The stoichiometry of photosynthetic phosphorylation \((P/2e^-)\) is of considerable theoretical significance, not only with respect to the mechanism of photosynthesis but also with respect to the relation between ATP formation and electron transfer in oxidative and substrate level phosphorylations. Of the two types of photosynthetic phosphorylation, cyclic and noncyclic (1, 2), only the noncyclic type lends itself to measurements of the number of molecules of ATP formed per pair of electrons transferred. In cyclic photophosphorylation, ATP formation is linked to an internal, light-induced electron flow that produces no change in any external electron donor or acceptor (3). In noncyclic photophosphorylation, the formation of ATP is coupled with a light-induced electron transfer from water to ferredoxin (4). Here the electron transfer that accompanies ATP formation can be determined by measuring the evolution of oxygen resulting from the photo-oxidation of water and the photoreduction of ferredoxin. The photoreduction of ferredoxin can be measured directly (5) or, more conveniently, by coupling its photoreduction with an enzymic reduction of NADP (4, 6). Alternatively, when ferredoxin is replaced by a nonphysiological electron acceptor such as ferricyanide, the stoichiometry of noncyclic photophosphorylation is determined by measuring the formation of ATP that is coupled with the photoreduction of ferricyanide and the accompanying oxygen evolution (1).

The first measurements of the stoichiometry of noncyclic photophosphorylation showed that 1 molecule of ATP is formed when illuminated spinach chloroplasts transfer 2 electrons from water to NADP or ferricyanide (1). This finding, confirmed by Jagendorf and Avron (7, 8), Stiller and Vennesland (9), and Turner, Black, and Gibbs (10), led to wide acceptance of the conclusion that the \(P/2e^-\) ratio for noncyclic photophosphorylation is 1. Recently, however, this stoichiometry was questioned in papers by Winget, Izawa, and Good (11) and Izawa, Winget, and Good (12) and by Lynn and Brown (13, 14). Winget et al. (11) obtained a \(P/2e^-\) ratio equal to 1 only when they used Tris-HCl buffer, the same buffer that was used in the earlier investigations. However, with glycglycine or tris(hydroxymethyl)methylglycine (15) buffer, the \(P/2e^-\) ratio increased up to 1.3. Lynn and Brown (13) obtained \(P/2e^-\) ratios approaching 4, not by using a special buffer (they used Tris-HCl), but by using selected electron acceptors and controlling the rate of addition of the electron acceptor. They found these high \(P/2e^-\) ratios using ferricyanide, benzoquinone, or chloranil (tetrachloro-p-benzoquinone) as electron acceptors, and then only when their rate of addition was slow. When these electron acceptors were added rapidly or when they were replaced by the physiological electron acceptor system, ferredoxin plus NADP (added either slowly or rapidly), the \(P/2e^-\) ratio was about 1.2.

The aim of this investigation was to examine the stoichiometry of noncyclic photophosphorylation in the light of recent investigations and to explore the possible existence of two different types of noncyclic electron transport, a phosphorylating and a nonphosphorylating type, postulated by Good (16, 17) and his associates (12) to explain their findings (12). Our results, obtained under the experimental conditions described by Winget et al. and Izawa et al. (11, 12) and by Lynn and Brown (13, 14), are consistent with the operation in noncyclic photophosphorylation of only one type of noncyclic electron transport, which, in the presence of \(Mg^{++}\), ADP, and orthophosphate, leads to ATP formation with a \(P/2e^-\) ratio of 1. The reported departures from this ratio were either not confirmed or, when confirmed, were found to be related to secondary factors that are extraneous to the stoichiometry of noncyclic photophosphorylation.
was carried out in test tubes placed in front of an incandescent lamp which supplied saturating illumination. The reaction mixture was stirred continuously with a magnetic stirrer as the ferricyanide was being added from a burette adjusted to the desired flow rate. Illumination was continued for 2 min after the addition of ferricyanide was completed. The reaction was carried out under air at 19°C. A cyclic photophosphorylation control (in which ferricyanide was replaced by 33 μM phenazine methosulfate) was run for 3 min and gave a phosphorylation rate of 1 μmole of organic 32P formed per min.

