I. INTRODUCTION

Mitchell (1985) has suggested a rolling well and turnstile hypothesis for the molecular mechanism of functioning of F<sub>0</sub>F<sub>1</sub> ATPases. The salient feature of this model are (for details see Chapter 1)

(i) Direct participation of H<sup>+</sup>-ions in ATP synthesis as given by the following equation,

\[
2H^+ + \text{ADP} \rightarrow H_2O + ATP
\]

(ii) Protons (or $\Delta\mu_{H^+}$) are involved in port-antiport processes i.e., porting ADP and $P_i$ to the hydrolytic site (from N to P site) and ATP to N domain from P domain,

(iii) Anticlockwise rotation of $\alpha$-and $\gamma$-subunits and clockwise rotation of $\beta$-subunit with the same angular velocity.

The energy dependent release of ADP is an activation phenomenon arising due to conformational changes.
in CF₁ induced by ΔµH⁺ across the membrane (Mitchell, 1984; Schlodder et al., 1982). The release process as influenced by subunit-subunit interactions and consequently the activation of CF₁ for phosphorylation activity is controlled by ΔµH⁺ (Rögner et al., 1986).

The electrochemical gradient of H⁺-ions across the membrane consists of ΔpH and Δψ

\[ ΔµH⁺ = ΔpH + Δψ \]

Uncouplers have been shown to dissipate ΔpH whereas ionophores decrease the Δψ and thus result in decrease of ΔµH⁺. Any change in ΔµH⁺ would affect both the energy dependent release of ADP and the photophosphorylation (Mills and Mitchell, 1984).

In this study we report here our observations on the effect of uncouplers and ionophores in the regulation of energy transduction in thylakoid membranes.

II. MATERIALS AND METHODS

A. Light-Induced Release of \([^{14}C]\)-ADP Bound to CF₁

The thrice washed thylakoid membranes were prepared as described in Chapter 2. The light-induced release of \([^{14}C]\)-ADP was also carried out as described previously
(See Chapter 2) with the only exception that uncouplers (NH₄Cl, m-CCCP, CH₃NH₂ and atebrin) or ionophores (valinomycin, gramicidin D and nystatin) in varying concentrations were added to assay mixture for the light-induced exchange. The final concentration of methanol or ethanol in the assay mixture did not exceed 1%.

B. Photophosphorylation

The ATP synthesis coupled to electron transport from water to methyl viologen in presence or absence of uncouplers or ionophores was determined under the same conditions as in light-induced release of [¹⁴C]-ADP with the only exception that [¹⁴C]-ADP was replaced by cold ADP (See Chapter 2).

C. Sources of Chemicals

Nystatin, atebrin, m-CCCP, valinomycin, gramicidin D and CH₃NH₂ were purchased from Sigma Chemicals Co., USA. NH₄Cl(AR grade) was purchased from BDH, India.

III. RESULTS

A. Energy Dependent Release of [¹⁴C]-ADP Bound to CF₁

(i) In presence of uncouplers

The rates of energy dependent release of [¹⁴C]-ADP were severely inhibited when salt-washed thylakoid membranes
Fig. 6.1.
The energy dependent release of $[^{14}\text{C}]$-ADP as a function of uncouplers concentrations. The salt-washed thylakoid membranes were prelabelled with $[^{14}\text{C}]$-ADP as described in MATERIALS AND METHODS. The energy dependent release of $[^{14}\text{C}]$-ADP was carried out as described in MATERIALS AND METHODS. The uncouplers were added to the assay mixture for light-induced release of $[^{14}\text{C}]$-ADP. Concentrations of methanol/ethanol did not exceed 1%. The control activity in absence of uncouplers was 0.85 nanomoles $[^{14}\text{C}]$-ADP released/mg Chl.

