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# LOCALIZATION OF PHOTOPHOSPHORYLATION AND PROTON TRANSPORT ACTIVITIES IN VARIOUS REGIONS OF THE CHLOROPLAST LAMELLAE*

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SUMMARY

Membrane fragments released by French pressure cell treatment of whole chloroplasts and isolated by differential centrifugation have been characterized structurally and with respect to phosphorylating and proton transport activities. In agreement with results of other workers, the heavy fraction released by pressure treatment was found by electron microscopy studies to be made up of mostly intact grana stacks while the light fraction was comprised of vesicles derived from the stromal lamellae. Both fractions were found to carry out rapid rates of cyclic photophosphorylation catalyzed by phenazine methosulfate (PMS). However, only the grana membranes demonstrated active proton accumulation in the presence of PMS. No light induced $H^+$ uptake could be detected in the stromal lamellae fraction; and as expected, proton gradient dissipating agents such as $NH_4Cl$, nigericin in the presence of $K^+$, and gramicidin were only slightly inhibitory to phosphorylation at concentrations which were very inhibitory in the grana membrane fraction.

Further evidence that stromal lamellae do not have active proton transport in the intact chloroplast was obtained by comparing various chloroplasts having different amounts of stromal and grana membranes. Comparative studies on young and old chloroplasts from lettuce, mesophyll and bundle sheath cell plastids from sorghum, and greening plastids from etiolated corn seedlings revealed a direct correlation between the extent of grana formation and the amount of proton transport activity. Samples which had larger amounts of stromal lamellae had high rates of ATP formation but a reduced capacity for $H^+$ accumulation.

## INTRODUCTION

There is currently much interest among biological scientists in what constitutes the driving force for ATP formation in chloroplasts and mitochondria. The classical

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; CCCP, *m*-chlorocyanocarbonyl phenylhydrazone; PMS, phenazine methosulfate.

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"chemical intermediate" hypothesis espoused by SLATER[1] and others has assumed that an energy-rich covalently bonded form of an electron carrier is the primary energy source for ATP formation. About ten years ago this concept was challenged by MITCHELL's[2] "chemiosmotic" hypothesis which suggested that an electrochemical potential gradient generated across the membrane during electron transport is the driving force for ATP formation.

As discussed by MITCHELL[3] the free energy, *i.e.* energy available to do useful work, of an electrochemical potential gradient, may be built up either as an electrically neutral concentration gradient of protons or by virtue of there being an electrostatic charge separation, or potential, across the membrane. Several lines of evidence have suggested that the energy coupling mechanism in chloroplasts is strongly dependent on the formation of a light-induced proton gradient (for recent reviews see refs. 4, 5).

With subchloroplast particles, the situation is very different, and massive proton movement is not correlated with ATP formation. MCCARTY[6] was the first to show that ATP formation in sonically prepared subchloroplast particles was active after proton uptake was largely inhibited by $NH_4Cl$ or nigericin + $K^+$. NELSON *et al.*[7] have shown a more dramatic effect with digitonin-derived subchloroplast particles which have active ATP synthesis rates but were virtually devoid of $H^+$ accumulation. Both of these experiments employed preparative procedures which apparently modified the coupling mechanism. The use of subchloroplast membrane preparations can be a useful tool in studies of the mechanism of ATP formation and ion transport, as the above examples demonstrate. Our interest was stimulated upon learning that morphologically distinct portions of spinach chloroplasts could be prepared without detergents, having greatly differing amounts of Photosystems I and II activity. We were interested in the ion transport and ATP synthesizing capacities of such fractions.

JACOBI AND LEHMANN[8] found that brief periods of sonication of chloroplasts followed by differential centrifugation allowed the recovery of a small portion of chlorophyll in a light fraction having only Photosystem I activity. Electron microscopy suggested that the manner of fragmentation resulted in a separation of grana stacks and intergrana membranes (stromal lamellae) with the latter being recovered in the light fraction. MICHEL AND MICHEL-WOLWERTZ[9] have employed the French press as a means of mechanically breaking chloroplasts and have also been able to isolate a membrane fraction containing only Photosystem I. SANE *et al.*[10] have used a modified procedure for the French pressure treatment and have examined the resulting fractions by freeze-etch and thin sectioning techniques for electron microscopy. Their results convincingly demonstrate that the light fraction released by this procedure (containing only Photosystem I) is derived from the intergranal or stromal lamellae. Our own structural evidence is also in agreement with their findings.

Biochemical characterization of the heavy fraction (UP-10 fraction of JACOBI AND LEHMANN[8], 10K fraction of SANE *et al.*[10], and 40K fraction of this paper) have shown that the grana membranes have both Photosystem I and II activities. The light fraction (UP-170 fraction of JACOBI AND LEHMANN[8], 160K fraction of SANE *et al.*[10], and 144K fraction of this paper), which is derived from stromal lamellae, has only Photosystem I activity.

We will demonstrate that the stromal lamellae fraction from mature spinach

chloroplasts and other types of chloroplasts which contain relatively greater amounts of stromal membranes, *i.e.* young lettuce leaf chloroplasts, bundle sheath cell chloroplasts from sorgum plants, and young corn seedling chloroplasts; have high photophosphorylation activities but have a reduced proton pump when compared to chloroplasts typified by a lesser proportion of stromal membranes.

Since the vesicular membrane fragments discussed in this paper have been prepared in the absence of any detergent (which can alter membrane permeabilities, *etc.*) and since these fragments represent clearly identifiable morphological parts of the overall chloroplast, we feel this system is a potentially very useful tool for testing the various hypotheses on the relation between ion transport, membrane potential, and phosphorylation.

