P/2e⁻ Ratios Approaching 4 in Isolated Chloroplasts*

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SUMMARY

P/2e⁻ ratios over 3 have been observed in isolated spinach chloroplasts with the use of low concentrations of ferricyanide and benzoquinone as the terminal electron acceptors.

Previous studies of Arnon, Whatley, and Allen (1), Kroghmann, Jagendorf, and Arnon (2), Good (3), and Stiller and Vennesland (4) have indicated that in noncyclic photophosphorylation only 1 adenosine triphosphate molecule is produced per pair of electrons. However, Winget, Izawa, and Good (5) have recently observed P/2e⁻ ratios of 1.3 with the use of chloroplasts from various species incubated at a high pH (pH 8.5). Also, Nishimura (6), from studies on delayed photophosphorylation in Rhodospirillum rubrum chromatophores, has calculated that the P/2e⁻ value may be as high as 4. The studies of Baltchefsky and Arwidsson (7), as well as those of Gorman and Levine (8), on mutants of Chlamydomonas reinhardi which have lost either cytochrome f or plastocyanin, also suggest that more than one site for phosphorylation exists along the linear electron chain of chromatophores and chloroplasts.

Current investigations in this laboratory have shown that the P/2e⁻ ratio for noncyclic photophosphorylation in isolated fresh market spinach (Spinacia oleracea) chloroplasts approaches 4, by the use of either benzoquinone or ferricyanide as the electron acceptor. These high P/2e⁻ ratios were not observed when nicotinamide adenine dinucleotide phosphate in the presence of NaCl or with the use of 0.2 mM NaCl in place of the 0.4 mM sucrose, routinely resulted in preparations which exhibited P/2e⁻ ratios of less than 3. Likewise, the preparations of chloroplasts had to be used immediately. Aging at 0° for as little as 3 hours often resulted in P/2e⁻ ratios of less than 3.

Assay Conditions—The assays were performed at 14° in an open cylindrical water-jacketed chamber (diameter, 1 inch) with constant stirring with the use of saturating white light. Four tungsten lamps were placed about the chamber.

Changes in pH and oxygen concentration were continuously monitored during the experiments. Changes in pH were assayed in open vessels and changes in oxygen content in closed air-free chambers, with aliquots of the same reaction mixture for each experiment. Unless otherwise indicated, the reaction mixture contained 0.4 mM sucrose, 0.001 mM sodium azide (sodium azide was added to eliminate all phosphorylation due to any contaminating mitochondria), 0.01 mM NaCl, 0.001 mM Tris-HCl, 0.01 mM MgCl₂, 0.002 mM sodium phosphate, and 0.001 mM ADP, all adjusted to pH 7.8. The reaction was usually carried out in a total volume of 10 or 16.7 ml with the use of 0.3 ml of chloroplasts (approximately 0.7 mg of chlorophyll). The pH was readjusted to 7.7 with NaOH after addition of chloroplasts. It was necessary to add the electron acceptors very slowly, usually at a rate of 0.5 μmole per min, to facilitate maximum phosphorylation.

Chloroplast Isolation—Spinach, which had been harvested 2 to 3 days previously, was purchased commercially and the leaves were packed in ice for 12 hours or more before use. Chloroplasts were isolated at 4° by the following procedure. The leaves were washed with cold sodium EDTA, 10⁻⁴ M, and the midrib was removed. One hundred grams of spinach in 100 ml of 0.4 M sucrose, containing 0.01 M NaCl, 0.025 M Tris (pH 7.65), 0.01 M sodium ascorbate, and 0.0001 M EDTA, were blended for 10 sec in a Waring Blendor. The final pH of the homogenate was between 7.2 and 7.4. The crude suspension was filtered through two layers of cheesecloth and then was centrifuged at 500 × g for 2 min. This supernatant was then centrifuged for 15 min at 800 × g. The chloroplast pellet was then resuspended by a very gentle homogenization in 100 ml of 0.4 M sucrose. This suspension was centrifuged again at 800 × g for 15 min. This washing procedure was repeated once more as above and the chloroplasts were again resuspended in 0.4 M sucrose at a concentration of about 2.0 mg of chlorophyll per ml. The chlorophyll content was measured by the method of Arnon (9). Preparation of chloroplasts under conditions other than the above procedure, i.e. in the absence of NaCl or with the use of 0.2 mM NaCl in place of the 0.4 mM sucrose, routinely resulted in preparations which exhibited P/2e⁻ ratios of less than 3.

