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# PHOTOSYNTHETIC PHOSPHORYLATION IN MITOCHONDRIA-FREE CHLOROPLAST SUSPENSIONS FROM LEAVES OF *VICIA FABA* L.

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## SUMMARY

Chloroplasts were prepared from leaves of *Vicia faba* L. Electron micrographs showed that crude chloroplast suspensions prepared by the classical differential centrifugation procedure were contaminated by mitochondria; the mitochondria were completely removed by a further centrifugation of the crude chloroplast preparations through a glycerol–sucrose density gradient. Most of the chloroplasts in the crude preparations and all the chloroplasts after centrifugation through the density gradient were devoid of external membranes and stroma.

The mitochondria-free chloroplast suspensions were able to mediate photophosphorylation with $3 \cdot 10^{-5}$ M FMN and $10^{-4}$ M vitamin $K_3$ as co-factors. The rate of photophosphorylation, the product (ATP) and the nonadditive effect of the co-factors compare closely with the results of other workers who used spinach chloroplasts prepared by differential centrifugation alone.

Thus, photophosphorylation would appear to be a real aspect of the metabolism of isolated chloroplast; mitochondria are not involved in the process.

## INTRODUCTION

Since 1954 the process of photophosphorylation has attracted considerable attention (see the review by KANDLER[1]). There now seems no reason to doubt that cyclic photophosphorylation is a biochemical attribute of chloroplast suspensions prepared from leaves of spinach[2–7] and other plants[8] according to the procedure of ARNON *et al.*[9]. This method involves the sedimentation of chloroplasts at $1000 \times g$ from leaf homogenates after removal of whole cells and cell debris. However, it has been shown that at a centrifugal force of $1000 \times g$ not only whole chloroplasts but also biochemically active mitochondria are sedimented from a suspension containing both types of particle[10]. Two further washings of the "chloroplast" pellet with the medium at $1000 \times g$ will not completely remove the mitochondria.

It is often assumed that, because the contamination of chloroplast preparations by mitochondria is relatively slight, it is negligible. This assumption may be dangerous for it ignores the possibility that the contaminating mitochondria, though few in number, may be very active biochemically. For this reason it cannot be categorically

stated that isolated chloroplasts possess the complete multienzyme system required for the incorporation of inorganic phosphate into ATP in the light (photophosphorylation) until this incorporation has been demonstrated in preparations of chloroplasts known to be free from mitochondria.

In the present work chloroplast suspensions from *Vicia faba* leaves were prepared according to the procedure of JAMES AND DAS[11] in an attempt to remove contaminating mitochondria. The chloroplast preparations were examined in the electron microscope and their capacity for photosynthetic phosphorylation determined in the presence of the co-factors FMN and vitamin $K_3$.

## METHODS AND MATERIALS

Seeds of *Vicia faba* L. (var. Green Windsor), obtained from Sutton's Seeds, Reading, were soaked for 24 h in tap-water and planted in John Innes' No. 1 Compost. The greenhouse temperature was maintained near 60° F and supplementary light given from 6 a.m. to 10 p.m. by a bank of fluorescent lights above the plants. The light intensity was between 500 and 600 foot-candles at the top of each plant. Young leaves which had just fully expanded were carefully selected from plants between two and three weeks old: only completely healthy, turgid, deep green leaves were used. The leaves were always picked at 9–10 a.m., washed in distilled water and dried on blotting paper. The washed leaves were stored in damp muslin at 4° for 1–2 h before use.

