Products of the Photophosphorylation Reaction*

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SUMMARY

Most determinations of the stoichiometric relationship between electron transport and phosphorylation have employed radioactive orthophosphate and have been based on the assumption that ADP-dependent incorporation of radioactivity can be safely equated to ATP synthesis. The validity of using this procedure to measure photophosphorylation in isolated chloroplasts has been challenged recently. However, the studies reported here show that estimates of photophosphorylation based on label incorporation are probably correct.

1. The amount of labeled orthophosphate incorporated does not differ significantly from the amount of orthophosphate which disappears from the medium. Thus, there is no evidence of an exchange reaction between orthophosphate and ADP or other unlabeled, phosphate-containing substances.

2. Nearly all of the incorporated 32P is found in ATP but a significant amount of label is sometimes found in ADP. However, the 32P in ADP is all or nearly all derived from ATP32P, presumably through the action of the ATP:AMP phosphotransferase (EC 2.7.4.3) known to be present in chloroplast preparations. Therefore, the sum of the radioactivities of ATP and ADP should be used in estimating the extent of the phosphorylation reaction.

It has been widely accepted that 1 and only 1 ATP molecule is formed by illuminated chloroplasts during the transfer of a pair of electrons from water to ferri cyanide or to other acceptors (1, 2). However, Winget, Izawa, and Good (3) routinely observed P/e2 ratios between 1.1 and 1.3. Very rarely, even in a single determination, did they obtain values as low as 1.0 unless the chloroplasts were deliberately uncoupled. More recently, Del Campo, Ramirez, and Arnon (4) have attempted to explain these higher values of P/e2 in terms of erroneous ATP measurements. Specifically, they have suggested that Good and his associates systematically overestimated the amount of ATP32P formed by overlooking a hitherto unsuspected ADP32P exchange reaction. Their contention is based on the following claims.

1. More labeled orthophosphate is incorporated into non-orthophosphate forms than can be accounted for by the decrease in total orthophosphate.

2. During the phosphorylation reaction labeled phosphate appears in ADP as well as in ATP.

3. When ATP formation is measured as the incorporation of radioactive orthophosphate, the apparent value of the ratio P/e2 increases with decreasing amounts of electron transport. This observation they attribute to the fact that the alleged exchange reaction becomes a more significant proportion of the total incorporation as the incorporation through phosphorylation becomes less.

The experiments described in this paper were undertaken in an attempt to reconcile the observations of Winget et al. (3) with the observations of other workers. Particular attention has been given to checking the reliability of using 32P incorporation as an assay for ATP formation. In the course of this work many of the experiments of Del Campo et al. (4) were repeated and extended.

METHODS

Preparation of Chloroplasts—Chloroplasts were isolated from market Spinach (Spinacea oleracea L.) by the following procedures. Petioles and the greater part of the midvein were removed from washed leaves which were then ground for about 10 sec in a Waring Blender with 0.3 M NaCl, 0.05 M tricine-NaOH, and 0.005 M MgCl2 (pH 7.5). The homogenate was squeezed through eight layers of cheesecloth and then centrifuged at about 2000 × g for 5 min. The sediment was resuspended in 0.1 M sucrose, 0.005 M tricine-NaOH, 0.002 M MgCl2, and 0.01 M KCl (pH 7.3). The suspension was centrifuged briefly (about 15 sec) at 2000 × g to remove cell debris and intact cells, then centrifuged again at about 2000 × g for 5 min. The pellet was washed once more in the same medium and was finally taken up in a small amount of the sucrose-tricine medium. Chlorophyll was estimated by the method of Arnon (5).

Photophosphorylation Reaction—Unless otherwise mentioned all reaction mixtures contained in a total volume of 2 ml, 0.1 M sucrose, 0.04 M tricine, 0.001 M KCl, 0.003 M MgCl2, labeled 0.01 M Na2HPO4, 0.001 M ATP, 1 mg of hexokinase, 0.01 M glucose, and chloroplasts with about 20 µg of chlorophyll. In addition, the reaction mixture contained potassium ferrocyanide at the concentration indicated in the legends of the figures and tables. All reactions were carried out in a 1-cm cuvette at 10°. The cuvette was illuminated from one side by broadband red light (>560 nm). Light was obtained from a 500-watt slide projector. The light was passed through a 1-liter round-bottomed flask.
containing 0.2% copper sulfate solution in water which acted as a combined heat filter and a condenser lens. The light intensity used was saturating.