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¹ The abbreviation used is: PMS, N-methylphenazonium methylsulfate.
phate esterification, as measured by phosphate uptake, ATP production, and alkali production in the absence of electron acceptors, was undetectable in these brief (10 min) experiments.

Assays and Materials—Phosphate was determined colorimetrically (10), as was ferricyanide (11). The formation of reduced benzoquinone was assayed at the various time intervals by adding excess ferricyanide in the dark to the reaction mixtures and following proton production with the use of the above pH meter. The amount of protons produced by reduction of ferricyanide by the generated hydroquinone was determined by titration of the reaction mixtures in the dark with standardized NaOH. Controls, incubated in the absence of quinone, reduced no ferricyanide in the dark.

ATP was measured by adding yeast hexokinase and glucose directly to the reaction vessel at the end of each experiment in the dark and monitoring the production of acid due to the formation of glucose-6-P in the dark. After production of acid ceased, a known amount of ATP was then added and acid production followed as a control. Also, controls were run in the absence of either ADP or P1, and in the presence of ATP and P1. Small amounts of ATP were produced in the controls containing ADP, presumably because of myokinase, but no P1 or protons were consumed.

The probable presence of a myokinase-like enzyme was also shown in these chloroplasts by the observation that the addition of AMP (in the presence of catalytic amounts of ATP) resulted in the conversion of all the AMP to ATP in the light, with the use of PMS as the electron acceptor. The rate of this reaction was slow (0.2 μmole of ATP per min at pH 8.8) and strongly pH dependent. The pH optimum was 8.9 and essentially no ATP was formed at pH 7.6. The OH:ATP ratio for this very slow rate of phosphorylation, with the use of AMP, was also quite low (0.51).

The consumption of known amounts of ADP was measured by adding PMS as cofactor at various times, and ATP formation was followed to completion by monitoring alkali production in the light. The ADP remaining in each of the noncyclic experiments at the end of incubations was also assayed by addition of PMS with the subsequent conversion of all the remaining ADP to ATP in the light. The ATP formed was measured by monitoring the alkali production and enzymatically. ATP was measured enzymatically in aliquots of each incubation, after removal of protein with perchloric acid, by the use of hexokinase, glucose, and glucose-6-phosphate dehydrogenase and following the reduction of NADP.

Oxygen production was measured polarographically, with the use of a vibrating Teflon-covered platinum electrode, as previously described by Kahn (12). Anaerobiosis was effected by bubbling all solutions before use with argon, and back diffusion previously described by Kahn (12). Anaerobiosis was effected by bubbling all solutions before use with argon, and back diffusion

