THE MECHANISM OF OXIDATIVE PHOSPHORYLATION
A hypothesis derived from studies of trimethyltin and triethyltin compounds

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1. Introduction

There is much information about the properties of the enzymic system in mitochondria which utilises the energy of oxidation of substrates for the synthesis of adenosinetriphosphate (ATP) from adenosinediphosphate (ADP) and inorganic phosphate (P_i). The electron transport chain as well as the energy-conserving mechanisms are dependent on the membranous structure of the mitochondria. Our present mental pictures of the system largely depend upon studies of the way various substances perturb it and there is no hypothesis to explain the mechanism in chemical terms. It is a reasonable assumption that those substances which inhibit the processes involved do so by interacting with a macromolecular component. When such interactions have high biological specificity (e.g. oligomycin and trialkyltins) it is likely that this reflects a high specificity for certain groups in a macromolecule. If these groups can be defined in chemical terms, it should help our understanding of the chemistry of oxidative phosphorylation. This paper describes studies with trialkyltin compounds. These compounds combine a high biological activity with a very limited chemical reactivity (i.e. little affinity for thiols [1], EDTA [2] and many biological molecules [3,4]). In addition they appear to be biologically very specific, for only oxidative phosphorylation [5] and photosynthesis [6] are affected by low concentrations. This paper describes the discovery of a site in mitochondria to which trimethyltin and triethyltin bind when they inhibit oxidative phosphorylation. The quantitative relationships between this inhibition and binding to this site, as well as experiments with model proteins, allow us to put forward a new hypothesis indicating the chemical basis of the mechanism by which electron transport is coupled to the synthesis of ATP.

2. The action of trialkyltins on oxidative phosphorylation

Trialkyltins are potent inhibitors of oxidative phosphorylation; concentrations of triethyltin as low as 0.1 μM are effective [5]. Since the oxidation of succinate is inhibited only when it is coupled to phosphorylation [1,5] and since adenosine triphosphatase (ATPase) stimulated by 2:4-dinitrophenol (DNP) is inhibited by the same concentrations which inhibit oxidative phosphorylation [5], it is clear that trialkyltins act on the energy-conserving mechanisms. Trialkyltins also stimulate an ATPase but the maximum activity is never as great as that produced by DNP [3,5] and differs with different homologues; for example triethyltin stimulates a maximal activity of approx. 5 μmole P_i/mg protein/hr whereas with tri-n-butyltin the maximal activity is about 1 μmole P_i [3]. Thus the stimulation of an ATPase by trialkyltins is distinct from the inhibition of oxidative phosphorylation. Nevertheless, these two actions influence one another because the concentration producing maximal ATPase activity is that necessary for complete inhibition of phosphorylation linked to NADH oxidation [3].

The action of trialkyltins is completely different

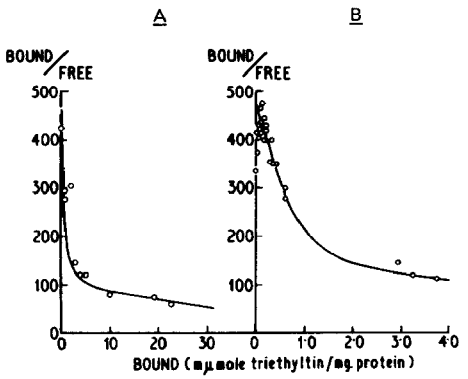

Fig. 1. The binding of triethyltin to rat liver mitochondria Graph B is a large scale plot of part of Graph A in order that all the points obtained using low concentrations of triethyltin may be shown. The solid line is a calculated line using the following constants: n_1 = 0.8 mμmole/mg protein, K_1 = 4.75 × 10^5 M^{-1}, n_2 = 66 mμmole/mg protein, K_2 = 1.4 × 10^3 M^{-1}.

from that of the dialkyltins and neither has the biological actions of the other [1].

