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UNCOUPLING AND INDUCTION OF ION PERMEABILITY CHANGES IN HEART MITOCHONDRIA BY N, N' -BIS (DICHLOROACETYL)-1, 12-DIAMINODODECANE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

Michael Warren Fowler, B.S.

* * * * * * * * * * *

The Ohio State University
1973

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ACKNOWLEDGEMENT

Acknowledgement is given to my adviser, Dr. A. J. Merola. His guidance and patience made this work both challenging and enjoyable.

I wish also to acknowledge the helpful discussions and suggestions of Dr. Gerald P. Brierley, Dr. Kathryn Scott, and Marriane Jurkowitz.

I wish also to thank my wife, Emilie, our family and friends for their support and encouragement which was necessary for this work to be successful.

Thanks are extended to those in our laboratory whose cooperation made this work most enjoyable.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>PUBLICATIONS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td><strong>Section</strong></td>
<td></td>
</tr>
<tr>
<td><strong>I. LITERATURE REVIEW</strong></td>
<td>1</td>
</tr>
<tr>
<td>Coupling Hypothesis</td>
<td>2</td>
</tr>
<tr>
<td>Uncouplers of Oxidative Phosphorylation</td>
<td>19</td>
</tr>
<tr>
<td><strong>II. STATEMENT OF PROBLEM</strong></td>
<td>23</td>
</tr>
<tr>
<td><strong>III. EXPERIMENTAL METHODS</strong></td>
<td>25</td>
</tr>
<tr>
<td>Preparation of Beef Heart</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>25</td>
</tr>
<tr>
<td>Alkalization and Acidification</td>
<td>25</td>
</tr>
<tr>
<td>ANS Fluorescence Measurements</td>
<td>25</td>
</tr>
<tr>
<td>Oxygen Consumption</td>
<td>25</td>
</tr>
<tr>
<td>Cytochrome b Reduction</td>
<td>26</td>
</tr>
<tr>
<td>Simultaneous Monitoring of Oxygen Usage, pH, and Cytochrome b Reduction or ANS Fluorescence</td>
<td>26</td>
</tr>
<tr>
<td>Picrate Extraction</td>
<td>26</td>
</tr>
<tr>
<td>Mitochondrial Swelling</td>
<td>26</td>
</tr>
<tr>
<td>$^{36}$Cl$^-$ Exchange</td>
<td>26</td>
</tr>
<tr>
<td>Metabolism of C-12</td>
<td>27</td>
</tr>
<tr>
<td>Synthesis of Tritiated C-12</td>
<td>27</td>
</tr>
<tr>
<td>Ca++ Uptake and Release</td>
<td>28</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>29</td>
</tr>
<tr>
<td>Transient Effects of C-12</td>
<td>29</td>
</tr>
<tr>
<td>Transient Effects of C-12 on Respiration, pH and Cytochrome b Reduction</td>
<td>31</td>
</tr>
<tr>
<td>Transient Effect of C-12 on Ca++ Uptake and Release</td>
<td>32</td>
</tr>
<tr>
<td>Transient Nature of C-12 on the Induction of Chloride Permeability in KCl</td>
<td>34</td>
</tr>
<tr>
<td>Effect of Preincubation on the Respiratory Rate and the Respiratory Control Ratio</td>
<td>36</td>
</tr>
<tr>
<td>Effect of Preincubation on the C-12-induced Chloride Ion Permeability</td>
<td>38</td>
</tr>
<tr>
<td>Metabolism Studies of C-12</td>
<td>42</td>
</tr>
<tr>
<td>Preincubation or Aggregation of C-12</td>
<td>45</td>
</tr>
<tr>
<td>Mechanism of C-12 Uncoupling</td>
<td>51</td>
</tr>
<tr>
<td>Passive ANS Fluorescence Changes and pH Changes Induced by C-12</td>
<td>53</td>
</tr>
<tr>
<td>Effect of KC1 Concentration on ANS Fluorescence Decay</td>
<td>58</td>
</tr>
<tr>
<td>C-12 Facilitated Transfer of 36Cl^-</td>
<td>66</td>
</tr>
<tr>
<td>Effect of pH on the Titration of the Respiratory Increase by C-12</td>
<td>68</td>
</tr>
<tr>
<td>V. DISCUSSION</td>
<td>71</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>79</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Types of Uncouplers</td>
<td>22</td>
</tr>
<tr>
<td>2. Dependency of the Decay and Half-time of Decay on the Concentration of KCl</td>
<td>63</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The Generation of the Common Intermediate</td>
<td>4</td>
</tr>
<tr>
<td>2. The Anisotropic Electron Transport Chain</td>
<td>12</td>
</tr>
<tr>
<td>3. Diagramatic Representation of ATPase I and II</td>
<td>15</td>
</tr>
<tr>
<td>4. Effect of C-12 on the Respiratory Rate, pH and Cytochrome b Reduction</td>
<td>30</td>
</tr>
<tr>
<td>5. Effect of CCP on the Respiratory Rate, pH and Cytochrome b Reduction</td>
<td>31</td>
</tr>
<tr>
<td>6. Effect of C-12 on the Energy-linked Accumulation of Ca++</td>
<td>33</td>
</tr>
<tr>
<td>7. Transient Induction of Chloride Permeability in KCl</td>
<td>35</td>
</tr>
<tr>
<td>8. Transient Effect of C-12 on the Respiratory Rate and Respiratory Control Ratio</td>
<td>37</td>
</tr>
<tr>
<td>9. Effect of Preincubation on the Chloride Permeability in NH₄Cl</td>
<td>39</td>
</tr>
<tr>
<td>10. Effect of CCP Incubation on the C-12-Induced Chloride Permeability in NH₄Cl</td>
<td>41</td>
</tr>
<tr>
<td>11. GLC of C-12, Mitochondrial Reaction Mixtures</td>
<td>43</td>
</tr>
<tr>
<td>12. TLC of C-12 Extracts</td>
<td>44</td>
</tr>
<tr>
<td>13. Effect of BSA and C-12-Cl⁻ on C-12 Precipitation</td>
<td>46</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>14.</td>
<td>48</td>
</tr>
<tr>
<td>Respiratory Stimulation by C-12 in Isosmotic Sucrose</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>49</td>
</tr>
<tr>
<td>Effect of C-12-C1\textsubscript{1} Precipitation on the Transient, C-12-induced Respiratory Stimulation</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>50</td>
</tr>
<tr>
<td>Effect of C-12-C1\textsubscript{1} on the C-12-induced Respiratory Stimulation</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>52</td>
</tr>
<tr>
<td>Effect of C-12 Preincubation with Buffer on the Transient Respiratory Stimulation</td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>54</td>
</tr>
<tr>
<td>ANS Fluorescence and pH Changes in KCl</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>57</td>
</tr>
<tr>
<td>ANS Fluorescence in Sucrose</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>60</td>
</tr>
<tr>
<td>Effect of KCl Concentration on ANS Fluorescence</td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>61</td>
</tr>
<tr>
<td>Effect of KCl Concentration on the Maximum Rate of Fluorescence Decay</td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>62</td>
</tr>
<tr>
<td>Effect of KCl Concentration on the Decay Constant and the $t_{\frac{1}{2}}$ of the ANS Fluorescence Decay</td>
<td></td>
</tr>
<tr>
<td>23.</td>
<td>65</td>
</tr>
<tr>
<td>Effect of KCl Concentration on C-12-induced pH Transitions</td>
<td></td>
</tr>
<tr>
<td>24.</td>
<td>67</td>
</tr>
<tr>
<td>$^{36}$Cl\textsuperscript{-} and Picrate Transfer in a Model System</td>
<td></td>
</tr>
<tr>
<td>25.</td>
<td>70</td>
</tr>
<tr>
<td>Effect of pH on the C-12 Titration of Respiratory Stimulation</td>
<td></td>
</tr>
<tr>
<td>26.</td>
<td>73</td>
</tr>
<tr>
<td>Mechanisms of Uncoupling</td>
<td></td>
</tr>
</tbody>
</table>

ix
ABBREVIATIONS

NAD+ - oxidized nicotinamide adenine dinucleotide
NADH - reduced nicotinamide adenine dinucleotide
ATP - adenosine triphosphate
ADP - adenosine diphosphate
AMP - adenosine monophosphate
P_i - inorganic phosphate
Tris - Tris-(hydroxymethyl)-aminomethane
HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate
EDTA - ethylenediamine-tetraacetic acid
DNP - 2, 4-dinitrophenol
CCP - m-chlorocarbonylcyanidephenylhydrazone
S-13 - 5-chlpro-3-t-butyl-2'-chloro-4-nitrosalicylanilide
C-12 - N, N'-bis (dichloroacety1)-1,12-diaminododecane
C-12-Cl - N,N'-bis(monochloroacety1)-1,12-diaminododecane
ANS - 1-anilinonaphthalene-8-sulfonic acid
cpm - counts per minute
Belitzer (1,2), as early as 1939, postulated that oxidative phosphorylation was coupled to the transfer of electrons down the electron transport chain from NAD+ to molecular oxygen. Little experimental evidence was presented to support this idea until Lehninger (3) was able to show aerobic oxidation of exogenous NADH by water-treated mitochondria with no tricarboxylic acid intermediates available. The NADH was rapidly oxidized to NAD+ at the expense of molecular oxygen. The transport of electrons from NADH to molecular oxygen was accompanied by the production of two to three molecules of ATP, if ADP and $P_i$ were added to the incubation medium. It can be seen from equations 1 and 2 that there is a sufficient decline in standard free energy ($\Delta G^0'$) in the transfer of electrons from NADH to molecular oxygen to account for the synthesis of at least three moles of ATP.

$$\text{NADH} + H^+ + \frac{1}{2}O_2 \rightarrow \text{NAD}^+ + H_2O \quad \Delta G^0' = -52.7 \text{ Kcal} \quad (1)$$

$$3\text{ADP} + 3P_i \rightleftharpoons 3\text{ATP} + 3H_2O \quad \Delta G^0' = 21.9 \text{ Kcal} \quad (2)$$
It has been demonstrated that there are three distinct sites of energy coupling along the electron transport chain. These three sites of energy coupling may be shown by varying the substrate. NADH, succinate and ascorbate provide energy necessary for the synthesis of three, two and one molecules of ATP, respectively, and show a differential effect toward various respiratory inhibitors. Energy derived at each of these sites may be used to synthesize ATP from ADP and inorganic phosphate, for transhydrogenation of pyridine nucleotides (4), and the uptake of metal cations by the mitochondrion (5, 6). As will be discussed later, each of these three activities appears to be driven by a common factor or intermediate. Another indication of this common intermediate is the ability of reversed electron transport to be driven by energy derived from a subsequent coupling site. This common intermediate could conceivably be a physical state, a chemical state or a combination of the two.