## MATERIALS AND METHODS

*Spinacea oleracea* chloroplasts were prepared from commercial spinach as described by SANE *et al.*[10] and were adjusted to a chlorophyll concentration of 0.5 mg/ml. An Aminco French press with an adjusting pressure regulator and a 40-ml pressure cell (Model 3398) was used for all treatments. Chloroplasts were subjected to various pressures as described in the figure legends. The treated material was fractionally separated by differential centrifugation at 40000 $\times$ *g* for 30 min and then at 144000 $\times$ *g* for 60 min. The resulting pellets were resuspended in 0.4 M sorbitol, 0.02 M sodium tricine (pH 7.8) and 0.01 M KCl (sorbitol–tricine–KCl buffer) for phosphorylation and electron transport assays. Portions of the samples were resuspended in 0.01 M NaCl for proton transport determinations.

Commercially obtained lettuce (*Lactuca sativa* var. romaine) leaves were ground and centrifuged by the procedure described by SANE *et al.*[10]. The chloroplast pellet was either resuspended in 0.15 M KCl, 0.05 M $K_2HPO_4$ (pH 7.4) for French pressure cell treatment, in sorbitol–tricine–KCl buffer for phosphorylation assays, or in 0.01 M NaCl for proton transport assays. French press treatments were conducted as described for spinach.

Seedlings of *Sorghum bicolor* were grown in a glasshouse for 2 weeks. Mesophyll and bundle sheath cell chloroplasts were prepared by a modification of the procedure of WOO *et al.*[11]. Leaves (10 g samples) were cut into short segments with scissors and blended in a Sorvall Omnimixer for 5 sec at 50 % line voltage in 100 ml of 0.4 M sorbitol, 0.1 M sodium tricine (pH 7.8), 0.05 M sodium ascorbate, and 2 mg/ml (final concentration) bovine serum albumin. Unbroken cells were removed with two layers of miracloth. The brei was centrifuged for 10 min at 1000 $\times$ *g* to yield a pellet containing mesophyll chloroplasts. The residue of unbroken cells remaining on the miracloth was resuspended in 100 ml of the same grinding mixture and ground for 5 min at full line current in the Omnimixer. This solution was passed through 1 layer of miracloth and the residue was again resuspended in 100 ml of the grinding mixture. This was ground once again for 5 min at full line current and the brei was passed through 1 layer of miracloth. The residue remaining on the miracloth was washed once more with 100 ml of the grinding solution and then resuspended in 75 ml of the same mixture. The bundle sheath cells which were retained in the residue by this procedure were then broken by hand homogenization in a Ten Broeck homogenizer. The homogenate was passed through 2 layers of miracloth and the solution was

centrifuged for 20 min at 10000 × *g*. Both types of chloroplasts were resuspended either in sorbitol–tricine–KCl buffer for phosphorylation assays or in 0.01 M NaCl for proton transport assays.

*Zea mays* seedlings were grown in the dark and were transferred to the light as has been described previously[12]. 50 g samples of leaves were collected at various times after illumination and were ground for 20 sec in a Waring blender in 100 ml of the same grinding mix described above for sorghum leaves. The resulting brei was passed through 4 and then 12 layers of cheesecloth. The plastids were collected by centrifugation at 1000 × *g* for 10 min and were resuspended in sorbitol–tricine–KCl buffer.

Chlorophyll concentrations were determined by the method of ARNON[13]. Electron transport activities were measured by following the reduction of either 2,6-dichlorophenolindophenol (DCIP) or $NADP^+$ in a spectrophotometer modified for direct illumination of the sample. ATP formation was measured according to the procedure of NEILSON AND LEHNINGER[14] as modified by AVRON[15]. Changes in pH were measured with Leeds and Northrup Model 124138 microelectrodes with yellow Corning filter (No. 3-68) placed between the light source and the sample. Acid–base transition induced phosphorylation was conducted as described by JAGENDORF AND URIBE[16].

## RESULTS

It has previously been shown by MICHEL AND MICHEL-WOLWERTZ[9] and by SANE *et al.*[10] that a light membrane fraction is released from the chloroplast by French pressure cell treatment, and that this fraction has a high chlorophyll *a/b* ratio and has only Photosystem I activity. To find the optimal pressure to use for our experimental conditions, a series of samples were passed through the press at various pressures and assayed for percent chlorophyll recovered in the light (144K) fraction, and for photochemical activities of the two fractions. As is shown in Table I, even very low cell pressures caused fragmentation of the chloroplasts as evidenced by the recovery of a small amount of chlorophyll in the light fraction. Increasing pressure resulted in an increasing yield of chlorophyll recovered up to a maximum of about 5 %. In all cases the heavy (40K) fraction was capable of carrying out both Photosystem I and II reactions. The reactions employed have been shown to be specific for the respective photoreactions by VERNON AND SHAW[17]. The light fractions carried out very rapid $NADP^+$ reduction but had almost no Photosystem II activity, in agreement with earlier work of SANE *et al.*[10].

It has previously been suggested that the light fraction released by pressure treatment[10] or sonication[8] was derived from the stromal membranes of the chloroplast. We have examined the fractions obtained by 4000 lb/inch$^2$ pressure treatment in our laboratory by both thin-sectioning and freeze-etching techniques for electron microscopy and have obtained essentially the same results as SANE *et al.*[10]. These data show the heavy fraction is comprised mostly of intact grana stacks with few remaining stromal membranes. The light fraction contains only small membranous vesicles which appear to be derived from the stromal lamellae. Examination of fractions obtained at lower pressures also revealed small vesicles in the 144K fraction; the 40K pellet in these cases contained grana stacks contaminated with many more

