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# Mitochondrial Oxidative Phosphorylation at Site I Involving a Fatty Aldehyde/Acid Couple

D. E. Weiss

*Division of Applied Chemistry, CSIRO*
*Box 4331 GPO, Melbourne 3001, Australia*

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*Abstract*

A mechanism for respiration and oxidative phosphorylation at Site I is proposed which involves reduction by NADH of a thioester of a fatty acid to aldehyde. Oxidation of the aldehyde by non-heme iron forms fatty acid which initially binds to a membrane base. As the non-heme iron reduces, entropy is lost through the stretching of a lipid bilayer attached to the non-heme iron and the membrane and to which the fatty acid chain binds. Simultaneously energy is expended in separating carboxyl ion from the protonated base. The charge separation induces movements of protons and reactants which result in the formation of ATP. Subsequent oxidation of non-heme iron relaxes the stretched lipid and the entropy gain in the fatty acid contributes to reformation of its thioester. The mechanism accounts in detail for many observations.

## I. *Introduction*

It was suggested in earlier communications from this laboratory that mitochondrial NADH-oxidase, which is associated with Site I oxidative phosphorylation of ADP, involves a thioester [1], the acyl component of which is fatty acid [2]. Reduction of the thioester by NADH was postulated to form fatty aldehyde via thiohemiacetal which is then oxidized to fatty acid by an oxidized electron carrier [2]. This hypothesis led to experimental work now in progress in this laboratory which so far supports the concept [3]. It will now be shown how a fatty acid/aldehyde couple can account for respiration and oxidative phosphorylation at Site I. Suggestions for new experiments based on some of the predictions of the hypothesis will also be given.

The basic process of energy conservation at an energy-conserving site

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in the mitochondrial electron-transport chain involves removing electrons from a donor and passing them to an acceptor while taking the energy available from the electrons and giving it to another membrane component to convert it into a "high energy state". The fundamental, but unanswered, question is—what is the nature of the "high energy state"? This paper suggests that at Site I it is a combination of an entropy loss in lipid and separation of charge within the membrane induced by a conformational change associated with non-heme iron.

According to the chemical hypothesis, first proposed by Slater in 1953 [4], the energized component is a chemical entity associated with the electron carrier. The "conformational hypothesis", proposed by Boyer in 1964 [5], attributes the high energy state to the formation of a thioester crosslink in a mechanico-chemical reaction of the electron carriers; subsequent hydrolysis of the thioester leads to phosphorylation of ADP. Storey pointed out that the hydrolysis of such a thioester is unlikely to provide sufficient energy for the phosphorylation of ADP under physiological conditions. He therefore proposed that additional "strain" may be added to a thioester crosslink by formation of a disulphide bridge in a reaction with the respiratory chain [6]. When the crosslinks rupture more free energy would then be available for phosphorylation. In recent years various forms of more generalized conformational concepts have been gaining prominence, particularly due to the extensive work of Green and his associates [7] and others. King *et al.* suggested in 1964 that a conformational change could provide a mechanism for regulating electron flow between components in the respiratory chain [8].

In 1961 Mitchell proposed the stimulating "chemiosmotic hypothesis" which incorporates an earlier concept of Robertson; Greville has critically analysed this concept [9]. In this hypothesis energy is considered to be stored in a membrane potential and proton gradient across the inner mitochondrial membrane. Williams proposed, in 1961, that since formation of ATP from ADP and Pi involves formation of water, oxidative phosphorylation might involve specific binding of the water by protons arising within the membrane from oxidation of respiratory carriers [10]. Boyer has systematically reviewed various mechanisms for phosphorylation, including the concept which I will use that the high-energy state may simply be a nucleophile which has been generated by separation of charges within a lipid phase [11].

The hypothesis to be presented below combines portions of these earlier hypotheses. Unlike some of the other conformational hypotheses, which do not show how a conformational change could phosphorylate, this hypothesis proposes a specific mechanism which can be tested experimentally. Accordingly, a number of suggestions for new experiments based on some of the predictions of the hypothesis will be given in the concluding section of the paper.

## II. *Membrane Rubbers*

The proposed mechanism exploits earlier conclusions that some lipoprotein membranes have lipid bilayers with the properties of rubber and are bound to critical structural and functional elements [12]. When such a bilayer is stretched by an energy-consuming process in the functional component the hydrocarbon components of the bilayer align, as in stretched rubber. The resultant loss in entropy gives rise to an elastic, rubber-like retractile force, the magnitude of which reflects the entropy difference between the relaxed and stretched bilipid. Consistent with this concept are the observations in nerve membranes that, like rubber, their stretching at the peak of the action potential gives rise to liberation of heat and greater ordering in the membrane [13]. It has been suggested also that general anaesthetics, which inhibit the stretching, do so by increasing lipid mobility in the relaxed membrane*; the resultant greater entropy loss on stretching increases the retractile tendency and so inhibits dilation [13]. Likewise, heating the membrane inhibits the stretching [13]. But if the membrane is cooled below a critical temperature (analogous with the glass-transition temperature of a rubber) the lipid chains freeze into a much less mobile state with greatly reduced extensibility [13].

Such lipid rubbers provide a mechanism for the conservation of energy in immobilized fatty-chains from a membrane reaction which stretches the lipid [12]. The significance of such phenomena has been discussed for the sodium pump and its association with an entropy effect in photoreceptors involving rhodopsin [14]. It will now be argued that related principles apply also to mitochondrial respiration at Site I; energy arising from an oxidation-reduction reaction at this site is postulated to be partly conserved as an entropy loss in an associated stretched lipid bilayer.

## III. *A Mechanism for Oxidative Phosphorylation at Site I*

Figure 1 shows, in schematic form, the proposed layout of Site I. It consists of flavin F, a thiol $R_2$ SH, a base B, a nucleophile YH, a binding site Pe for a proton, a binding site for MgPi, a fatty acid ($R_1$COOH) forming portion of a bilayer with lipid firmly bound at one end to a structural element of the membrane and to electron-carrier A at the other end†, non-heme iron structures A and electron-acceptor X. X

* The concentration of anaesthetic to produce a 50% block in a cat nerve correlates with that which increases $\Delta S$ in a liposome membrane by 1.7 cals $mole^{-1}$ $deg^{-1}$. Hence anaesthetics probably act by increasing the disorder of the lipid components of a membrane [93].

† The possibility cannot be excluded that the lipid attached to A may bind directly with a hydrophobic component of a structural protein rather than a structurally-bound lipid.

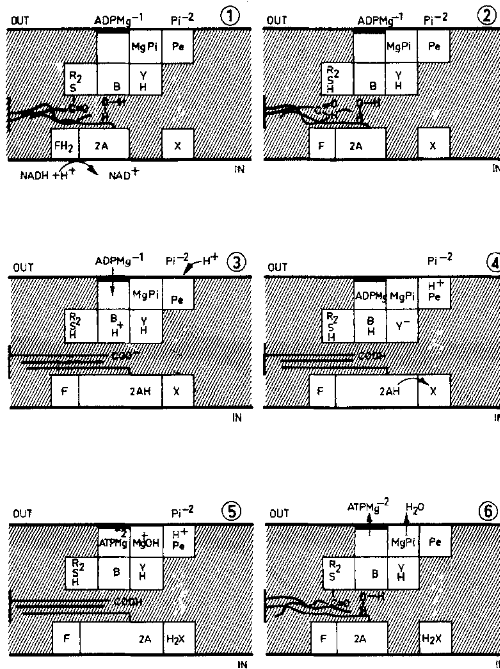
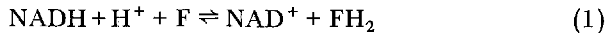



Figure 1. A schematic diagram of the oxidative phosphorylation model at Site I.

represents the branched complex of cytochrome b, iron proteins and coenzyme $Q_{10}$ (CoQ). The shading in the diagram indicates a general lipid environment. The critical lipid bilayer is shown as a pair of lines. The space connecting $ADPMg^{-1}$ and MgPi to their translocases is not shown.

1. *Flavin Reduction*

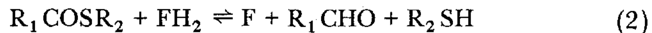
NADH reduces flavin F.



$$NADH + H^+ + F \rightleftharpoons NAD^+ + FH_2 \qquad (1)$$

2. *Thioester Reduction*

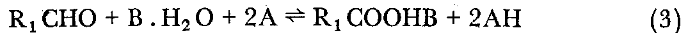
Reduced flavin (and associated iron and sulphur structures) reduce a thioester which has been previously formed between membrane thiol $R_2SH$ and fatty acid $R_1COOH$:



$$R_1COSR_2 + FH_2 \rightleftharpoons F + R_1CHO + R_2SH \qquad (2)$$

3. *Aldehyde Oxidation and Membrane Energization*

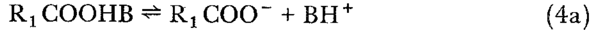
Fatty aldehyde reduces non-heme iron in a reaction involving water. The water may be free, bound to a special hydration site such as molybdenum or, as shown in Fig. 1, bound to B. The overall reaction is:



$$R_1CHO + B.H_2O + 2A \rightleftharpoons R_1COOHB + 2AH \qquad (3)$$

As non-heme iron A reduces it stretches. In so doing it motivates three processes.

(a) The stretching tends to dissociate $R_1COOHB$ to create a carboxylate ion within lipid:



$$R_1COOHB \rightleftharpoons R_1COO^- + BH^+ \quad (4a)$$

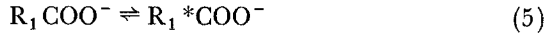
(b) $ADPMg^{-1}$ moves in over its translocase to neutralize $BH^+$ and $H^+$ moves passively in to Pe to balance the charge:

$$BH^+ + ADPMg^{-1} \rightleftharpoons BHADPMg \quad (4b)$$

$$Pe + H^+ \rightleftharpoons PeH^+ \quad (4c)$$

$ADPMg^{-1}$ therefore facilitates the dissociation of $R_1COOHB$.