The critical aspect of the experiments in this report is the rate of addition of the electron acceptors. As indicated in Fig. 1, the rapid addition of either benzoquinone or ferricyanide leads to rapid exhaustion of the available acceptor. After reduction of the acceptors phosphorylation completely stops. The observed P/2e- ratio under these conditions is approximately 1.2, as previously reported (5). However, the constant infusion of either benzoquinone (0.5 μmole per min) or ferricyanide (approximately 0.5 μmole per min) at saturating light intensities results in P/2e- ratios of well over 3 (Figs. 2 and 3). The over-all stoichiometry of this type of photophosphorylation under the above optimal conditions is indicated in Table I. The P/2e ratios were usually lower (3.1) at the onset of the incubation, but rose to over 3.5 on continued infusion of the electron acceptors. Optimum P/2e- ratios were obtained by maintaining the rate of ATP (OH-) formation, with either ferricyanide or benzoquinone as acceptor, at a rate approximately 25% of the maximal rate of cyclic (PMS) phosphorylation. Infusion of the electron acceptors at slower rates (0.1 μmole per min) or higher rates (2 μmole per min) resulted in much lower P:O ratios (below 2 in both instances). In fact, if the acceptors were infused very slowly (less than 0.05 μmole per min), no phosphorylation could be observed. Since the cyclic rate varied somewhat with each preparation of chloroplasts, the cyclic rates of ATP synthesis with the use of PMS were of necessity predetermined for each batch of chloroplasts. In these experiments (Figs. 1, 2, and 3), no phosphorylated products other than ATP were produced, and the amount of ATP produced, as measured enzymatically, was equal to the amount of phosphate consumed,
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effect of varying intensities of light on P/2e- ratios under the
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production of alkali and O2 as well as phosphate consumption
were essentially equal to the rate of ATP formation, only the rate of formation of ATP is shown on the graph. The reaction mixtures were back titrated with HCl in the dark at the end of each incubation period to obtain micromoles of protons consumed.
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These high P/2e- ratios could be observed only at very high light intensities (saturating). For quantitative assays of the effect of varying intensities of light on P/2e- ratios under the conditions of this report, see the following paper (14).
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Nishimura (6) has previously shown that approximately 0.9 µmole of OH- is produced in chromatophores subsequent to the formation of 1.0 µmole of ATP at pH 8.0. The average OH- : ATP ratio with the use of spinach chloroplasts and PMS as electron acceptor was approximately 0.72 when assayed in open chambers and the amount of alkali is measured in the dark by titration of the reaction mixtures at the end of the experiment. However, when experiments were performed in closed air-free chambers with the use of aged chloroplasts (12 hours at 5°) and titrations were performed in the light after consumption of all the ADP, OH- : ATP ratios of about 0.9 (average 0.87) were usually observed. The reduction of ferricyanide by fresh chloroplasts in closed chambers in the absence of ADP is also associated with the production of more protons than can be accounted for by the reduction of ferricyanide (Fig. 5). Since chloroplasts are known to exchange external protons for internal monovalent cations during nonphosphorilative electron transport (15), and since higher OH- : ATP ratios (0.82) are observed
3 Unpublished data.
TABLE I