TABLE I

PHOTOCHEMICAL ACTIVITIES OF FRENCH PRESS-DERIVED CHLOROPLAST FRAGMENTS

Chloroplasts were diluted to 0.5 mg chlorophyll/ml and were passed twice through the cell at the pressure indicated. Chlorophyll recovery in the light fraction was determined by measuring the volume and chlorophyll concentration in the supernatant of the 40000 × *g* step since essentially all of the membranes remaining in the 40 K supernatant were sedimented by high speed centrifugation. Photosystem II assays contained (in μmoles): sucrose 500, sodium phosphate (pH 6.7) 60, DCIP 0.2, and 1,5-diphenylcarbazide 1.0 in a total volume of 2 ml. DCIP reduction was followed at 590 nm. Photosystem I assays contained sucrose, phosphate (pH 6.7), and DCIP in the same concentrations and also included (in μmoles) $NADP^+$ 0.8, sodium ascorbate 15, 0.1 % Triton X-100 and saturating amounts of plastocyanin and a crude ferredoxin preparation in a total volume of 2 ml. $NADP^+$ reduction was followed at 340 nm.

| *Pressure** (*lb/inch²*) | *% Chlorophyll recovered in 144 K fraction* | *$NADP^+$ reduction (Photosystem I activity)*** | | *DCIP reduction (Photosystem II activity)**** | |
|---|---|---|---|---|---|
| | | *40 K fraction* | *144 K fraction* | *40 K fraction* | *144 K fraction* |
| 0 | 0 | 468 | — | 225 | — |
| 500 | 0.8 | 370 | 300 | 196 | 12 |
| 750 | 1.5 | 320 | 665 | 191 | 7 |
| 1000 | 2.9 | 315 | 680 | 184 | 10 |
| 2000 | 3.2 | 310 | 575 | 163 | 6 |
| 4000 | 5.4 | 294 | 530 | 188 | 7 |
| 6000 | 5.2 | 295 | 424 | 198 | 10 |
| 8000 | 5.4 | 322 | 450 | 174 | 20 |

* Pressure in the cell compartment computed from the gauge pressure.
** μmoles $NADP^+$ reduced/mg chlorophyll per h.
*** μmoles DCIP reduced/mg chlorophyll per h.

stromal lamellae remaining attached to the grana membranes. We would concur with the previous authors[10] in suggesting that the light fraction in all cases is derived from fragmentation and release of the stromal lamellae from the heavier stacked membranes.

Since all fractions obtained in Table I showed good electron transport activities, it was of interest to test the photophosphorylation capacities of the various samples. Preliminary experiments indicated that the French pressure treatment caused some reduction in rates of ATP formation. Table II therefore shows the effect of pressure treatment on whole chloroplasts. Both cyclic phosphorylation catalyzed by phenazine methosulfate (PMS) and non-cyclic phosphorylation catalyzed by methyl viologen were inhibited by treatment, with the latter being reduced to a slightly greater extent. The greater loss of non-cyclic activity may be due to inactivation of Photosystem II during treatment.

The effect of pressure treatment on proton uptake by chloroplasts is shown in Fig. 1. The results indicate that both the initial rate and extent of proton transport was inhibited by treatment. The percent inhibition of proton transport can be seen to be slightly greater at various pressures than that of the phosphorylation shown in Table II, although there is a general correlation in the pattern of loss of the two activities.

At this point it was of interest to determine whether both fractions derived by pressure treatment had photophosphorylating activity, and if so, whether either of the fractions was preferentially inhibited by the treatment. The results, shown in

TABLE II

THE EFFECT OF FRENCH PRESSURE TREATMENT ON CYCLIC AND NON-CYCLIC PHOSPHORYLATION

Isolated chloroplasts were diluted to 0.5 mg chlorophyll/ml and passed once through the pressure cell at the pressure indicated. This sample was assayed directly without centrifugation. The cyclic phosphorylation reaction mixture contained (in $\mu$moles): sodium tricine (pH 8.0) 50, KCl 50, $MgCl_2$ 10, $Na_3PO_4$ 10, ADP 4, PMS 0.15, sodium ascorbate 20, and 50 $\mu$g chlorophyll in 3 ml total volume. The non-cyclic phosphorylation reaction mix was identical except that PMS and ascorbate were replaced by methyl viologen and $NaN_3$ (each at 2.5 $\mu$moles/3 ml). Illumination for 2 min was provided by white light at an incident intensity of $5 \cdot 10^4$ ergs·cm$^{-2}$·sec$^{-1}$.

| *Pressure in French pressure cell (lb/inch²)* | *ATP formed ($\mu$moles/mg chlorophyll per h)* | |
|---|---|---|
| | *Cyclic phosphorylation* | *Non-cyclic phosphorylation* |
| 0 | 502 | 292 |
| 500 | 379 | 224 |
| 750 | 369 | 215 |
| 1000 | 376 | 202 |
| 2000 | 328 | 150 |
| 4000 | 233 | 57 |
| 8000 | 190 | 46 |

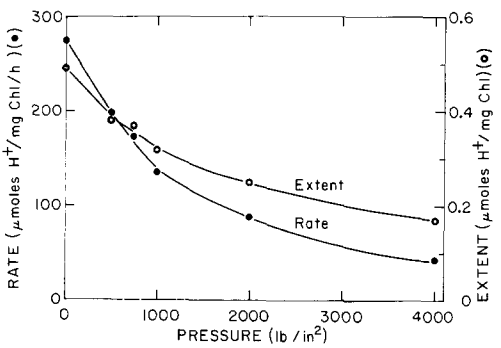


Fig. 1. The effect of French pressure treatment on proton transport in chloroplasts. Pressure treatment was conducted as described in Table I. The assay mixture for proton transport determinations contained (in $\mu$moles): PMS 0.1, KCl 0.33, choline chloride 33, dithioerythreitol 1.2, and 50 $\mu$g chlorophyll in a total volume of 2 ml. The initial pH of each reaction mix was adjusted to pH 6.0. Illumination was by white light passed through a yellow filter (Corning No. 3-69) providing an intensity of $4 \cdot 10^5$ ergs·cm$^{-2}$·sec$^{-1}$. The initial rate and maximal extent of proton transport are plotted.