3. The binding of trimethyltin and triethyltin to rat liver mitochondria

The binding of [^{113}Sn] triethyltin to rat liver mitochondria has been measured [7] under identical conditions to those used for the examination of oxidative phosphorylation [8]. In fig. 1, results are plotted according to Scatchard [9] and the solid line is that calculated for two classes of binding sites with the constants n_1 = 0.8 mμmole/mg protein, K_1 = 4.75 × 10^5 M^{-1} and n_2 = 66 mμmole/mg protein, K_2 = 1.4 × 10^3 M^{-1} where n_1 and n_2 are the concentration of sites and K_1 and K_2 are the respective intrinsic or microscopic affinity constants for sites 1 and 2. The inhibitory power of triethyltin would require an affinity constant of approx. 10^6 M^{-1}; therefore site 1 could be the site involved in the action of triethyltin on oxidative phosphorylation. The constants for the binding of trimethyltin to rat liver mitochondria are n_1 = 0.8 mμmole/mg protein, K_1 = 1.2 × 10^4 M^{-1}; n_2 = 120 mμmole/mg protein, K_2 = 1.0 × 10^2 M^{-1}; n_3 = 0.0042 mμmole/mg protein, K_3 = 4 × 10^6 M^{-1}. Since trimethyltin is 30–40 times less active than triethyltin as an inhibitor of oxidative phosphorylation, the characteristics of site 1 are those expected for the inhibitory power of both compounds. That the binding defined by the constants of site 1 reflects true binding and is not associated with intramitochondrial penetration is shown by the fact that an insoluble fraction from rat liver mitochondria (prepared by treatment with Triton TX-100 in phosphate buffer pH 7.4) contains all of site 1. This fraction contains about 15% of the mitochondrial protein and n_1 is increased 6–7 times with the affinity constants for trimethyltin and triethyltin substantially unaltered (table 1) [7]. Therefore site 1 has the required characteristics for a site involved in the inhibitory action of trimethyltin and triethyltin on oxidative phosphorylation [7].

Table 1
Binding and inhibition of oxidative phosphorylation. For binding, insoluble fraction (see text), for inhibition, rat liver mitochondria.

	Binding		Inhibition of oxid. phosph.
	K_1 (M^{-1})	n_1 (mμmole/mg) protein	I_{50} (μM)
Triethyltin	4 × 10^5	5.9	0.2
Trimethyltin	1 × 10^4	6.2	6.0
Ratio of K_1		(Et_3Sn/Me_3Sn)	40
Ratio of I_{50}		(Me_3Sn/Et_3Sn)	30

Using the constants derived above the relationship between inhibition of oxidative phosphorylation and binding to site 1 may be examined (fig. 2). There is not a simple relationship between % inhibition of P_i uptake with pyruvate as substrate and % of site 1 complexed to triethyltin. At all concentrations of triethyltin, % inhibition is much greater than % of site 1 complexed; if the relationship at low inhibition is extrapolated linearly to 100% inhibition, a value of 10–15% of site 1 complexed is obtained. Similar relationships between binding and inhibition are obtained for the effects of both triethyltin and trimethyltin on the ATPase stimulated by DNP and on P_i uptake with both pyruvate and succinate as substrate [10]. This is consistent with site 1 containing 7–10 positions for triethyltin all involved in oxidative

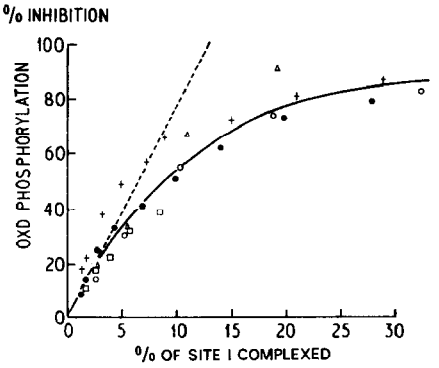

Fig. 2. The relation of the inhibition of oxidative phosphorylation by triethyltin and its binding to site 1. Pyruvate was used as a substrate with rat liver mitochondria. The different symbols represent different experiments in the same conditions.

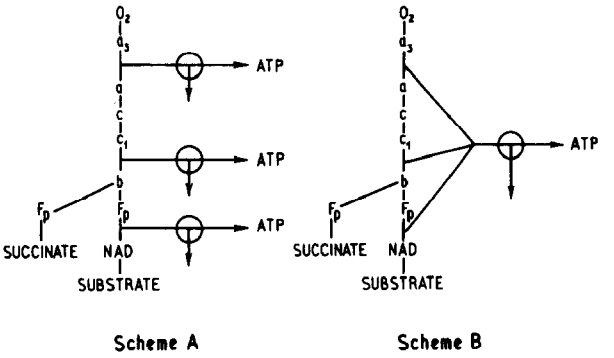

Fig. 3. Two schemes for oxidative phosphorylation. The vertical arrows indicate the site of action of DNP and the circles the area where triethyltin has been thought to act. The cytochromes are indicated by a_3,a,c,c_1 and b and the flavoproteins by Fp.

phosphorylation, complexing of triethyltin with any one position leading to complete inhibition. Another possible explanation of these findings could be that site 1 contains 7–10 positions for triethyltin and only one position is involved in the processes of oxidative phosphorylation. That this latter explanation cannot be the case is shown by the linear 1 to 1 relationship between % inhibition and % of site 1 complexed when phosphorylation is linked to the oxidation of ascorbate [10]. Therefore the whole of site 1 is involved in oxidative phosphorylation. As will become apparent later, there are other compelling reasons for this conclusion.