Estimation of Labeled Organic Phosphate—Organic-\(^{32}\)P was estimated by a method adapted from the methods of Ahrens, V. (6) and of Nielsen and Lehninger (7). The details of the procedure are as follows. 

**Acetone** (1.2 ml), 1.0 ml of 10% ammonium molybdate, and 10.0 ml of butanol-benzene (1:1, v/v, saturated with 10% perchloric acid) were added to a 1.0-ml aliquot of the reaction mixture which had been diluted with 10 ml of 10% perchloric acid (saturated with butanol-benzene). The two-phase mixture was stirred thoroughly by an up and down movement of a glass rod flattened at the end. After separation of the layers, the upper organic layer containing the phosphoromolybdate was carefully sucked out of the test tube through a pasteur pipette connected to a vacuum line with two traps. The aqueous solution was then filtered into another test tube through a Whatman No. 4 filter paper soaked immediately before use with 0.5 ml of distilled water. The wet filter paper held back chloroplast and any minute droplets of organic phase remaining suspended in the aqueous phase. To the clear filtrate were added 0.5 ml of distilled water. The wet filter paper held back chloroplast and any minute droplets of organic phase remaining suspended in the aqueous phase.

| Table 1 |
| --- |
| Comparison of amount of labeled organic phosphate formed and amount of orthophosphate removed from medium during photophosphorylation |

The 2.0 ml of reaction mixture contained chloroplasts with 20 \(\mu g\) of chlorophyll, 10\(^{-3}\) M sucrose, 10\(^{-3}\) M KCl, 3 \(\times\) 10\(^{-3}\) M MgCl\(_2\), 2.5 \(\times\) 10\(^{-3}\) M Na\(_2\)HPO\(_4\) labeled with \(^{32}\)P, 10\(^{-3}\) M ATP, 10\(^{-3}\) M glucose, 1 mg of hexokinase, 6.25 \(\times\) 10\(^{-7}\) M potassium ferricyanide, and 4 \(\times\) 10\(^{-7}\) M tricine buffer adjusted to pH 8.4 with NaOH. The reaction was stopped as soon as all of the ferricyanide (1.25 \(\mu\) moles) had been reduced.

| Experiment | P\(_i\) removed from the medium | Organic \(^{32}\)P formed |
| --- | --- | --- |
| 1 | 0.645 | 0.630 |
| 2 | 0.648 | 0.627 |
| 3 | 0.643 | 0.628 |
| 4 | 0.673 | 0.630 |
| 5 | 0.648 | 0.629 |
| 6 | 0.637 | 0.634 |
| 7 | 0.633 | 0.649 |
| 8 | 0.637 | 0.658 |
| 9 | 0.623 | 0.644 |
| 10 | 0.617 | 0.627 |
| 11 | 0.607 | 0.621 |
| 12 | 0.613 | 0.631 |
| 13 | 0.610 | 0.626 |
| 14 | 0.627 | 0.640 |
| 15 | 0.613 | 0.622 |
| 16 | 0.580 | 0.596 |
| 17 | 0.590 | 0.620 |
| 18 | 0.633 | 0.632 |
| 19 | 0.631 | 0.626 |
| 20 | 0.643 | 0.640 |
| 21 | 0.647 | 0.629 |
| 22 | 0.627 | 0.620 |

Averages: 0.628 \(\mu\) moles for organic \(^{32}\)P.

Measurements of Ferricyanide Reduction—The concentration of ferricyanide in the reaction mixture was followed spectrophotometrically by continuously recording the optical density of 420 nm, using a Bausch and Lomb Spectronic 505 spectrophotometer modified for actinic illumination. Ordinarily the light was turned off as soon as the ferricyanide had been reduced.