Chloroplasts were extracted according to the procedure of ARNON *et al.*[9]. The medium used was 0.05 M Tris–HCl buffer (pH 8.0) containing NaCl (0.35 M). No sand was used in the initial homogenization. The unwashed chloroplast suspension obtained after the first centrifugation at 1000 × *g* was then washed once in the medium, the pellet resuspended and termed "crude" chloroplast suspension. Alternatively, the unwashed chloroplast suspension was centrifuged through a glycerol–sucrose density gradient by the procedure of JAMES AND DAS[11]. The glycerol concentration required for the gradient was made up with the medium. 10 ml of 60 % (v/v) glycerol-medium was placed in the bottom of a 50-ml centrifuge tube and 10 ml of 25 % (v/v) glycerol-medium layered carefully on top. 5 ml of an unwashed chloroplast suspension, ground in a glass homogenizer to disperse the clumps of chloroplasts, was placed on top of the prepared gradient. The tube and contents were centrifuged at 1000 × *g* for 12 min in a horizontal swing-out head and the deep green layer, lying just above the division between the original 60 % and 25 % glycerol layers removed. Observations with the light microscope showed that this deep green layer consisted almost exclusively of non-fragmented chloroplasts. After dilution with the medium, the chloroplasts were sedimented at 1000 × *g* for 12 min. The pellet obtained was taken up in the medium and termed "purified" chloroplast suspension.

Photophosphorylation experiments were carried out in Warburg flasks in a perspex Warburg bath. The bath was controlled thermostatically at 15° ± 0.1° by pumping ice-cold water through the bath while operating the heater. The bottom of each flask was illuminated with light focussed from a 500-W projector bulb under the bath. The light intensity at the bottom of each flask was 30000 Lux (measured in foot-candles by an EEL photometer). All reagents and flasks were pre-cooled to between +2 and +4° and to each Warburg flask was added (in μmoles) $K_2HPO_4$, 10;

ADP (pH 6.0), 10; $MgCl_2$, 10; sodium ascorbate, 10; FMN (pH 6.0), 0.1; vitamin $K_3$, 0.3; and chloroplasts as indicated. The volume was made up to 3 ml with 0.05 M Tris (pH 8.2). Vitamin $K_3$ (menadione) was dissolved in 100% methanol; 0.05 ml of a $6 \cdot 10^{-3}$ M solution was added to each flask. Ascorbic acid was adjusted to pH 6.0 with NaOH 10 min before use.

The flasks were flushed with nitrogen (British Oxygen Co. "white spot" containing less than 10 parts per million $O_2$) for 10 min, and the reaction started by tipping ADP from the side-arm and switching on the lights. After the reaction period each flask was removed from the bath, an aliquot from the flask pipetted into a centrifuge tube and trichloroacetic acid added to a final concentration of 2% (w/v). The contents of the centrifuge tube were spun at 10000 × *g* for 10 min and the amount of inorganic phosphate in the clear supernatant determined by the method of ALLEN[13]. The control was a flask, containing chloroplasts and all reagents, removed from the bath at zero time. In most experiments a control in the dark was also run.

Chlorophyll was determined as a solution in 80% acetone by the method of ARNON[14].

Chromatograms were run on Whatman 3 MM and No. 1 paper by descending chromatography at 25°. The solvent[15] was isobutyric acid–1 N $NH_4OH$–0.1 M EDTA (100:60:1.6). Equal aliquots removed from the reaction mixtures were cleared by the addition of trichloroacetic acid (final concentration 2%) and centrifugation. 20-100 µl of the cleared supernatant was applied to the chromatogram as a spot. The chromatograms were run until examination under the ultraviolet lamp revealed a reasonable separation of the ATP, ADP and AMP markers (8–10 h). Some chromatograms were sprayed with the phosphate reagent recommended by HANES AND ISHERWOOD[16].

*Preparation of the material for electron microscopy*

To the chloroplast pellet were added 2 ml osmic acid fixative (1% $OsO_4$ buffered at pH 7.0 and containing 0.35 M NaCl). Fixation, carried out in the centrifuge tube, was for 30 min, at 2°, followed by 90 min at room temperature.

The pellet was then carefully released from the bottom of the tube, washed twice in glass-distilled water and dehydrated in two changes each of 20%, 50%, 75% and 100% alcohol. The time for each change was approx. 5 min except for the final change in 100% alcohol which was for 1 h. In the 100% alcohol, small pieces (2 mm × 1 mm × 1 mm) were cut from the pellet.