Table III, show that both the light and heavy fractions can carry out cyclic ATP formation, although the 144K fraction always has higher specific activities. Both types of membrane fragments were reduced in activity to about the same extent with increasing pressure, indicating that there was no selective inactivation of either one of the fractions. The proton transport of the light and heavy fractions was also determined. As is shown in Table IV, the 40K fraction showed some reduction in both rate and extent of ion transport with increasing treatment pressure. The amount of inhibition is very similar to that observed with whole chloroplasts in Fig. 1. The

TABLE III

THE EFFECT OF FRENCH PRESSURE TREATMENT ON CYCLIC PHOSPHORYLATION IN THE GRANA MEMBRANE (40K) AND STROMAL LAMELLAE (144K) FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION

For pressure treatment, chloroplasts were diluted to 0.5 mg chlorophyll/ml and passed once through the pressure cell at the indicated pressure. Reaction conditions for PMS-mediated phosphorylation were as described in Table II.

| *Pressure in French pressure cell (lb/inch²)* | *ATP formed (μmoles/mg chlorophyll per h)* | |
|---|---|---|
| | *40K fraction* | *144K fraction* |
| 0 | 380 | — |
| 500 | 360 | 631 |
| 750 | 287 | 625 |
| 1000 | 258 | 450 |
| 2000 | 194 | 255 |
| 4000 | 96 | 133 |
| 6000 | 50 | 85 |
| 8000 | 39 | 76 |

TABLE IV

THE EFFECT OF FRENCH PRESSURE CELL TREATMENT ON PROTON TRANSPORT IN THE GRANA MEMBRANE (40K) AND STROMAL LAMELLAE (144K) FRACTIONS

Pressure treatment was as described in Table III, and reaction conditions were as in Fig. 1.

| *Pressure in French pressure cell (lb/inch²)* | *Initial rate of $H^+$ uptake (μmoles $H^+$/mg chlorophyll per h)* | | *Extent of $H^+$ uptake (μmoles $H^+$/mg chlorophyll)* | |
|---|---|---|---|---|
| | *40K fraction* | *144K fraction* | *40K fraction* | *144K fraction* |
| 0 | 280 | — | 0.50 | — |
| 500 | 200 | <17 | 0.43 | <0.10 |
| 750 | 142 | <13 | 0.41 | <0.10 |
| 1000 | 130 | <25 | 0.40 | <0.12 |

144K fraction, however, did not show any detectable $H^+$ transport in any of the fractions obtained at different pressures. Since the buffer capacity of the sample was high due to large amounts of contaminating soluble protein, the values expressed in the table give the lowest level of activity which could have been detected.

The lack of an active proton uptake in the light fraction is also indicated by the relative insensitivity of phosphorylation in this sample to the uncoupling action of $NH_4Cl$ (which has been suggested to dissipate the proton gradient by uptake of $NH_4^+$ (ref. 18)). In Fig. 2, the uncoupling action of a range of $NH_4Cl$ concentration is plotted for both the light and heavy fractions obtained by French pressure treatment. It should again be noted that the specific activity of the 144K fraction was approximately twice that of the 40K fraction. The rate of formation of ATP in the light fraction even in the presence of 10 mM $NH_4Cl$ was therefore greater than the control phosphorylation in the heavy fraction.

The effect of various other uncoupling compounds on ATP formation in the two fractions is shown in Table V. The demonstration of a reduced proton pump in the

144K fraction is consistent with the observations that nigericin and gramicidin, which are thought to dissipate the proton gradient by allowing ion exchange[5,19], are less effective in inhibiting phosphorylation in the 144K fraction. Dio-9, atebrin, *m*-chlorocyanocarbonyl phenylhydrazone (CCCP) and valinomycin in the presence of $NH_4Cl$ were found to act as effective inhibitors of the ATP formation in both fractions, although valinomycin + $NH_4Cl$ was less effective in the stroma preparation.

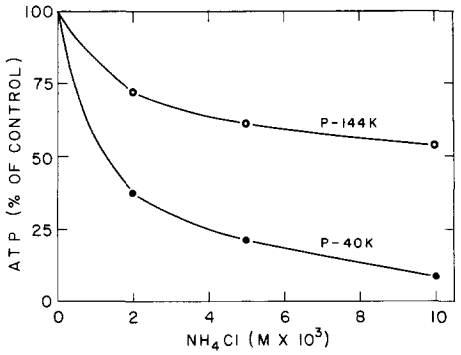


Fig. 2. The effect of $NH_4Cl$ on cyclic phosphorylation in grana membranes (40 K fraction) and stromal lamellae (144K fraction). Particles were prepared as described in Table III using a pressure of 750 lb/inch$^2$. Reaction conditions for PMS-mediated phosphorylation were as in Table II. Control rates of ATP formation were 700 and 330 $\mu$moles ATP/mg chlorophyll per h for the 144K and 40 K fractions, respectively.

TABLE V

SENSITIVITY OF PMS-MEDIATED PHOSPHORYLATION IN THE 40K AND 144K FRACTIONS TO VARIOUS INHIBITORS OF ATP FORMATION

Membrane fractions were prepared as described in Table III using a pressure of 750 lb/inch$^2$. The reaction mix was as described in Table II. Control rates of phosphorylation ($\mu$moles ATP formed/mg chlorophyll per h) were 645 for the 144K fraction and 368 for the 40K fraction.

| *Inhibitor* | *Concn.* | *ATP formed (% of control)* | |
|---|---|---|---|
| | | *40K fraction* | *144K fraction* |
| Nigericin | 0.1 $\mu$M | 8 | 49 |
| Gramicidin-D | 0.7 $\mu$M | 5 | 49 |
| Dio-9 | 20 $\mu$g/ml | 1 | 5 |
| CCCP | 20 $\mu$M | 2 | 3 |
| Atebrin | 17 $\mu$M | 4 | 4 |
| Valinomycin | 1 $\mu$M | 85 | 88 |
| Valinomycin + $NH_4Cl$ | 1 $\mu$M<br>1.5 mM | 28 | 44 |