(c) Lipid attached to A stretches and stretches also the hydrocarbon chain $R_1$ of the fatty acid which binds to it. This lipid rubber loses entropy as a result and gains free energy from the reduction of A:



$$R_1COO^- \rightleftharpoons R_1{}^*COO^- \quad (5)$$

Thus energy is conserved from the aldehyde/A couple in two ways: (a) as a membrane potential, since charge has been transferred over the membrane without an adjacent cation, and (b) as an entropy loss represented by $R_1 \rightarrow R^*$. Consequently reactions 4 and 5 will be termed the "energizing reactions". When 2AH becomes fully extended $R_1^*COO^-$ contacts YH from which it abstracts one proton:

$$R_1^*COO^- + YH \rightleftharpoons R_1^*COOH + Y^- \quad (6)$$

In this reaction, negative charge transfers to $Y^-$.

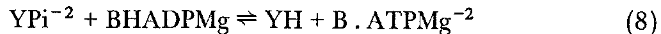
## 4. *Phosphorylation*

Being in a lipid environment, $Y^-$ is a strong nucleophile and forms a phosphorylated derivative by reacting with MgPi:

$$Y^- + MgPi \rightleftharpoons YPi^{-2} + MgOH^+ \quad (7)$$

(This reaction is based on a suggestion of Boyer [11].)

The phosphoryl group now transfers to ADP:



$$YPi^{-2} + BHADPMg \rightleftharpoons YH + B\,.\,ATPMg^{-2} \quad (8)$$

As may be seen from Figs. 2 and 3, energy for phosphorylation comes from recombination of the separated membrane charges $BH^+$ and $Y^-$ through successive movements of charge. Abstraction of the proton from $BH^+$ in BHADPMg by $YPi^{-2}$ to form YH eventually neutralizes these charges; energy thus liberated is postulated to liberate transient metaphosphate which adds to $ADPMg^{-1}$.

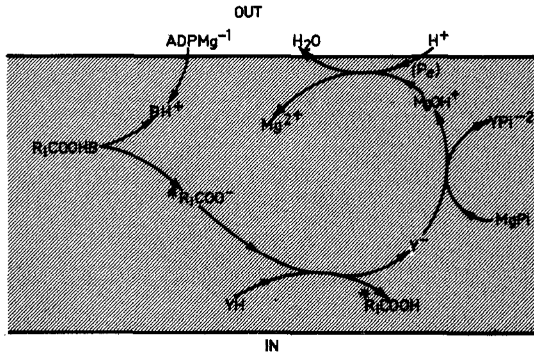



Figure 2. Energized dissociation of $R_1COOHB$ induces movements of positive and negative charge through the system.

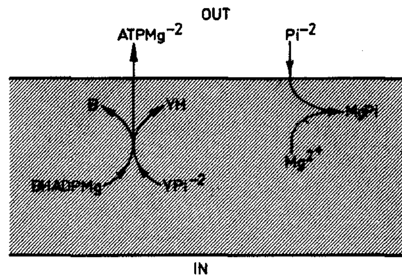
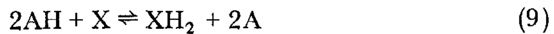



Figure 3. The phosphorylation of $ADPMg^{-1}$ produces further movements of charge which result in outward movement of $ATPMg^{-2}$ in exchange for inward movement of $Pi^{-2}$.

5. *Respiratory-chain Reduction*

Whilst phosphorylation is occurring, the stretched 2AH has met the next component in the respiratory chain, X, which it reduces:



$$2AH + X \rightleftharpoons XH_2 + 2A \qquad (9)$$

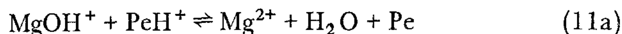
6. *Membrane Relaxation*

Non-heme iron A, and with it stretched fatty acid, now contracts into its oxidized conformation and the gain in entropy from the relaxing fatty acid chain permits the formation of thioester. Water thus liberated is shown tentatively as being bound by B (see III, 3).

$$R_1^{*}COOH + R_2SH + B \rightleftharpoons R_1COSR_2 + B.H_2O \qquad (10)$$

The stretching-relaxing membrane rubber may also provide a mechanical mechanism for inducing favourable conformations at the adenine nucleotide and $Pi^{-2}$ binding sites. The conformation of the adenine nucleotide binding site is postulated to change when it exchanges $ADPMg^{-1}$ for $ATPMg^{-2}$ such that $R_1COOH$ cannot contact B when $ATPMg^{-2}$ is combined with B. Otherwise $R_1COOHB$ would form, rather than thioester, when the membrane relaxes.

As shown in Fig. 3 the $H^+$, which follows $ADPMg^{-2}$ into the membrane, reacts with $Mg^+OH$ to form water:



$$MgOH^+ + PeH^+ \rightleftharpoons Mg^{2+} + H_2O + Pe \qquad (11a)$$

$Pi^{-2}$ then enters to neutralize $Mg^{2+}$:

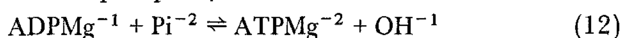
$$Mg^{2+} + Pi^{-2} \rightleftharpoons MgPi \qquad (11b)$$

Simultaneously $ATPMg^{-2}$ dissociates from its binding site B and enters the outer aqueous phase to balance the charges.

### 7. *Stoichiometry and Operation of Translocases*

The energizing reactions induce two flows of opposite charge (Fig. 2). Positive charge flows from $BH^+$ to $PeH^+$ as $ADPMg^{-1}$ enters and induces passive movement of $H^+$ to Pe thereby bringing two key reactants into the membrane. Simultaneously negative charge flows from $R_1^*COO^-$ to $Y^-$ and sets the stage for phosphorylation. At the completion of phosphorylation $MgOH^+$ has been produced. Its reaction with $H^+$ from $PeH^+$ provides $Mg^{2+}$ and the driving force which enables $Pi^{-2}$ to enter for the next cycle in exchange for $ATPMg^{-2}$ (Fig. 3).

The overall result is that $Pi^{-2}$ has exchanged with $ATPMg^{-2}$ and $ADPMg^{-1}$ has been replaced by $OH^-$ in the outer phase. This is consistent with the overall phosphorylation reaction:



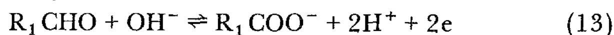
$$ADPMg^{-1} + Pi^{-2} \rightleftharpoons ATPMg^{-2} + OH^{-1} \qquad (12)$$

It is also consistent with the known exchange of $Pi^{-2}$ for $OH^-$ over its translocase [15].

### 8. *Reversed-flow Operation*

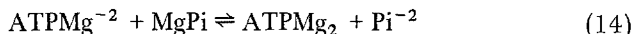
The oxidation-reduction state of the respiratory chain is a sensitively-poised function of the [ATP]/[ADP] [Pi] ratio. This is also related to the oxidation-reduction couples involving thioester, NADH and $R_1COOH$. Reduction of $NAD^+$ requires ATP and the addition of succinate to supply a source of electrons and protons with which to reverse aldehyde oxidation.

The oxidation of aldehyde produces carboxylate rather than carboxyl since it involves hydroxylation:

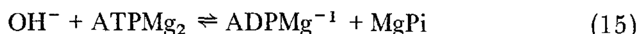


$$R_1CHO + OH^- \rightleftharpoons R_1COO^- + 2H^+ + 2e \qquad (13)$$

As may be seen from the top portion of Fig. 4, $OH^-$ from reversal of reaction 13 hydrolyses $ATPMg_2$; entry of $ATPMg^{-2}$ involves reaction with MgPi and exchange with $Pi^{-2}$:



$$ATPMg^{-2} + MgPi \rightleftharpoons ATPMg_2 + Pi^{-2} \qquad (14)$$



$$OH^- + ATPMg_2 \rightleftharpoons ADPMg^{-1} + MgPi \qquad (15)$$

Aldehyde, from reversal of reaction 13, forms thioester and reduces $NAD^+$ (Fig. 4). Hydrolysis of the thioester liberates fatty acid which provides $H^+$ with which to balance the charge on $ADPMg^{-1}$ so that both may leave the membrane.

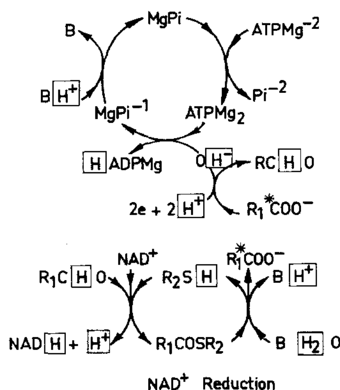



Figure 4. The proposed mechanism for ATP-induced energy-linked reduction of $NAD^+$.

Thus by acting as hydroxyl acceptor, $ATPMg_2$ induces a flow of protons out of the membrane; the resultant increase in the ratio $R_1COO^-/R_1COOH$ raises the extent of reduction of the pyridine nucleotide couple provided succinate is present to donate the necessary electrons. The aldehyde/acid couple thus provides a pH-control point in the electron-carrier system.

It should be noted that this mechanism for reversed-electron flow is not simply reversal of phosphorylation and does not require Y. Also, as shown in Fig. 1, the conformational change is postulated to bring AH in contact with X, and A contacts the forward pathway through X only when it is reduced. There must therefore be another pathway for reversed-electron flow and this might result from the complex spatial changes which seem to occur under different conditions of the system cyt. b-$b_T$, CoQ and flavoprotein.