**Coupling Hypotheses**

A mechanism proposed by Slater (7) in 1953 to explain the coupling between electron transport, or oxidation, and phosphorylation of ADP was patterned after the mechanism proposed for the substrate-level phosphorylation, e.g., the reaction catalyzed by the enzyme glyceraldehyde-3-phosphate
dehydrogenase. Because this mechanism proposes that the energy from oxidation is coupled to phosphorylation through a strictly chemical pathway, the hypothesis is best known as the chemical intermediate hypothesis. Slater's original hypothesis has been expanded by additional evidence and may now be represented by equations 3 - 6:

\[
\begin{align*}
\text{A}_{i,\text{red}} + \text{B}_{i,\text{ox}} + \text{I} & \rightleftharpoons \text{A}_{i,\text{ox}} \text{I} + \text{B}_{i,\text{red}} \quad (3) \\
\text{A}_{i,\text{ox}I} + \text{X} & \rightleftharpoons \text{A}_{i,\text{ox}} + \text{X}\sim\text{I} \quad (4) \\
\text{X} \sim \text{I} + \text{P}_{i} & \rightleftharpoons \text{X}\sim\text{P} + \text{I} \quad (5) \\
\text{X} \sim \text{P} + \text{ADP} & \rightleftharpoons \text{ATP} + \text{X} \quad (6)
\end{align*}
\]

Where \( \text{A}_{i} \) and \( \text{B}_{i} \) are adjacent carriers at a given coupling site in the respiratory chain and \( \text{X} \) and \( \text{I} \) are energy-transfer carriers common to all three coupling sites. Equation 3 may be thought of as the "energy-coupling reaction" and equations 4 - 6 as the "energy-transfer reactions" (8). \( \text{X} \sim \text{I} \) is postulated to be the common intermediate for the three coupling sites (9), as well as the common factor which drives ATP synthesis, cation translocation and pyridine nucleotide transhydration as shown in Figure 1. Inhibition of respiration observed when oligomycin is present during state 4 is explained as the accumulation of this common intermediate, \( \text{X} \sim \text{I} \), which will decrease the rate of the reaction of equation 3, if the reactions of equations 3 and 4
are reversible. The slow respiration of state 4, which is considered to be a high-energy state, is explained as that respiration necessary to maintain any spontaneous breakdown of $X\sim I$ or the depletion of $X\sim I$ required for the support of:

\[
\begin{align*}
\text{NADH} & \quad \text{Site I} & \quad \text{Site II} & \quad \text{Site III} & \quad \text{O}_2 \\
\text{uncouplers} & & & & \\
\text{Cation Translocation} & & \text{X$\sim I$} & & \text{NAD Transhydrogenation} \\
\text{oligomycin} & & & & \\
\text{ADP} + P_i & \quad \text{ATP} \\
\end{align*}
\]

Fig. 1. The generation of the common intermediate and its utilization by the energy linked processes.

on-going energy-requiring reactions. Experimental evidence is available to suggest that the common intermediate is a non-phosphorylated intermediate such as $X\sim I$, as opposed to $X\sim P$ in equations 5 and 6. Four lines of evidence support this view:

1) Respiration in the presence of DNP does not require $P_i$.

2) Reversed electron transport through one coupling site by energy derived from another
coupling site is not sensitive to oligomycin (10) or $P_i$ (11).

3) Energy derived from electron transport due to substrate which can drive energy-linked pyridine nucleotide transhydrogenation is not sensitive to oligomycin and does not require $P_i$. When ATP drives the reaction it is sensitive to oligomycin.

4) The pyridine nucleotide transhydrogenation has been demonstrated in "nonphosphorylating" submitochondrial particles (12).

Equations 3 and 4 indicate that $P_i$, and not ADP, react with the common intermediate, $X \sim I$. Boyer (13) showed by means of exchange reactions with $H_2^{18}O$ into $P_i$ and ATP that the bridge oxygen of the terminal pyrophosphate bond of ATP must have come from the terminal oxygen of ADP and not $P_i$. This observation necessitates the existence of a $\sim P$ intermediate, rather than a $\sim ADP$ intermediate, in the sequence leading to ATP. The chemical intermediate hypothesis is compatible with the following observations:

1) ATP-$P_i$ exchange (13, 14) which may be given by equation 7

$$AMF \sim P \sim P + ^{32}P_i \quad AMF \sim P \sim ^{32}P_i + P \quad (7)$$
2) \( P_1-H_2^{18}O \) exchange after Boyer (13) and given by equation 8

\[
\text{HPO}_4^{-2} + H_2^{18}O \rightleftharpoons \text{HP}^{18}O_4^{-2} + H_2O \quad (8)
\]

3) ATP-ADP exchange as first observed by Rose and Ochoa (14) and shown in equation 9

\[
^{14}\text{C}-\text{ADP} + \text{ADP}\sim P \rightleftharpoons^{14}\text{C}-\text{ATP} + \text{ADP} \quad (9)
\]

4) ATPase activity as shown by equation 10 which was first suggested to be associated with oxidative phosphorylation by Lardy and Elvehjem (15) in 1945.

\[
\text{ATP} + H_2O \rightleftharpoons \text{ADP} + P_i \quad (10)
\]

This activity is relatively low in intact preparations but is increased markedly by the addition of an uncoupler such as DNP or by aging the mitochondria.

5) Uncoupling of phosphorylation by arsenate. This effect of arsenate is competitively inhibited by \( P_i \) and the arsenate induces an oligomycin-sensitive ATPase that is much slower than the rate of the DNP-induced ATPase. The rate of respiratory stimulation due to arsenate is also
much slower than that due to arsenate and ADP (16, 17). Idada and Cohn (18) showed that the arsenate-induced ATPase reaction is accompanied by an arsenate-\(^{18}\)O exchange which has a rate 50 to 100 times greater than the ATPase activity. All of these observations cannot be accounted for by the interaction of arsenate with the non-phosphorylated intermediate, \(X\sim I\), and its subsequent hydrolysis as is proposed for the mechanism of arsenate uncoupling of substrate-level phosphorylation. Ernster, Lee and Janda (9) suggest a mechanism compatible with the chemical intermediate hypothesis in which the arsenate is proposed to react with \(X\sim I\) to form a stable intermediate, \(X\sim\)arsenate. ADP is then arsenyalted by this stable intermediate to give ADP\(\sim\)arsenate which is then rapidly hydrolyzed. This scheme accounts for the slow arsenate-induced ATPase, the ADP requirement and the high rate of arsenate-\(H_2^{18}\)O exchange.

While the chemical intermediate hypothesis serves to explain many of the observations concerning the coupling of oxidation and phosphorylation, its weakness lies in the fact
that no such high-energy chemical intermediates have been isolated despite a great volume of research. Also difficult to reconcile with this hypothesis is the apparent need for an intact membrane and for the numerous respiratory chain components. It has been suggested by Slater (19) that the membrane is necessary to organize the multienzyme complex catalyzing oxidative phosphorylation.

Since no high-energy intermediates of a chemical nature have been isolated, Boyer (20) has suggested that the free energy provided by the oxidation of substrates by the electron transport chain may be conserved by a conformational change in one of the electron transport chain components or some associated structure. The idea of energy conservation by means of a conformational change is based on an analogy with the interaction of ATP and myosin. Gillis and Marechal (21) have reported the synthesis of ATP by stretching previously contracted glycerinated-muscle fibers. In the case of muscle contraction covalent bond breaking is used to cause a conformational change and in the case of oxidative phosphorylation it is hypothesized that a conformational change caused by electron transport could result in covalent bond formation in the form of ATP synthesis (22). Two types of experimental evidence have been put forward to support the conformational hypothesis:
1) Electron micrographs showing a morphological change in the membrane of mitochondria in response to different energy states

2) Fluorescence changes of 1-anilinonaphthalene-8-sulfonic acid (ANS) in response to the energy state of the mitochondrion

Hackenbrock (23, 24) has presented evidence by means of electron micrographs that the mitochondrion can exist in two distinct morphological forms, these two forms, the "orthodox" and "condensed" forms, correspond to the high-energy state 4 and the low energy state 3, respectively. Green et al. (25, 26) have extended these observations and have designated three different mitochondrial conformations: the nonenergized, energized and energized-twisted. The nonenergized state obtained when the mitochondria were treated with antimycin A and rotenone. The energized form obtained when the mitochondrial suspension was allowed to respire in the presence of substrate. The energized-twisted conformation obtained when the mitochondria respiring with substrate were supplemented with $P_i$. The energized and energized-twisted forms were shown to be dissipated by the addition of uncouplers, $Ca^{++}$, or ADP, when $P_i$ was present. Antimycin A and rotenone blocked the transition from the nonenergized to the energized conformations when pyruvate.
plus malate, or succinate was utilized as substrate, but not when N, N, N', N' - tetramethyl-p-phenylenediamine-ascorbate was used. Cyanide blocked the transformation when the latter substrate was used. Chance et al. (27) and Hunter and Brierley (28) have been critical of these electron microscopic studies suggesting that the fixation techniques are not sufficiently rapid to accurately monitor the energy changes of the mitochondrion. Hackenbrock (24), by means of freeze-fracture techniques, has suggested that this technique is rapid enough to fix suspensions of mitochondrion on a time scale comparable to that shown for energy changes by other techniques. According to Hackenbrock fixation by this quick-freeze method is capable of fixing the structure in one second or less. This corresponds closely with the half-time of both NAD\(^+\) and cytochrome b reduction.