One final assay was employed to determine the relative contribution of a proton gradient to phosphorylation in the two French press-derived membrane fragments. Since JAGENDORF AND URIBE[16] have shown dark ATP formation in acid–base transition experiments with whole chloroplasts, we followed their assay procedures for each of our two fractions. As is shown in Table VI, both whole chloroplasts and the 40K fraction demonstrated dark formation of ATP, although the latter sample had

TABLE VI

ATP FORMATION BY THE 40K AND 144K FRACTIONS IN THE DARK DUE TO ACID–BASE TRANSITION

Chloroplasts were treated as described in Table III at a pressure of 750 lb/inch$^2$. The fractions collected by centrifugation were resuspended in 10 mM NaCl. The reaction mixture contained 150 $\mu$g chlorophyll and 5 mM succinate at either pH 4.0 or 8.4 in a total volume of 0.9 ml for the initial stage. This was incubated for 20 sec and then injected into 0.9 ml containing 0.11 M tricine (pH 8.4), 5.5 mM $MgCl_2$, 0.22 mM ADP, 2.2 mM inorganic phosphate containing $^{32}P$ and 4.4 $\mu$M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The reaction was stopped after 20 sec with 0.2 ml of 30% trichloroacetic acid.

| *Sample* | *ATP formed (nmoles/mg chlorophyll) at pH of initial stage* | |
|---|---|---|
| | *4.0* | *8.4* |
| Control chloroplasts | 154 | 1.0 |
| 40 K fraction (grana membranes) | 74 | 1.0 |
| 144 K fraction (stromal lamellae) | 2 | 1.0 |

reduced activity presumably due to inactivation during pressure treatment. The 144K fraction, however, exhibited almost no incorporation of $^{32}P$ label into ATP.

Since our own structural studies and the earlier work of SANE *et al.*[10] have demonstrated that the 144K fraction is derived from stromal lamellae within the chloroplast, we chose to further investigate phosphorylation and proton transport in various types of chloroplasts which had different percentages of grana and stromal membranes. The simplest means of doing this was to isolate chloroplasts from leaf tissue of different ages, as suggested to us by Dr. R. B. Park. This was accomplished by selecting leaves from different portions of a head of Romaine lettuce. Yellowish,

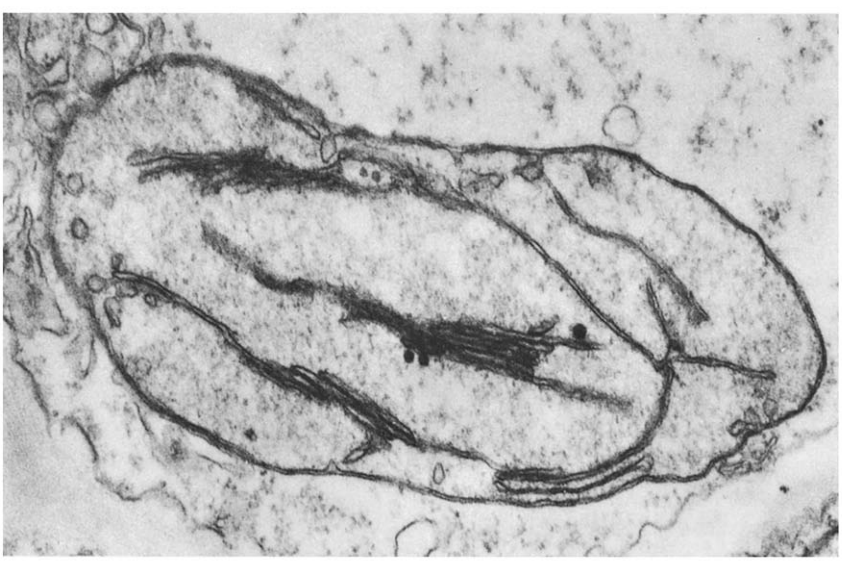

Fig. 3. A portion of an immature lettuce leaf showing a young chloroplast containing few grana stacks. 30550 × magnification.