The embedding medium used was Epon prepared according to LUFT[17]. The mixtures A and B of LUFT were mixed in the proportions 3A:1B. The diluent, propylene oxide was not used. After fixation and dehydration, the tissue blocks were transferred from absolute alcohol into an Epon – absolute alcohol (1:1) mixture for 1 h, left overnight in 100% Epon and finally embedded in fresh Epon in gelatine capsules. Polymerisation took place at 60° for 48 h. The sections were cut with the glass knife in a Porter-Blum microtome and viewed in an EM6 electron microscope.

## RESULTS

*Examination of the chloroplast preparations with the light microscope*

An examination of the *crude* chloroplast preparations under the light microscope confirmed the observations of JAMES AND DAS[11]: the preparation was heterogeneous

and consisted of whole chloroplasts, chloroplast fragments and numerous particles approx. 1 $\mu$ in diameter exhibiting vigorous Brownian movement. These particles could not be stained with iodine or Janus Green B. By contrast, the purified chloroplast preparation consisted almost exclusively of unfragmented chloroplasts, which appeared to be undamaged. Very few chloroplast fragments were present and smaller particles were almost completely absent.

No whole nuclei or whole cells were found in either type of chloroplast preparation.

*Examination of chloroplast suspensions with the electron microscope*

The visual observations of the chloroplast preparations were extended by examination in the electron microscope of thin sections from fixed and embedded chloroplast pellets. Representative electron micrographs of the chloroplast preparations are shown in Plates A, B and C.

In the crude chloroplast preparations, whole chloroplasts (Chl. m.) were present together with chloroplast fragments, mitochondria (M), membranes (Mb) and unidentifiable cell debris (Plates A and C). The mitochondria did not appear to be greatly damaged structurally and characteristic internal membranes were clearly discernible. These mitochondria present in the crude chloroplast preparations closely resembled the mitochondria seen in thin sections of the mesophyll and palisade cells of the *Vicia faba* leaf[18].

In several sections, groups of mitochondria and chloroplast were enclosed in free membranes of unknown origin.

In the heterogeneous crude chloroplast suspensions a very distinctive feature of most of the unfragmented chloroplasts was the complete absence of their external chloroplast membranes. A survey of a section would usually reveal some chloroplasts with intact, or almost intact, external membranes but most of the chloroplasts had no external membranes at all. In other respects, the whole chloroplasts closely resembled the free chloroplasts detached from the cytoplasm of the cells which are sometimes seen in sections of *Vicia faba* leaves.

In the extracted chloroplasts the internal lamellae remain ordered in granal units. However, where the bounding membranes of the chloroplast have disappeared, no stroma material can be detected. It appears that in the absence of the external membrane virtually all the stroma is leached from the lamellar systems, which themselves remain substantially intact.

In contrast, sections of purified chloroplast preparations showed that these suspensions consisted almost entirely of unfragmented chloroplasts (Plate B). Almost every chloroplast was devoid of its external membrane and of stroma material. A few chloroplast fragments were also present but mitochondria were absent. In a survey of several sections about 100 chloroplasts and 20 chloroplast fragments were counted but only one particle was seen which was possibly a mitochondrion. Further, in a survey of sections from chloroplast pellets prepared on five different occasions, 1000 chloroplast profiles were counted but no mitochondria. None of the free membranes characteristic of the crude chloroplast preparations and no cellular debris, other than fragmented chloroplasts, was seen.