It has been shown that the intensity of the fluorescence of ANS and the maximum wavelength of the emission spectrum decreases when the ANS is bound to proteins (29) or is dissolved in nonpolar solvents (30, 31). Azzi et al. (32) showed a similar result when ANS was added to sub-mitochondrial particles. These workers also showed that a further fluorescence increase obtained with the addition of an oxidizable substrate or ATP. This increase in fluorescence was reversed by the addition of uncouplers. Chance et
al. (33) have shown that fluorescence of ANS also responds to the energy state of the intact mitochondrial suspension. Slater and Berden (34) have shown a half-time of about 10 seconds for the ATP-induced ANS fluorescence increase, and a 0.5 second half-time for the ATP-induced red shift of cytochrome b. Brockelhurst et al. (35) found a half-time of 2.5 seconds for the fluorescence increase after the addition of NADH. These measurements were extrapolated and given as the half-time at infinite particle concentration. Because of these discrepancies in the response time of ANS and the energy changes monitored by the half-time for the oxidation and reduction of cytochrome b doubt has been cast on the usefulness of ANS to monitor the energy state responsible for the immediate conservation of the energy derived from the oxidation of substrate (32, 33).

In 1961 Mitchell (36) presented a hypothesis which is an alternative to the chemical intermediate and conformational hypotheses of energy coupling. The chemiosmotic hypothesis, in brief, provides for the energy derived from electron transport to be conserved in a pH and membrane potential gradient. This gradient then is used to drive ATP synthesis by the dehydration of ADP and P_i. The chemiosmotic hypothesis requires an anisotropic electron transport system, a coupling membrane, a proton-translocating reversible ATPase
and certain exchange diffusion carriers. Experimental evidence for the chemiosomotic hypothesis has been provided by Mitchell and Moyle (37, 38, 39, 40, 41, 42, 43, 44).

The anisotropic electron transport chain proposed by Mitchell is shown in Fig. 2. It consists of alternating hydrogen and electron carriers. The oxidation of substrates on the inner side of the cristal membrane is proposed to result in the translocation of protons from the inner to the outer aqueous phase and the subsequent transfer of

\[ \text{OUT} \quad \text{IN} \]

\[
\begin{align*}
2H^+ & \quad \text{F} \\
2H^+ & \quad \text{NHFe} \\
2H^+ & \quad \text{F} \\
2H^+ & \quad \text{cyt b} \\
2H^+ & \quad \text{Co Q} \\
2H^+ & \quad \text{cyt c, c}_1 \\
2H^+ & \quad \text{cyt a, a}_3 \\
2H^+ + \frac{1}{2} O_2 & \quad H_2O
\end{align*}
\]

Fig. 2. The anisotropic electron chain. F represents a flavoprotein and NHFe refers to nonheme iron protein. Co Q is used to designate coenzyme Q. The IN and OUT refer to the inner and outer side of the inner mitochondrial membrane. Ref. 8.
electrons down the chain. The chain is shown to be folded into three loops with each loop containing an electron carrier and a hydrogen carrier. The translocation of $2H^+$ out is equivalent to the production of $OH^-$ inside and this situation is equivalent to the splitting of $H_2O$ across the inner and outer phases. This splitting results in pH disequilibrium across the membrane and an electrical gradient due to the apparent inward movement of electrons. The combination of pH gradient and membrane potential tend to force protons back through the membrane and has been called the "proton motive force" (PMF) by Mitchell.

Mitchell's hypothesis requires that the membrane separating the inner and outer phases, i.e., the inner mitochondrial membrane which contains the components of the electron transport chain, must be impermeable to protons and hydroxide ions.

Racker (45, 46) has shown that the coupling factor, $F_1$, i.e., the membrane-bound ATPase, is implicated in the synthesis of mitochondrial ATP. In equation 11 the equilibrium lies far to the right and requires some special mechanism to drive the reaction to the left in a direction opposite that which is thermodynamically favorable.

$$ATP + H_2O \rightleftharpoons ADP + P_i$$  (11)
It can be seen from equation 12, where \( K \) is the hydrolysis constant and \( (H_2O)_{aq} \) is the activity of water in the aqueous phase, that the lowering of the activity of water in the aqueous phase will make the reverse reaction i.e., the synthesis of ATP, more favorable (8).

\[
(H_2O)_{aq} = (ADP) \times (P_i)/(ATP) \times 1/K \quad (12)
\]

Mitchell (41) has shown that to obtain an ATP/ADP concentration ratio of unity, with a \( P_i \) concentration of 10 mM, the activity of water would have to be reduced to about 5 uM from its normal 55mM. If the synthesis of ATP is strictly coupled to the translocation of 2 H\(^+\) equations 11 and 12 may be rewritten as shown in equations 13 and 14 and show that the proton distribution markedly affects the effective activity of water and the equilibrium position of the ATPase reaction.

\[
\begin{align*}
ATP + H_2O + 2H^+_{in} & \rightarrow ADP + P_i + 2H^+_{out} (13) \\
(H_2O)_{aq} \times (H^+_{in})^2/(H^+_{out})^2 & = (ADP) (P_i)/(ATP) \times 1/K
\end{align*}
\]

The ATPase reaction of equation 11 may be rewritten as shown in equations 15 and 16. The formulation of equations 15 and 16 depend on whether one or two protons are being translocated per ATP synthesized.

\[
\begin{align*}
ATP + H^+ + OH^- & \rightarrow ADP + P_i \quad (15) \\
ATP + 2H^+ + O^{2-} & \rightarrow ADP + P_i \quad (16)
\end{align*}
\]
Figure 3, part A and B, provide diagrammatically an explanation for the translocation of the OH\(^-\) or O\(^{-2}\) of the proposed ATPase I or ATPase II, respectively. These formulations contain an "X-I" hydrolase and an "X-I" synthetase.

\[ \text{INMEMBRANE} \]

\[ \text{OUT} \quad \text{MEMBRANE} \quad \text{IN} \]

\[ \begin{align*}
\text{H}_2\text{O} & \quad \rightarrow \quad \text{OH}^- \\
\text{H}^+ & \quad \rightarrow \quad \text{ATP} + \text{H}^+ \\
\text{H}_2\text{O} & \quad \rightarrow \quad \text{X-I} \\
\text{H}^+ & \quad \rightarrow \quad \text{XH} + \text{IO}^- \\
\end{align*} \]

\[ \text{A} \]

\[ \begin{align*}
\text{H}_2\text{O} & \quad \rightarrow \quad \text{O}^{-2} \\
2\text{H}^+ & \quad \rightarrow \quad \text{ATP} + 2\text{H}^+ \\
\text{H}_2\text{O} & \quad \rightarrow \quad \text{X-I} \\
2\text{H}^+ & \quad \rightarrow \quad \text{X}^- + \text{IO}^- \\
\end{align*} \]

\[ \text{B} \]

Fig. 3. Diagramtic representation of the proposed ATPase I and ATPase II. Ref. 38.

These components have not been isolated or identified. The "X-I" hydrolase catalyzes the reaction shown in equation 17 and the "X-I" synthetase catalyzes the reaction shown...
in equation 18 and 19 for ATPase I or ATPase II, respectively. Mitchell suggests that the "X\textasciitilde I" synthetase corresponds to Racker's factor $F_1$ and that the "X\textasciitilde I" hydrolase is the oligomycin-sensitivity conferring factor, i.e., Racker's factor $F_0$ (45, 46).

\[
X - I + H_2O \rightleftharpoons XH + IOH \tag{17}
\]
\[
XH^- + IO^- + ATP + H^+ \rightleftharpoons X\textasciitilde I + ADP + P_i \tag{18}
\]
\[
X^- + IO^- + ATP + 2H^+ \rightleftharpoons X\textasciitilde I + ADP + P_i \tag{19}
\]

The fourth postulate of the chemical intermediate hypothesis is the existence of exchange diffusion carriers. The best characterized of these carriers is the adenine nucleotide translocase. Brierley and O'Brien (47) and Klingenberg and colleagues (48, 49, 50, 51) have characterized this diffusion system and have shown it to be specific for adenine nucleotides. This ATP/ADP exchanger is found to be strongly inhibited by atractyloside (52, 53, 54). Studies by Schnaitman, et al. (55) and Meisner and Klingenberg (56) have located the adenine nucleotide carrier on the outside of the inner membrane.

The work of Chappell and Haarhof (57), Tyler (58) and Fonyo (59) support the existence of a phosphate carrier.
Tyler (60), Brierley, Knight and Settlemire (61) and Fonyo and Bessman (62) have shown mercurial compounds that specifically inhibit a phosphate transport system. Further work by Chappell and coworkers (57, 63, 64, 65), Ferguson and Williams (66) and de Haan and Tager (67) have indicated the existence of not only a phosphate exchanger but also a malate/$P_i$ and substrate/malate exchanger (68).

Experiments to show that the membrane of mitochondria is actually impermeable, or only slightly permeable, to protons was reported by Mitchell and Moyle (40, 69). By injecting small pulses of HCl into anaerobic suspensions of mitochondria they were able to calculate the proton conductance of the inner membrane. They found a value of about 0.45 umho/cm$^2$, or 0.50 ug ions/sec * gm protein. Mitchell and Moyle (71) have also shown that the protons are ejected by anaerobic suspensions of rat liver mitochondria when pulsed with small amounts of oxygen. The ratio of protons produced per oxygen molecule utilized was shown to approach 6 for 3-hydroxybutyrate and 4 for succinate. Further work with submitochondrial particles prepared by sonication showed an inward movement of protons when pulsed with oxygen (72,73). These results are consistent with the idea that sonicated submitochondrial particles possess an inside-out orientation as compared to intact mitochondria. Anaerobic mitochondria
given a small pulse of ATP also show proton appearance in the suspending medium (74). In these experiments Mitchell has shown a corrected proton for ATP ratio of about two.

The chemiosomotic hypothesis not only requires that protons be produced under conditions of substrate oxidation but also that this proton disequilibrium and membrane potential be capable of synthesizing ATP. Experiments by Jagendorf and Uribe (75, 76, 77, 78, 79) have shown that it is possible to synthesize ATP by inducing a pH differential across the membrane of isolated chloroplasts. This synthesis is inhibited by uncouplers and by delays in the addition of ADP which allows equilibration of the pH gradient.

Mitchell's hypothesis has prompted much significant research in the field of bioenergetics and is compatible with much of the experimentally observed phenomena of oxidative phosphorylation, but it also has some weak points. The chemiosmotic hypothesis offers no explanation for the exchange reactions of Boyer. The "X~I" hydrolase and synthetase have been as elusive as the high energy chemical intermediate of the chemical intermediate hypothesis. Slater (81) has suggested that the membrane potential required by the chemiosmotic hypothesis is much higher than for any other membrane system studied so far. A list of objections to and difficulties of the chemical and chemiosmotic
hypotheses has also been compiled by Slater (80).