## 9. *Respiratory Control and Uncoupling*

The phenomenon of "respiratory control" [16] is accounted for in the model by the difficulty of separating $R_1COO^-$ from $BH^+$ during the energizing reactions when there is no permeant anion to neutralize $BH^+$ so produced. Addition of $ADPMg^{-1}$, being a permeant anion, facilitates the dissociation by neutralizing the charge on $BH^+$ and by having a significant binding affinity for $BH^+$. Respiration is therefore enhanced.

Lipid-soluble acids, such as dinitrophenol (DNP), uncouple phosphorylation but enhance respiration.

Four possible mechanisms require to be considered in accounting for uncoupling in the model. (a) Uncoupling acid anions could compete with entry of substrate anions as has been suggested by others [17]. (b) Uncoupling acids, being able to carry protons through lipid [18], could provide an alternative pathway to that leading to Pe for passive entry of $H^+$. (c) Uncoupling acid anions could compete with $ADPMg^{-1}$ for combination with $BH^+$. This seems unlikely since the binding of ATP or ADP to their translocase in a de-energized membrane is not affected by uncoupling acids [19]. (d) Protonated uncoupling acids acting as acids, or uncoupling acid anions functioning as bases, could catalyse decomposition of $YPi^{-2}$. Recent evidence, based on the pH-dependence of various uncoupling reactions [20], shows that uncoupling involves an acid-base catalysis which either results in the decomposition of a phosphorylated intermediate [20] or prevents its formation.

The following mechanism is therefore proposed for the uncoupling activity of agents, such as DNP, which uncouple through acid catalysis although they also induce a bypassing flow of $H^+$.

Uncoupling acid HU, rather than YH, protonates $R_1^{\ddagger}COO^-$ since it is a stronger acid; hence $YPi^{-2}$ cannot form and phosphorylation is inhibited. The uncoupling acid anion $U^-$ combines with $H^+$ from the external solution so that the latter does not combine with Pe.

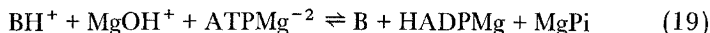
$$R_1^{\ddagger}COO^- + HU \rightleftharpoons R_1^{\ddagger}COOH + U^- \quad (16)$$

$$U^- + H^+ \rightleftharpoons HU \quad (17)$$

Uncoupling acid is now ready for the next cycle.

Because the internal flow of protons via Pe is now cut off the reaction described by Eq. (11) reverses and leads to hydrolysis of ATP:

$$MgPi + H_2O \rightleftharpoons MgOH^+ + HPO_4^{-2} + H^+ \quad (18)$$



$$BH^+ + MgOH^+ + ATPMg^{-2} \rightleftharpoons B + HADPMg + MgPi \quad (19)$$

In such reactions exit of $Pi^{-2}$ is balanced by entry of $ATPMg^{-2}$ and the outer phase receives $ADPMg^{-1}$ and one $H^+$. Respiratory energy is necessary to dissociate $R_1COOHB$. Acid uncoupling therefore induces a proton flow from the medium to $R_1^{\ddagger}COO^-$ which bypasses the phosphorylating mechanism.

Base-catalysed uncoupling involves competition between $Y^-$ and the anionic uncoupling acid in the reaction with MgPi to form a phosphorylated derivative of $U^-$ which hydrolyses so that oxidation phosphorylation is uncoupled. The base then ionizes for the next cycle of reaction.

Both the acid- and base-catalysed uncoupling mechanisms give an ATPase which differs from that involved in ATP energy-linked reduction of $NAD^+$. Neither involve YH. The ATPase resulting from uncoupling keeps the ratio $R_1COO^-/R_1COOH$ low and so induces only forward-electron flow from NADH. Under ATP energy-linked conditions addition of uncoupling acid therefore inhibits $NAD^+$ reduction because $R_1COO^-$ is protonated.

## 10. *Inhibitors*

Oligomycin is a characteristic inhibitor of oxidative phosphorylation and all the partial reactions involving ATP.

Oligomycin is postulated to bind to a component of the lipoprotein membrane which is near $BH^+$ and YH so as to prevent reaction between MgPi and $Y^-$. In ATPase situations its binding inhibits reaction between $ATPMg^{-2}$, when bound at B, and $MgOH^+$. By hindering such reactions oligomycin inhibits phosphorylation and reversed reactions involving ATP. Elimination of reaction 7, where $YPi^{-2}$ forms from MgPi, inhibits further Pi and adenine nucleotide movements and so inhibits respiration in tightly-coupled mitochondria. Addition of uncoupling acid protonates $R^*_1COO^-$; since a proton flow can now bypass the phosphorylation site respiration is relieved. The inhibitor dicyclohexylcarbodiimide behaves similarly.

Aurovertin can also bind to the same site as oligomycin but its different shape is such that it is postulated to prevent reaction between $YPi^{-2}$ and BHADPMg. Thus oligomycin inhibits phosphorylation of $Y^-$ whilst aurovertin inhibits dephosphorylation of $YPi^{-2}$.

Alkyl guanidines and biguanidines (BG) are postulated to inhibit phosphorylation and respiration at Site I by the following reaction:

$$R^*_1COO^- + YH + BG \rightarrow R^*COOHBG + Y^- \qquad (20)$$

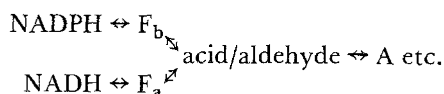
$Y^-$ completes one phosphorylation cycle, with concomitant entry of $Pi^{-2}$ and subsequent binding as MgPi, after which further phosphorylation and respiration are inhibited by the binding of BG to fatty acid. Addition of uncoupling acid relieves the respiratory inhibition by providing a proton to enable $R^*_1COOH$ to separate from $BGH^+$.

## 11. *Transhydrogenase Reaction*

The hypothesis suggests two possible reaction pathways for the energy-linked transhydrogenase reaction between $NADP^+$ and NADH

under physiological conditions where the NADPH couple is considerably more reducing than the NADH couple. There is insufficient experimental evidence to decide between these alternatives.

One proposal is that there are two distinct pathways leading from the acid/aldehyde couple to the pyridine nucleotides via separate thioesters:




Such a scheme has been proposed for *Torulopsis utilis* [21], for which there is some evidence, but it may not apply to all organisms. The second possibility is that NADH and NADPH react with a common thioester, but on opposite sides, as proposed by Storey [6].

As may be seen from Fig. 4, reversed, energy-linked electron flow to $NAD^+$ involves reduction of $NAD^+$ by aldehyde. The reducing power of the aldehyde couple is considerably greater than that of the NADH couple. Consequently there is an excess of free energy for reduction of $NAD^+$ and this makes reduction of $NADP^+$ possible even at highly reducing concentrations of the latter couple. The transfer of hydride between NADH and $NADP^+$ therefore requires ATP and the respiratory chain to form $R_1CHO$ which then reduces $NADP^+$ and forms thioester; the latter then oxidizes NADH. In a reversed situation NADPH can reduce fatty acid and synthesize ATP; some of the aldehyde so produced can also reduce $NAD^+$. The net result is that NADPH has reduced $NAD^+$ and synthesized some ATP. Hence the model shows how the transhydrogenation reaction can be in reversible equilibrium with the energy conserving and phosphorylating systems of the respiratory chain.

## IV. *Evidence for Entropy Coupling at Site I*

A key feature of the model is that energy liberated from the oxidation of fatty aldehyde by non-heme iron is conserved partly as a membrane potential for driving phosphorylation, and partly in the stretched lipid chains. Energy derived from the latter is recycled to enable electrons to flow from NADH at a potential which is comparable with that of the fatty acid-aldehyde couple. This section analyses the experimental evidence on which this postulate is based.

### 1. *Flavin*

The simplest interpretation is that flavoprotein is involved in Eq. (1) as an acceptor of an hydride ion from NADH without formation of measurable amounts of one-electron intermediates, although the latter possibility cannot be excluded [22]. This step is inhibited by rhein [23].

2. *Fatty Acid*

The rate of respiration, and the amount of Pi ⇌ ATP exchange during oxidative phosphorylation, when plotted as functions of fatty acid concentration show sharp maxima at very low concentrations [24, 25]. Fatty acid is therefore intimately associated with oxidative phosphorylation.

3. *Fatty Aldehyde*

Labelled oxygen atoms appear in fatty acids [26, 27] when oxidative phosphorylation occurs in the presence of $H^{18}OH$. This is accounted for by oxidation of fatty aldehyde in a reaction (reaction 3) involving water which can exchange with water in the external phase.

Mackler has shown that rat liver mitochondria can oxidize acetaldehyde, the addition of which results in immediate reduction of cytochromes b and $c + c_1$ [28]. Oxidation is inhibited by antimycin, Seconal or Amytal. The addition of fatty aldehydes markedly stimulates respiration, particularly that which is supported by succinate [3]. The latter is accounted for by reduction of endogenous CoQ via the respiratory chain by the aldehyde; such reduction is known to stimulate succinate-oxidase activity [23]. Inhibitor studies with Amytal, rotenone, and piercidin A have shown, for both forward and reversed-electron flow conditions, that fatty aldehyde substrates with varying chain lengths are probably oxidized at a site in the respiratory chain which is close to the site at which NADH is oxidized [3]*. The specific aldehyde reagent, dimedon, inhibits respiration supported by NADH [3], although it has not been shown that it does so by reacting with endogenous aldehyde.

There is therefore some reason to believe that a fatty acid/aldehyde couple may be present in the mitochondrial respiratory chain close to NADH.