From these observations, it would appear that the centrifugation of the crude chloroplast preparation through a glycerol–sucrose density gradient does remove contaminating mitochondria from the chloroplast suspension. The removal of the

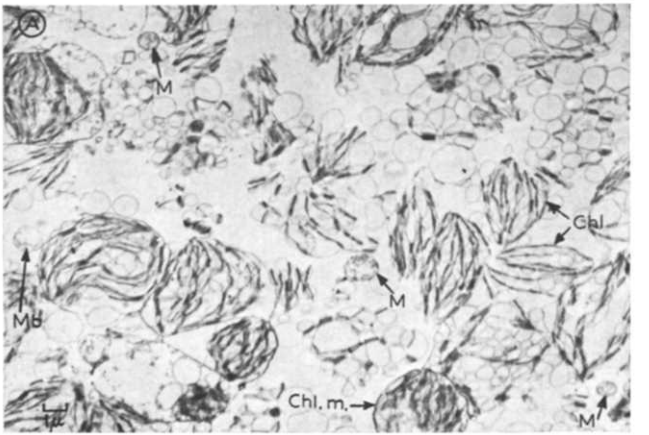


Plate A. Electron micrograph of a crude chloroplast preparation from *Vicia faba*. Fixation in 1 % $OsO_4$ (buffered pH 7.0) in 0.35 M NaCl; embedding medium Epon. Magnification 4250. Chloroplasts with external membranes (Chl. m.), chloroplasts without membranes (Chl), mitochondria (M) and unidentified cytoplasmic membranes (Mb) can be seen.

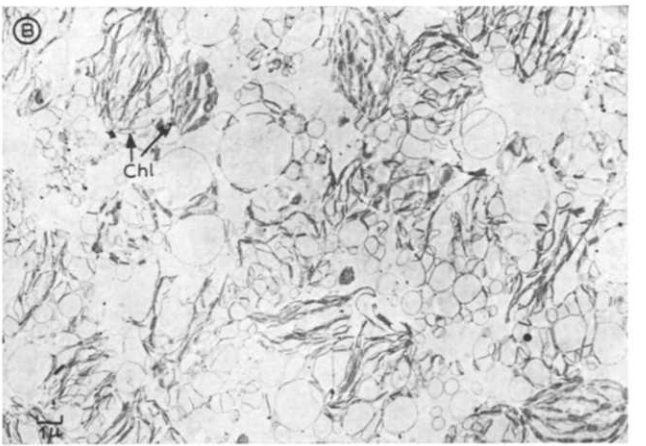


Plate B. Electron micrograph of a purified chloroplast preparation from *Vicia faba*. Fixation in 1 % $OsO_4$ (buffered pH 7.0) in 0.35 M NaCl; embedding medium Epon. Magnification 4250. Only chloroplasts devoid of membranes (Chl) and fragments of chloroplasts are seen.


Plate C. Electron micrograph of a crude chloroplast preparation from *Vicia faba*. Conditions of fixation as in Plate A. Magnification 8000.

mitochondria was consistent and complete in the ten separate experiments in which chloroplasts were fixed and embedded.

*Photophosphorylation by crude and purified chloroplast suspensions*

Chloroplast suspensions prepared from *Vicia faba* leaves possessed the capacity for photophosphorylation and this capacity was retained even after the chloroplasts had been freed from contaminating mitochondria by centrifugation through the glycerol–sucrose density gradient. The results for both crude and purified suspensions are shown in Table I.

In further experiments crude and purified chloroplast suspensions were prepared from the same batch of leaves and the two types of preparation compared. In a series of five experiments it was found that centrifugation of the chloroplasts through the glycerol–sucrose density gradient never reduced their capacity for photophosphorylation by more than 10–20%. The result of one experiment in which the reduction in activity was 11% is shown in Fig. 1.

Great variation was found in the capacity for photophosphorylation of different chloroplast suspensions. This variation was found both for crude and also for purified

TABLE I

CYCLIC PHOTOPHOSPHORYLATION BY CHLOROPLAST PREPARATIONS FROM *Vicia faba*

Reaction mixture contained in μmoles: NaCl, 170; Tris, 80; ADP (pH 6.0), 10; $K_2HPO_4$, 10; $MgCl_2$, 10; sodium ascorbate (pH 6.0), 10; FMN, 0.1; vitamin $K_3$, 0.3. Chlorophyll as given in the table. Final volume, 3.0 ml. Temperature, 15°; light intensity, 30000 Lux.