$P/2e^-$ ratios with slow infusion of noncyclic electron acceptors

Incubations were performed as in Figs. 2 and 3. The electron acceptors were added slowly and sequentially as in Figs. 2 and 3. At the end of the incubations, the amounts of proton consumption or proton production, oxygen production, ATP formation, reduced acceptor production, $P_i$ consumption, and ADP consumption were assayed (see "Methods") on aliquots of each incubation, and are tabulated in the table. The pH was maintained between 7.8 and 8.1 by titration after completion of the infusion of the various amounts of the electron acceptors. $P/2e^-$ ratios (ATP formed per reduced acceptor formed) are also tabulated. The data in the table were obtained with one preparation of chloroplasts from spinach harvested 3 days before use. However, similar data have been obtained repeatedly over the past year. The commercial spinach has been obtained from suppliers in Delaware, Texas, and Florida and from that harvested immediately from local greenhouses.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Time of incubation (min)</th>
<th>Reduced acceptor produced (µmoles)</th>
<th>$O_2$ produced (µmoles)</th>
<th>$\Delta H^+$ (µmoles)</th>
<th>ATP formed (µmoles)</th>
<th>$P_i$ used (µmoles)</th>
<th>ADP used (µmoles)</th>
<th>$P/2e^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. None</td>
<td>6</td>
<td>0</td>
<td>0.0</td>
<td>+0.3</td>
<td>0.8</td>
<td>0.2</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Quinone, 2 µmoles</td>
<td>4</td>
<td>1.5</td>
<td>2.05</td>
<td>-4.3</td>
<td>6.6</td>
<td>6.2</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Quinone, 1 µmole</td>
<td>2</td>
<td>0.97</td>
<td>1.02</td>
<td>-2.2</td>
<td>3.9</td>
<td>3.6</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Quinone, 1 µmole</td>
<td>1.5</td>
<td>0.95</td>
<td>0.95</td>
<td>-2.0</td>
<td>3.9</td>
<td>3.5</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>II. Ferricyanide, 2 µmoles</td>
<td>2</td>
<td>2.0</td>
<td>1.04</td>
<td>+0.25</td>
<td>3.8</td>
<td>3.2</td>
<td>3.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Ferricyanide, 2 µmoles</td>
<td>1</td>
<td>1.05</td>
<td>0.52</td>
<td>+0.04</td>
<td>4.1</td>
<td>3.8</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Ferricyanide, 1 µmole</td>
<td>1.5</td>
<td>0.68</td>
<td>0.48</td>
<td>+0.00</td>
<td>2.0</td>
<td>1.9</td>
<td>2.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Inhibition of cyclic photophosphorylation by benzoquinone and potassium ferricyanide. Reaction mixtures containing chloroplasts (1.05 mg of chlorophyll), 0.4 M sucrose, 0.01 M MgCl$_2$, 0.003 M sodium phosphate (pH 7.7), 0.001 M Tris-HCl (pH 7.8), 0.001 M sodium azide, and 10.8 µmoles of sodium ATP (pH 7.8) were incubated in saturating white light at 14°. PMS, $10^{-5}$ M, was added where indicated, and proton consumption was constantly monitored (see "Methods"). To duplicate experiments, ferricyanide, 1 µmole, or benzoquinone, 1 µmole, was added at the points indicated. The broken lines represent the change in proton consumption or production after the addition of the noncyclic electron acceptors. After exhaustion of the added ADP, the addition of more ADP (10 µmoles) resulted in further consumption of proton. Similarly, the addition of ferricyanide after consumption of the available ADP resulted in the immediate reduction of all of the ferricyanide.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Acid production by chloroplasts, accompanying reduction of ferricyanide. Duplicate experiments were performed, with the use of ferricyanide as electron acceptor, in the presence and absence of chloroplasts (0.8 mg of chlorophyll) under the conditions of Fig. 1. Hydrobenzoquinone, 10 µmoles, was added when chloroplasts were omitted. Hydrobenzoquinone was not added when chloroplasts were present. Potassium ferricyanide, 1 µmole, was added where indicated (arrows) and acid production followed as indicated. $P_i$, $2 \times 10^{-3}$ M, was present, but no ADP was added. All incubations were performed at 14° with saturating white light, both in the presence and absence of chloroplasts.

3 µmoles of acid can be released from chloroplasts containing 1.05 mg of chlorophyll and approximately 90 mg of water (Fig. 5) which are incubated in the presence of high concentrations of magnesium ($10^{-5}$ M). Water content of the chloroplasts was estimated by weighing the chloroplasts (isolated by centrifugation at 10,000 × g for 10 min) before and after vacuum desiccation. It is of interest to note that this loss of acid from the chloroplasts with ferricyanide reduction is also accompanied by a progressive decline in the PMS-catalyzed phosphorylatable ability of the chloroplasts. In fact, prolongation of ferricyanide reduction by chloroplasts (under the conditions of Fig. 4) to the point at which 3 extra µmoles of acid have appeared in the reaction mixture results in complete inhibition of PMS-catalyzed phosphorylation. This inhibition is not caused by the generated ferrocyanide, since the addition of equivalent amounts of ferrocyanide to fresh chloroplasts which have not been forced to reduce...
findings on cation-H+ exchange, see Reference 16. In fresh preparations of chloroplasts which subsequently exhibit high P/2e- ratios, very little acid appears on addition of these to the reaction mixtures (pH 7.7) results in the immediate liberation of acid (1.5 to 3 pmole) to the medium. Of these chloroplasts to the reaction mixtures of the chloroplasts. In preparations which exhibit low P/2e-ratios (either aged chloroplasts or chloroplasts prepared from the OH-:ATP ratio also seems to depend largely on the initial state ferricyanide has no effect on the rate of PMS-catalyzed phos- ferredoxin to obtain a maximum rate of oxygen evolution. Aerobiosis was obtained by bubbling the incubation, exclusive of the chloroplasts, for 10 min with argon. At 20 sec, NADP, 5 amoles, was added and at 3 min ADP, 12 amoles, was added. The ADP and NADP solutions were anaerobic. Acid production or consumption and oxygen evolution were constantly monitored (see “Methods”). Control incubations in the absence of NADP or ferredoxin were also run (see two bottom traces). No acid or oxygen was produced in the absence of NADP or ferredoxin. Formation of ATP was measured at 8 min in all incubations, both spectrophotometrically and by acid production, with the use of hexokinase and glucose. No detectable ATP was present at zero time. The addition of AMP, 3 amoles, at zero time completely abolished the formation of ATP in the absence of ferredoxin or NADP, but had no effect on the rate of ATP production, acid production, or oxygen evolution. The numbers on the graph represent the amount of ATP (micromoles) formed at 8 min. The calculated P/2e-ratio was 1.32. The amount of reduced NADP formed at 3 min and 8 min was also assayed, with the spectrophotometric method of Levine and Smillie (17), and was found to agree very well with the amount of oxygen evolved. Proton production due to NADP reduction in the absence of ADP was, like ferricyanide reduction, greater than could be accounted for by the redution of NADP only. The factor was 1.58 (protons produced to NADP reduced) in the absence of ADP.