Fig. 6.2
The effect of uncouplers on non-cyclic photophosphorylation. See MATERIALS AND METHODS and Fig. 6.1. for details. Control activity was 147.8 μmoles ATP formed/mg Chl/hr.
were incubated with increasing concentrations of uncouplers (See Fig. 6.1). The inhibition, by uncouplers, in the energy dependent release of $[^{14}\text{C}]-\text{ADP}$ was biphasic in nature with faster phase at lower concentrations and slower phase at higher concentrations of uncouplers (Fig. 6.1 and Tables 6.1 and 6.2). The maximum inhibition in the activity was 51\%, 62\%, 66\% and 56\% in presence of 20mM $\text{NH}_4\text{Cl}$, 20mM $\text{CH}_3\text{NH}_2$, 20\(\mu\text{M}\) atebrin and 20\(\mu\text{M}\) m-CCCP respectively (Table 6.1). The values for the faster phase of inhibition of energy dependent release of $[^{14}\text{C}]-\text{ADP}$ by uncouplers were 48\%, 56\%, 41\% and 40\% in presence of 1.25mM $\text{NH}_4\text{Cl}$, 1.25mM $\text{CH}_3\text{NH}_2$, 1.25\(\mu\text{M}\) atebrin and 1.25\(\mu\text{M}\) m-CCCP respectively (Table 6.2).

(ii) In presence of ionophores

The addition of increasing concentrations of ionophores in the assay also caused inhibition in the rate of energy dependent release of $[^{14}\text{C}]-\text{ADP}$ from salt-washed thylakoid membranes (Fig. 6.3). However, the rate and the degree of maximum inhibition of the activity was less as compared to those observed with uncouplers (compare Figs 6.1 and 6.3). The maximum inhibition of activity was 47\%, 42\% and 21\% in the presence of 10\(\mu\text{M}\) each of gramicidin D, valinomycin and nystatin respectively (Table 6.3).
Table 6.1
THE MAXIMUM INHIBITION IN THE RATES OF ENERGY DEPENDENT RELEASE OF $[^{14}\text{C}] $-ADP AND PHOTOPHOSPHORYLATION BY UNCOUPLERS.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Percentage of Maximum Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20mM NH$_4$Cl</td>
</tr>
<tr>
<td>Energy Dependent Release of $[^{14}\text{C}]$-ADP</td>
<td>51</td>
</tr>
<tr>
<td>Non-cyclic Photophosphorylation</td>
<td>65</td>
</tr>
</tbody>
</table>

Control activities were 0.85 nanomoles $[^{14}\text{C}]$-ADP released/mg Chl and 147.8 μmoles ATP formed/mg Chl/hr.
TABLE 6.2
THE INHIBITION OF ENERGY DEPENDENT RELEASE OF $^{14}$C-ADP AND PHOTOPHOSPHORYLATION BY UNCOUPLERS

<table>
<thead>
<tr>
<th>Activity</th>
<th>Percentage of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_4$Cl</td>
</tr>
<tr>
<td>Energy Dependent Release of $^{14}$C-ADP</td>
<td>48 (1.25mM)</td>
</tr>
<tr>
<td>Non-cyclic Photophosphorylation</td>
<td>58 (2.5mM)</td>
</tr>
</tbody>
</table>

The inhibition values are shown for the faster phase. The values in parentheses indicate concentration of a given uncoupler at which the inhibition takes place. Control activities were 0.85 nanomoles $^{14}$C-ADP released/mg Chl and 147.8 μmoles ATP formed/mg Chl/hr.
The inhibition of the rate of energy dependent release of $[^{14}C]-$ADP by ionophores was also biphasic with a faster phase at lower concentrations of ionophores and slower phase at higher concentrations of ionophores (Fig. 6.3). The degree of inhibition in the faster phase were $22\%$, $17\%$ and $15\%$ in presence of $0.5\mu M$ gramicidin D, $0.5\mu M$ valinomycin and $0.5\mu M$ nystatin respectively (Table 6.4).