| *Material** | *Expt.* | *Chlorophyll (mg/flask)* | *Uptake of $P_i$ (μmoles/mg chlorophyll/h)* |
|---|---|---|---|
| A. "Unwashed" chloroplast suspension | 1 | 0.28 | 85 |
| | 2 | 0.49 | 37 |
| | 3 | 1.03 | 22 |
| B. As A but washed once ("crude") | 4 | 0.32 | 94 |
| | 5 | 1.01 | 41 |
| | 6 | 1.6 | 22 |
| C. As A but centrifuged through a sucrose–glycerol density gradient ("purified") | 7 | 0.18 | 72 |
| | 8 | 0.19 | 92 |
| | 9 | 0.28 | 138 |
| | 10 | 0.37 | 85 |
| | 11 | 0.4 | 40 |

* For details of preparation see under METHODS.

chloroplasts. Some of the variation was shown to be due to the amount of chlorophyll per flask. 0.25–0.35 mg chlorophyll per flask gave the greatest rate of decrease in phosphate in the light over a period of 10 min, irrespective of the time of year when the experiment was performed. In addition, in order to obtain maximal rates of photophosphorylation, it was necessary to prepare the chloroplasts with the greatest possible rapidity, to maintain the chloroplasts and reagents at not more than −2° before illumination and to use leaves from plants under three weeks old. Chloroplasts from leaves of older plants were often completely inactive.

The conditions of incubation were also very important: no phosphorylation occurred in experiments where the light intensity at the bottom of each flask was only 2500 Lux and in any experiments where the temperature was raised above the standard 15° the rate of photophosphorylation was reduced.

Photophosphorylation by the purified mitochondria-free chloroplast suspensions was further investigated. Uptake of inorganic phosphate from the medium was linear over a period of 12–15 min and then began to fall off when about three quarters of the inorganic phosphate supplied had disappeared from the reaction mixture. The disappearance of inorganic phosphate was dependent on light (Fig. 2).

The decrease in inorganic phospnate was determined for different amounts of chlorophyll per flask. A 6-min time period was chosen because experience had shown

that over this short period, whatever the chlorophyll concentration, the decrease in inorganic phosphate was linear. The result is shown in Fig. 3. The uptake of inorganic phosphate increases linearly with increasing chlorophyll concentration to 0.6 mg chlorophyll per flask but falls off at higher chlorophyll concentrations. The reason for this fall off in activity at higher chlorophyll concentrations is unknown. It may be due to the build up of an inhibitor such as the one demonstrated by HEBER[19] and by SIMONIS AND FÜCHTBAUER[20], to inhibition by the accumulated ATP, or even to increased mutual shading of the chloroplasts at the higher chlorophyll concentrations.

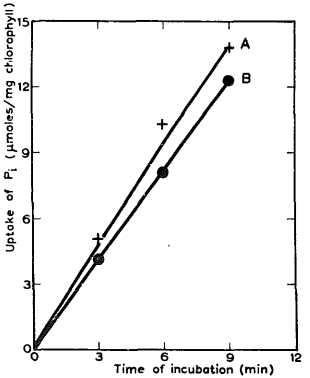


Fig. 1. Photophosphorylation by chloroplast preparations from *Vicia faba* L. A, crude chloroplast preparation; B, purified chloroplast preparation. Reaction mixture contained in μmoles: NaCl, 170; Tris, 80; ADP (pH 6.0), 10; $K_2HPO_4$, 10; $MgCl_2$, 10; sodium ascorbate (pH 6.0), 10; FMN, 0.1; vitamin $K_3$, 0.3 and chloroplasts containing 0.58 mg chlorophyll. Final volume, 3.0 ml. Temperature, 15°; light intensity, 30000 Lux; gas phase, nitrogen.