4. Consideration of the relationship between inhibition by triethyltin and complexing to sites for formulations of oxidative phosphorylation

In fig. 3 are shown two arrangements of oxidative phosphorylation. In scheme A each of the energy transfer chains between electron transport and ATP formation is linked to each of the three coupling sites independently and the binding site for triethyltin has 3 positions. When one of these positions in any of these chains is occupied by triethyltin, phosphorylation linked to NADH oxidation would be completely stopped. On the other hand, when the reverse reaction is studied (ATPase stimulated by DNP) all three positions in the chains would have to be occupied for complete inhibition. Scheme A is therefore incompatible with our experimental results which show the equal sensitivity to triethyltin of phosphorylation linked to NADH oxidation and ATPase stimulated by DNP, and the 8:1 relationship between inhibition and binding. In scheme B there is one position for triethyltin, and phosphorylation linked to NADH oxidation and ATPase stimulated by DNP would be equally affected when this position is occupied. Scheme B is also incompatible with the observed relationship between inhibition and binding (i.e. occupation of only one position out of 7–10 being required for complete inhibition). The chemiosmotic theory of Mitchell [11] states that all three coupling sites in electron transport give rise to a general pH gradient and electric potential across a membrane. This is incompatible with the differing sensitivities to triethyltin of phosphorylation linked to the oxidation of NADH and ascorbate.

A new approach is required and our proposal is shown in fig. 4. The three coupling sites in the electron transport chain are linked to an energy transfer chain, at one end of which is an enzymic system for the formation of ATP and at the other end a mechanism upon which DNP acts. The energy transfer chain itself consists of 7–10 components (X); when any of these are complexed with trimethyltin or triethyltin the chain cannot operate, either to allow the formation of ATP or for ATP to be broken down under the

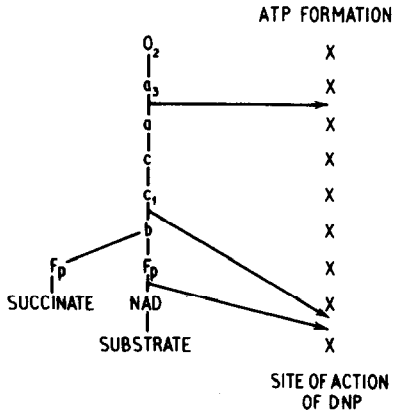

Fig. 4. A scheme linking the electron transport chain to an energy transfer chain which has at one end a system for ATP formation and at the other the site of action of DNP. Other symbols are as in fig. 3.

influence of DNP. The experimental finding that, using triethyltin, the relationship between inhibition and binding for P_i uptake linked to oxidation of pyruvate is identical to that with succinate, means that coupling sites 1 and 2 must feed in at the same point in the energy transfer chain. For inhibition of phosphorylation linked to the oxidation of ascorbate, triethyltin must occupy the position proximal to the system forming ATP. To ensure that this position is occupied in all energy transfer chains will require that all other positions are also filled, thus giving rise to the observed relationship between inhibition and binding. The new scheme provides a framework for known reactions to take place. We now have to consider the nature of X and the nature of the intermediate in the chain.

5. The nature of X in fig. 4.

It is obviously difficult to discover the identity of X in such a complex structural entity as a mitochondrion. Even the fraction obtained by treatment with Triton TX-100 and phosphate is very complex; it contains all of site 1 from the untreated mitochondria and also other nonspecific binding. This fraction still contains macro-structure essential for the binding of triethyltin for when it is sonicated site 1 disappears leaving the nonspecific binding [10]. An attempt has therefore been made to define the chemical specificity of triethyltin and trimethyltin from their reactions with model proteins.