Colorimetric Determination of Orthophosphate—Orthophosphate was estimated by the method of Ames (8). An 0.3-ml aliquot of the reaction mixture (containing 0.04 to 0.15 \(\mu\) mole of phosphate) was placed in a test tube containing 1.2 ml of water, 0.3 ml of 10% ascorbic acid, and 3.0 ml of 0.42% ammonium molybdate in 1 N H\(_2\)SO\(_4\) (total volume 5 ml). The reaction mixture was incubated for 20 min at 50\(^\circ\)C and then cooled to room temperature (20\(^\circ\)C). The optical density of the blue-colored solution was measured at 820 nm using a Beckman model DU spectrophotometer with Gilford electronics. The amounts of orthophosphate in the reaction mixture were determined by comparing the optical densities from the samples with the optical densities from carefully prepared standard concentrations of phosphate in identical reaction mixtures (including chloroplasts). Standards were run simultaneously with each sample analysis and the concentrations of the standards were arranged to bracket the phosphate concentrations in samples being measured. Every sample was run in duplicate and the average was taken. The difference between the initial and final concentrations of orthophosphate was taken as a measure of amount of organic phosphate formed. An aliquot (1.0 ml out of a total of 2.0 ml of reaction mixture) from the same sample was directly estimated for organic \(^{32}\)P for comparison.

Paper Chromatography of Products of Photophosphorylation—After the photophosphorylation reaction, the reaction mixture was transferred from the cuvette to a centrifuge tube wrapped in black tape to prevent light entry. It was then centrifuged to spin down the chloroplast fragments. Unlabeled ATP (0.1 mg) and unlabeled ADP (0.1 mg) were added to the supernatant to facilitate detection of the labeled substances on the paper. An aliquot was chromatographed on a Whatman No. 1 filter paper using a mixture of 95% ethanol and 1 M ammonium acetate (pH 7.5) in the ratio of 7.5:3.0, v/v, as solvent. The ADP spot was eluted in 20% ethanol and rechromatographed in methanol, ammonia, and water in the ratio of 6:1:3, v/v. After drying, the areas containing ADP and ATP were located in ultraviolet light and were then cut out. These and any other radioactive spots were counted by immersing the piece of paper
TABLE II
Distribution of labeled phosphate in ADP and ATP of phosphorylation reaction mixtures

The 2.0-mL reaction mixture contained chloroplasts with 20 μg of chlorophyll, 10^{-2} M sucrose, 10^{-3} M KCl, 3 × 10^{-2} M MgCl₂, 10^{-3} M Na₂HPO₄ labeled with ³²P, 10^{-2} M ADP, 5 × 10^{-4} M potassium ferricyanide, and 4 × 10^{-2} M tricine buffer adjusted to pH 8.4 with NaOH. Aliquots of arbitrary size from the reaction mixture were spotted on paper and chromatographed in the indicated solvents.

| Portion of reaction mixture chromatographed | cpm | cpm | % |
|-------------------------------------------|-----|-----|---|
| once in 95% ethanol-1 M ammonium acetate pH 7.5, 7.5:3.0, v/v | 5346 | 312 | 5.5 |
| Eluate of ADP area rec chromatographed in methanol-ammonia-water, 6:1:3, v/v | 463 | 582 | 52.0 |

* Maximum proportion of label actually in ADP was 2.8%.

TABLE III
Distribution of labeled phosphate in glucose-6-phosphate and combined "ADP + ATP" of phosphorylation reaction mixture

The reaction mixture was as in Table II except that 10^{-3} M ATP, 10^{-4} M glucose, and 1 mg of hexokinase replaced the ADP.

| Portion of reaction mixture chromatographed once in methanol-ammonia-water, 6:1:3, v/v | cpm | cpm | % |
|--------------------------------------------------------------------------------------------|-----|-----|---|
| Eluate of "ADP + ATP" region rec chromatographed in the same solvent | 8440 | 211 | 2.4 |

* Maximum proportion of label actually in ADP was 1.2%.

RESULTS

Disappearance of Orthophosphate and Appearance of ³²P in Other Substances—Both measurements utilized in this experiment depend on the formation of phosphomolybdate from orthophosphate. In the direct analysis of orthophosphate, excess molybdate is added, the phosphomolybdic acid is reduced, and the resulting blue color is measured spectrophotometrically. Obviously, determination of the amount of orthophosphate disappearing cannot be very precise unless a considerable proportion of the total orthophosphate is consumed. Consequently, very low (decidedly suboptimal) concentrations of phosphate were used in these experiments.

For measurement of the incorporation of radioactive orthophosphate into other substances an excess of molybdate is again added, but in this case the phosphomolybdic acid is quantitatively removed by organic solvents and discarded. Phosphate incorporation is measured as the residual radioactivity. The great advantage of such determinations is that they can be very accurate even if huge amounts of unused orthophosphate are still present after the reaction. For this reason the method has been used in most studies of photophosphorylation. Therefore, the report of Del Campo et al. (4) throws doubt on many data in the literature.