**Uncouplers of Oxidative Phosphorylation**

Certain chemical agents have been shown to uncouple phosphorylation from the oxidation of substrates by the mitochondrion and have also been shown to affect other energy requiring reactions including the energy-linked adenine nucleotide transhydrogenase (81, 82), the uptake of divalent cations (83, 84), ATP-supported reversed electron transport (85) and the monovalent cation transport facilitated by various antibiotics (86).

For each coupling hypotheses there exists at least one uncoupling hypothesis put forward to explain the separation of oxidation and phosphorylation in the light of that hypothesis. The chemical intermediate hypothesis requires uncoupling agents to dissipate a high energy chemical intermediate. This may be accomplished by processes causing energy drain on the system or by direct hydrolysis of one of the proposed high energy intermediates such as $A_1H_2O$ or $XH_2O$ (87, 88, 89, 90). Recent work by Wilson (91) with 5-chloro-3-t-butyl-2'-nitrosalicylanilide (S-13), the most potent uncoupler discovered, was interpreted to show a site specificity in the cytochrome oxidase region. He further proposes that this agent uncoupled by hydrolysis of the $A_1H_2O$ compound at site 3 in the electron transport chain and that
uncoupling at the other two coupling sites could be accomplished by equilibration through X \sim I, the common intermediate of the chemical hypothesis.

Weinbach and Garbus (92) propose a mechanism for uncoupling in which the uncoupling agent binds to membrane protein. This binding results in a conformational change in the catalytic proteins which couple oxidation to phosphorylation. These workers suggested that uncoupling efficiency of certain uncouplers was related to their ability to bind to protein (93, 94).

Mitchell proposes that uncouplers act by carrying protons back through the coupling membrane (38) and thus decrease or equilibrate the pH and/or electrical gradient produced by oxidation of substrate or the hydrolysis of ATP. Mitchell points out that most of the effective uncouplers are lipid-soluble, weak acids. After proton transfer the anion form of the uncoupler is able to pass back out through the membrane without an accompanying cation because the negative charge is delocalized in a \pi-orbital system (38). This concept of uncoupling has gained support from the work on synthetic bilayers (95, 96, 97, 98, 99). Here it was shown that dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrozone (CCCP) can cause an increased conductance in phospholipid bilayer membranes.
van Dam and Slater (100) and Slater (19) have proposed a mechanism which is basically the reverse of Mitchell's mechanism. They propose that a high-energy chemical intermediate splits water inside the mitochondrion. Slater and van Dam propose that uncouplers enter the inner phase in exchange for OH⁻ produced by the splitting of water. The uncoupler exits as the uncharged acid.

A great many agents have been found to act as uncoupler of oxidative phosphorylation. A partial list of the many compounds which uncouple oxidative phosphorylation is shown in Table 1. While many may fit the description of uncouplers as given by the mechanisms proposed by Mitchell, van Dam and Slater and Wilson, i.e., lipid soluble, weak acids with a delocalized π-orbital system, others seem to be exceptions to at least a portion of this description and include possibly the organotin compounds (101), the bis-dichloroacetamides (102) and the fatty acids (103).
### Table 1
Types of Uncouplers

<table>
<thead>
<tr>
<th>Group</th>
<th>Example</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylhydrazones</td>
<td>Carbonyl cyanide m-chloro-phenylhydrazone</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>105</td>
</tr>
<tr>
<td>Nitrophenols</td>
<td>2, 4-Dinitrophenol</td>
<td>89</td>
</tr>
<tr>
<td>Thiophenes</td>
<td>2-(2', 6'-dimethylanilino)-3, 4-dinitro-5-chlorothiophene</td>
<td>106</td>
</tr>
<tr>
<td>Halophenols</td>
<td>Pentachlorophenol</td>
<td>107</td>
</tr>
<tr>
<td>Organotins</td>
<td>Trialkyltin chloride</td>
<td>101</td>
</tr>
<tr>
<td>Dichloroacetamides</td>
<td>N, N'-bis (dichloroacetyl) 1, 12-diaminododecane</td>
<td>102</td>
</tr>
<tr>
<td>Salicylanilides</td>
<td>5-chloro-3-t-butyl-2'-chloro-4-nitrosalicylanilide</td>
<td>108</td>
</tr>
<tr>
<td>Coumarols</td>
<td>3, 3'-methylenebis (4-hydroxy-coumarin)</td>
<td>109</td>
</tr>
<tr>
<td>Phenothiazines</td>
<td>2-chloro-10 (3-dimethylamino-propyl) - phenothiazine</td>
<td>110</td>
</tr>
<tr>
<td>Benzimidazoles</td>
<td>4,5,6,7-tetra-2-trifluoromethylbenzimidizole</td>
<td>111</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>Oleic Acid</td>
<td>103</td>
</tr>
</tbody>
</table>
II

STATEMENT OF PROBLEM

Whether uncoupling agents act by hydrolyzing a high-energy chemical intermediate, inducing a conformational change in the coupling system or discharging a potential gradient, an understanding of the uncoupling mechanism is of prime importance in delineating the mechanism for the coupling of oxidation to phosphorylation in the mitochondrion. The investigation into the mechanism of uncoupling has been complicated by the fact that the action of most effective couplers can not be easily reversed and it has therefore not been possible to adequately study the important phenomenon of recoupling. The bis-dichloroacetamide, C-12, has been shown to be effective as an uncoupler and to possess a transient nature. In view of these characteristics, studies to better characterize the transient nature of this agent have been undertaken. Procedures have been developed to determine whether this transient effect is dependent on oxidative processes within the mitochondrion or is due to purely physical phenomena.

Studies have also been carried out to better define the mechanism of C-12 action on the induction of anion
permeability and its uncoupling mechanism. Possible connections between these phenomena have been explored and are discussed. This research, then, will attempt to better define the properties of C-12 in hope that this agent may prove to be of use in better defining the mechanism of energy coupling.
III
EXPERIMENTAL METHODS

Preparation of Beef Heart Mitochondria

Beef heart mitochondria were prepared by the Nagarse method of Hatefi et al. (112) as modified in this laboratory (113). Protein concentration was determined by a biuret method.

Alkalization and Acidification

Changes of proton ion concentration in lightly buffered suspensions of mitochondria was monitored by a glass electrode and a recording pH meter. These changes were quantitated by the addition of standard HCl.

ANS Fluorescence Measurements

ANS fluorescence in suspensions of mitochondria subjected to various agents was monitored in an Aminco-Chance spectrophotometer with a fluorescence attachment. The excitation wavelength was 360 μm and the emission wavelength was 460 μm.

Oxygen Consumption

Oxygen consumption was monitored by a Yellow Springs Instrument electrode.
Cytochrome b Reduction

The oxidation and reduction state of cytochrome b was monitored in an Aminco-Chance spectrophotometer in the dual wavelength mode at 430 μ vs. 490 μ.

Simultaneous Monitoring of Oxygen Usage, pH, ANS or Cytochrome b Reduction

The simultaneous measurement of oxygen consumption, pH, ANS or cytochrome b reduction was carried out in a specially designed multiparameter cell for use in the Aminco-Chance Spectrophotometer (114).

Picrate Extraction

The concentration of picrate extracted was determined spectrophotometrically in a Beckman DU at 820 μ. The molar extinction coefficient, ε, of picrate in aqueous solution was taken to be 13,700 (115).

Mitochondrial Swelling

The swelling of mitochondria was followed by light scattering changes at 546 μ in an Eppendorf photometer or a Beckman Model B spectrophotometer adapted for this purpose.

36Cl⁻ Exchange

36Cl⁻ was monitored in a Packard Tricarb Scintillation counter with the settings maximized for 36Cl⁻.
Metabolism of C-12

Tests for possible metabolism of C-12 were carried out by incubating tritiated drug under conditions used in the studies when the transient changes were noted and under appropriate control conditions. The reaction mixtures were extracted with 3 x 10 ml volumes of ethyl acetate and aliquots of the organic and aqueous phases were counted by liquid scintillation spectroscopy. Gas-liquid chromatography of C-12 extracts from mitochondrial reaction mixtures was carried out on 3% OV-1 in all glass columns on a Varian Aerograph Model 200 chromatograph with a flame ionization detector. The injector, column, and detector temperature were 245°, 227°, and 242°, respectively.

Synthesis of Tritiated C-12

All the acetamides were synthesized, crystallized and analyzed as previously described (102). Tritium-labeled C-12 was prepared from commercially labeled 1, 12-diaminododecane dihydrochloride. The 1, 12-diaminododecane dihydrochloride was purified by thin-layer chromatography on silicic acid. It was then combined with crystalline carrier dihydrochloride and acetylated with dichloroacetylchloride. The tritiated compound was judged to be pure on the basis of thin-layer chromatography, melting point determination, gas-
liquid chromatography and by comparison to unlabeled drug in
in vitro systems.

**Ca++ Uptake and Release**

Ca++ movements were followed by measuring the
accompanying pH transitions and verified by atomic absorp­
tion spectroscopy of perchloric acid diluted samples of
supernatant in a Perkin Elmer atomic absorption spectro­
photometer. Specific details for the composition of the
various media are given in the figure legends of individual
experiments.
SECTION IV
RESULTS

Transient Effects of C-12

It has been shown by Merola and Brierley (116) that the C-12 induced respiratory stimulation and pH transitions accompanying the uncoupling of oxidative phosphorylation in beef heart mitochondria were transient. It was also shown that, in addition to the uncoupling, C-12 induced a passive chloride permeability. This was shown by the C-12 induced swelling in KCl with valinomycin and in NH₄Cl facilitated by CCP. It will be the purpose of this section to verify and extend these studies on the transient nature of C-12.