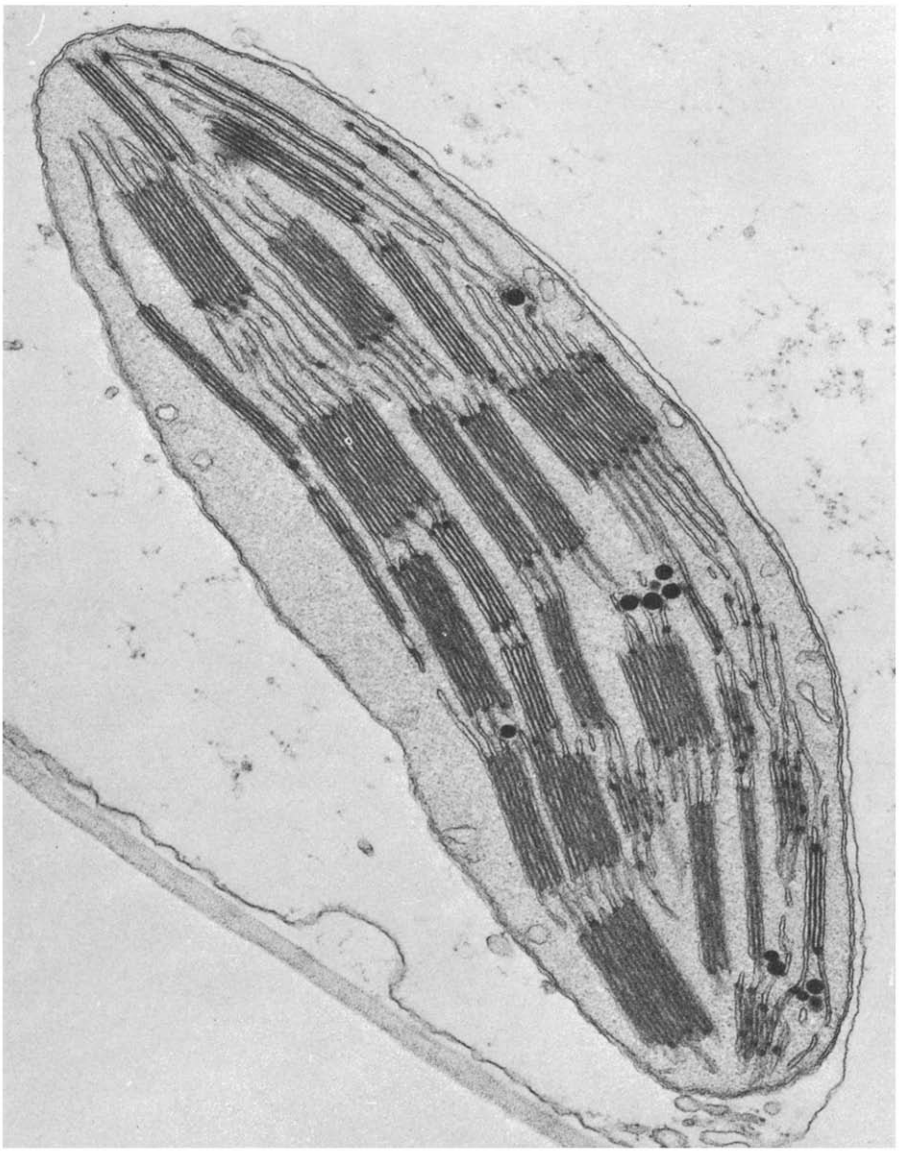

Fig. 4. A portion of a mature lettuce leaf showing a fully developed chloroplast containing large and numerous grana stacks. 44 200 × magnification.

small (less than 4 inches length) leaves were collected from the center of a tight head of lettuce for isolation of young, immature chloroplasts. These were compared to the older, mature plastids isolated from the dark green outer leaves. Representative samples of the types of chloroplasts were examined by thin sectioning for electron microscopy. In general, the most obvious difference in the older chloroplasts was an increase in number and size of grana stacks, and a relative decrease in the percentage of membrane material in the stromal membranes (see Figs. 3 and 4). Additional

evidence for the greater amount of stromal lamellae in the young plastids was obtained by subjecting the two samples to the French press procedure described above for spinach chloroplasts. In all cases, two to three times more chlorophyll was recovered in the 144K fraction from the young plastids. This is in agreement with SESTAK[20] who found a greater release of photosystem I particles from chloroplasts isolated from the younger leaves than from older leaves of spinach and radish. The younger leaves of lettuce also have a higher chlorophyll *a/b* ratio, again in agreement with the presence of larger amounts of stromal lamellae which have higher *a/b* ratios.

The $NH_4Cl$ sensitivity of photophosphorylation in young and old lettuce leaf chloroplasts shows that where there are more stromal lamellae (in young leaves) the ATP synthesis is more resistant to $NH_4Cl$ (Fig. 5). There is about 15 % of the control activity which is resistant to 15 mM $NH_4Cl$ in young leaf chloroplasts compared to less than 3 % in the old leaf chloroplasts. This is consistent with our finding that the younger leaf chloroplasts always exhibited higher specific activities of phosphorylation but had the same or lower "proton pump" activities when compared with old leaf plastids.

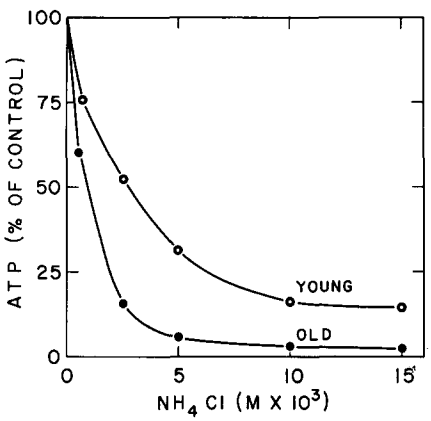


Fig. 5. The effect of $NH_4Cl$ on PMS-mediated phosphorylation in chloroplasts isolated from young and old leaves of lettuce. Conditions for assay were as in Table II. Control rates of ATP formation were 545 and 484 $\mu$moles/mg chlorophyll per h in the young and old leaf chloroplasts, respectively.

Chloroplasts from various plants with the $C_4$-dicarboxylic acid pathway of photosynthesis have been shown to have dimorphic plastids[21,22]. The chloroplasts of the mesophyll cells have the normal grana structures with some stromal lamellae whereas the plastids in the bundle sheath cells exhibit mainly unpaired lamellae with little or no grana formation. WOO *et al.*[11] have recently discovered that the lack of grana formation is correlated with a Photosystem II deficiency in the bundle sheath cells. We have used a modification of their procedure to isolate the two types of plastids from sorghum and have characterized them with regard to phosphorylation and ion transport. The results of these experiments are tabulated in Table VII. Electron transport assays for the two samples revealed that the bundle sheath plastids had very low Photosystem II activity as compared with those chloroplasts from the mesophyll cells. The bundle sheath chloroplasts demonstrated a higher rate of Photosystem I activity than the mesophyll plastids, and also showed a higher chlorophyll

TABLE VII

CHARACTERIZATION OF MESOPHYLL AND BUNDLE SHEATH CELL CHLOROPLASTS FROM SORGHUM

Photosystems I and II assays were conduced as described in Table I, cyclic phosphorylation with PMS and non-cyclic with methyl viologen as in Table II and proton uptake measurements as in Fig. 1.

| *Sample* | *Chloro-phyll a/b ratio* | *Photo-system II activity** | *Photo-system I activity*** | *ATP formed (μmoles/mg chlorophyll per h)* | | *Proton transport* | |
|---|---|---|---|---|---|---|---|
| | | | | *Cyclic* | *Non-cyclic* | *Initial rate (μmoles $H^+$/mg chlorophyll per h)* | *Extent (μmoles/ mgchlorophyll* |
| Mesophyll chloroplasts | 2.84 | 271 | 180 | 614 | 237 | 131 | 0.48 |
| Bundle sheath chloroplasts | 3.94 | 39 | 426 | 570 | 23 | 18 | 0.05 |

* Photosystem II activity: μmoles DCIP reduced/mg chlorophyll per h.
** Photosystem I activity: μmoles $NADP^+$ reduced/mg chlorophyll per h.