It has been claimed recently that molybdenum, a known component in soluble aldehyde oxidase [29], is present also in the NADH portion of the mitochondrial respiratory chain [30], although this observation has not been confirmed by others. In soluble aldehyde oxidase molybdenum participates as a Lewis acid in the hydroxylation reaction of the enzyme [29]. It may fulfil a similar role in mitochondria if it is present.

4. *Non-heme Iron*

There is evidence showing that non-heme iron proteins accept only single electrons [22]. Hence Eq. (3) is written as involving 2AH rather than $AH_2$.

Since forward mitochondrial oxidation of decylaldehyde is inhibited

* However, it has not yet been established whether the respiratory stimulation observed upon addition of long chain aldehyde is due to direct electron donation from aldehyde to the respiratory chain or due to an indirect donation via an aldehyde oxidase producing NADH and fatty acid which stimulates respiration through uncoupling.

by rotenone and Amytal, but reduction of $NAD^+$ by the aldehyde is not inhibited by the presence of rotenone, Amytal or piericidin A, the site of aldehyde oxidation lies close to the NADH-flavin component of the respiratory chain [3].

Recent studies show the presence of a non-heme iron component in *C. utilis,* with a low oxidation potential which is similar to that of NADH, following the NADH flavoprotein in the respiratory chain [21]. This flavoprotein is essential for energy conservation at Site I in this yeast and is associated with a second, high potential non-heme iron component to which piericidin A and Amytal binds; interaction between the two non-heme iron components gives rise to an epr signal at g = 1.94 [21]. The physiological significance of the second non-heme iron component is obscure. There are indications that the NADH dehydrogenase of *C. utilis* resembles that of the mammalian enzyme in many respects [23]. Studies with the latter also suggest that coupling Site I is located between the point in the non-heme iron complex which gives the g = 1.94 epr signal and the specific binding site for rotenone [23, 31]. With rotenone or piericidin A present a chromophore, which seems to be associated with non-heme iron, remains permanently bleached even after exhaustion with NADH; ATP causes rapid and complete reoxidation of the chromophore [31].

It is therefore legitimate to involve a non-heme iron component A as the energy-conserving site in the model. Is there any evidence to show that A may change its conformation on reduction and so stretch lipid and $R_1$ ?

The only model compound so far described with absorption spectra characteristic of the oxidized non-heme iron components of NADH dehydrogenase is a $Fe^{3+}$-mercaptoethanol-inorganic sulphide octahedral complex [32]. It does not show an epr signal at g = 1.94. Reduction of the iron decomposes the complex. The similarity of the absorbances of the model and non-heme iron, and the fact that NADH-dehydrogenase which has been inactivated by oxygen can be reactivated by treatment with $Fe^{3+}$, mercaptoethanol and sodium sulphide [32], shows that at least some of their iron-sulphur coordination structures are closely related. At least portion of the non-heme iron protein may therefore have a similar basic conformation and exhibit the same conformational change on reduction to that of the model complex. Electron microscopy [7, 33], circular dichroism [34] and optical rotatory dispersion studies [34] provide further evidence for conformational changes in the mitochondrial membrane arising from its energization.

There is therefore some basis for postulating a conformational change when A is reduced and a reasonable chemical mechanism for achieving it.

### 5. *Lipid*

Is there evidence that lipid is bound to the non-heme iron component close to the site where piericidin A inhibits and that such lipid forms a

bilayer with lipid which is bound to a structural element in the membrane and which is essential for activity as postulated in the model?

The NADH-CoQ enzyme reaction in mitochondria requires phospholipid but the latter's composition is not critical [35, 36]. Digestion of mitochondrial particles with very small amounts of phospholipase A inhibits NADH-CoQ oxidase [36]; this is partly because of the depletion of essential phospholipids and partly because the lysolecithins so formed are strong inhibitors [23]. Larger amounts of the phospholipase liberate the NADH dehydrogenase; piericidin A strongly inhibits such solubilization [23, 37]. Specifically bound piericidin A is therefore close to the sensitive phospholipids and its binding site has been shown (Section IV, 4) to be adjacent to A. The specific binding of rotenone and piericidin A appears to involve strong non-covalent bonds to lipid and protein [38] and the lipid concerned is involved in the binding of NADH dehydrogenase to the membrane [23]. These observations therefore support the model.

If $R_1 \rightarrow R_1^*$ involves an entropy change then this should be a function of the chain length and stereochemistry of $R_1$ and the lipid chain in the postulated critical lipid bylayer. It is an experimental fact that respiratory rate [24, 25, 39], ATP $\rightleftharpoons$ Pi exchange [24, 25], and incorporation of $^{18}O$ from $H^{18}OH$ into fatty acid [26, 27], are all functions of the chain length of the fatty acid. The rate of oxidation of fatty aldehyde is also a function of its chain length [3]. The concentration of fatty acid for optimal oxidation rate in rat liver shows a maximum at a chain length of $C_{14}$ for saturated fatty acids but $C_{18}$ unsaturated acids show approximately the same activity as the $C_{14}$-$C_{16}$ saturated acids [39]. Whilst oleic acid strongly influences ATP $\rightleftharpoons$ Pi activity its *trans* isomer, elaidic acid, is only weakly active [26]. $C_3$ and $C_4$ fatty acids, the methyl esters of myristic, oleic and arachidaric acids and triolein are without effect on ATP $\rightleftharpoons$ Pi activity [26]; similarly $C_3$ and $C_4$ fatty aldehyde substrates do not induce the accelerated respiratory rate found with longer chain fatty aldehydes [3]. The chain length, stereochemistry and presumably binding of fatty acid/aldehyde to a membrane component, are therefore important elements in the NADH-CoQ enzyme system and in oxidative phosphorylation. The following experiments suggest that the binding site for the hydrocarbon chain of the fatty acid/aldehyde is most likely lipid rather than protein.

Arrhenius plots of mitochondrial respiration rate with NADH-linked substrates show a marked transition temperature above which there is a considerable increase in activity [40]. Although phosphorylation rate is influenced by temperature, P : O and respiratory control ratios are independent of temperature between 1.5 and 25°C [41]. Lipid components of the membrane undergo thermal transitions at temperatures which correlate with the transition in the Arrhenius plots obtained with intact mitochondria [42]; unsaturation of the lipid

depresses the transition temperature. Spin labelling also shows that there is a marked increase in lipid mobility above the transition temperature and that this increased mobility is related to enhanced respiration and phosphorylation rates [42].

It is therefore likely that the element to which fatty acid and aldehyde binds is lipid, rather than protein, as postulated in the model.

## 6. *Entropy Coupling*

Why is respiratory rate so sharply increased above the transition temperature? The correlation of lipid mobility with the transition temperature of the fatty acid and with enzymic activity establishes the importance of lipid chain stereochemistry. This is also shown by the observation that oleic, but not elaidic, acid has a large influence on $Pi \rightleftharpoons H_2O$ exchange and $^{18}O$ incorporation from $H^{18}OH$ into the fatty acid. Such stereochemistry recalls the behaviour of rubbers. Thus at room temperature *cis* polyisoprene (natural rubber), but not its *trans* isomer (gutta percha), exhibits the elastic properties of a rubber.

The observation that there is a thermal transition in mitochondria, and that high respiration rates occur only above this temperature, meets the crucial requirement of the model, as deduced from the analogous glass/rubber transition in polymers, that the large postulated entropy change $R_1 \rightarrow R_1^*$ can occur only if $R_1$ is in a state of maximum mobility before it is mechanically stretched. When hydrocarbon chains "freeze" below their transition temperature they align [43] to form a structure which is very similar to that which they attain when they are stretched at a temperature above the transition; there is therefore little rubber retractibility and entropy change between $R_1$ and $R_1^*$ when such "frozen" chains are stretched.

At a very low concentration vitamin A greatly stabilizes the mitochondrial membrane and produces a state with a maximum rubber-like retractile tendency which opposes swelling; at much higher concentrations swelling is greatly facilitated [44]. This is the typical behaviour of a "general" anaesthetic [12, 13, 44]. Low concentrations of such anaesthetics, which are swelling solvents for lipids, maximize the difference between the mobility of the relaxed and the stretched lipid chains; high concentrations prevent alignment of the adjacent chains when they are stretched. Consistent with such phenomena are the facts that general anaesthetics, at $2 \times 10^{-3}$ molar concentrations, inhibit NADH oxidation† but do not significantly alter P : O ratios and that the inhibition is not released by uncoupling acids [45]. The inhibition by halothane of $NAD^+$ energy-linked reduction [45] is also consistent with the reaction pathway shown in Fig. 4 which involves $R_1^*$.

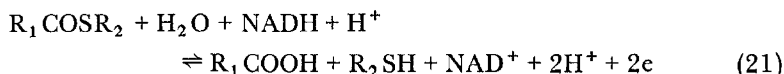
† Steroid anaesthetics also uncouple oxidation in brain tissue to an extent which parallels their potency as anaesthetics [94].

The behaviour of anaesthetics on NADH-CoQ reductase, the existence of a transition temperature and the influence of chain length and the stereochemistry of $R_1$ on this system, provide strong support for the concept of entropy coupling associated with a lipid bilayer and a fatty acid/aldehyde in this enzyme system. This is reinforced by the following thermodynamic considerations.