Transient Effects of C-12 on Respiration, pH Transitions and Cytochrome b Reduction

Fig. 4 shows the simultaneous recording of respiration, pH and cytochrome b reduction. From this experiment it can be appreciated that C-12 behaves as a moderate uncoupler as seen by the stimulation of respiration, the accompanying alkalization of the external medium and the rapid oxidation of cytochrome b. Fig. 5 serves to show the effect of the well-known uncoupler, CCP, on the same parameters. Fig. 4
Fig. 4 -- The effect of C-12 on the respiratory rate, pH transitions and cytochrome b reduction. The medium as 150 mM KCl, 1 mM Tris-Cl pH 7.2, 2 mM K-succinate and 5.4 μM rotenone in a total volume of 10 ml. Mitochondria were present in a concentration of 1 mg per ml. C-12 and CCP were added in ethanol to a final concentration of 20 μM and 0.5 μM, respectively. All three parameters were monitored simultaneously as described in the methods section.
Fig. 5 -- The effect of CCP on the respiratory rate, pH transitions and cytochrome b reduction. The conditions are the same as described in Fig. 4.
also shows the transient nature of C-12 on all the parameters monitored in this experiment. It can be seen that the respiratory rate, external alkalization and the oxidation of cytochrome b remain maximal for about 2 minutes after the addition of C-12. After 2 minutes there is a slow but steady return of these parameters to their state 4 levels. This is shown most clearly on the cytochrome b trace. After about 4 minutes a second addition of C-12 is shown to cause another cycle of uncoupling and release with a somewhat shorter time course. After this second addition of C-12, it is seen that an addition of CCP results in the expected uncoupling with its respiratory increase, alkalization and cytochrome b oxidation.

**Transient Effect of C-12 on Ca++ Uptake and Release**

It has been shown that Ca++ can be accumulated by an energy-linked mechanism in mitochondria (5, 6) and that this accumulated Ca++ can be released by uncoupling agents (84, 85). Fig. 6 shows the transient nature of C-12 on the release of Ca++ accumulated by energy-linked uptake. It can be seen that C-12 will cause the release of about 40% of the Ca++ taken up by the mitochondria under the conditions of this experiment. It is also shown by the pH trace that after 2 minutes there begins a slow external acidification.
Fig. 6 -- The effect of C-12 on the energy-linked accumulation of Ca++. The medium contained 100 mM KCl, 5 mM Tris-Cl pH 7.3, 5 mM K-succinate and 5.4 μM rotenone in a total volume of 10 ml. Mitochondria were present at a final concentration of 0.75 mg protein per ml. 300 nmoles Ca++ were added as the chloride salt. C-12 and CCP were added in ethanol to final concentrations of 20 μM and 0.5 μM, respectively. 1 ml samples were spun down in an Eppendorf microcentrifuge for 2 minutes. The supernatant was appropriately diluted with perchloric acid and the Ca++ concentration was determined by atomic absorption spectroscopy. The numbers in parentheses refer to the nmoles of Ca++ in 10 ml of supernatant.
indicating a reuptake of Ca++ by the mitochondrion. After
about 4 to 5 minutes this acidification stops, suggesting
that all of the Ca++ has been reaccumulated. Verification
by atomic absorption spectroscopy shows that nearly all of
the Ca++ which had been released by C-12 has been reaccumu­
lated as indicated by the pH trace. A second addition of
C-12 at this point results in the release of about 27% of
the accumulated Ca++.

Transient Nature of C-12 on the Induction of Chloride
Permeability in KCl

The experiment in Fig. 7 shows the effect of preincu­
bation on the induction of chloride permeability in valino­
mycin-treated mitochondria. In part A mitochondria treated
with rotenone and antimycin A to inhibit all respiration
swell when C-12 and valinomycin are added simultaneously.
Part B shows the effect of incubating C-12 with the mito­
chondria for about 5 minutes before the addition of valino­
mycin. It can be seen that after this incubation with C-12
there is little swelling observed when the valinomycin is
added to induce the potassium ion permeability. Part C
shows that a second addition of C-12 after the addition of
the valinomycin results in the same extensive swelling seen
when the two agents are added simultaneously as in part A.

This study as well as those on respiratory stimulation
Fig. 7 -- The transient induction of chloride ion permeability in KCl. The medium was 100 mM KCl, 2 mM Tris-Cl pH 7.0, 5.4 μM rotenone, in a total volume of 10 ml. The mitochondrial concentration was 0.5 mg protein per ml. C-12 and valinomycin (val) were added in ethanol to a final concentration of 10 μM, respectively. Antimycin A and oligomycin were added to a concentration of 1μg/mg protein and 2μg/mg protein, respectively. The swelling changes were followed by the changes in light scattering at 546 μm.
and Ca++ release and reuptake, helps show that the transient effect is common to all of the C-12 induced parameters monitored to date.

**Effect of Preincubation on the Respiratory Rate and the Respiratory Control Ratio**

In order to determine if the transient nature of C-12 requires oxidation to be manifested on the respiratory rate and the respiratory control ratio the experiment summarized in Fig. 8 was undertaken. In this experiment a concentration of C-12 necessary for a maximum effect was added to a rotenone treated suspension of mitochondria and allowed to incubate with the non-respiring suspension for 0, 1, 2, 3, 4, 5 and 6 minutes. After this preincubation with the C-12, K-succinate (8 mM) was added to induce respiration. The maximal rate of respiration was calculated from the polarographic measurements and plotted against the time of preincubation in minutes. As shown in Fig. 8 the respiratory stimulation remained maximal (0.130 μatoms O₂/min/mg protein) for two minutes after which a sharp decline is seen to a preincubation time of 5 minutes. At 5 minutes the respiratory rate had returned to within 95% of the state 4 rate seen with no C-12 added i.e., about 0.040 μatoms O₂/min/mg protein. Also shown in Fig. 8 are the results of an experiment showing the transient effect of C-12 on the respiratory
Fig. 8 — The transient effect of C-12 on the respiratory rate and the respiratory control ratio. The medium for the determination of the respiratory rate was 150 mM KCl, 2 mM Tris-C1 pH 7.2, and 5.4 μM rotenone in a total volume of 10 ml. The medium for the determination of the respiratory control ratio was 150 mM KCl, 2 mM Tris-C1 pH 7.2, 3 mM K-Pi and 5.4 μM rotenone in a total volume of 10 ml. Mitochondrial protein concentration was 0.5 mg per ml. C-12 added in ethanol to a final concentration of 10 μM was added and incubated for a time (t) after which K-succinate was added to final concentration of 8 mM. The maximum respiratory rate (—O—) was determined after the substrate was added. For the determination of the respiratory control ratio (—X—) 1 μmole ADP was added 1 minute after the substrate. The preincubation time for this experiment was taken as (t − 1) minutes. The oxygen utilization with no C-12 was 0.040 μatoms O₂/min/mg protein and the respiratory control ratio with no C-12 was 2.7.
control ratio. In this experiment the mitochondrial sus-
ension was incubated with C-12 in a medium capable of
supporting phosphorylation of ADP. After an incubation time
of 0, 1, 2, 3, 4, and 5 minutes, K-succinate was added and
allowed to incubate for 1 minute after which the ADP was
added and allowed to be phosphorylated. The calculated
respiratory control ratio was plotted versus time. The time
was taken as the time from the addition of C-12 to addition
of the ADP. As can be seen from Fig. 8 the respiratory
control ratio is 1 for no preincubation and shows a slow rise
to 1.5 after 3 minutes. There is a marked restoration in
the respiratory control between the third and fourth minutes
from 1.5 to 2.4 and a further slow increase to a control
value of 2.5 after 6 minutes. This return is to about 94% of
the control value of 2.7 for the KC1 buffer system with
no C-12 present.

These experiments show that the transient effect on the
respiratory rate and the respiratory control ratio occur on
essentially the same time scale and that the transient effect
is independent of oxidative processes.

**Effect of Preincubation on the C-12 Induced Chloride
Ion Permeability**

Fig. 9 demonstrates the effect of preincubation on the
induction of passive chloride ion permeability in NH4Cl.
Fig. 9 -- The effect of preincubation of C-12 on the chloride permeability in NH₄Cl. The medium was 150 mM NH₄Cl, 5 mM Tris-Cl pH 7.2 and 5.4 μM rotenone in a total volume of 10 ml. Mitochondrial protein concentration was 0.5 mg per ml. C-12 and CCP were added in ethanol to a final concentration of 10 μM and 0.5 μM, respectively. The swelling was followed by changes in light scattering at 546 μm.
Though less dramatic than the respiratory studies it can be seen that when C-12 and CCP are added simultaneously there is a marked swelling seen as a decrease in the optical density at 546 μ. If, however, the C-12 is added but the addition of CCP is delayed, there is a time dependent decrease in the swelling rate. This decrease is on the same time scale as the return of respiratory control in the previous study. Part B shows that a second addition of C-12 after 6 minutes results in the initiation of a maximal rate of swelling.

The experiments shown in Fig. 10 serve to demonstrate that the transient nature of the chloride permeability is due to the C-12 and not the CCP or simply an effect due to the incubation of the mitochondria. While a somewhat slower rate of swelling is observed when CCP is allowed to incubate with mitochondria for 10 minutes prior to the C-12 addition, there is still an extensive swelling seen when C-12 is added. This slower rate of swelling is possibly due to an alteration in the existing gradients by CCP which results in somewhat less favorable conditions for the movement of NH₄⁺ into the interior of the mitochondrion as NH₃ and H⁺ via CCP when the chloride ion is made permeable by C-12. These studies further show a correlation between the various parameters that C-12 affects and show that the transient nature is seen in both oxidative and passive systems.
Fig. 10 -- The effect of CCP incubation on the C-12-induced chloride permeability in NH₄Cl. The incubation medium was the same as described in Fig. 9.
Metabolism Studies of C-12

C-12 was incubated with the mitochondria in the presence of substrate for 10 minutes, a time sufficient for the transient effect to occur, after which the suspension was extracted with ethyl acetate as described in the experimental methods section. Fig. 11 presents the GLC traces of the three extracts. The conditions for the chromatography are given in the methods section. As can be seen there is no detectable metabolic transformation of the C-12 in this system. The efficiency of extraction by ethyl acetate of tritiated-C-12 and possible metabolites was found to be between 93 - 99%. To verify the experiment shown in Fig. 11 another series of incubations were carried out using tritiated C-12. In the experiment summarized in Fig. 12 tritiated C-12 was incubated with buffer (part A), with mitochondria and buffer but no substrate (part B) and with mitochondria, buffer and substrate (part C) as described in the figure legend. After 10 minutes incubation each suspension was extracted 3 times with 10 ml of ethyl acetate. These extracts were concentrated by evaporation and spotted on thin-layer plates of Silica Gel G. The thin-layer plates were developed in hexane/acetone (2:1) and visualized by cutting 1 cm strips and counting the tritium label as described in the figure legend. With the exception of a small number of counts at
Fig. 11 — GLC chromatography of C-12, mitochondria and C-12 plus a mitochondrial reaction mixture. The exact conditions for the chromatography are given in the experimental methods section. The buffer was 150 mM KCl, 2 mM Tris-Cl pH 7.2 and for the trace labeled C-12 - mitochondria 8 mM K-succinate. Rotenone was present in a final concentration of 5.4 µM and C-12 was added in ethanol to a final concentration of 15 µM. Mitochondrial protein was 0.5 mg per ml in a total volume of 10 ml. All systems were incubated for 10 minutes.
Fig. 12 — The localization of tritiated C-12 and any tritiated metabolic products in mitochondrial and C-12 extracts on TLC. Thin layer chromatography on silica gel G of ethyl acetate extracts were developed in hexane/acetone (2:1). One centimeter strips were scraped beginning 1 cm before the origin and placed in 10 ml of Brays counting solution. The samples were then counted by scintillation spectroscopy.