Triethyltin reacts with very few proteins [4,12,13]. Only two have been found which bind triethyltin with an affinity constant of 10^5-10^6 M^{-1}-rat haemoglobin and a protein in the supernatant fraction from liver homogenate [4]. Much detailed work has shown that two molecules of triethyltin combine with one molecule of rat haemoglobin and that the triethyltin is located between two pairs of histidines [12], this complex involving 5-coordinated tin. The study of the liver protein is not complete but the following facts are known [14]:

(1) only a small proportion of the protein in the supernatant fraction from rat and guinea pig liver homogenates binds triethyltin;

(2) binding is extremely sensitive to photo-oxidation in the presence of methylene blue or rose bengal. Only histidine and thiol are lost by this treatment and the rate of loss of thiol is 1st order and unrelated to the loss of binding;

(3) the rate of loss of binding is not first-order – the rate may be separated into two first order components; the rate of the slower component being approx. twice the rate of loss of the bulk histidines;

(4) the loss of binding by the fast component appears to be related to an initial fast loss of histidine.

Therefore, evidence is now accumulating that triethyltin and trimethyltin bind to very few proteins and that when they do with an affinity constant of 10^5-10^6 M^{-1} two histidines are involved. The following information reinforces this view. Triethyltin reacts with imidazole in non-aqueous solvents to produce a polymer in which the tin atom is sandwiched between the nitrogens of the imidazole rings [15]. The tendency to 5-coordination in the group 4B metals is maximal for lead, high for tin but very slight for germanium [16]. In agreement with this, the rate of combination of triethyl-lead with rat haemoglobin was too fast to measure, for triethyltin very rapid (approx. bimolecular rate constant, $k = 4 \times 10^4$ l mole^{-1} min^{-1}) and for triethylgermanium very slow (k=7 l mole^{-1} min^{-1}) [17]. It is interesting that triethyl-lead inhibits oxidative phosphorylation in a way which cannot be distinguished from the action of triethyltin, whereas triethylgermanium has, at most, a very low activity [18,38]. It is concluded

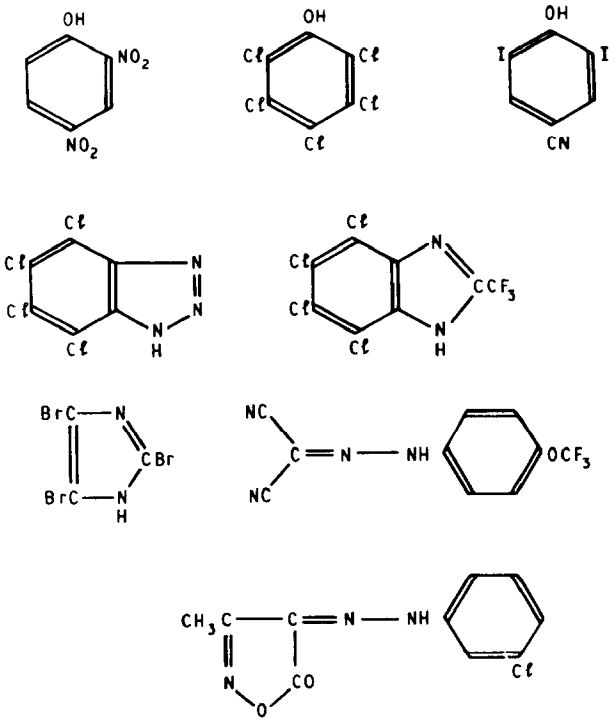

Fig. 5. Chemical structures of compounds which uncouple oxidative phosphorylation.

therefore, that when triethyltin attaches itself to site 1 in mitochondria, it is reacting with two histidines in such a conformation that 5-coordination can take place: therefore we consider that in fig. 4 X is histidine.

6. Nature of the intermediate using the energy transfer chain

DNP and the various molecules shown in fig. 5 react with the oxidative phosphorylation system in a defined way [19–22]. This interaction leads to inhibition of phosphorylation, stimulation of state 4 respiration, stimulation of an ATPase system, but does not cause swelling [19]. All of these compounds behave identically [20] and the above three effects are produced by one primary action. The common factors between all these compounds are their lipophilic properties and their possession of an acidic hydrogen essential for activity [20].

Trimethyltin and triethyltin also stimulate an ATPase activity [3,5] which is associated, unlike DNP-induced ATPase, with swelling of the mitochondria [3,20]. There is a correlation between the ATPase stimulated and swelling of the mitochondria [3]: both are temperature-dependent and the swelling is prevented by DNP [3] and therefore is probably a metabolic event. Trimethyltin and triethyltin are lipophilic to some extent and have pKs of 6–6.5. The uncharged molecules can penetrate lipoprotein membranes and can also combine with H^+ ions to form positively charged trialkyltin ions.