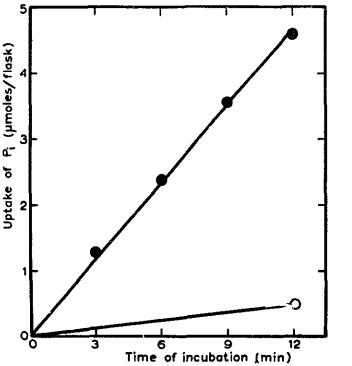


Fig. 2. Photophosphorylation by a purified chloroplast preparation from *Vicia faba*. O—O, control in the dark; ●—●, incubated in the light. Chloroplasts contained 0.29 mg chlorophyll. Other conditions as in Fig. 1.

An experiment was also performed to determine whether the two co-factors FMN and vitamin $K_3$ had an additive stimulatory effect on photophosphorylation. The decrease in inorganic phosphate was determined after 15 min in the presence of increasing amounts of vitamin $K_3$ from 0 to 0.3 μmole per flask. For each level of vitamin $K_3$, the stimulatory effect was determined both in the presence and in the absence of 0.1 μmole FMN. The result of such an experiment is shown as a histogram in Fig. 4. In the presence of either 0.1 μmole FMN alone or 0.03 μmole vitamin $K_3$ alone, the decrease in inorganic phosphate was the same; there was only a very small additive effect when both co-factors were present together. In the absence of FMN, an increase in the concentration of vitamin $K_3$ from 0 to 0.03 μmole per flask increased the uptake of inorganic phosphate: further increase in vitamin $K_3$ to 0.3 μmole gave only a very slight additional increase in phosphate uptake. This agrees with the general finding that FMN and vitamin $K_3$ can function independently as co-factors of photophosphorylation in spinach chloroplasts[1]. It will be noted that photophos-

phorylation will still proceed in these purified chloroplast preparations in the complete absence of either co-factor. This is shown in the far left-hand column of the histogram.

Photophosphorylation in the absence of FMN and vitamin $K_3$ has also been demonstrated for spinach chloroplasts by FORTI AND JAGENDORF[21].

ARNON *et al.*[2] and AVRON AND JAGENDORF[3,22] have identified ATP chemically as the sole product of the photophosphorylatic a reactions in crude suspensions of spinach chloroplasts. In the present investigation of the nature of the product(s) of the photophosphorylation reaction in chloroplast preparations from *Vicia faba*, identi-

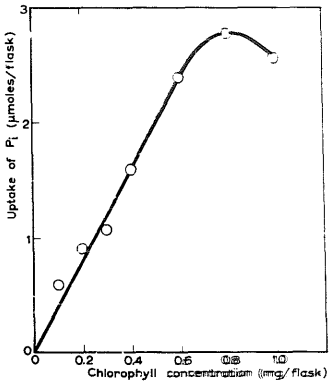


Fig. 3. Effect of chlorophyll concentration on photophosphorylation by a purified chloroplast preparation from *Vicia faba*. Time of incubation 6 min. Chlorophyll concentration per flask as indicated in the figure. Other reaction conditions as in Fig. 1.

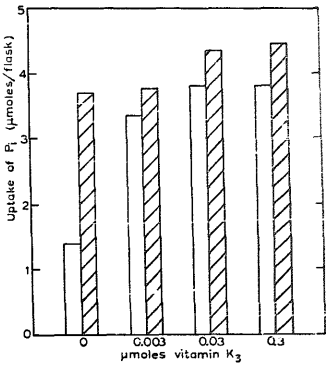


Fig. 4. Effect of the co-factors FMN and vitamin $K_3$ on photophosphorylation by a purified chloroplast preparation from *Vicia faba*. □, no FMN; ▨, plus 0.1 μmole FMN. Time of incubation 15 min. Chloroplasts contained 0.19 mg chlorophyll. Other conditions as in Fig. 1.

fication was on the basis of known markers only: no attempt was made to identify the products of the reaction chemically.

In purified chloroplast preparations from *Vicia faba*, after 10–20 min incubation, in the light, the formation of a single new compound was detected. This compound ran in the position of marker ATP, gave the phosphate reaction with the HANES-ISHERWOOD[16] reagent and showed ultraviolet absorption. The result was the same whether both vitamin $K_3$ and FMN or vitamin $K_3$ alone (0.3 μmole per flask) were present as co-factors. A similar result was obtained with the unpurified chloroplast preparation. The chromatographic results are consistent with the view that ATP is the major or sole product of the photophosphorylation reactions in the light in chloroplast preparations from *Vicia faba*.