### METHODS

Chloroplasts (C) were prepared from greenhouse-grown spinach leaves essentially as described by Whatley and Arnon (18), except that Tricine-NaOH buffer was used instead of Tris-HCl. Midrib-free leaves, 10 g, were ground with 30 ml of a solution containing 0.35 M NaCl, 0.05 M Tricine (pH 8.5), and 0.01 M sodium ascorbate. The isolated chloroplasts were washed once in the same solution but without the ascorbate. Chlorophyll was measured by the method of Arnon (19).

Spinach ferredoxin (more than 90% pure) was prepared as described by Tagawa and Arnon (20). The amount used was calculated with the extinction coefficient at 420 nm of 0.732 (mg per ml)−1·cm−1 (21).

Unless otherwise indicated, the reactions were performed in Warburg manometer vessels under argon at 19°C. Filtered yellow light (λ > 460 nm) from a bank of 300-watt incandescent lamps provided illumination of about 17,500 lux at the bottom of the vessels.

Ferricyanide was estimated by absorbance measurements at 420 nm, after the reaction was stopped with trichloroacetic acid and the proteins were removed by centrifugation. NADPH was measured by absorbance at 340 nm (22), after the reaction was stopped with NaOH to prevent the oxidation of the reduced dinucleotide. ATP formation was measured either by the enzymatic method (23), or by the increase in labeled organic phosphate, determined by the method of Hagihara and Lardy (24).

### RESULTS AND DISCUSSION

#### Effect of Rate of Addition of Electron Acceptors on Stoichiometry of Noncyclic Photophosphorylation

The P/2e− ratios obtained by Lynn and Brown (13) depended on special experimental conditions: (a) the use of 0.4 M sucrose and 10 mm NaCl both in the grinding of the leaves and in the reaction mixture; (b) the use of saturating light intensity; and (c) the slow addition of the terminal acceptor, ferricyanide or benzoquinone, during the reaction. According to Lynn and Brown, the slow addition of the electron acceptor was the "critical aspect" (13) in obtaining high P/2e− ratios; it had to be adjusted to give a rate of noncyclic ATP formation approximately one-fourth of the maximum rate of cyclic photophosphorylation catalyzed by phenazine methosulfate.

As shown in Table I, we have been unable to repeat these results under experimental conditions as close as possible to those described by Lynn and Brown (13). Varying the rate of ferricyanide addition to give a rate of noncyclic photophosphorylation within a range from 5 to 25% of the rate of cyclic ATP formation gave no significant variations in the P/2e− ratios, which were in no case higher than 0.92 (Table I).

Unlike Lynn and Brown (14), we obtained essentially the same results (P/2e− ratios not higher than 1) when 3 × 10−4 M chloranil was used as electron acceptor for noncyclic photophosphorylation under air and in the presence of sodium azide. The consistency of our results provides no explanation for the data of Lynn and Brown (13, 14).

#### One or Two Electron Transport Pathways?

In isolated chloroplasts, as in mitochondria, the concurrence of phosphorylation greatly increases the rate of electron flow (1, 26). Arnon, Whatley, and Allen (1) interpreted this enhancement as evidence that in intact chloroplasts the physiological electron transport is normally coupled with phosphorylation with a P/2e− ratio of 1. Accordingly, the rapid rate of electron flow that results from the addition of ADP, Pi, and Mg2+ to isolated chloroplasts results from the acceleration of the existing electron transport pathway, not from the initiation of a new one. A different interpretation, however, was recently put forward by Iwata et al. (12) on the basis of experiments with phloridzin (12) and Dio-9 (27), two inhibitors that inhibit ATP formation and the concurrent enhancement in the rate of electron flow.

