# THE CATALYSIS BY MODIFIED CHLOROPLASTS OF THE $P_i \rightleftarrows$ ATP, $P_i \rightleftarrows$ HOH AND ATP $\rightleftarrows$ HOH EXCHANGE REACTIONS IN THE ABSENCE OF LIGHT*

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The absence of definitive evidence for the participation of phosphorylated or non-phosphorylated intermediates in oxidative or photosynthetic phosphorylation has given increasing importance to isotopic exchange techniques for study of the phosphorylation and water-formation reactions of the overall processes (1). If photophosphorylation shares mechanistic features of oxidative phosphorylation, one prediction is that measurable $P_i \rightleftarrows$ HOH, $P_i \rightleftarrows$ ATP and ATP $\rightleftarrows$ HOH exchanges should accompany the process. This has recently been demonstrated by Shavit et al. (2) for net phosphorylation in the light. A distinctive property of photophosphorylation remained, however, in that the exchanges were weak or absent in the dark. In analogy with mitochondria that show prominent exchange apparently independent of oxidation and reduction (3), chloroplasts under appropriate conditions might be expected to show exchanges in absence of light. The observations that ATPase activity could be induced in chloroplasts by incubation in the light with thiols (4,5) and that such preparations had capacity for a $P_i \rightleftarrows$ ATP exchange (6) suggested that such modified preparations might show oxygen exchanges independent from light. The results presented in this paper demonstrate the occurrence in the dark of $P_i \rightleftarrows$ HOH and ATP $\rightleftarrows$ HOH exchanges, as well as the $P_i \rightleftarrows$ ATP exchange, and some effects of $P_i$, ADP and ATP on these exchanges.

Methods. Chloroplasts were prepared from 8 g batches of Romaine lettuce leaves, essentially as described by Avron (7) and adjusted to give approximately 1.2 mg of chlorophyll per ml of the suspension. Chloroplasts were modified to induce ATPase as described by Carmeli and Avron (6). For each sample, chloroplasts equivalent to approximately 60 μg of chlorophyll were incubated at 20° for 2 minutes in the light (intensity approximately 180,000 lux) in an 0.8 ml

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volume containing 20 μmoles of Tris-Cl, pH 7.8, 20 μmoles of NaCl, 60 μmoles of $MgCl_2$, 0.005 μmoles of phenazine methosulfate, 15 μmoles of dithiothreitol and with $P_i$ as given with the tables. ATPase or exchange measurements were initiated by appropriate additions of ADP or ATP to bring the final volume to 1.0 ml. The water had a final $^{18}O$ content of 0.850 atom per cent excess. Measurements of capacity for photophosphorylation, separations, and analyses were made as described elsewhere (2).

Results. Preliminary experiments showed that chloroplasts modified by incubation in the light with dithiothreitol so as to induce an ATPase also showed capacity for the $P_i \rightleftarrows HOH$ and $ATP \rightleftarrows HOH$ exchanges as well as the $P_i \rightleftarrows ATP$ exchange. On basis of these results, the experiments reported herein were performed using multiple incubations with a single batch of chloroplasts so that the various results would be directly comparable. Precision of measurement of the $^{18}O$ exchanges was limited by the amount of $^{18}O$ present in the water, but the expense of additional excess of $^{18}O$ did not appear warranted at this stage because the results suffice to clearly demonstrate the occurrence of the exchange reactions.

Data in Table I show the extent of incorporation of $^{32}P$ from $P_i$ into

TABLE I

CATALYSIS IN THE DARK BY MODIFIED CHLOROPLASTS OF A $P_i \rightleftarrows ATP$ EXCHANGE AND OF ATP CLEAVAGE AND THE EFFECT OF $P_i$ AND ADP ADDITIONS

Chloroplasts were incubated for 16 minutes in the dark in 1 ml volumes under conditions as described in the text with 4 mM ATP, other additions as indicated in the table, and with either $^{32}P_i$ ($4.5 \times 10^5$ cpm) or $AT^{32}P$ ($4.5 \times 10^5$ cpm in the γ-phosphoryl group).

| $P_i$ | ADP | $AT^{32}P$ $P_i$ | $^{32}P_i$ ATP | NET ATP HYDROLYSIS |
|---|---|---|---|---|
| mM | mM | mM | mM | mM |
| 0 | 0 | 0.24 | - | 0.24 |
| 0 | 2 | 0.16 | - | 0.16 |
| 0 | 8 | 0.11 | - | 0.11 |
| 2 | 0 | 0.52 | 0.12 | 0.40 |
| 8 | 0 | 0.46 | 0.12 | 0.34 |
| 8 | 2 | 0.46 | 0.13 | 0.33 |

ATP and the release of $^{32}P$ from $AT^{32}P$ to $P_i$ as catalyzed by the modified chloroplasts. The release of $^{32}P$ from $AT^{32}P$ reflects both exchange and hydrolysis; the incorporation of $^{32}P_i$ into ATP gives a measure of hydrolysis. The demonstration of occurrence of both hydrolysis and exchange corroborates the previous report of Carmeli and Avron (6). An unexpected result was that

the extent of ATP hydrolysis was nearly doubled by addition of $P_i$. In the absence of $P_i$, ADP inhibited the hydrolysis rate. In presence of $P_i$, ADP did not inhibit either the ATPase or the $P_i \rightleftarrows$ ATP exchange.