B. Non-Cyclic Photophosphorylation

(i) In presence of uncouplers

Like energy dependent release of $[^{14}C]-$ADP, similar biphasic decline in the rate of non-cyclic photophosphorylation was observed when salt-washed thylakoid membranes were treated with increasing concentrations of uncouplers (See Fig. 6.2 and compare it with Fig. 6.1). The decline in the photophosphorylation activity was quite fast at lower concentrations of the uncoupler compared to the slower rates at higher concentrations of uncouplers. The maximum inhibition in the rate of non-cyclic photophosphorylation in presence of uncouplers were $65\%$, $63\%$, $60\%$ and $71\%$ in presence of $20mM NH_4Cl$, $20mM CH_3NH_2$, $20\mu M$ atebrin and $20\mu M$ m-CCCP respectively (Table 6.1). The inhibition in the phosphorylation activity in the faster phase was $58\%$, $62\%$, 
Fig. 6.3.
The energy dependent release of $[^{14}\text{C}]$-ADP as a function of ionophores concentrations. See Fig. 6.1. for details. Control activity was 0.85 nanomoles $[^{14}\text{C}]$-ADP released/mg Chl. GmcD, Nys. and Val. stand for Gramicidin D, Nystatin and Valinomycin, respectively.

Fig. 6.4.
The effect of increasing concentrations of ionophores on the rate of non-cyclic photophosphorylation. See Fig. 6.1. and MATERIALS AND METHODS for details. Control activity was 147.8 μmoles ATP formed/mg Chl/hr.
61% and 49% in presence of 2.5mM NH₄Cl, 1.25mM CH₂NH₂, 2.5µM atebrin and 2.5µM m-CCCP respectively (Table 6.2).

(ii) In presence of ionophores

Fig. 6.4 shows that the addition of ionophores to the assay mixture led to decrease in the rate of non-cyclic photophosphorylation (See Fig. 6.4). The rate of decline in the photophosphorylation activity was quite slow in presence of gramicidin D as compared to that observed in presence of other ionophores like nystatin and valinomycin. Nevertheless, a biphasic inhibition of photophosphorylation was observable in presence of ionophores and the rate of inhibition in the faster phase was 14%, 66% and 52% in presence of 0.3µM gramicidin D, 5µM valinomycin and 1.25µM nystatin respectively (Table 6.4). The maximum inhibitions in the photophosphorylation activities were 21%, 66% and 55% in presence of 10µM each of these ionophores (Table 6.3).

IV. DISCUSSION

NH₄Cl and CH₂NH₂ have been known to cause uncoupling in the chloroplasts by decreasing the H⁺-ion gradient generated due to electron transport or acid-base transition induced ΔpH across the thylakoid membranes.
TABLE 6.3

THE INHIBITION OF ENERGY DEPENDENT RELEASE OF $^{14}$C-ADP AND PHOTOPHOSPHORYLATION BY IONOPHORES

<table>
<thead>
<tr>
<th>Activity</th>
<th>Percentage of Maximum Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10μM Gramicidin D</td>
</tr>
<tr>
<td>Energy Dependent Release of $^{14}$C-ADP</td>
<td>47</td>
</tr>
<tr>
<td>Non-cyclic Photophosphorylation</td>
<td>21</td>
</tr>
</tbody>
</table>

Control activities were 0.85 nanomoles $^{14}$C-ADP released/mg Chl and 147.8 μmoles ATP formed/mg Chl/hr.
TABLE 6.4

THE INHIBITION OF ENERGY DEPENDENT RELEASE OF $[^{14}C]$-ADP AND PHOTOPHOSPHORYLATION BY IONOPHORES

<table>
<thead>
<tr>
<th>Activity</th>
<th>Percentage of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gramicidin D</td>
</tr>
<tr>
<td>Energy Dependent Release of $[^{14}C]$-ADP</td>
<td>22 (0.5µM)</td>
</tr>
<tr>
<td>Non-cyclic Photophosphorylation</td>
<td>14 (0.3µM)</td>
</tr>
</tbody>
</table>

The values in parentheses indicate the concentration of a given ionophore at which the faster phase of inhibition is defined.