Iwata et al. (12) found that the P/32P2− ratio, i.e. the ratio between ATP formed and the increment in ferricyanide reduction occasioned by the concurrent phosphorylation, was essentially constant and close to 2.0 at different levels of phloridzin. Iwata et al. (12), following earlier suggestions by Krohnmann, Jagendorf, and Avron (28) and Good (16, 17), explained these observations as an indication that two types of electron flow operate independently, one superimposed on the other: a "basal," nonphosphorylating type (unaffected by Dio-9 and phloridzin)

### Table I

<table>
<thead>
<tr>
<th>Rate of ferricyanide addition</th>
<th>μmol incorporated</th>
<th>Ferricyanide reduced</th>
<th>P/2e−</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol/min</td>
<td>μmole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.45</td>
<td>1.0</td>
<td>0.90</td>
</tr>
<tr>
<td>0.25</td>
<td>0.98</td>
<td>2.5</td>
<td>0.78</td>
</tr>
<tr>
<td>0.5</td>
<td>2.30</td>
<td>5.0</td>
<td>0.92</td>
</tr>
</tbody>
</table>

1 The abbreviations used are: Tricine, tris(hydroxymethyl)-methylglycine; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea.

ATP and ADP were separated by descending chromatography on Whatman No. 1 paper by the technique of Paladini and Leloir (25). Aliquots of 0.2 ml each of the reaction mixture were centriuged after addition of 0.1 ml of 5 M HCl, and 0.1 ml of the supernatant fluid was used for chromatography. Spots corresponding to ATP and ADP were localized with ultraviolet light, and their radioactivity was counted on the paper with a Geiger-Müller counter.
and a "phosphorylating" type (sensitive to phloridzin and Dio-9) that is accompanied by ATP formation. They suggested that the true stoichiometry of noncyclic photophosphorylation is measured not by the over-all P/2e− ratio which includes the postulated, nonphosphorylating basal electron flow but by the P/Δ2e− ratio which is always 2.0 and which gives the stoichiometry only of the postulated phosphorylating pathway.

If the P/Δ2e− ratios were indeed constant at all levels of electron flow, then the coexistence of a phosphorylating electron transport superimposed on a basal electron transport would appear more likely. We undertook, therefore, to measure P/Δ2e− ratios under experimental conditions that varied the rate of electron flow as a function of the intensity of illumination or the concentration of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, an inhibitor of electron transport in noncyclic photophosphorylation.

Table II shows that the enhancement of electron transport by concurrent phosphorylation was not constant but decreased with a decrease in the rate of electron transport (ferricyanide reduction) brought about by decreasing light intensity. Likewise, Table III shows that the enhancement of electron transport by concurrent phosphorylation decreased with decreasing rates of electron transport brought about by increasing concentration of DCMU. Similar effects of light intensity and DCMU were obtained when ferredoxin plus NADPf replaced ferricyanide as the terminal electron acceptor.

Fig. 1 gives a comparison of the over-all P/2e− ratios and P/Δ2e− ratios at the different rates of electron transport determined by light intensity or concentration of DCMU. The P/Δ2e− ratios varied from 1.6 (at the highest light intensity or absence of inhibitor, when the rate of electron flow was not limited) to 3.5 (at the lowest light intensity and highest DCMU concentration, when the rate of electron flow was limited) thus, the ratios between the ATP formed and the concomitant increment in electron transport were variable, depending on the rate of electron flow. The variability of the P/Δ2e− ratio was in marked contrast to the constancy of the ratio between ATP formation and total electron transport (P/2e−) which remained close to 1.0 at all the different rates of electron flow. To account for the observed constant over-all P/2e− ratio of 1 by a combination of the two postulated pathways, it would be necessary to make the unlikely assumption that each is responsible for 50% of the total electron flow, regardless whether the rate of electron flow is restricted or not.

These results do not support the postulation (12) of a phosphorylating pathway of electron flow with a constant stoichiometry (P/Δ2e− = 2) that is superimposed on a basal electron flow pathway. The constant P/Δ2e− ratio of 2 observed by Izawa et al. (12) at different phloridzin concentrations was obtained under special conditions when phosphorylation in the absence of phloridzin happened to double the rate of electron flow. In experiments not included here, we have also observed an inhibition by phloridzin of photophosphorylation and the concomitant increment in electron flow, as reported by Izawa et al. (12). However, we interpret these results as an indication that phloridzin impedes the utilization of orthophosphate and consequently suppresses the concomitant increment in the electron flow. The latter explanation is consistent with the evidence for a partial competition between orthophosphate and phloridzin, reported recently by Izawa et al. (29).