## 7. *Thermodynamics*

If formation of thioester involves an entropy gain then a thermodynamic analysis of oxidation of NADH by the oxidized respiratory-chain should show a loss in entropy. Calorimetric studies of the oxidation of NADH by a nonphosphorylating submitochondrial preparation show this [46]. An entropy loss corresponding with $T\Delta S = -9.2$ kcal/mole† is observed at Site I but the entropy change for succinate oxidation is only $T\Delta S = -0.5$ kcal/mole. When a hydrocarbon chain assumes a straight, immobile state from a state of maximum mobility both theory and experiment show that there is an entropy loss of R log 3 per methylene carbon; unsaturated groups would not contribute [43]. Consequently the observed entropy loss corresponding with $T\Delta S = -9.2 \pm 1.2$ kcal/mole at 25°C can be accounted for if about 14 carbon atoms change from a mobile to an immobile state in the lipid-fatty acid rubber when $R_1$ changes to $R_1^{\ddagger}$. Oleic acid, with one unsaturated double bond, would be expected to show approximately the activity of a $C_{16}$ saturated acid whilst linoleic acid would be expected to behave more like a $C_{14}$ saturated acid; their observed influence on respiratory rate is consistent with these predictions which are approximations.

The overall oxidation-reduction reaction associated with NADH is:



$$R_1COSR_2 + H_2O + NADH + H^+ \rightleftharpoons R_1COOH + R_2SH + NAD^+ + 2H^+ + 2e \quad (21)$$

This has two components: oxidation of NADH itself under midpoint potential conditions at neutral pH contributes −320 mV whilst hydrolysis of thioester probably yields a further −210 mV. Thus the total potential change associated with the reaction is about −530 mV. As shown by the calculations of Devault [47], these values can vary by 60 mV either way from the midpoint potential through changes in reactant concentration, without losing transfer efficiency between the reacting couples. The actual free energy change for reaction 21 [40] will be modified by the binding of water to an hydration site such as B, and of $R_1COOH$ to B, although these are opposed rather than additive effects, so that the above values can give only an approximate indication

† The sign reported was in error as clearly shown by the other data ($\Delta H = -61.6$ kcal/mole; $\Delta G = -51.9$ kcal/mole) which check with the work of others [46].

of the true situation. Nevertheless it can be seen that the potential of the NADH/thioester couple will be more reducing than that of the NAD/$NAD^+$ couple itself. The midpoint potential at pH 7.0 of the acetaldehyde/acetate couple is −580 mV, which is probably not too different from that of the longer chain homologues. This is of the right magnitude for efficient acceptance by a fatty aldehyde/acid couple of free energy from the NADH-thioester couple as postulated in the model. Involvement of thioester therefore accounts for a more reducing component occupying a position on the oxygen side of the weaker reductant NADH in the electron-transport sequence. A theoretical analysis of such a situation has been made recently by Devault [47].

The ability of the electron chain to reduce the more reducing NADPH/$NADP^+$ endogenous couple under energy-linked, reversed-electron flow conditions (see Sections III, 11; IV, 9) provides further thermodynamic arguments for the existence of an intermediate oxidation-reduction couple having higher reducing power than the NADH/$NAD^+$ couple.

The model is therefore supported by the available thermodynamic data.

## 8. *Thioester*

Is there evidence to support involvement of fatty acid with a thiol to form thioester in NADH-dehydrogenase?

A number of thiols of different reactivity are present in the NADH-dehydrogenase of mitochondria [23, 48]. One or more thiols are essential for NADH-ferricyanide reductase activity, reduction of flavin by NADH and the formation of the NADH-linked epr signal ($g = 1.94$) [49]. Titration with mercurials, under conditions which expose the thiols, results in the loss of one of the two specific binding sites of piericidin A and rotenone [48]. There is however no evidence to show that fatty acid reacts, under respiratory-energized conditions, to form a thioester with any of the thiols although the evidence does not exclude such a possibility. The fact that the thiols in NADH-dehydrogenase are only observed under conditions which suggest that conformational changes have occurred in the enzyme [23] suggests that if a thioester does form it will be well shielded and will most likely be inaccessible to external reagents. The hindered ester resonance of a thioester does however provide a well known mechanism for mediating the reduction of NADH [50].

## 9. *Transhydrogenase Reaction*

The proposed mechanism for pyridine nucleotide transhydrogenase activity accounts for its thermodynamic properties, its state of reversibility with the respiratory chain and phosphorylating systems

[51], for participation of thiols [48], for the consumption of one high-energy bond for the reduction of one molecule of $NADP^+$ by NADH [52] and for hydride transfer without participation of water provided the hydride ion from the pyridine nucleotide reacts at only one position in the thioester [6].

### 10. *Conclusions*

The proposed concept of lipid stretching at Site I, and the use of such a source of energy to facilitate oxidation of NADH via a fatty acid/aldehyde couple, is strongly supported by experiments concerning the thermodynamics of the situation and the known presence and behaviour of the postulated participants (F, A, $R_1CHO$, $R_1COOH$, $R_2SH$ and $R_1^\ddagger$) at the proposed sites of action in the respiratory chain. Evidence for the postulated conformational change on reduction of non-heme iron is less convincing, but the available information is consistent with the hypothesis. There is no evidence to show that a thioester participates.

## V. *Evidence for a Membrane Potential Associated with a Protomotive Force and Oxidative Phosphorylation at Site I*

The second postulated mechanism for conserving energy from aldehyde oxidation by non-heme iron is the creation of a membrane potential through movements of charges and protons. It is energy so conserved which is postulated to drive the phosphorylation of ADP. Phosphorylation occurs when a proton moves from $BH^+$ in BHADPMg to $YPi^{-2}$ to form YH. The membrane potential is created when respiratory energy stretches AH and separates $R_1COO^-$ from $BH^+$. This induces, as can be seen in Figs. 2 and 3, sequential movements of negative charge by a series of carriers in the order $ADPMg^{-1}$, $R_1COO^-$, $Y^-$ and $OH^-$; the latter is cleaved from $Pi^{-2}$ when $YPi^{-2}$ forms. Compensatory movements of positive charge, which is carried at first by $H^+$, and then by $Mg^{2+}$, occurs in the opposite direction. The movements of charge terminate when $OH^-$ is captured by $Mg^{2+}$ and the $MgOH^+$ so formed reacts with the proton current to liberate $Mg^{2+}$, and form water. The termination reaction is therefore:

$$MgOH^+ + PeH^+ \rightleftharpoons Mg^{2+} + H_2O + Pe \qquad (22)$$

In such a reaction sequence $Mg^{2+}$ plays a key role. By binding to $Pi^{2-}$ it neutralizes negative charge and produces a neutral complex which permits close approach of the nucleophile $Y^-$ to the phosphorous atom. $Mg^{2+}$, and $BH^+$, fulfil a similar function with $ADP^{-3}$ so that approach by $YPi^{-2}$ during the phosphorylation reaction is not subjected to

electrostatic repulsion. $Mg^{2+}$ also functions as the hydroxyl acceptor from $Pi^{-2}$. Studies of metal-ion activation of ATPases, including mitochondrial ATPase, show that $Mg^{2+}$ is well suited to its postulated task of functioning as hydroxyl acceptor because of its small ionic radius [53]. Formation of $Mg^{2+}$ causes $Pi^{-2}$ to move into the membrane.

The proposed phosphorylation mechanism therefore has some resemblance in principle to the ATPase of Mitchell's chemiosmotic hypothesis, since both are driven by a proton/membrane potential gradient [9]. However, there are important distinctions. The Mitchell hypothesis postulates the movement of two protons, per phosphorylation site, right across the membrane from the outer to the inner aqueous phase (see Fig. 6, reference [9]); however, despite assiduous searching, the postulated acidification of the inner aqueous phase during phosphorylation has never been observed (for a discussion see references [1, 9]). The present hypothesis involves no such acidification of the inner aqueous medium; the ion and proton currents are confined to the membrane and the outer aqueous phase. The hypothesis therefore resembles in this respect that proposed by Williams [10]. However only one $H^+$ is extracted from the outer solution for each ATP produced.

This Section analyses the experimental justification for the postulated phosphorylation mechanism.

### 1. *Adenine Nucleotide Translocation*

An adenine nucleotide translocase is a known, important element in oxidative phosphorylation [15, 19]. Is there evidence for the postulate in Section III, 7 that respiratory energization of Site I induces the inward movement of $ADPMg^{-1}$ which establishes a membrane potential and that some of the respiratory-energy is conserved by creating this potential gradient?

The specificity of oxidative phosphorylation in intact mitochondria for ADP as phosphate acceptor resides in the adenine nucleotide translocase in the inner membrane [54]. The normally high affinity of oxidative phosphorylation mechanisms in intact mitochondria for both ADP (and Pi) is greatly decreased when membrane structure is disrupted by exposure to digitonin or to sonic energy, although high P : O ratios are preserved [55]. Studies with de-energized systems show that the binding affinities of ADP and ATP to the highly specific site from which they can be displaced by atractyloside are similar [19]. The apparent specificity for ADP exhibited under phosphorylation conditions must therefore derive from the membrane potential and the associated phosphorylation reaction.

The behaviour of ATP $\rightleftharpoons$ ADP exchange through the membrane translocase has been shown to conform with theoretical predictions based on a model invoking a membrane potential; the membrane

potential is poised positively outside [15]. Adenine nucleotide translocation also contributes a significant part of the phosphorylation energy of ATP delivered from the mitochondria in addition to the energy generated in the mitochondria by the formation of the anhydride bond in ATP [15]. Such observations show the creation within the membrane of a positive charge during the energizing reactions followed by inward movement of $ADPMg^{-1}$ and the absorption of respiratory energy in the establishment of a membrane potential.

If the energizing reactions occur in the absence of adenine nucleotides, there will be a tendency for other anions to move into the membrane to neutralize $BH^+$. This will be governed by their ability to penetrate the inner membrane and the region in the vicinity of $BH^+$. How are aqueous inorganic anions excluded from $BH^+$?