*a/b* ratio. These findings are all in agreement with the data recently published by Woo *et al.*[11].

Phosphorylation activity in the two sorghum samples also reflected an unequal distribution of Photosystem II. Non-cyclic phosphorylation catalyzed by methyl viologen, which requires complete electron transport through both photosystems, was ten fold more active in the mesophyll plastids than in the bundle sheath cells although cyclic phosphorylation mediated by only Photosystem I was approximately the same in the two types of plastids. It should be noted that the preparative procedure for isolation of the bundle sheath plastids was much longer and less gentle than for the mesophyll chloroplasts. The specific activities for ATP formation in the former may therefore be slightly reduced due to partial uncoupling during the isolation procedure. The true comparative values for cyclic phosphorylation in the bundle sheath plastids might therefore be somewhat higher than those for the mesophyll cells if conditions of identical isolation were available. However, in spite of the fact that PMS-catalyzed photophosphorylation was approximately equal in both samples, proton transport mediated by PMS was strikingly different. Again both the rate and extent of proton uptake was reduced in the plastids having fewer grana stacks. The values for $H^+$ transport reported here were determined at pH 7.0 since this was optimal condition for assay. The same pattern was also observed at higher and lower pH values. Sensitivity to uncoupling action by a variety of uncouplers was also tested with both samples. As can be seen in Fig. 5, the mesophyll chloroplasts were much more sensitive to the action of $NH_4Cl$ than were the bundle sheath plastids; again in agreement with the reduced proton pump activity of the latter. It was also observed that the bundle sheath cells were more resistant to the action of nigericin and gramicidin-D but not Dio-9 or CCCP just as was shown for French press-derived 144K fraction particles in Table V.

In our examination of plastids with varying extents of grana formation, we have also tested phosphorylation in chloroplasts isolated at various times after transfer of dark-grown corn seedling into the light. We have previously shown[12] that PMS-catalyzed photophosphorylation can be detected after only 1 h of illumination of etiolated seedlings but grana formation cannot be detected prior to 4 h.

Since the plastid samples have high buffer capacity due to low chlorophyll to protein content, it was impossible to directly assay low levels of proton movement. We have therefore tested the sensitivity of chloroplasts isolated from 2-, 4-, 6- and 72-h illuminated seedlings to uncoupling by $NH_4Cl$. The specific activity of each sample and the changing pattern of sensitivity to the uncoupler are shown in Table VIII.

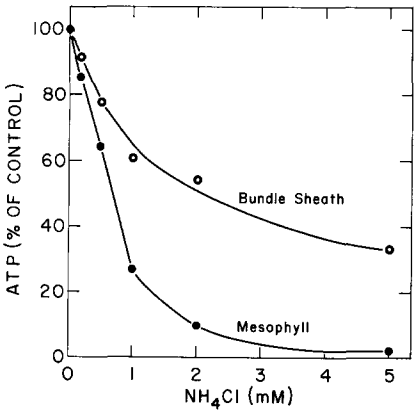


Fig. 6. The effect of $NH_4Cl$ on PMS-mediated phosphorylation in mesophyll and bundle sheath chloroplasts from sorghum. Reaction conditions were as described in Table II. Control rates of ATP formation ($\mu$moles formed/mg chlorophyll per h) were 570 for bundle sheath and 614 for mesophyll plastids.

TABLE VIII

THE EFFECT OF $NH_4Cl$ ON CYCLIC PHOSPHORYLATION IN GREENING MEMBRANES OF *Zea mays* CHLOROPLASTS

Conditions for the PMS-catalyzed phosphorylation were as described in Table II. The time of illumination indicated in the table refers to the duration of constant illumination received by the dark-grown seedlings prior to grinding of the leaves for plastid isolation.

| *Time of illumination (h)* | *ATP formed ($\mu$moles/ mg chlorophyll per h)* | *ATP formed in the presence of $NH_4Cl$ (% of control)* | |
|---|---|---|---|
| | | *+2.5 mM $NH_4Cl$* | *+5.0 mM $NH_4Cl$* |
| 0 | 0 | — | — |
| 2 | 78 | 86 | 51 |
| 4 | 164 | 66 | 43 |
| 6 | 175 | 43 | 27 |
| 72 | 254 | 6 | 2 |

Since there was some variability in activity of the samples isolated on different days, each value reported is the average of several individual experiments. The data reported here showing increased uncoupling action by $NH_4Cl$ over longer times of illumination are consistent with a gradual increase in proton pumping activity during membrane differentiation. This onset of proton transport roughly parallels the time of appearance of grana stacks in corn seedlings[12].

## DISCUSSION

The main point of this paper is that stromal lamellae, isolated by a gentle method without the use of detergents, showed high rates of ATP formation, but had little

or no $H^+$ uptake activity. The lack of proton uptake could not have been due to the French Press treatment *per se*, since the grana fraction derived from the separation did show active proton uptake (Table IV).

A major question was whether the differences observed could be in some way an artifact of the isolation procedure. This problem was somewhat complex since higher pressure treatment itself was found to inhibit both phosphorylation and proton transport in the whole chloroplast preparations. Two lines of evidence suggest that specific inactivation of the proton pump in the 144K fraction did not occur. The first indication is that the inactivation of both ATP formation and $H^+$ transport in the original chloroplast preparation is a function of increasing pressure during treatment (Table II and Fig. 1). The decrease in phosphorylation with increasing pressure was also observed in both the light and heavy fractions obtained by differential centrifugation (Table III). By contrast, however, a gradual decrease in the proton pump with increasing pressure was only observed with the 40K fraction (Table IV). The 144K fraction did not show any detectable proton transport at any of the pressure treatments employed indicating that these stromal membranes did not have an active proton pump even prior to their release and isolation.