Extract A was obtained in a system containing only tritiated C-12 (15 μm) and the KCl buffer described in Fig. 11. Extract B was obtained from a system containing tritiated C-12, mitochondria (5 mg protein) and 10 ml of the KCl buffer. Extract C was identical to system B except that succinate was added and it was allowed to incubate for 10 minutes.
the solvent front all of the counts were localized between
the 8th and 11th centimeter mark for each extract. These
studies show rather conclusively that the transient changes
described above are not due to any metabolic or other
chemical change in the C-12 molecule.

Prepcipitation or Aggregation of C-12

Because of the relatively low solubility of the bis-
dichloroacetamides in aqueous solution, the possibility of
precipitation and/or aggregation as an explanation for the
cause of the transient nature of C-12 must be considered.
This idea gains support from attempted binding studies in
which it was not possible to separate mitochondria from
the C-12 by either centrifuation or rapid filtration tech-
niques. The C-12 appeared at all times and at very high
concentrations to be bound to the mitochondrion. This
result would be expected if the C-12 were precipitating or
aggregating on the mitochondrion. The studies of Fig.13
were undertaken to investigate this possibility. Part A
of Fig. 13 demonstrates the effect of incubation of C-12
with a KCl buffer system similar to those used in the pre-
vious studies. As can be seen there is a time-dependent
increase in the optical density at 546 μm which suggests
that the C-12 is precipitating.
Fig. 13 -- The effect of BSA and C-12-Cl\textsubscript{1} on the precipitation or aggregation of C-12. The precipitation or aggregation of C-12 was followed as an increase in the optical density at 546 m\textmu. The buffer consisted of 120 mM KC1, 25 mM Tris-Cl pH 7.0 and was present in a total volume of 10 ml. C-12-Cl\textsubscript{1} and C-12 were added in ethanol to a final concentration of 20 \(\mu\)M and 15 \(\mu\)M, respectively. The bovine serum albumin (BSA) was added to a final concentration of 0.75 mg per ml.
Previous studies have shown that the addition of albumin to the mitochondrial suspension was effective in decreasing the C-12-induced swelling in NH₄Cl. It was of interest then to see the effect, if any, of albumin on the observed C-12 precipitation. It is shown in Fig. 13, part B that the addition of bovine serum albumin (BSA) resulted in a slight inhibition of the optical density increase when C-12 was added to the system. Since the albumin does not result in a significant increase in the rate of C-12 precipitation, a mechanism other than the enhancement of C-12 precipitation by the albumin must be sought for the observed inhibition of the in vitro swelling in NH₄Cl due to C-12.

The slight, but observable, initial inhibition of the C-12 precipitation by the albumin indicates that some interaction between the protein and the C-12 is taking place. The inhibition of C-12 by the albumin may possibly be due to a greater affinity of the C-12 for the albumin as compared to mitochondria concomitant with a diminution of the effective C-12 concentration by its normal precipitation.

It has been demonstrated that the monochloro derivative of the bis-acetamides (C-12-Cl₁), though inactive itself, is capable of decreasing the extent of alkalization due to the addition of C-12 to anaerobic suspensions of
mitochondria (102). The studies presented in Fig. 14, 15, and 16 verify and extend this observation. Fig. 14 serves as a control for this study and shows only slight decrease in the C-12-induced respiratory stimulation after about 4 minutes. The effect of C-12-C1 on the transient nature of C-12 on the respiratory stimulation is demonstrated in Fig. 15. As can be seen there is little or no stimulation due to the C-12-C1 addition. The transient cycle due to a subsequent addition of C-12 is seen to be much more rapid and is over in less than 1 minute. If the C-12 is added first, followed by C-12-C1 after maximum respiratory stimulation has been induced, it can be seen in Fig. 16 that the C-12-C1 causes a rapid cut-off in the C-12-induced respiratory increase. It was of interest to see if the C-12-C1 had any effect on the rate of C-12 precipitation in a system without mitochondria. The results of this study are presented in Fig. 13, part C. As shown there, the addition of C-12-C1 results in only a slight increase in the optical density. The subsequent addition of C-12, however, results in a rapid precipitation of the C-12 as seen by the rapid increase in the optical density. It is probable that the decrease in the time scale of the transient effect of C-12 by C-12-C1 is due to the increased rate of
Fig. 14 -- The respiratory stimulation and transient nature of C-12 in buffered sucrose. The buffer was 0.25 M sucrose, 10 mM Tris pH 7.4, 3 mM K-succinate and 5.4 μM rotenone in a total volume of 10 ml. Mitochondria were present in a concentration of 0.5 mg per ml. C-12 was added in ethanol to a final concentration of 15 μM.
Fig. 15 -- The effect of C-12-Cl\textsubscript{1} preincubation on the transient respiratory increase induced by C-12. The experimental conditions were the same as described in the legend of Fig. 14. C-12-Cl\textsubscript{1} was added in ethanol to a final concentration of 15 \textmu M.
Fig. 16 -- The effect of C-12-Cl₁ on the C-12 induced respiratory stimulation. The conditions were the same as described for Fig. 14 and Fig. 15.
precipitation which lowers the effective concentration available to the mitochondria.

If these interpretations are correct it should be possible to preincubate C-12 in the buffer system with no mitochondria present, allowing it to precipitate, and show a decreased effect on the respiratory stimulation when mitochondria are added. Fig. 17 demonstrates this point. C-12 was incubated for 10 minutes with the buffer before the mitochondria were added to the system. It can be observed that the respiratory rate is essentially normal as compared to the control of Fig. 14. The subsequent addition of C-12 results in the induction of the expected respiratory stimulation followed by a return to a near normal state 4 rate.

**Mechanism of C-12 Uncoupling**

It has been suggested that C-12 uncouples by inducing an anion permeability which subsequently discharges the potential and pH gradient resulting in the loss of phosphorylation and an increase in respiration (116). It will be the purpose of this section to present additional evidence concerning this anion permeability and its relationship to the uncoupling mechanism.
Fig. 17 -- The effect of preincubation of C-12 with the buffer on the respiratory rate and subsequent respiratory rate increases induced by further additions of C-12. The buffer was the same as described in Fig. 14. *C-12 was added to a final concentration of 15 μM and incubated with the buffer for 10 minutes before mitochondria were added to the system.
Passive Fluorescence Changes of ANS and pH Transitions Induced by C-12

Skulachev (117) has shown that the fluorescence of the dye, 1-anilino-8-naphthalene sulfonic acid (ANS), is increased when the interior of mitochondria become more positive. Fluorescence is decreased under conditions reversing this positive membrane potential. According to this hypothesis the movement of OH\(^-\) out of the interior should result in the uptake of ANS and a subsequent increase in fluorescence. Fig. 18 shows the effect of C-12 and S-13 on the fluorescence of ANS in a mitochondrial suspension in buffered KCl. The data of Fig. 18, part A show that C-12 induces a rapid fluorescence increase concomitant with extensive alkalization of the external medium which is followed by a rapid decay of the fluorescence enhancement. A subsequent addition of S-13 causes no further change in either pH or fluorescence. The changes induced by C-12 in this system are interpreted to be an initial rapid efflux of OH\(^-\) followed by a slower and greater than stoichiometric movement of Cl\(^-\) into the interior with little further evidence of a pH change in the external medium. Apparently this agent sufficiently collapses the potential and pH gradient so that the subsequent addition of S-13 has no effect. Part B demonstrates the effect of reversing the
Fig. 18 — The effect of C-12 and S-13 on the pH transitions and ANS fluorescence in KCl. The medium was 150 mM KCl, 2 mM Tris-Cl pH 7.2, 23 μM ANS and 5.4 μM rotenone in a total volume of 10 ml. Mitochondrial protein concentration was 0.75 mg per ml. C-12 was added in ethanol to a final concentration of 20 μM and S-13 in ethanol to a final concentration of 0.2 μM. The fluorescence of ANS was given in relative fluorescence units.
order of addition of C-12 and S-13. In this trace it can be seen that the addition of S-13 results in a more extensive fluorescence increase and a less extensive alkalization of the external medium than does C-12. It is also seen that there is only a slow decay of the ANS fluorescence as compared with the initial addition of C-12 in part A. The addition of C-12 following S-13 results in a rapid decrease in the ANS fluorescence and a further alkalization of the external medium. It is proposed that the addition of S-13 causes a rapid influx of $H^+$, seen as an increase in pH in the external medium, and an increase in the uptake of ANS which results in the increased fluorescence. Since S-13 does not significantly induce a compensating anion symport or cation antiport in this system, there is no rapid decay seen as with C-12 in part A. The marked decrease in fluorescence and the further alkalization of the external medium seen with the addition of C-12 is explained as the collapse of this relative positive potential by the greater than stoichiometric movement of Cl$^-$ into the mitochondrion and either OH$^-$ out or $H^+$ in. Since S-13 should have allowed sufficient equilibration of the pH gradients by allowing the free movements of $H^+$, little OH$^-$ should be available inside the mitochondria to move out as
the Cl" moves in. It is perhaps more reasonable to visualize the decay of the S-13 induced fluorescence enhancement as being due to a greater than stoichiometric symport of Cl" and H⁺ into the mitochondrion. It is also noticed in Fig. 12 that the extent of the fluorescence enhancement induced by S-13 is greater than that induced by the initial addition of C-12. It is proposed that the decreased fluorescence enhancement due to C-12 is the result of the superimposition of the slower Cl"-induced decay on the rapid OH"-induced increase with the net result being a decreased fluorescence. Since there are no compensating movements possible when S-13 is added there is a much larger fluorescence change observed.