The presence of lipid around B would protect $BH^+$ from access to aqueous anions; however lipid-soluble acids or anions could penetrate. The fact that the inhibitors oligomycin and dicyclohexylcarbodiimide (see Section V, 8) bind to lipid shows that lipid is present in this region. The formation of $BH^+$ in the energizing reactions in an environment of low dielectric constant would create an electric field which would carry some distance through lipid thereby creating a membrane potential gradient which could direct permeant anions, such as $ADPMg^{-1}$, to $BH^+$ through the lipid.

By studying the movement across the mitochondrial membrane of anionic phenyl dicarbaundecarborane ($PCB^-$), which can readily penetrate lipid membranes, Skulachev has shown that in the absence of ADP and Pi addition of substrate in the presence of NADH creates a positive potential on the outside of the intact membrane; since addition of rotenone abolishes the effect, the potential is associated with membrane energization at Site I [56]. Such a potential can be dissipated by adding lipid-soluble anions which can transport protons through lipid from the outer to the inner aqueous solution on opposite sides of the membrane [18]. If $ADPMg^{-1}$ is not present some anions, being lipid-permeable, can probably neutralize $BH^+$ directly. Various fluorescent probes have also shown the existence of a membrane potential and its close association with energizing reactions [57, 58, 59].

There is therefore good experimental support for the postulated electrogenic movement of $ADPMg^{-1}$ into the energized membrane.

### 2. *Phosphate Translocation*

Is there evidence for the postulated proton-binding site Pe and for proton uptake being induced under the influence of the positive potential in the outer phase during the energizing reaction?

A controlling system is present in the mitochondrial membrane which regulates the entry of Pi or arsenate [60]. The stimulatory action of Pi

on ATP transport [61] is consistent with the proposal (Fig. 3) that Pi entry accompanies ATP exit. There is also evidence [15] that Pi crosses the membrane in association with the movement of a proton. Also consistent with the proposed binding of a proton to Pe is the observation that when ATP is added to the uncoupled system containing endogenous ADP, the ADP $\rightleftharpoons$ ATP exchange which is thus initiated involves an initial uptake of protons which is followed by an efflux of protons once ATP hydrolysis sets in [15]. There is therefore a proton binding site Pe.

Mersalyl and *p*-hydroxymercuribenzoate inhibit entry of Pi as does formaldehyde [60]. The former inhibition has been attributed to reaction with a thiol. However the observations are also consistent with the postulation of Pe as a base provided it contains an active hydrogen atom; this would be so if it were a primary or secondary amine.

It has been observed that, in intact mitochondria, $Mg^{2+}$ inhibits the reversal of oxidative phosphorylation but not the forward reaction; the effect has been attributed to inhibiting access of ATP to the phosphorylation site [62]. Formation of $MgOH^+$ from $Y^-$ and MgPi in reaction 7 will be favoured by a rising concentration of $Mg^{2+}$ exceeding the stoichiometric requirement. Addition of $Mg^{2+}$, by promoting formation of $MgOH^+$, will therefore promote inward movement, but will inhibit outward movement, of $Pi^{-2}$ through the postulated reaction between $MgOH^+$, $Pi^{-2}$ and $H^+$ (reaction 11). Since $ATPMg^{-2}$ can enter only when $Pi^{-2}$ leaves the membrane, $Mg^{2+}$ inhibits entry of $ATPMg^{-2}$ by inhibiting the outward compensating movement of $Pi^{-2}$. The experimental observation for the postulated involvement of $Mg^{2+}$ in $Pi^{-2}$ translocation [62] therefore supports the model.

The inhibition of Site I phosphorylation by addition of alkyl biguanidines parallels the binding of $Pi^{-2}$ to the membrane [63]. This is accounted for by formation of MgPi which binds to the membrane (see Sections III, 10; V, 8).

The proposed movements of $ADPMg^{-1}$, $H^+$, $Pi^{-2}$ and $ATPMg^{-2}$ from, and into, the external solution under energizing conditions are therefore supported by experiment.

### 3. *Nucleophile $Y^-$*

Reaction 6 postulates that negative charge from $R_1^*COO^-$ is transferred to YH in exchange for a proton. The initial role of such a nucleophile in the model is to transfer negative charge to an inner, protected phosphorylation site in a non-polar environment. Histidine is particularly suited for such a role because charge can move between its two nitrogen atoms. In α-chymotrypsin, histidine-57 provides such a pathway for transferring charge away from a buried aspartate group [64]. Is there such a nucleophile at Site I and, if so, is it histidine, or histidine in association with a carboxyl group?

Triethyltin is a potent inhibitor of oxidative phosphorylation linked with NADH oxidation [65]. Evidence so far shows that when triethyltin binds to proteins it attaches to a pair of histidines [65]. The behaviour of triethyltin therefore suggests that B and YH are either an adjacent pair of histidines or that YH is a carboxyl group in association with another histidine which pairs with B. Evidence that there may be present an array of 7-10 pairs of such histidines will be considered in Section VI.

The alkylating agent N,N-dichloroethyl-*p*-aminophenylacetic acid also inhibits Site I phosphorylation and stimulates ATPase and respiration in State 4 [18]. By combining with the postulated histidines B and YH phosphorylation would be inhibited. Respiration in State 4 would be relieved because $R_1COOH$ can no longer combine with B. ATPase need involve only reactions 14 and 15 when B and YH are alkylated; this situation should be distinguished from DNP-induced ATPase where B is available.

Imidazole inhibits ATP-dependent succinate-linked reduction of $NAD^+$, the ATP $\rightleftharpoons$ Pi exchange reaction, and reduces the P : O ratio obtained during NADH-linked substrate oxidation by mitochondria [66]. Tetrachlorotrifluoromethylbenzylimidazole (TTFB) also inhibits phosphorylation, and stimulates ATPase and respiration in State 14 [18]. The reduced P : O ratio in the presence of these inhibitors with NADH-linked substrates suggests that they may also involve competition with YH for reaction with $R_1COO^-$ during phosphorylation.

There is therefore good experimental support for the postulate that a nucleophile $Y^-$ is present at Site I and that it may be histidine associated with a second histidine although a carboxyl could be involved as well (see Section V, 4).

#### 4. *Phosphorylated Intermediate*

Equation 7 postulates the formation of a phosphorylated derivative $YPi^{-2}$ of the nucleophile $Y^-$. Alternatively, reactions 7 and 8 could be concerted:

$$Y^- + MgPi + BHADPMg \rightleftharpoons B + YH + ATPMg^{-2} + MgOH^+ \qquad (23)$$

Is there any evidence for the phosphorylated intermediate $YPi^{-2}$? If so, does it involve histidine, or histidine and a carboxyl group?

Oligomycin and aurovertin inhibit oxidative phosphorylation at different sites [67]. In terms of the model oligomycin prevents formation of $YPi^{-2}$ but aurovertin prevents the latter's reaction with BHADPMg. Direct observation of the binding, in the presence of aurovertin, of $Pi^{-2}$ in what may be a phosphorylated intermediate has been made recently [67]. Its properties suggest that it may be acyl phosphate [68]. If this can be confirmed then YH would need to be a nucleophile, such as histidine (to account for inhibition by trialkyl tin)

in association with a carboxyl group, in which case the acyl phosphate may react directly with BHADPMg or indirectly via transient formation of phosphohistidine. Since the proposed phosphorylation mechanism is energized by proton transfer between $YPi^{-2}$ and $BH^{+}$, $YPi^{-2}$ must be acid-labile; it must also be discharged by uncoupling acid. Phosphohistidine, but not acyl phosphate, meets this requirement [69].

There are therefore some indications that a phosphorylated intermediate, corresponding to $YPi^{-2}$, may exist.

### 5. *Exchange Reactions*

An unusual ATP $\rightleftharpoons$ HOH exchange reaction occurs during oxidative phosphorylation [11, 70]. Pi $\rightleftharpoons$ HOH, ADP $\rightleftharpoons$ ATP and Pi $\rightleftharpoons$ ATP exchanges also occur [11, 70], as does an exchange of oxygen atoms between water and fatty acid [27]. The Pi $\rightleftharpoons$ HOH and ATP $\rightleftharpoons$ HOH exchanges are more rapid than the Pi $\rightleftharpoons$ ATP exchange. The exchange reactions have been reviewed by Boyer [11, 70]. Such information provides a useful tool for selecting possible phosphorylation mechanisms; a number of such possibilities have been examined critically by Boyer [11]. Is the proposed phosphorylation mechanism consistent with such data? Is the phosphorylated intermediate more likely to be a derivative of an hydroxylated compound YOH such as a carboxyl group, or a derivative of a non-hydroxylated compound YH like histidine?

$^{18}O$ exchange data show that the bridge oxygen atom between the terminal phosphoryl group in ATP is furnished by ADP[71]. Direct reaction between BHADPMg and an acyl phosphate leading to this result would require spatial selectivity on the part of the oxygen atoms involved to ensure that $OH^{-}$ leaves with $Pi^{-2}$. A simpler mechanism, where special spatial selectivity need not be assumed, would be for the phosphorylated derivative of a non-hydroxylated intermediate, such as phosphohistidine, to react with BHADPMg. This does not exclude the possibility that an acyl phosphate could phosphorylate BHADPMg via phosphohistidine. Consequently the model leaves open this question and simply postulates a reaction involving YH (which may have more than one component) where $OH^{-}$ passes from $Pi^{-2}$ to water via $MgOH^{+}$ and where $YPi^{-2}$ is acid-labile.