Thus, two classes of compounds which stimulate the hydrolysis of ATP – (1) trimethyltin, triethyltin and (2) the uncoupling agents such as DNP – can accept H^+ ions. The underlying common factor linking the action of uncoupling agents, the stimulation of an ATPase by trimethyltin and triethyltin and the utilisation of histidines in the energy transfer chain may be that H^+ ions are the intermediate and are produced by the electron transport chain. This attractive idea which removes the difficulty of finding a common chemical intermediate which would react at the three different coupling areas in the electron transport chain has been considered by others [11, 23–25].

7. The mechanism of oxidative phosphorylation

In fig. 6 is a reaction scheme incorporating the above ideas and utilising eight histidine pairs (it could be 7–10). All three coupling sites in the electron transport chain produce H^+ ions which are passed into the energy transfer chain which acts as a conducting medium for their movement as required. These H^+ ions are never free in the medium and our hypothesis differs in this respect from that of Mitchell [26]. We have considered two arrangements of the histidines in the chain, one consisting of eight pairs (fig. 6) and the other nine histidines in a linear array (cf fig. 4; X = histidine). There are a number of possible mechanisms whereby protons and histidines may be involved in coupling electron transport to phosphorylation. Actual proton flow need not be obligatory and at our present state of knowledge it would be premature to attempt to be precise. We have chosen in fig. 6 to present one suggestion which in-

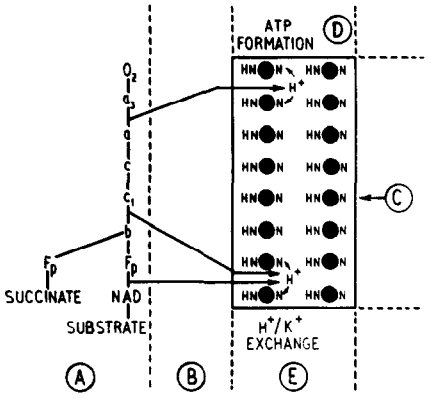

Fig. 6. A mechanism for oxidative phosphorylation. HN ● N represents the imidazole ring of histidine. The dotted lines divide up, for the purpose of discussion, the mechanism into sections A–E. Other symbols are as in fig. 3.

volves proton flow, but we do not consider that this is necessarily correct. The arguments which follow do not depend on an exact formulation. For the purpose of discussion the whole process shown in fig. 6 is divided into five sections (A–E).

Section A. This is the electron transport chain. From detailed measurements of the oxidation-reduction states of coenzymes and respiratory pigments their order in the chain is almost settled [27]. However, we do not know if the hydrogens or electrons pass directly from one pigment to another or through intervening steps.

Section B. This is the mechanism whereby H^+ ions are produced linked to three oxidation-reduction steps in the electron transport chain. The general position of these linkages is known [27] and there is little doubt that the production of H^+ ions is possible in these regions and has been considered by others [23,26].

Section C. This is the energy transfer chain and consists of histidines so arranged as to facilitate the movement of H^+ ions within the chain. If this arrangement is to operate efficiently there must be insulation of the histidines from the general aqueous environment and the whole of sections B and C must be a hydrophobic area.

Section D. This is the site of synthesis of ATP. This enzymic mechanism will catalyse the reaction: $ADP^{3-} + P_i^{2-} + H^+ = ATP^{4-} + H_2O$. The mechanism of this is not known but it could be through a phospho-enzyme intermediate or a concerted reaction [28].

Section E. This is the site of action of DNP and other uncoupling agents. DNP moves into the inner region of the mitochondrion as a negatively charged species [29–31], which will be able to collect an H^+ ion from the end of the energy transfer chain. The unionised species will be able to migrate out through the lipoprotein membranes. This end of the chain is considered to be involved with ion movement and possibly with an H^+/K^+ exchange.

There is no doubt of the importance of mitochondrial cation content in balancing the concentration of substrate anions [29,32]. Substrates compete with one another [29,32] and the system seems admirably organised to ensure a mixture of tricarboxylic acid cycle substrates within mitochondria.

The placing of an ion-exchanging system at one end and an ATP-forming system at the other end of the energy transfer chain fits the known competition of both systems for a common intermediate [33].

8. The interaction of oligomycin, DNP, rotenone and trialkyltins

Oligomycin [34] acts on the ATP synthesizing mechanism (Section D) and will inhibit the formation of ATP coupled to any of the three positions in the electron transport chain. It will inhibit the ATPase stimulated by DNP and will be ineffective against the DNP-stimulated state 4 respiration. Trialkyltins prevent the formation of ATP and ATPase or respiration stimulated by DNP [5]. Rotenone is specific for the NAD/Fp region (sections A and B) and does not prevent phosphorylation with succinate as a substrate, does not inhibit the ATPase stimulated by DNP but does inhibit respiration stimulated by DNP when an NAD-requiring dehydrogenase is being utilised [35]. All of these interactions are expected from the proposed mechanism (fig. 6).