If chloride is responsible for the decay phase of the fluorescence enhancement, it should be possible to eliminate this phase by observing the effect of C-12 on the ANS fluorescence in a Cl" free medium such as buffered sucrose.

The experiment in Fig. 19 shows the results of these studies, which were run in 0.25 M sucrose and 2 mM Tris-HEPES pH 7.2. The only chloride present in this system would be the small amount present endogenously in the mitochondrial suspension. As can be seen from part A, the addition of C-12 results in an extensive and rapid fluorescence increase which is proposed to be due to the in-
Fig. 19 — The effect of C-12 and S-13 on ANS fluorescence in buffered sucrose. The medium was 0.25 M sucrose, 2 mM Tris-HEPES pH 7.2, 23 μM ANS and 5.4 μM rotenone in a total volume of 10 ml. Mitochondrial protein concentration was 0.75 mg per ml. C-12 and S-13 were added in ethanol to a final concentration of 20 μM and 0.2 μM, respectively.
creased interior positive potential caused by the efflux of OH\(^{-}\) as in Fig. 18, part A, but uncompensated for in this case. When compared to Fig. 18, part A, it can be seen that not only is the fluorescence change more extensive but the decay rate is greatly decreased. This trace also shows that the subsequent addition of S-13 has no further effect. Fig. 19, part B, shows that the effect of S-13 on the fluorescence is an increase similar to that seen for the chloride containing system of Fig. 18. The addition of C-12 after this S-13 addition results in no further effect. These results are compatible with the view that S-13 causes an increased relative positive interior potential similar in extent as the obtaining when C-12 is added to the sucrose system. It is proposed, however, that the S-13 induces this positive potential by moving H\(^{+}\) into the interior while C-12 moves OH\(^{-}\) out of the interior. The lack of a rapid fluorescence decay when C-12 follows S-13 is expected on the basis of the absence of a large Cl\(^{-}\) concentration gradient for the neutralization of the relative positive potential as in the KC1 system of Fig. 18.

**Effect of KC1 Concentration on the ANS Fluorescence Decay**

If the above interpretations are correct, both the extent of the fluorescence enhancement and the rate of its decay should be dependent on the KC1 concentration in the suspending medium. In the series of experiments shown in Fig.
20 it is seen that the extent of the ANS fluorescence increase and the rate of decay of this fluorescence is dependent on the concentration of KCl in the external medium. In this experiment the KCl concentration was varied as shown and the osmolarity was maintained by the addition of appropriate concentrations of sucrose. As shown, there is little difference between the traces for 0 mM and 50 mM KCl in regard to either the extent of fluorescence or the rate of decay. Between 50 mM and 100 mM KCl there is, however, a significant decrease in the extent of the ANS fluorescence induced and an increase in the decay rate of this fluorescence. Fig. 21 shows graphically this dependence of the maximum rate of decay of the fluorescence on the KCl concentration. Because of these observations concerning the increased rate of decay, it was of interest to determine the decay constant, k, and the half-time, \( t_{1/2} \), of the decay as a function of KCl concentration. The points of Fig. 22 were obtained from a trace similar to those in Fig. 20. Zero time was taken as the point of maximum fluorescence. The points were fit to the curves using the method of least-squares. The decay constant, k, is obtained by multiplying the slope of the line by 2.303. The half-time, \( t_{1/2} \), of the decay is found by equation 20. Table 2 serves to show the dependency of the two parameters on the KCl concentration.

\[
t_{1/2} = 0.693/k
\]  

(20)
Fig. 20 -- Effect of KCl concentration on the C-12 induced ANS fluorescence. The medium contained KCl as indicated in the figure and buffered with 2 mM Tris-Cl pH 7.2. The milliosmolarity was maintained with sucrose to 250 milliosmolar. In the case of 0 mM KCl, 2 mM Tris-HEPES pH 7.2 was used to buffer the 0.25 M sucrose medium. Mitochondria at 0.75 mg protein per ml were blocked with 5.4 µM rotenone. ANS as the Mg++ salt was added in water to a final concentration of 23 µM. C-12 was added in ethanol to a final concentration of 20 µM. The total incubation volume was 10 ml.
Fig. 21 -- The effect of KCl concentration on the maximum rate of C-12 induced ANS fluorescence decay. Experimental conditions were the same as given in Fig. 20.
Fig. 22 -- The effect of KC1 concentration on the decay constant and the half-time of the decay of the ANS fluorescence induced by C-12. The incubation conditions were the same as described in Fig. 20. The rate of fluorescence decay was determined with the point of maximum fluorescence taken as the zero time point, the curves were fit by the method of least-squares.
Table 2

Dependency of the Decay Constant and the Half-time of Decay on the Concentration of KC1

<table>
<thead>
<tr>
<th>KC1 Concentration</th>
<th>Slope</th>
<th>k (sec(^{-1}))</th>
<th>t(_{1/2}) (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.0078</td>
<td>0.018</td>
<td>38.5</td>
</tr>
<tr>
<td>25</td>
<td>-0.0068</td>
<td>0.016</td>
<td>43.3</td>
</tr>
<tr>
<td>50</td>
<td>-0.0091</td>
<td>0.021</td>
<td>33.0</td>
</tr>
<tr>
<td>100</td>
<td>-0.0293</td>
<td>0.068</td>
<td>10.2</td>
</tr>
<tr>
<td>150</td>
<td>-0.0323</td>
<td>0.074</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Data from Fig. 22 was used to calculate the parameters. The calculation of k and t\(_{1/2}\) is described in the text.
the decay constant or the half-time of decay for 25 mM and 50 mM KCl as compared to the values for 0 mM KCl. There is, however, a marked effect on the two parameters for 100 mM and 150 mM KCl. For a KCl concentration of 150 mM the decay constant showed a value of 0.074 sec\(^{-1}\) as compared to 0.018 for no KCl. The half-time showed a decrease from 38.5 sec for the system with no KCl to 9.4 sec for 150 KCl.

The experiment in Fig. 23 shows the effect of KCl concentration on the extent of alkalization induced by C-12. The extent of alkalization is almost doubled when the KCl concentration is increased from 25 mM to 150 mM.

All of the fluorescence data are compatible with the idea that C-12 induces a rapid movement of OH\(^-\) ions out of the mitochondrion in both sucrose and KCl systems. This exit of OH\(^-\) results in the interior of the mitochondrion becoming relatively more positive. ANS moves into the mitochondrion in response to this relative positive interior potential and a fluorescence increase is observed. In sucrose where OH\(^-\) is the only major anion that can move in response to C-12 there is only a slow decay of the ANS fluorescence. In KCl, however, the C-12 induces a permeability to Cl\(^-\) as well as OH\(^-\). Therefore, in KCl the extent of the ANS fluorescence is decreased while the rate of its decay is increased as a function of the external chloride concentration.
Fig. 23 -- Effect of KCl concentration on the C-12 induced pH transitions. Incubation conditions were the same as described in Fig. 20.
C-12 Facilitated Transfer of $^{36}\text{Cl}^-$ in a Model System

In order to determine if C-12 induces the anion permeability by a carrier mechanism or by a direct alteration of the membrane the experiment summarized in Fig. 24 was undertaken. The model system consisted of two aqueous phases separated by an organic phase in a specially constructed cell. The organic phase was stirred by a small magnetic stirrer so that it and the interfaces between it and the aqueous phases were adequately mixed. It has been shown by Eisenman et al. (115) and Brierley et al. (118) that the amphipathic, colored anion, picrate, can be extracted into an organic phase if provisions are made to allow simultaneous movement of the cation. The experiment showing the extraction of picrate and its subsequent equilibration into a second aqueous phase was carried out to support the validity of the model system and to determine the effective concentration of valinomycin necessary to carry the cation in the system, i.e. $K^+$. The summary of the experiment in Fig. 24 demonstrates that little picrate is extracted and equilibrated during the control period of 0-240 minutes when no valinomycin was present. After the valinomycin was added to a final concentration of 1 mM at 240 minutes there was a significant transfer of picrate from the one aqueous phase to the other aqueous phase of distilled water. This extraction and equilibration of picrate was observed to reach a maximum at about 420 minutes.
Fig. 24 — $^{36}$Cl$^{-}$ transfer and picrate transfer through a bulk organic phase in a model system. The $^{36}$Cl$^{-}$-containing phase consisted of 10 mM K$^{36}$Cl, 1 mM Tris-Cl pH 7.2 in a total volume of 2.5 ml. $^{36}$Cl$^{-}$ was present at 260,000 cpm per ml. Valinomycin and C-12 were added in ethanol to a final concentration of 1 mM each. The organic phase was 6 ml of CC1. The second aqueous phase (III) was distilled water (2.5 ml). In the experiment using picrate the medium was the same with the addition of $2 \times 10^{-4}$M picrate to the KC1 buffer solution. Valinomycin was added at 240 minutes in the picrate experiment. In the $^{36}$Cl$^{-}$ study the valinomycin was added at the start of the experiment and the C-12 was added at 240 minutes. The insert provides a diagram of the cell adapted for this study.
Using the valinomycin concentration found to be effective in the above experiment and using $^{36}\text{Cl}^-$ in place of picrate, the ability of C-12 to transfer the $^{36}\text{Cl}^-$ in this system was investigated. With the valinomycin present during the control period of 0-240 minutes it is seen in Fig. 23 that there is no $^{36}\text{Cl}^-$ transfer during either the control period or after the addition of C-12 at 240 minutes. It can be concluded that in this system where valinomycin is capable of transferring the potassium cation C-12 is ineffective in transferring $^{36}\text{Cl}^-$ from one aqueous phase to the other aqueous phase. These studies and those to be discussed subsequently give support to the idea that C-12 is acting on the membrane and not on the ion in its induction of anion permeability.