It is interesting to note that in the dark controls there was formation of traces of both ATP and AMP after 20 min incubation. This suggests the presence of adenylic kinase in the chloroplast suspension. Such an enzyme has already been studied by MAZELIS[23] in spinach chloroplasts.

The reactions taking place in the dark were not further investigated.

## DISCUSSION

The incorporation of inorganic phosphate into ATP in the light by chloroplast preparations shown to be completely free from mitochondria, demonstrates the self-sufficiency of the chloroplast to carry out photophosphorylation. HONGLADARUM, HONDA AND WILDMAN[24] have made the interesting suggestion that in the leaf the ATP produced in photophosphorylation may be formed not in the chloroplasts themselves but in the enveloping layers of cytoplasm. This suggestion is not supported by the results of the present studies of chloroplasts *in vitro*. Nor is there any evidence from the present work that mitochondria can affect the photophosphorylation process in any way. A crude chloroplast suspension considerably contaminated by mitochondria and a chloroplast suspension known to be entirely free from these particles were prepared from the same batch of leaves. The uptake of inorganic phosphate in the light ($\mu$moles $P_i$/mg chlorophyll/h) was similar for the two chloroplast suspensions (Fig. 1).

The highest rates of photophosphorylation (130–150 $\mu$moles $P_i$/mg chlorophyll/h) catalysed by Vicia chloroplasts, compare favourably with the highest rates found for chloroplasts of other species. JAGENDORF AND AVRON[4] record rates of 130–200 $\mu$moles $P_i$/mg chlorophyll/h and NAKAMOTO *et al.*[25] 100–250 $\mu$moles $P_i$/mg chlorophyll/h for spinach chloroplasts in the presence of FMN and vitamin $K_3$. (Rates five times higher have been obtained with phenazine methosulphate as co-factor[4,26].) With chloroplasts of pea (*Pisum sativum*), SMILLIE AND KROTKOV[27] obtained a rate of 270 $\mu$moles $P_i$/mg chlorophyll/h.

From *Vicia faba* there appears to be no previous record of the successful preparation of chloroplasts active in photophosphorylation reactions. HEBER[19] only obtained very low rates of phosphorylation of 0–0.4 $\mu$mole $P_i$/mg chlorophyll/h, even with phenazine methosulphate as co-factor. HEBER's failure to demonstrate active photophosphorylation could be accounted for by the age of the plants he used; they were between eight and twelve weeks old[28]. The author could only obtain active chloroplast preparations from *Vicia faba* plants less than three weeks old. It is possible that the acetone-soluble inhibitor of photophosphorylation extracted from leaves of eight to twelve weeks old bean plants[19] may not be present in the leaves of younger plants.

In the present work, the reactions were carried out in nitrogen (containing less than 10 parts per million $O_2$) and high rates of photophosphorylation were obtained in the presence of $3 \cdot 10^{-5}$ M FMN alone (Fig. 4). NAKAMOTO *et al.*[25] have recently demonstrated, in spinach chloroplast preparations almost certainly contaminated by mitochondria, that in the presence of $3 \cdot 10^{-5}$ M FMN photophosphorylation is partially oxygen-dependent. It would be of considerable interest to determine whether this is also the case in the mitochondria-free chloroplast preparations used in the present investigation.