9. The stimulation of ATPase by trimethyltin and triethyltin

When trimethyltin and triethyltin inhibit oxidative phosphorylation binding to histidine in the energy transfer chain (section C) is involved. In contrast we

consider that when these compounds stimulate an ATPase, they do so by penetrating as an uncharged molecule into the energy transfer chain (section C) and combining with H^+ ions thus dissipating energy. To completely prevent the ATPase, the position next to ATP formation (section D) must be occupied by bound triethyltin. Statistically, in order to achieve this, all positions in the chain must be filled. This has been experimentally verified and trimethyltin- or triethyltin-stimulated ATPase approaches zero as the % of site 1 complexed approaches 100 [10]. The same argument and finding applies to the stimulation of state 4 respiration by these compounds and its inhibition by higher concentrations [5]. This is further evidence for the involvement of the whole of site 1 in oxidative phosphorylation.

The complex changes taking place when triethyltin reacts with mitochondria goes some way to explain some of the differences encountered between the sensitivity to triethyltin of the initial rates of oxygen uptake measured polarographically [36] when compared with steady state measurements or with measurements of P_i uptake [5].

It is important to appreciate that the stimulation of ATPase by trimethyltin and triethyltin requires the ability of the uncharged molecule to accept an H^+ ion. In contrast, the classical uncouplers such as DNP accept an H^+ ion only when the molecule carries a negative charge. For this reason we believe the classical uncouplers act in section E in contact with the aqueous environment and cannot uncouple in section C.

10. The sensitivity of the oxidative phosphorylation system to structural damage

Quite mild treatment of intact mitochondria reduces the efficiency of oxidative phosphorylation, stimulates ATPase and reduces respiratory control. The proposed mechanism (fig. 6) requires a high degree of structural integrity and the energy transfer chain (section C) has to be insulated from the aqueous medium. Damage to the chain will lead to leaks of H^+ ions, thus producing the above effects. The ATPase produced by structural damage could be due to loss of H^+ ions from many areas (sections B, C, D and E respectively).

11. Conclusions and advantages of the proposed mechanism

This first chemical mechanism for oxidative phosphorylation has been deduced from quantitative correlations between the independently determined parameters of binding to a site in mitochondria and the inhibition of certain activities associated with oxidative phosphorylation. Once the primary arrangement had been deduced (figs. 4 and 6) many other important consequences emerged. Listed below are some of the known properties of oxidative phosphorylation which the new mechanism will explain.

(1) The effects of trialkyltins excepting the high sensitivity of the P_i-ATP exchange reaction [37].

(2) The ability of uncoupling agents to inhibit ATP formation and to stimulate ATPase and state 4 respiration by one primary action.

(3) The inhibition by oligomycin of ATP formation, ATPase stimulated by DNP and the lack of effect on DNP stimulated state 4 respiration.

(4) The sensitivity of oxidative phosphorylation to structural damage.

(5) The close connection between the formation of ATP, respiration and ion movement and the effects of oligomycin and uncoupling agents upon these systems.

(6) The differing properties of ATPase produced by structural damage, trimethyltin and triethyltin, and that produced by uncoupling agents.

(7) The differing effects of oligomycin and uncoupling agents upon the energy-linked reduction of NAD by succinate.

We have presented a new hypothesis which has considerable advantages and is precise in certain areas. Figs. 4 and 6 are merely representations of the type of system involved. Our experimental results require an energy transfer chain linking ATP formation and the site of action of DNP and containing histidines so arranged that there are 7–10 triethyltin-binding sites. Coupling sites 1 and 2 must feed in at a common point at one end of the chain and coupling site 3 at the other end adjacent to ATP formation. The whole system depends on protons as the common intermediate; these protons are insulated from the aqueous environment. The chemistry of ATP formation (section D), the mechanism of formation of H^+ ions (section B), and the chemistry of the ion exchange system (section E) are not understood. In

addition, the schemes proposed in figs. 4 and 6 represent the simplest type of arrangement but more than one electron chain may be linked to the same energy transfer chain. The framework described will allow definitive experiments to be designed to test the accuracy of the hypothesis, particularly whether it applies to photosynthetic phosphorylation.

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