Our results are consistent with the idea that the basal noncyclic electron transport in chloroplasts in vivo is always coupled with photophosphorylation (1). In experiments with isolated

### Table II

Effect of light intensity on enhancement of electron flow caused by concurrent photophosphorylation

<table>
<thead>
<tr>
<th>Light intensity</th>
<th>Ferricyanide reduction</th>
<th>Ferricyanide reduced, P1 present/ferricyanide reduced, P1 omitted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1 omitted</td>
<td>P1 present</td>
</tr>
<tr>
<td>μmol/mg chlorophyll/hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>204</td>
<td>790</td>
</tr>
<tr>
<td>20%</td>
<td>152</td>
<td>452</td>
</tr>
<tr>
<td>11%</td>
<td>120</td>
<td>235</td>
</tr>
<tr>
<td>5%</td>
<td>67.5</td>
<td>105</td>
</tr>
</tbody>
</table>

### Table III

Effect of DCMU concentration on enhancement of electron flow caused by concurrent photophosphorylation

<table>
<thead>
<tr>
<th>DCMU concentration</th>
<th>Ferricyanide reduction</th>
<th>Ferricyanide reduced, P1 present/ferricyanide reduced, P1 omitted</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>μmol/mg chlorophyll/hr</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>296</td>
<td>672</td>
</tr>
<tr>
<td>0.2</td>
<td>214</td>
<td>530</td>
</tr>
<tr>
<td>0.35</td>
<td>192</td>
<td>209</td>
</tr>
<tr>
<td>0.5</td>
<td>78</td>
<td>110</td>
</tr>
</tbody>
</table>
chloroplasts, noncyclic electron flow will proceed with or without a concurrent phosphorylation. However, maximum rates of noncyclic electron flow are obtained only when the system is under "photophosphorylation control," i.e. when experimental conditions are so arranged that a concurrent phosphorylation can take place.

**P/2e⁻ Ratios Obtained by Different Methods for Measuring Photophosphorylation**

In the experiments represented by Fig. 1, the over-all P/2e⁻ ratios were very close to 1, despite the fact that the buffer used was Tricine. We observed, however, that as the amount of the reduced ferriyanide decreased, the P/2e⁻ ratios increased slightly above 1, approaching the values of the P/2e⁻ ratios obtained by Winget et al. (11), who used a very low concentration of ferriyanide (0.8 μmole/2 ml) in the reaction mixture. We undertook to investigate, therefore, the relation between P/2e⁻ ratios and ferriyanide concentration.

**Fig. 2.** P/2e⁻ ratios for different amounts of ferriyanide reduced. The reaction mixture included, in a final volume of 3.0 ml, chloroplasts (see "Methods") containing 150 μg of chlorophyll and the following (in micromoles): Tricine-NaOH buffer (pH 8.5), 150; MgCl₂, 10; Na₂HPO₄, 10; ADP, 5; and K₃Fe(CN)₆ as indicated. The reaction time (4 min) was sufficient to reduce completely the highest amount of ferriyanide added. Other conditions are under "Methods."

