiratory rate by regulating oxidation–reduction potentials or ion transport.

Whilst it is believed that the hypothesis accounts for the major features of mitochondrial oxidative phosphorylation and ion transport, it fails to account for a number of details. This may in part stem from the assumption that all the phosphorylating sites are identical, which is probably an oversimplification, and in part from neglecting details of the electron-transport carrier train and its associated specificities.

This series of papers has considered only three major processes of energy transduction in order of increasing complexity, and striking similarities in their operating principles have been noted. There are probably many other biological systems utilizing combinations, or special adaptations of parts of them. For example, in both the sodium pump and reversed operations of mitochondria, evidence has been cited suggesting that ATP energizes a contraction by formation of thioester crosslinks via an intermediate acyl phosphate. Is the mechanism of muscle contraction, which which involves Ca^{2+} and ATP, related to that for the contraction induced in mitochondria by addition of ATP and Ca^{2+}? Mitochondrial ATP-energized Ca^{2+} transport may be similar to that in sarcoplasmic reticulum particles where an ATP-driven calcium pump transports two Ca^{2+} for each P_i released from ATP on formation of ADP (see Weber 1966). *N*-Ethylmaleimide inhibits the calcium pump, but ATP protects the pump against such inhibition (Hasselbach and Seraydarian 1966). An acyl phosphate intermediate forms whose dephosphorylation is facilitated by subsequent addition of Mg^{2+} (Martonosi 1969). These observations are remarkably similar to the behaviour of the sodium pump and suggest that a related, thioester crosslinking mechanism may be involved. By analogy with the proposed mitochondrial mechanism, the dephosphorylating effect of Mg^{2+} may be due to its interaction with ADP, resulting from ATP hydrolysis, and stimulation of thioacylation by reaction of HADPMg with a membrane base analogous with B.

In all the energy-transducing processes considered, a dilatory process is opposed by a contractile process and ion pairing and lipid is involved. Three major postulates have been made. Heald's phospholipopeptide has been utilized as a model to illustrate principles for achieving changes in Na^+–K^+ selectively as a result of a conformational change. Energized crosslinking processes, involving thioesters or salt links, have been proposed to enable energy associated with the crosslinking reaction to interact with that associated with a dilatory process. Rubber-like elasticity has been invoked for some membrane bilipids to provide a site and mechanism for hormonal control, drug activity, and the operation of some sensory receptors. From these postulates a unifying interpretation of energy transduction follows which is consistent with by many experimental observations, and which suggests a number of new experiments.

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