Control activities were 0.85 nanomoles $[^{14}C]$-ADP released/mg Chl and 147.8 µmoles ATP formed/mg Chl/hr.
Similarly m-CCCP also makes the thylakoid membranes permeable to $H^+$-ion leading to the collapse of proton gradient. Though the exact mechanism of uncoupling by atebrin is not clear; it is known to bind to thylakoid membranes (Kraayenhof and Slater, 1974). Addition of atebrin to the chloroplasts suspension leads to the collapse of internal space of thylakoid membrane due to massive efflux of salts and water (See Good, 1977).

The decrease in energy dependent release of ADP and photophosphorylation by uncouplers may be explained on the basis of decrease in $\Delta \mu_{H^+}$ leading to decrease in fraction ($\phi$) of activated CF$_1$ molecules. Our results are in agreement with the proposals of Mills and Mitchell (1984) that $\Delta \mu_{H^+}$ is the driving force for activation of ATPase as well as photophosphorylation (also see Gräber et al., 1977; Schlodder et al., 1982).

The biphasic decline in the rates of both energy dependent release of ADP and non-cyclic photophosphorylation indicates that low concentrations of uncouplers are sufficient enough to cause maximum inhibition of energy dependent release of ADP and photophosphorylation (See Figs. 6.1 and 6.2). Our results on the effect of higher concentration of uncouplers suggest that either
(1) Higher concentration of uncouplers are not effective in the complete collapse of $\Delta \mu_{H^+}$ across the thylakoid membranes; or

(2) there are localized pathways of proton transfer from the site of its production to the site of its utilization; the so-called protoneural network (Laszlo et al., 1984). And such protoneural network is inhibited maximally but not completely by 100% by uncouplers.

Our observations on the effect of various concentrations of ionophores on the energy dependent release of ADP and non-cyclic photophosphorylation show that (See Figs 6.3 and 6.4 and Tables 6.3 and 6.4) both these activities were affected less severely as compared to those with uncouplers.

According to the chemiosmotic theory the driving force for the synthesis of ATP consists of $\Delta \rho \text{H}$ and $\Delta \psi$ according to the equation

$$\Delta \mu_{H^+} = - (2.3 \text{ RT } \Delta \rho \text{H} + F \Delta \psi),$$

where $R$ is gas constant

$F$ is Faraday constant

$\psi$ is transmembrane electric potential

$\Delta \rho \text{H}$ is pH difference across the membrane

$T$ is absolute temperature
Ionophores, in general are known to collapse the $\Delta \psi$ thereby reducing $\Delta \mu_{H^+}$. This would result in decrease of the fraction ($\phi$) of activated $\text{CF}_1$ as shown by decrease in energy dependent release of ADP (Fig. 6.3) according to the equation (Junesch and Gräber, 1984).

$$E_i + bH^+_{\text{in}} \rightleftharpoons E_a + bH^+_{\text{out}} \quad \ldots \ldots \ldots (1)$$

$$\frac{E_a}{E_t} = \frac{e^x}{1 + e^x}; \quad x = \ln \frac{K^0_E - \frac{b \Delta \mu_{H^+}}{RT}}{\ln K^0_E}$$

where,

- $K^0_E$ is the equilibrium constant of reaction 1
- $E_a$ is the active $\text{CF}_1$
- $E_t$ is total number of $\text{CF}_1$
- $b$ is number of protons involved for activation of $\text{CF}_1$

This would result in the decrease of the rate of photophosphorylation as shown in Fig. 6.4.

However, as shown with uncouplers (See above), we did not find complete inactivation of the energy dependent release of ADP and non-cyclic photophosphorylation, even at very high concentration of ionophores (10$\mu$M). Therefore, we conclude that either
(i) these ionophores which are capable of collapsing \( \Delta \psi \), do not affect \( \Delta p\text{H} \). So \( \Delta p\text{H} \) would still be available for \( \Delta p\text{H}^+ \) and consequently these activities are noticeable, or

(ii) there are localized pathways of transfer of protons (Protoneural network, see above) which can not be completely inactivated by these ionophores. This is further shown by the fact that we do not observe one to one relationship between these two activities and also different ionophores behave very differently (See Tables 6.3 and 6.4).

V. REFERENCES


Chapter 7

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