Table II shows the ability of the modified chloroplasts to catalyze an

TABLE II

CATALYSIS IN THE DARK BY MODIFIED CHLOROPLASTS OF A $P_i \rightleftarrows$ HOH EXCHANGE AND EFFECTS OF ADP AND ATP

Chloroplasts were incubated in the dark under conditions as described in the text with additions as indicated. When ATP was present a correction for $^{18}O$ introduced by net ATP hydrolysis was made (see Table I). Duplicate samples for exchanges given in this and subsequent tables are from incubations as in Table I but with $^{32}P$ added either as $P_i$ or ATP.

| | | | $P_i \rightleftarrows$ HOH | |
|---|---|---|---|---|
| $P_i$ | ATP | ADP | 8 MINUTES | 16 MINUTES |
| mM | mM | mM | μatoms water oxygen incorporated | μatoms water oxygen incorporated |
| 2 | 0 | 0 | <0.05 | <0.05 |
| 2 | 0 | 2 | 0.2 | 0.3 |
| 2 | 0 | 8 | 0.1 | 0.2 |
| 2 | 4 | 0 | 0.6 | 1.1 |
| 2 | 4 | 0 | 0.4 | 0.8 |
| 8 | 4 | 0 | 0.8 | 1.5 |
| 8 | 4 | 0 | 0.6 | 1.6 |
| 8 | 4 | 2 | 0.7 | 1.5 |
| 8 | 4 | 2 | 0.9 | 1.7 |

appreciable $P_i \rightleftarrows$ HOH exchange. Control experiments showed that the ability to catalyze the exchanges depended on prior exposure to light and dithiothreitol. Values are reported to only 1 or 2 significant figures because a difference of only 0.01 atom percent $^{18}O$ found in the $P_i$ was equivalent to about 0.1 μatoms of water oxygen incorporated into the $P_i$. The results show that a weak $P_i \rightleftarrows$ HOH exchange may occur in the absence of added ADP or ATP, but further experimentation will be necessary to quantitate such a possibility. Addition of ADP results in a definite stimulation of the $P_i \rightleftarrows$ HOH exchange. A much greater stimulation is shown by ATP addition.

Data in Table III show the ability of the modified chloroplasts to catalyze an ATP $\rightleftarrows$ HOH exchange. Analytical limitations were such that a variation of 0.01 in the observed atom percent excess was equivalent to about 0.4 μatoms of water oxygen incorporated into ATP. Nonetheless, the agreement between similar samples and the comparison of 8 and 16 minute values gives

TABLE III

CATALYSIS IN THE DARK BY MODIFIED CHLOROPLASTS OF AN ATP ⇄ HOH EXCHANGE AND THE EFFECT OF $P_i$ AND ADP

Chloroplasts were incubated in the dark in 1 ml volumes under conditions as described in the text with 4 mM ATP and with other additions as indicated.

| $P_i$ | ADP | ATP ⇄ HOH | |
|---|---|---|---|
| | | 8 MINUTES | 16 MINUTES |
| mM | mM | μatoms water oxygen incorporated | μatoms water oxygen incorporated |
| 0 | 0 | 0.2 | 0.2 |
| 0 | 2 | 0.1 | 0.2 |
| 0 | 8 | 0.2 | 0.3 |
| 2 | 0 | 0.6 | 1.1 |
| 2 | 0 | 0.5 | 0.9 |
| 8 | 0 | 0.3 | 0.8 |
| 8 | 0 | 0.5 | 0.8 |
| 2 | 2 | 0.5 | - |
| 2 | 2 | 0.5 | 0.9 |
| 8 | 2 | 0.4 | 0.8 |
| 8 | 2 | 0.5 | 0.9 |

assurance that prominent effects on the exchange are detectable under conditions used. ADP addition has little or no effect on the weak exchange shown in the absence of added $P_i$. $P_i$ addition results in a pronounced stimulation of the ATP ⇄ HOH exchange. The ATP ⇄ HOH exchange observed in presence of added $P_i$ is not appreciably modified by addition of ADP.

Table IV summarizes the rates of the maximum exchanges observed in the

TABLE IV

COMPARATIVE RATES OF MAXIMUM OBSERVED EXCHANGES AND NET REACTIONS

Chloroplast preparations were incubated as given in the experimental section or with the other tables. Activities are expressed as μmoles or (for oxygen exchanges) as μatoms/mg chlorophyll/hour.

| REACTION | EXPT 1 | EXPT 2 |
|---|---|---|
| ATP synthesis, untreated chloroplasts, light | 501 | 547 |
| ATPase, treated chloroplasts, dark [a] | 8 | 15 |
| $P_i$ ⇄ ATP " " " | 12 | 8 |
| ATP ⇄ HOH " " " | 47 | 64 |
| $P_i$ ⇄ HOH " " " | 75 | 100 |

[a] No added $P_i$

results as reported in this paper (Experiment 2) and in one of the preliminary experiments (Experiment 1). The $P_i \rightleftarrows HOH$ exchange appears to be the most prominent and approaches a value 1/6 to 1/7 of that of the net capacity for ATP synthesis in the light prior to exposure to dithiothreitol.