DISCUSSION

It is possible that some of the observed phosphorylation in these experiments, performed at very low concentrations of electron acceptors, is the result of a true cyclic type of phosphorylation which perhaps uses some endogenous electron acceptor within the chloroplasts. Perhaps the slow addition of these electron acceptors maintains sufficient amounts of this postulated endogenous electron carrier at its midpoint of oxidation so that it can serve both as an electron donor and acceptor, and thus effect cyclic photophosphorylation. Since only 1 to 2% of the available light energy is being used for phosphorylation (6, 14), ample energy is available to drive this postulated cyclic phosphorylation. Although this possibility does exist, it seems unlikely for the following reasons. (a) Phosphorylation promptly ceases when the noncyclic carriers are fully reduced. (b) Addition of the noncyclic acceptors immediately stops cyclic phosphorylation, as mediated by PMS or pyocyanine, and this inhibition persists for over 20 sec after reduction of the electron acceptor (ferricyanide) is essentially complete. (c) Essentially no phosphorylation can be observed in these chloroplasts, either aerobically or anaerobically, unless some acceptor is added externally. The presence or absence of a Mehler reaction in these chloroplasts apparently depends on the length of the storage time of the harvested spinach. Chloroplasts, prepared as above immediately after harvesting the spinach, do exhibit a slow Mehler reaction (0.1 to 0.2 pmole of ATP formed per min under the conditions of Fig. 1, in the absence of any added electron acceptors). However, in the commercial spinach used in these experiments (Figs. 1,
2, and 3), essentially no Mehler reaction was observed in the short time periods of these experiments. Finally, the reduced acceptors (ferrocyanide and hydrobenzoquinone) are totally incapable even at high concentrations of supplying electrons to the phosphorylative segments of the electron chain.

Furthermore, at a quantum yield of 1 (14), ample energy is available in 1 mole of the absorbed light, 640 μ, 44,000 calories, to effect the reduction of ferricyanide ($E'_o$ +0.4 volts) by H₂O ($E'_o$ +0.8 volts) and to form considerably more than 2 moles of ATP (standard free energy of hydrolysis of ATP = −7,000 calories per mole in the presence of magnesium (21)). Thus, noncyclic P/2e− ratios of over 3 are thermodynamically feasible.

Since in these experiments both Photosystem I and Photosystem II are absorbing light, it is also possible that the energy from both protons is used in the production of ATP. This assumes that ferricyanide and benzoquinone can accept electrons from Photosystem I as well as from Photosystem II, as proposed by Rumberg (22).

REFERENCES
P/2e\textsuperscript{−} Ratios Approaching 4 in Isolated Chloroplasts
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