The exchange reactions directly associated with oxidative phosphorylation, and their different reaction rates, have been accommodated in a quantitative model by Boyer [70, 72]. This is shown in its simplest form, without reaction intermediates, in Fig. 5 which adapts the reaction sequence of the present model to that presented by Boyer (Fig. 5 in reference [72]). Charge neutralization between $BH^{+}$ and $Y^{-}$ provides energy for phosphorylation. $BH^{+}$ and B provide binding sites for $ADPMg^{-1}$ and $ATPMg^{-2}$ respectively. Enzyme-bound Pi is shown as MgPi. Enzyme-bound water in Boyer's model is replaced by $MgOH^{+}$.

This is allowable since the $^{18}O$ exchange studies reported do not distinguish between $H^{18}OH$ and $^{18}OH^-$. Boyer has shown [68, 72] that dynamic reversal of steps 3, 4 and 5 accounts for ATP $\rightleftharpoons$ HOH exchange without net reaction change and requires ADP. Rapid dynamic reversal of steps 1, 3 and 5, but slow reversal of 2, accounts for the observed rapid Pi $\rightleftharpoons$ ATP exchange and the slower ADP $\rightleftharpoons$ ATP exchange [70, 72]. Higher affinity of the membrane with intact mitochondria for ADP than for ATP is attributed to the membrane potential associated with $BH^+$; dissociation of $ATPMg^{-2}$ from neutral B may therefore have a lower activation energy than separation of $ADPMg^{-1}$ from $BH^+$. As is observed, Pi $\rightleftharpoons$ HOH exchange in such a system cannot occur without Pi $\rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges [68].

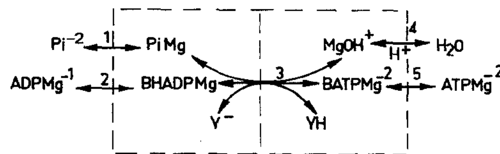



Figure 5. A simplified representation of the phosphorylation process which formally resembles a proposal by Boyer [72].

Pi $\rightleftharpoons$ HOH exchange requires the presence of some ADP [73] and some free magnesium [72]. This is accounted for by involvement of $MgOH^+$ and the need for some ADP to accept metaphosphate from $YPi^{-2}$ in the reaction which produces $MgOH^+$ (reactions 7 and 8).

Equation 11 accounts for the need for some free $Mg^{2+}$ in the production of ATP [72]. As may be seen from Fig. 4, ATPase associated with energy-linked reduction of $NAD^+$ does not require free $Mg^{2+}$. However Pi $\rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH involve $MgOH^+$ and so require some free $Mg^{2+}$. This accounts for the observation that only sufficient $Mg^{2+}$ for combination with ATP suffices to give a rapid ATPase and reduction of $NAD^+$ whereas higher $Mg^{2+}$ concentrations are required for maximum stimulation of the Pi $\rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges [72].

Different responses of the various exchange reactions to DNP and *p* mercuribenzoate show the presence, under some conditions, of a second Pi $\rightleftharpoons$ HOH exchange which is not associated with oxidative phosphorylation [72]. This is accounted for by the mechanism for ATP cleavage during energy-linked reduction of $NAD^+$ (Fig. 4). In this pathway oxygen atoms from water enter fatty acid. Because, under both reversed and forward conditions, $^{18}O$ from $Pi^{-2}$ does not enter fatty acid [27] the pathway shown for reversed-flow conditions involves reaction of $BH^+$ with $MgPi^{-1}$ rather than with the alternative, viz. $ADPMg^{-1}$. This mechanism accounts for the stimulating effect of ATP in Pi $\rightleftharpoons$ HOH exchange [72] and for the fact that the latter requires less $Mg^{-2}$ than is

required for the Pi $\rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges [72] where involvement with $MgOH^+$ is postulated.

It is concluded that the model accounts for the observed exchange reactions.

## 6. *Stoichiometry*

The hypothesis accounts for the uptake of one proton for each ATP synthesized at Site I. Although the pulse experiments of Mitchell and Moyle originally suggested that two protons are taken up for each ATP produced [9], more recent work with mitochondria [74], and also with photophosphorylating systems [75], shows that only about one proton is involved per ATP.

## 7. *Uncoupling*

Addition of uncoupling acid to coupled mitochondria induces an ATPase [76]. The involvement of $R_1^{\ddagger}COO^-$ in ATPase reaction 16, and the need for respiratory energy to dissociate $R_1COOHB$ to provide a source of $R_1COO^-$, are in accordance with the observation that ATPase activity is inhibited when electron-transfer is blocked with Amytal or antimycin or both [26, 76]. Recent work does not support the concept that uncouplers act simply as proton carriers through lipid since the pH-activity profiles for uncoupling acids differ substantially from those relating to their ability to decrease the electrical resistance of a lipid layer [20]. The acid-base catalysed uncoupling mechanisms which have been proposed account for this. Current flows in the outer aqueous phase associated with the movements of $ATPMg^{-2}$, $ADPMg^{-1}$ and $H_2PO_4^{-1}$ would be facilitated by the presence in this phase of monovalent cations. These have been shown to facilitate DNP-induced ATPase [77].

DNP does not inhibit the fatty acid $\rightleftharpoons$ HOH exchange reaction [27]; this is consistent with the mechanism proposed for forward-electron flow and the effect of uncoupling acid on it. Uncoupling acids inhibit succinate-linked reduction of $NAD^+$ by ATP[18]. Formation of $R_1COOH$, through reaction with HU, rather than $R_1COO^-$ inhibits formation of aldehyde in the fatty acid/aldehyde couple (reaction 13). Consequently uncoupling acids inhibit energized reduction of $NAD^+$.

## 8. *Phosphorylation Inhibitors*

Oligomycin inhibits phosphorylation in coupled mitochondria, the Pi $\rightleftharpoons$ HOH and Pi $\rightleftharpoons$ ATP exchange reactions, and the ATPase induced by uncoupling acid and arsenate [78]. These effects are consistent with the postulate that oligomycin inhibits reaction between MgPi and $YPi^{-2}$, and

between B . $ATPMg^{-2}$ and $MgOH^+$ in reversed situations, and with data [67] showing that it probably inhibits formation of $YPi^{-2}$. Oligomycin interacts with membrane phospholipid in "oligomycin-sensitivity-conferring-protein" [79]. This is consistent with its postulated binding near $BH^+$ and YH and with the presence of lipid at B.

Aurovertin also inhibits oxidative phosphorylation at a point which is thought to prevent reaction between a phosphorylated intermediate and ADP [67]. This can be accounted for if it binds at or near the oligomycin binding site so as to prevent reaction between $YPi^{-2}$ and BHADPMg.

Dicyclohexylcarbodiimide (DCCD) and oligomycin A have very similar functional effects and operate at the same site [80, 81]. DCCD binds to proteolipids [82]. Recent evidence suggests that the binding site is probably phosphatidylserine [83].

Alkyl guanidines and biguanidines inhibit respiration and oxidative phosphorylation at Site I [63], and also the transhydrogenase reaction [84], in a process which involves the binding of a proton within the membrane when in an energized state and where the inhibition parallels binding of Pi to the membrane [63, 84]. They act at a point nearer the electron chain than the oligomycin-sensitive site [85]. Such behaviour is consistent with the mechanism proposed in Section III, 10 and with the fact that inhibition induced by alkyl guanidines is relieved by addition of DNP [63] or fatty acid [86].

Trialkyltin is a potent inhibitor of oxidative phosphorylation and the ATPase stimulated by uncoupling acids like DNP [65]. This is consistent with the postulate that by binding to the unpaired electrons on the nitrogen atoms of B and Y, which are most likely histidines, it inhibits the phosphorylation reactions 7 and 8, and the ATPase reaction 19.

### 9. *Conclusions*

There is strong experimental support for the presence of sites B and YH and for the conclusion that these may be a pair of histidines. There is tentative experimental evidence for the existence of the phosphorylated intermediate $YPi^{-2}$. The phosphorylation reaction sequence accounts for complex exchange reaction data and can explain the effects of uncouplers and inhibitors. There is good evidence for the existence of an energized membrane potential which, as postulated in the model, is associated with energy conservation, the movement of reactants into the membrane, and phosphorylation. The model explains why there is no change in the pH of the inner aqueous phase during oxidative phosphorylation. It accounts for the different behaviour of $Mg^{2+}$ in forward and reversed operations, for the pyridine nucleotide transhydrogenase reaction and for the uptake of only one proton for each ATP molecule synthesized.

## VI. *A Possible Relationship Between Phosphorylating Sites*

Detailed studies of the stoichiometry of the inhibition of oxidative phosphorylation by trialkyltin, its ability to stimulate an ATPase when it is bound to only portion of its binding sites, and its ability to inhibit such an ATPase when it is bound to all its binding sites, led Aldridge and Rose to reconcile these and other observations in the following way [65]. The binding studies suggest the presence of 7-10 pairs of histidines; this may account for the basic sites observed in coupling factors Fc, $F_4$ and $F_3$ [87]; Aldridge and Rose proposed that these form an "energy-transfer chain" which enables protons to enter from any of the three coupling sites and move from histidine to histidine to one end of the chain where ATP is produced. Trialkyltin is considered to bind to pairs of the histidines and trap movement of $H^+$.

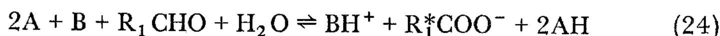
The present model of Site I phosphorylation readily fits such a scheme. The fatty acid/B complex, and its associated YH, could be the first pair of such a chain of pairs of histidines leading to the formation of the phosphorylated intermediate at the other end of the chain. Phosphorylation at Sites II and III might be accounted for if the respiratory chain offered mechanisms for transferring a proton from YH to B at the two appropriate levels in the energy-transfer chain.