Discussion. The results demonstrate that chloroplasts can be modified so as to show capacity for the $P_i \rightleftarrows HOH$ and $ATP \rightleftarrows HOH$ exchange reactions as well as the $P_i \rightleftarrows ATP$ exchange and ATPase activities in the dark. The incubation in light with the thiol has in some manner disengaged the phosphorylation and water-formation reactions from a compulsory coupling with light-dependent reactions. It appears plausible that in these preparations a dynamic reversal of ATP formation and accompanying exchange reactions are occurring independently from concomitant oxidation and reduction.

The pronounced stimulation of the $P_i \rightleftarrows HOH$ exchange by addition of ATP suggests that this exchange either results from the dynamic reversal of ATP cleavage and resynthesis, or that an activated compound or state resulting from ATP cleavage can induce $P_i$ activation independent of ATP formation. The increase in the $ATP \rightleftarrows HOH$ exchange by addition of $P_i$ is consistent with the view that all exchanges result from dynamic reversal of ATP formation. The stimulation would result from increased occupancy of the $P_i$ locus at the catalytic site. The unexplained stimulation of ATPase by addition of $P_i$ may mean, however, that additional factors need consideration.

The $ATP \rightleftarrows HOH$ exchange as catalyzed by chloroplasts in the light is inhibited by addition of either $P_i$ or ADP (10,2). As pointed out elsewhere (1), such inhibition could result from a requirement of both $P_i$ and ADP for the $ATP \rightleftarrows HOH$ exchange. When ADP is added in the light, $P_i$ is removed; when $P_i$ is added ADP is removed. Results presented herein show that when conditions for the exchanges in the absence of light are attained, the inhibitory effects are no longer observed. Indeed, $P_i$ stimulates the $ATP \rightleftarrows HOH$ exchange and ADP has little or no effect. Any ADP requirement could be met by ATPase action.

The exchange rates observed as summarized in Table IV are roughly comparable to those shown by spinach chloroplasts during net photophosphorylation (2), except that the $P_i \rightleftarrows HOH$ exchange appears somewhat greater. Indeed, the rate of the $P_i \rightleftarrows HOH$ exchange is sufficiently great to suggest the possibility of an additional source of this catalytic activity akin to observations with mitochondria (11).

Bacterial chromatophores exposed to light show a $P_i \rightleftarrows ATP$ exchange and ATPase activity in the dark (12). Probably these particles will also have the capacity to catalyze the $^{18}O$ exchanges in the dark.

Summary. $P_i \rightleftarrows HOH$, $ATP \rightleftarrows HOH$ and $P_i \rightleftarrows ATP$ exchanges, probably reflecting the phosphorylation and water formation reactions of photosynthetic phosphorylation, are catalyzed in the dark by lettuce chloroplasts after exposure to light and dithiothreitol. A marked stimulation of the $P_i \rightleftarrows HOH$ exchange by ATP and of the $ATP \rightleftarrows HOH$ exchange by $P_i$, and a lack of ADP inhibition of the $P_i \rightleftarrows ATP$ and $ATP \rightleftarrows HOH$ exchanges are consistent with all exchanges occurring by dynamic reversal of ATP cleavage at the catalytic site.

## References

1. Boyer, P. D. in D. R. Sanadi (Editor) Advances in Bioenergetics, Vol. 2, in press, 1967.
2. Shavit, N., Skye, G. E. and Boyer, P. D., J. Biol. Chem., in press, 1967.
3. Boyer, P. D., Bieber, L. L., Mitchell, R. A. and Szabolcsi, G., J. Biol. Chem. 241, 5384 (1966).
4. Petrack, B., Craston, A., Sheppy, F. and Farron, F., J. Biol. Chem., 240, 906 (1965).
5. Avron, M., J. Biol. Chem., 237, 2011 (1962).
6. Carmeli, C. and Avron, M., Biochem. Biophys. Res. Communs., 24, 923 (1966).
7. Avron, M., Anal. Biochem., 2, 535 (1961).
8. Lindberg, O. and Ernster, L., Methods of Biochem. Anal., 3, 1 (1956).
9. Shavit, N. and Boyer, P. D., J. Biol. Chem. 241, 5738 (1966).
10. Avron, M., Grisara, V. and Sharon, M., J. Biol. Chem., 240, 1381 (1965).
11. Mitchell, R. A., Hill R. D. and Boyer, P. D., J. Biol. Chem., 242, 1793 (1967).
12. Zuagg, W. S. and Vernon, L. P., Biochem. 5, 34 (1966).