Evidence for a decreased level of proton transport in stromal lamellae also came from comparative studies of several different types of plastids having different proportions of stacked *vs.* unstacked membranes. Comparison of phosphorylating activity, proton uptake, and uncoupler sensitivity in mesophyll and bundle sheath chloroplasts from sorghum, and in developing corn plastids revealed a striking correlation between the occurrence of grana stacks and high proton transport activity or high sensitivity to uncoupling by proton gradient dissipating agents such as $NH_4Cl$ (Tables VII and VIII). Young leaf chloroplasts from lettuce have a higher proportion of stroma compared to grana membranes than chloroplasts from older leaves as determined by thin sectioning of stained material and they also are more resistant to $NH_4Cl$ uncoupling (Fig. 5).

A consistent pattern has been revealed by these studies; it is that unpaired stromal membranes are deficient in proton transport and accumulation. Generally these same membranes are devoid or deficient in Photosystem II activity and cytochrome 559 (refs. 10–12). It is not clear whether the lack of proton uptake is more closely correlated to the pattern of membrane organization in the plastid or the presence or absence of some electron transport component associated with Photosystem II. The stroma membranes are therefore similar to the digitonin subchloroplast particles[7] and sonicated chloroplast fragments[6], none of which show the close correlation between $H^+$ uptake and phosphorylation exhibited by intact chloroplasts (intact is not used here to indicate the presence of the outer membrane). The stromal membrane preparation did not exhibit acid-base-induced phosphorylation[16] (Table VI), an observation consistent with their lack of light-induced proton movement. Digitonin subchloroplast particles also have much reduced acid-base phosphorylation and light dependent proton uptake[7]. Since subchloroplast preparations which have little capacity for $H^+$ transport show little ATP formation by the acid-base transition, it may be that a proton gradient is not involved as the driving force for phosphorylation in such preparations.

This issue is not entirely clear, however, since McCARTY[23] has shown that $H^+$ uptake and post illumination phosphorylation were both stimulated by valinomycin

+ $K^+$ in digitonin subchloroplast particles. McEVOY AND LYNN[24] found that digitonin subchloroplast particles gave a neutral red dye response indicative of an internal acidification, although the external pH changes were barely detectable. The neutral red response may not be strictly diagnostic of just internal pH changes, but if it is, then one would have to assume that some membrane parameter is altered during preparation of the subchloroplast particles so as to cause the loss of capacity to hold large amounts of protons within the membrane, but that the proton transport mechanism still operates. The similarities of the stroma lamellae vesicles and the digitonin subchloroplast particles, could mean that the above interpretation also holds for the stromal lamellae.

If counter ion (*i.e.* $K^+$) exchange or co-ion (*i.e.* $Cl^-$) uptake could not occur in stroma or digitonin subchloroplast particles to as great an extent as they may in intact chloroplasts[25], then an electrogenic $H^+$ uptake would develop a large electrical potential with a barely detectable external pH change (*e.g.* about 1 $H^+$ per 40 chlorophylls could generate a poteintial of about 300 mV if the charges were not neutralized[4]). The inhibition of phosphorylation in the stroma vesicles by $NH_4^+$ + valinomycin and the lack of inhibition by $NH_4Cl$ alone (Table V) is consistent with a membrane potential being generated in the light. Diffusion of $NH_3$ into the vesicles would lead to $NH_4^+$ formation and the maintenance of the positive inside potential. Addition of valinomycin would make $NH_4^+$ much more permeable, leading to a rapid collapse of the electrical gradient[25].

It is an intriguing and unanswered question as to why intact chloroplasts and certain subchloroplast vesicles show such great differences in the correlation of massive proton uptake to the phosphorylation mechanism. We cannot lightly dismiss the large proton uptake in chloroplasts as a "side reaction," for if it were $NH_4Cl$ or nigericin + $K^+$ should not be inhibitory to ATP synthesis, assuming their sole action is to dissipate a proton gradient. It can also be argued by similar reasoning that the massive proton uptake must do more than generate an electrical potential. Thus, an hypothesis made earlier[26,4] is still attractive, that for grana regions internal proton accumulation provides for the protonation of certain chemical groups on macromolecules which are required to be in the protonated state for the ATP synthesis mechanism to operate. Changing the degree of protonation of fixed charges could lead to large changes in the conformation of membrane macromolecules. It may be that stroma regions normally have this protonated state or conformation at all times and that digitonin or sonic prepared subchloroplast particles have such a state induced during the preparation. In any event, the various subchloroplast membrane preparations should provide valuable tools in the continued attempts to elucidate the mechanism of energy transduction.

One final overall question unanswered by this and previous investigations concerns the basic pattern of membrane organization in the chloroplasts. Why do the stromal lamellae contain only Photosytem I, and why is their energy conserving mechanism apparently different in that there is much less proton accumulation? An obvious physiological correlation may be made by assuming that the stromal membrane cyclic phosphorylation produces the "extra" ATP needed to complete the Calvin–Benson cycle requirements (see ref. 27 for a discussion of this). This point does not touch on the underlying reason for differences between the stroma and grana membranes. One possible reason is suggested by the several points of similarity

between stromal lamellae and plastid membranes during the early stages of greening. Both membranes have high chlorophyll *a/b* ratios, have little or no cytochrome 559, have only Photosystem I activity (cyclic but not non-cyclic phosphorylation)[12], and do not have an active proton pump. It could be suggested that the stromal membranes are immature or incomplete in terms of overall chloroplast membrane differentiation. Additional research is necessary to clarify this critical question.

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