Effect of pH on the Titration of the Respiratory Increase by C-12

Wilson et al. (119) have suggested that there are two potential sites of pH dependency in the uncoupling of phosphorylation. Possibilities include an extrinsic pH dependency due to a pH effect on the uncoupler molecule and an intrinsic pH dependency on the respiratory system within the mitochondrion. Those uncouplers which are weak acids should show a combination of these two pH effects. Hemker (120) showed that if the uncoupling activity of DNP were titrated at different pH, an increase in the concentration of DNP was required to obtain the maximal stimulation as the pH
increased. It is possible that this large pH effect is due to a predominant effect on the ionizable group of DNP. If C-12 were acting as DNP is postulated to act a similar pH dependency should be expected. Fig. 25 shows this not to be the case, however, when the respiratory rate increase is titrated with C-12 at various pH values it is shown that the maximal rate of respiratory stimulation attainable by C-12 at a given pH titrates to a maximum at essentially the same concentration of C-12 i.e. about 10 μM. The decrease in the maximal respiratory stimulation with increasing pH is possibly due to the intrinsic pH dependency mentioned above. These studies strongly suggest that there is no pH effect on the C-12 molecule and it, therefore, is likely not behaving as a proton conductor as postulated by Mitchell (38) for the so-called classical uncouplers. It appears from these studies and the extraction studies discussed previously that C-12 has its effect directly on the membrane of the mitochondrion and does not itself carry ions across the membrane.
Fig. 25 -- Effect of pH on the titration of the respiratory increase by C-12. The medium was 120 mM KCl, 25mM Tris-Cl and 8 mM K-succinate. Rotenone was present in a final concentration of 5.4 μM. The mitochondrial protein concentration was 0.5 mg per ml in a 10 ml system. C-12 was added in ethanol to a final concentration as indicated on graph. The points (not shown) were fit to the best line by the method of least-squares.
V.

DISCUSSION

Merola and Brierley (116) demonstrated that the bis-dichloroacetamide, C-12, was an effective uncoupler of oxidative phosphorylation and also possessed the ability to induce a permeability to anions in mitochondria. It was also observed by these workers that C-12 possessed a transient nature as witnessed by the return of the stimulated respiratory rate to a near normal state 4 rate after about 4 minutes. In the long run this characteristic of C-12 may prove to be the most useful in the study of the coupling mechanism of oxidative phosphorylation.

It was also shown that the respiratory rate increase (see Fig. 25) and the accompanying alkalization of the external medium (121) and the induction of chloride permeability (122) titrate over the same concentration. Merola et al. (102) reported that there was an identical structure-activity profile for the acetamides on all the observed effects on the mitochondrion. There is an absolute requirement for the secondary amide with the monochloroacetyl and trichloroacetyl derivatives showing no and weak effects, respectively. The dichloroacetyl derivative, C-12, showed a maximal effect on all systems studied as compared to the
other analogs of the bis-acetamides.

Like C-12 the trialkyltins are capable of moderate uncoupling and induction of passive chloride permeability but under more restrictive conditions (123, 124). The trialkyltins require not only valinomycin but also an uncoupler for the induction of swelling in KCl. An obligatory Cl\(^-\)/OH\(^-\) exchange has been postulated to account for these observations. If the actions of trialkyltin involves an obligatory Cl\(^-\)/OH\(^-\) exchange, a pH imbalance would result which, if not equilibrated by an uncoupler, would inhibit further uptake of Cl\(^-\) and therefore retard the swelling in KCl with valinomycin. Further support for this idea comes from the observation that the uncoupling due to the trialkyltins is seen only in chloride-containing systems.

In contrast, C-12 does not require an uncoupler to induce swelling in KCl with valinomycin (Fig. 7) and is as effective an uncoupler in chloride-free systems as in chloride-containing systems (compare Fig. 3 and Fig. 14) which suggests that the two agents are acting by different mechanisms.

If the ANS anion moves into areas of increased positive potential as postulated, the ANS data suggest that C-12 induces the independent movements of anions in response to the existing pH or concentration gradients with no evidence for an obligatory Cl\(^-\)/OH\(^-\) exchange. These ANS studies further suggest that there is a very rapid movement of OH\(^-\) out of
the mitochondrion and a slower movement of $\text{Cl}^-$ into the interior. The half-time for the alkalization is about 10 seconds while the half-time for the swelling in KCl and $\text{NH}_4\text{Cl}$ was much longer. It is concluded from these studies that C-12 induces the independent movements of $\text{Cl}^-$ and $\text{OH}^-$ and this movement is in response to the existing gradients, be they pH or concentration gradients, and unlike the trialkyltins no obligatory $\text{Cl}^-/\text{OH}^-$ exchange is operable.

Mitchell (38), van Dam and Slater (19,100) and Wilson (91) have all emphasized the importance of uncouplers possessing a weakly acidic group. Mitchell (38) and Slater (19) propose that the ionizable group of the uncoupler is responsible for the transfer of a proton either into or out of the mitochondrion, respectively. A diagrammatic representation of these two proposals is given by Slater and shown in Fig. 25. Wilson (119) visualizes the

![Diagram of respiratory chain hypotheses](image_url)

Fig. 26 -- Mechanism of uncoupling of the respiratory-chain
phosphorylation, according to the chemical hypothesis (C) and the chemiosmotic hypothesis (C-0). See ref. 19.

uncoupling to be due to a general acid-catalyzed hydrolysis of a high-energy chemical intermediate. Wilson bases this proposal on observations on the effect of pH on the solubility of the uncoupler in aqueous solution, the in vitro uncoupling activity and the ability of the agents to effect a resistance drop across synthetic bilayer membranes.

The data here does not support these concepts of uncoupling for C-12. It is shown by the additional effects of C-12 on the ANS fluorescence and the alkalization of the external medium after a maximal concentration of S-13 in Fig. 18, that C-12 and S-13, a weak acid uncoupler of the sort mentioned above, are clearly acting by different mechanisms. The ANS data and the inability of C-12 to induce extensive swelling in NH$_4$Cl without CCP and in K-acetate with valinomycin but no CCP (116) indicates that C-12 is responsible for the movement of anions and not H$^+$ or other cations. Further support for the proposal that C-12 is acting by a different mechanism than the more classical uncouplers is the lack of a pH effect on the titrable end-point of the respiratory stimulation as would be expected if the C-12 molecule possessed an ionizable group which was participating in the uncoupling mechanism and the induction of anion permeability.

Since there is no apparent pH effect on the C-12 molecule and since it was not possible to demonstrate any trans-
fer or exchange of $^{36}\text{Cl}^-$ in the model system, it was concluded that the effects of C-12 are due to its direct interaction with the inner mitochondrial membrane and not to a carrier-mediated mechanism as postulated for the classical uncouplers such as DNP, S-13 and CCP.

Considering all of the data here it is proposed that C-12 uncoupling results from the direct interaction of the C-12 with the inner membrane which leads to an alteration in the membrane with the subsequent permeability to anions. The OH${}^-$, free to move out of the mitochondrion down a pH and electrical gradient, effectively short circuits the membrane potential and causes separation of oxidation from phosphorylation and therefore an increase in the respiratory rate.

Perhaps more useful in the delineation of the uncoupling mechanism and the coupling of oxidation to phosphorylation, is the transient nature of C-12. Several possibilities exist for the mechanism of this phenomenon. It was suggested early by Merola and Brierley (116) that the transient effect could possibly be due to the active transport of C-12 to some inert compartment or site or possibly the metabolism of the agent. Several lines of evidence argue against these suggestions and include (a) the inability to isolate any metabolic breakdown products using GLC or TLC of tritiated-C-12 extracts, (b) the observed transient effect in the passive systems used to demonstrate C-12-indu-
ced swelling, and (c) the ability of C-12 to induce a rapid, and transient, decrease in resistance across a synthetic phospholipid bilayer (125), a system where little metabolic transformation would be expected. It seems likely from the studies on the precipitation of C-12 and the effects of C-12-Cl\(\text{I}\) and BSA on this precipitation and on the C-12-induced changes \textit{in vitro} that any explanation for the transient effect must consider both precipitation and binding to sites other than the active site. The precipitation studies, though on a time scale somewhat longer than the \textit{in vitro} effects, suggest that the effective concentration of C-12 in the suspending medium may be lowered over a period of time. The effects of C-12-Cl\(\text{I}\) on the transient nature of C-12 add particularly strong support to this idea. It was seen that the C-12-Cl\(\text{I}\) increased the rate of precipitation of C-12 and decreased the time of the transient C-12 effect \textit{in vitro}. While precipitation very likely plays an important role in the transient nature of C-12, the studies showing the effect of albumin and the observation by Alkaitis (125) that phosphatidylethanolamine is a necessary component of the synthetic bilayer membrane for the transient effect, it should not be thought of as the only possibility. While albumin only caused a slight delay in the precipitation of C-12, it is effective in causing a decrease in the action of C-12 \textit{in vitro}. It is possible, then, that C-12 binds preferentially to the albumin in these \textit{in vitro}
studies and thus decreases the effect of C-12.

It seems likely then that C-12 initially interacts with a phospholipid component of the inner membrane as evidenced by its effect on the synthetic phospholipid bilayer. This interaction results in the induction of the anion permeability as evidenced by the alkalization accompanying the respiratory stimulation, the changes in the ANS fluorescence indicating an alteration of the membrane potential, the rapid resistance decrease of the synthetic bilayer and the initiation of swelling in NH₄Cl and KCl with valinomycin. The transient phase of C-12 action appears to involve the decrease in the effective concentration of C-12 in the external medium and the movement of the initially-bound C-12 to an inactive site. This inactive site could possibly be a hydrophobic area in the membrane itself. Evidence from the bilayer studies implicates the phosphatidylethanolamine in the membrane as being involved in this inactive site. Once the effective concentration has been lowered sufficiently, there is the regeneration of the membrane potential by electron transport and recoupling of oxidation to phosphorylation. These proposals are compatible with many parts of the mechanism postulated by Mitchell for the coupling and uncoupling of oxidative phosphorylation. If Mitchell's hypothesis for uncoupling is correct i.e. the uncoupler carries protons into the interior of the mitochondrion and thus short circuits the membrane potential,
one should expect to be able to cause uncoupling by moving anions, OH− in this case, out of the mitochondrion, as proposed here for the action of C-12. These studies then support the contention that the maintenance of the membrane potential is of prime importance in the coupling of oxidation to phosphorylation.

Only one other group of compounds have been shown to have a cyclic effect on uncoupling. These are the thiophene derivatives reported by Schafer and Buchel (106). They propose that these compounds are accumulated by the mitochondrion in an energy-requiring reaction much like Ca++. No further explanation for the transient nature of the thiophenes has been put forward to date. This leaves C-12 as the only well-defined agent available for the study of transient coupling and recoupling. The studies here have opened many interesting questions as to the use of C-12. C-12 could possibly be used to study the uptake and release of Ca++ in view of the mitochondrial role in muscle contraction, as well as to better define the primary energy coupling within the mitochondria.