Aldridge and Rose postulated that only the end of the energy-transfer chain remote from the ATPase has access to water [65]. Ion transport was postulated to occur within this aqueous region. This also conforms with the requirements of the model. Water is involved in aldehyde oxidation and the fatty acid is postulated to interact only at the first member of the chain. Ion transport is discussed in the next section.

## VII. *Ion Transport*

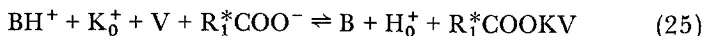
Although a detailed discussion of ion transport is beyond the scope of this paper, the following description serves to show in principle how such phenomena can be accommodated by the model.

The overall energizing process at Site I, when driven by the respiratory chain, can be summarized in the following equation:



$$2A + B + R_1CHO + H_2O \rightleftharpoons BH^+ + R_1^*COO^- + 2AH \qquad (24)$$

On addition of $K^+$ and valinomycin, in the absence of ADP, respiration is stimulated and $K^+$ crosses the membrane. $K^+$ can enter the lipid as the valinomycin complex ($K^+V$) and form an ion pair with $R_1^*COO^-$ under energizing conditions. However a $K^+$ ion can only enter from outside ($K_0^+$) if a proton leaves $BH^+$ in exchange:



$$BH^+ + K_0^+ + V + R_1^*COO^- \rightleftharpoons B + H_0^+ + R_1^*COOKV \qquad (25)$$

However the loss of a proton from $BH^+$ tends to reverse Eq. (24); this is facilitated by $R_1COO^-$ being kept as an anion. (The situation should be contrasted with phosphorylation where the conversion of $R_1COO^-$ into $R_1COOH$ helps to shift the equilibrium of Eq. (24) to the right.) In the reversal, KOH rather than water forms since only B is available:

$$R_1^*COOKV + 2AH \rightleftharpoons R_1CHO + 2A + KOHi + V \qquad (26)$$

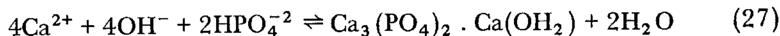
It will be postulated that the KOH enters the matrix (denoted by KOHi). Energy is available from $R_1^* \rightarrow R_1$ to eject $K^+$ into the matrix against its electrochemical gradient. At the end of the first of one such cycle the potential of the aldehyde/acid and AH/A couples will usually have fallen to a lesser extent than in a phosphorylation cycle since less work is performed. As a result further cycles are possible with the transfer of more $K^+$ over the membrane. This will continue until the tendency for reducing equivalents to flow back from AH to aldehyde in Eq. (26) has reached a steady state with their tendency to pass along the respiratory chain to X and beyond. A steady state is ultimately established between the ion and electron gradients. This mechanism implies that there is no fixed stoichiometry between the amount of $K^+$ transferred per ~.

The formation of $Y^-$ and $BH^+$ at coupling Sites II and III in the energy-transfer chain could also drive cations if the energizing reactions could produce $R_1^*$. A proton passes to the outside from $BH^+$ in exchange for entry of $K^+$; simultaneously charge transfers from $Y^-$ to $R_1COOH$ to yield YH and $R_1COOKV$ after which the ion-pumping cycle commences as before.

ATP can also energize cation movements. As discussed in Section III, 8, ATP tends to oxidize AH and increase the ratio $R_1COO^-/R_1COOH$. Addition of $K^+$ and valinomycin will increase this ratio and $R_1COOKV$ can then participate in the pumping cycle.

Such a mechanism accounts for the exchange of one $H^+$ per $K^+$ taken up [1], for an inverse relationship between oxidative phosphorylation and ion transport [1], and for the variable stoichiometry [88]. The ability of $K^+V$ to partially overcome respiratory inhibition induced by alkyl biguanidines, and evidence that monovalent metal cations bind to the same site as alkyl biguanidines [86], provide further support for the concept.

An analogous mechanism might account for the energized movement of $Ca^{2+}$ over the membrane for which a specific carrier exists [89]. If one molecule of $Ca^{2+}$ in this carrier behaved like $K^+V$ then, in four cycles, four $Ca^{2+}$ and four $OH^-$ would enter the matrix. The outer solution would gain $4H^+$ and entry of two $Pi^{-2}$ over the membrane would balance the charges. The net result would be formation of hydroxy apatite in the matrix:



$$4Ca^{2+} + 4OH^- + 2HPO_4^{-2} \rightleftharpoons Ca_3(PO_4)_2 \cdot Ca(OH_2) + 2H_2O \qquad (27)$$

A stoichiometry of 1.7-2 $Ca^{2+}$ and one $Pi^{-2}$ per ~ has been reported for $Ca^{2+}$ transport [89]. In the absence of permeant anions, one $H^+$ enters the outer solution for each $Ca^{2+}$ taken up and equivalent alkalization of the medium occurs [89]. Unless such stoichiometry follows from a thermodynamic requirement related to formation of hydroxy apatite, the model does not account for such limited stoichiometry with respect to ~. Consistent with the role of X, and CoQ as a buffer in the electron chain, are spectroscopic observations showing the important role played by X in calcium transport; the speed of response of cytochrome b in the State 4 to 3 transition caused by $Ca^{2+}$ is very rapid [90].

The observation that alkalization of the medium, and acidification of the outer solution, disappear when there is a permeant anion [91] can be accounted for if the anion is transferred over its translocase bound to the protonated form of a base within the translocase [2]. The proton enters the permease in response to liberation of a proton by the cation. Hydroxyl entering the matrix neutralizes the proton in the permease and releases the anion. The net result is therefore transfer of the cation and anion over the membrane.

## VIII. *Discussion and Predictions*

A mechanism has been proposed for respiration and oxidative phosphorylation at Site I which attempts to account at a molecular level for some of the more generalized concepts embodied in the chemical, conformational, William's and chemiosmotic hypotheses, and in an earlier proposal from this laboratory [1, 2]. Such an hypothesis is of utility only if it suggests new experiments. Utility has already been demonstrated by the predicted involvement of a fatty acid/aldehyde couple [2] which led to the experiments now in progress, and which so far are consistent with the predictions [3]. Some other experiments are now suggested.

If lipid stretching is indeed involved in formation of thioester along the lines proposed, then the entropy change which has been observed in calorimetric studies should be a function of fatty acid/aldehyde chain length and should change by R log 3 for every unit change in chain length. The entropy change should also reflect the state of osmotic swelling of the inner membrane; a highly swollen membrane should exhibit a smaller entropy loss in the transition $R_1 \rightarrow R_1^{\ddagger}$ than an unswollen one. General anaesthetics, and vitamin A, should exhibit a biphasic effect on NADH oxidation if such a study were extended to sufficiently low concentrations of the anaesthetics, provided the endogenous lipid composition is not already optimal, and provided the effect is not obscured by a rate-limiting step, such as adenine nucleotide translocation, which is insensitive to the anaesthetic. The latter possibility could be eliminated by using particles.

The steady-state concentration of free $R_2SH$ should reflect the relative rates of formation and reduction of thioester. Consequently, if all other factors can be kept constant, the concentration of free $R_2SH$ should diminish with increasing chain length of fatty acid/aldehyde up to the optimum value for maximum respiration. This is $C_{14}$ for rat liver but this may vary with the species. Isotope hydrogen exchange studies with labelled NADH or fatty aldehyde might be able to establish if hydride ion from NADH does in fact reversibly incorporate into fatty aldehyde and a thiol. If so, this would establish the existence of the postulated thioester.

If dimedon is inhibiting electron flow at the specified site of the aldehyde/acid couple, then it should influence the epr signal (g = 1.94), the absorbances of which are characteristic of non-heme iron protein, and the state of reduction of flavoprotein, CoQ and cyt. b. These could be observed in the presence and absence of dimedon under forward and reversed-flow conditions. The use of labelled dimedon, coupled with a search for it in inhibited particles, should show that it is covalently bound to an endogenous fatty aldehyde if the hypothesis is correct. Dimedon should be a more effective inhibitor if a long fatty chain were attached to it to improve its ability to penetrate lipid.

Decylaldehyde should be able to reduce a $NADP^+$/NADPH couple at the latter's physiological potential in sonicated particles, and such reduction should not be inhibited by rotenone, Amytal and piericidin A.

The hypothesis suggests that the best conditions for observing the postulated phosphorylated intermediate would be in the presence of aurovertin, to prevent reaction with BHADPMg, and an alkyl biguanidine to make discharge of $YPi^{-2}$ with respiratory-derived fatty acid more difficult; uncoupling acids should however discharge $YPi^{-2}$ if their concentration is adequate to dissociate fatty acid from the alkyl biguanidine.

If a nucleophile ($Y^-$) is involved in phosphorylation, and the inhibitory effect of the alkylating agent N,N-dichoroethyl-*p*-amino-phenylacetic acid is due to covalent combination of this reagent with $Y^-$, then it should be possible to isolate and identify such a compound by using a labelled version of the alkylating agent and causing it to react with mitochondria. This would also enable the nature of $Y^-$ to be ascertained. It is expected to be histidine, or histidine in association with aspartate or glutamate.

If the hypothesis is correct then it is necessary to maintain a very low concentration of free endogenous fatty acid within the inner membrane to prevent uncoupling. This is achieved by keeping fatty acid in a bound form with CoA, carnitine, or bound thiols with CoA characteristics as proposed by Garland [92]. In such a system it is likely that $R_1COSR_2$ would be carefully shielded to prevent exchange reactions between $R_1COSR_2$ and endogenous fatty acid-CoA and acetyl-CoA since it seems

that in such situations in rat liver and rat brain mitochondria fatty acid is activated by ATP and not directly by respiratory energy [25]. This may be why the thiols in NADH-dehydrogenase are not readily accessible.

## IX. *Acknowledgements*

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