The appearance of the chloroplasts, as revealed in Plates A–C, is at first sight disconcerting and requires further consideration. Approx. 90 % of the chloroplasts in the crude preparations and virtually all the chloroplasts in the purified preparations are devoid of external membranes. There is now general agreement[29] that in the cell the chloroplast is surrounded by a limiting membrane. There is, however, no convincing evidence that their membranes remain intact when chloroplasts are isolated

from the cell. The external chloroplast membrane cannot be definitely distinguished in the light microscope, but in the present work it had been assumed that because the isolated chloroplasts were discrete in shape and had clearly distinct outlines, the external chloroplast membrane was probably intact. It was only on examination of the preparations in the electron microscope that the absence of external membranes was revealed. The few published electron micrographs of other isolated chloroplasts also suggest that the external membranes are extremely labile. JACOBI AND PERNER[30] examined chloroplasts of Spinacia extracted in 0.35 M NaCl buffered in 0.1 M phosphate buffer (pH 7.0) and washed once. These chloroplast suspensions are comparable with the crude chloroplasts of the present work. The pictures published by JACOBI AND PERNER show that the majority of their chloroplasts also have been stripped of external membranes; there is little evidence for the retention of stroma although the internal lamellar systems of the chloroplasts have remained largely intact. The earlier pictures of sections of isolated chloroplasts from Nitella[31] are not easy to interpret. Electron micrographs are only given of the chloroplasts isolated in water and the diffuse osmiophilic region bounding some parts of the lamellate structures is not very convincingly interpreted as a chloroplast membrane. A similar appearance would be shown by the outermost granal lamellae of a chloroplast which had lost its stroma.

PARK AND POX[32] surprisingly do not state whether their isolated chloroplasts from spinach were devoid of external membranes although their picture of a very small portion of a single isolated chloroplast suggests the presence of such a membrane. STOCKING[33] prepared chloroplasts from *Nicotiana rustica* in carbon tetrachloride–cyclohexane mixtures. These chloroplasts appeared to be intact when examined in the light microscope but on examination in the electron microscope were seen to be stripped of their membranes[34]. STOCKING AND ONGUN[34] state that chloroplasts isolated in buffered 0.4 M sucrose containing EDTA and fixed in 2.5% $KMnO_4$ may retain their membranes.

It is possible the stripping of the chloroplast membrane takes place during the fixation or embedding procedures. This seems unlikely because if the limiting membranes and stroma are removed during fixation, the remains of the membranes and stroma should be found in sections of the final embedded pellet. Whilst it cannot be ruled out that some of the membranes free in the preparations of crude chloroplasts are in fact chloroplast membranes, in preparations of purified chloroplasts free membranes and stroma are completely absent. Further, if the chloroplast membranes disappear during fixation, it is difficult to account for the consistent presence of a few chloroplasts with intact membranes in the crude chloroplast suspensions but no intact chloroplasts in the purified suspensions.

The striking absence of stroma from all the chloroplasts which had lost their external membranes has already been considered above. A similar situation was revealed in the work of JACOBI AND PERNER[30].

The chloroplasts in Plates A–C also show "ballooning" of the outermost units of the lamellae of the granal stacks. This ballooning is characteristic of chloroplasts isolated in 0.35 M NaCl or 0.35 M NaCl buffered in 0.02 M Tris (pH 8.0) and subsequently fixed in 1% $OsO_4$ buffered at pH 7.0 in the corresponding medium. Similar "ballooning" is found in chloroplasts isolated in 0.125 M KCl but not in chloroplasts isolated in buffered sucrose solutions over a wide range of tonicity (0.3 M–1.0 M).

It is likely the "ballooning" takes place before fixation because it is found in chloroplasts which have been fixed for only 5 min and can be partially reversed if chloroplasts prepared in 0.35 M NaCl are fixed in 0.3 M sucrose containing the 1 % $OsO_4$ (see ref. 35).

Thus it seems reasonable to assume that the condition of the chloroplasts shown in Plates A–C is a real reflection of the state of the chloroplasts used in the studies of photophosphorylation described in this paper.

The presence of an intact external chloroplast membrane is not essential for active photophosphorylation to proceed. The apparent absence of stroma from these chloroplasts is particularly interesting; it seems likely that only the lamellate regions of the chloroplast are involved in the photophosphorylation reactions.

Further study of the relationship between the ultrastructure and biochemical function of the chloroplast is in progress.

## ACKNOWLEDGEMENTS

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