Figure 1 shows the results of 22 consecutive experiments, each value given representing the average of dupli-
catalysts. (In this table values should be compared horizontally, not vertically.) During the reduction of 1.25 μmoles of ferricyanide, an average of 0.628 μmole of orthophosphate disappeared, while an average of 0.629 μmole of labeled phosphate appeared in the non-orthophosphate fraction. It is, therefore, probably safe to assume that there was no significant exchange of labeled orthophosphate with unlabeled phosphate compounds such as ADP.

It should be noted that the ratio P/e₂ in these experiments was almost precisely 1.0. However, it should also be noted that in order to obtain reliable estimates of orthophosphate disappearance the concentration of phosphate used was only about 3% of the concentration which is optimal for phosphorylation. From other studies of the dependence of phosphorylation on phosphate concentration, it can be calculated that with these chloroplasts P/e₂ ratios of about 1.2 would have been possible.

**Distribution of ^32P in Products of Photophosphorylation Reaction**

Chromatographic analysis of the reaction mixture also showed that there was no significant ADP-^32P₁ exchange during phosphorylation. It is true that a small amount of AD^32P was formed (see Table I) but this minor reaction depends on the continued presence of labeled ATP. Thus, when hexokinase and glucose kept the level of ATP very low, much less AD^32P was formed (Table III). Conversely, when hexokinase and glucose were absent and the reaction mixture was incubated in the dark after phosphorylation had ceased, there was an increase in the amount of labeled ADP at the expense of labeled ATP (see Fig. 1). It seems clear that most if not all of the trivial amount of label in ADP is derived from ATP through the action of the ATP:AMP phosphotransferase (adenylate kinase, myokinase) known to be present in chloroplast preparations

\[ \text{ATP} + \text{AMP} \leftrightarrow \text{ADP} + \text{ADP} \]

and, because the label is derived from ATP, this form of organic ^32P must also be included in estimating the extent of the phosphorylation reaction.

**Apparent P/e₂ as Function of Amount of Ferricyanide Reduced**

As already pointed out, Del Campo et al. (4) have observed that the use of low ferricyanide concentrations results in apparent P/e₂ values higher than 1.0 and the lower the ferricyanide concentration the higher the apparent P/e₂. However, it would seem from the published description of their experiments that they illuminated the chloroplasts for a constant period of time (4 min), which was greater by varying degrees than the time actually required for complete reduction of the amount of ferricyanide added. Therefore, it is reasonable to suspect that the absence of an exogenous electron acceptor permitted some cyclic electron transport catalyzed by endogenous carriers in the manner described by Mehler (9). Maximum "endogenous" phosphorylation would therefore occur with minimum ferricyanide and the computed ratio of P/e₂ would rise, because an increasing proportion of the electron transport would be unrecognized and unrecorded. We find that the ratio of ATP formed to ferricyanide reduced is independent of the amount of ferricyanide reduced when the light is turned off as soon as the ferricyanide is reduced. Only when there is a constant period of illumination does the ratio rise with decreasing amounts of ferricyanide (Fig. 2).

**DISCUSSION**

If Winget et al. (3) erred in their estimates of the stoichiometry of photophosphorylation, they must have either overestimated ATP formation or underestimated electron transport. This study shows that their measurements of phosphorylation are very probably correct. We cannot be so sure about the measurements of electron transport because light-driven electron movements can never be measured unambiguously; electrons raised to a higher potential by a photochemical reaction can always return to the lower level unnoticed. Therefore, there is no way of eliminating the possibility of some unmeasured cyclic electron flow. However, it should be noted that phosphorylation supported by cyclic electron flow is usually quite slow (although probably never negligible). This low rate can be subtracted from the rate observed in the presence of ferricyanide and still leave a ratio of P/e₂ well over 1.0. Therefore, unless the presence of ferricyanide or other electron acceptors actually increases the cyclic flow of electrons (which seems improbable), the true value of P/e₂ must be greater than 1.0. Indeed, for other reasons Izawa and Good (10) have suggested that the phosphorylating part of the electron transport may make 2 ATP molecules for each pair of electrons transferred.

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J. Biol. Chem. 1970, 245:5017-5021.

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