**TABLE IV**

<table>
<thead>
<tr>
<th>System</th>
<th>Organic ³²P formed</th>
<th>ATP ³²P formed</th>
<th>ADP ³²P formed</th>
<th>P₁ utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncyclic</td>
<td>2.11</td>
<td>2.0</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Noncyclic, dark</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cyclic</td>
<td>2.57</td>
<td>2.44</td>
<td>0.13</td>
<td>2.31</td>
</tr>
<tr>
<td>Cyclic, dark</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

As shown in Fig. 2, the P/2e⁻ ratios declined as the amount of ferriyanide reduction increased. Similar results were obtained when ferredoxin and NADP replaced ferriyanide. In checking the extent to which a possible endogenous esterification of orthophosphate, independent of added electron acceptors, might have influenced the P/2e⁻ ratios, we found that continuing the illumination for 6 min after the ferriyanide was completely reduced gave no increase in the ³²P incorporation. However, a small incorporation of ³²P occurred in the controls without any added ferriyanide, despite the use of argon atmosphere in the reaction vessels. A computation of the possible contribution of such an endogenous ³²P incorporation shows that it would have increased the total ³²P incorporation by 7.8% at the lowest concentration of ferriyanide and by only 2.2% at the highest concentration of ferriyanide used. It appeared, therefore, that P/2e⁻ ratios somewhat higher than 1 might have resulted from small errors in the estimation of ATP formation. Such errors would, in turn, most likely result in P/2e⁻ ratios above 1 when the magnitude of the total electron transfer was small.

To test this possibility, ATP formation was determined in the same experiment by two methods, the uptake of orthophosphate and the formation of labeled organic phosphate (see "Methods"). Measurements of ATP formation by the orthophosphate uptake method consistently gave P/2e⁻ ratios of 1 (Table IV). On the other hand, the labeled organic phosphate method gave higher values for ATP formation that resulted in P/2e⁻ ratios approaching 1.2. Table IV shows that the labeled organic phosphate method also gave higher values for ATP formation in cyclic photophosphorylation, included here for comparison, than the orthophosphate uptake method. The higher values for ATP formation by the organic phosphate method were obtained despite the care that was exercised to correct for errors resulting from overlapping counts (see "Methods"). Without such corrections, even higher values for ATP formation are obtained, particularly when the incorporated ³²P represents only a small fraction of a high total ³²P count.

The reproducibility and reliability of the orthophosphate uptake method was established by the linearity of the standard curve that covered the range of all the phosphate determinations.
Moreover, each experiment included a control in which a known amount of orthophosphate was incubated with the complete reaction mixture in the dark. The dark controls gave orthophosphate uptake values that in no case differed by more than 1% from the measurements of the same amount of orthophosphate that was not combined with the reaction mixture.

To account for the higher ATP values that were obtained by measuring the incorporation of $^{32}$P into organic phosphorus compounds (Table IV), the labeled products of photosynthetic phosphorylation were analyzed by paper chromatography. Table V shows that, although the main labeled product was ATP, there was also a small but significant amount of ADP formed in the course of photophosphorylation by a reaction which does not involve a net uptake of orthophosphate. The mechanism of this reaction is not yet clear.

The present results suggest that the P/2e$^-$ ratios higher than 1 may have resulted from small analytical errors in the ATP determination by the radioactive method, errors that become relatively more important when the total magnitude of electron transfer is small. These errors include but are not limited to an endogenous incorporation of $^{32}$P and the formation of small amounts of ADP$^{32}$P by a reaction that does not involve a net uptake of orthophosphate (Table V). At any rate, these errors have little effect on the P/2e$^-$ ratio when the total amount of ATP formed is high. In these circumstances, the P/2e$^-$ ratios become very close to 1 even when ATP formation is measured by the radioactive method (Fig. 2). P/2e$^-$ ratios of the order of 1.2 are obtained when the total magnitude of ATP formation is limited by a low amount of the electron acceptor, as was the case in the experiments of Winget et al. (11).

In other experiments, not included here, the radioactive method gave higher values for ATP formation than the orthophosphate uptake method when Tris buffer replaced Tricine, both in the preparation of chloroplasts and in the reaction mixture. In these experiments, however, P/2e$^-$ ratios greater than 1 were not obtained, even when ATP formation was measured by the radioactive method. These results are consistent with the observation of Good (17) that Tris buffer partly uncouples noncyclic photophosphorylation. By using buffers such as Tricine, which give optimal coupling between photophosphorylation and electron transport, P/2e$^-$ ratios equal to 1 are consistently obtained under suitable experimental conditions.

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