Uncoupling Phosphorylation in Spinach Chloroplasts by Absence of Cations [1, 2, 3]

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During attempts to prepare active fragments of spinach chloroplasts, we observed major losses of phosphorylative activity following suspension of the chloroplasts in water. This damage was traced to treatments which probably had the effect of removing cations from the chloroplasts, rather than to a simple physical disruption. Indeed, chloroplast fragments have been used successfully in studies of phosphorylation for a considerable time by now (3, 18). Evidence for the basis of the observed damage and characteristics of the inactivated chloroplasts will be described below.

Materials & Methods

Spinach leaves were ground for 15 seconds at 75 v in a Waring Blendor, in 0.40 M sucrose, 0.02 M tris[4] pH 8.0, and 0.01 M NaCl; or in the same buffer with the addition of EDTA to 0.005 M. Chloroplasts were collected by centrifuging and were washed once as described previously (7). Following the wash, the chloroplast pellets were resuspended in various test media as indicated in the text (either distilled water, more sucrose-tris-NaCl, or various low concentrations of salts, etc.). Control chloroplasts resuspended in sucrose-tris-NaCl were not handled further; experimental series were centrifuged in the Servall at 15,000 × *g* for 15 minutes. The pellets from the Servall centrifugation were finally resuspended in sucrose-tris-NaCl in all cases. All suspensions were adjusted to 0.015 mg of chlorophyll per 0.1 ml. Chlorophylls *a* plus *b* were determined by the method of Arnon (2).

All standard assays for phosphorylative activity were performed in 0.5 ml final volume, containing in μmoles: pH 8.0 tris, 3.33; NaCl 11.5; pH 8.0 ADP 1.65; $MgCl_2$ 3.30; PO_4 1.65, and phenazine methosulfate 0.008. Chloroplasts containing 0.015 mg of chlorophyll were used in each vessel. Light was provided by a row of incandescent spot lamps, with 5,000 ft-c at flask height. The reaction was run for 5 minutes at 15 C, under nitrogen atmosphere, in 25 ml Erlenmeyer flasks. Residual phosphate was measured by the method of Taussky and Shorr (17).

Light intensities were varied, where indicated, by wrapping each flask with a different piece of wire screening (purchased from C. O. Jeliff Mfg. Corp., Southport, Conn.) The percentage transmission through each screen was measured both in a Beckman spectrophotometer and with the use of a thermopile. In the light intensity series phosphorylation was measured with the aid of P^{32} as described previously (9).

Reduction of ferricyanide was carried out in a reaction mixture of 3.0 ml final volume containing 0.040 mg of chlorophyll and, in μmoles: pH 8.0 tris, 40; NaCl 70, and potassium ferricyanide 1.5. Other additions are indicated in the tables. Reactions were run in test tubes, exposed to 5,000 ft-c of light from an incandescent lamp shielded by a water bath, for 2 minutes at room temperature. For the pH series the concentration of tris was increased to 150 μmoles per 3 ml, and all reaction mixtures contained 150 μmoles of potassium phosphate. After illumination 0.3 ml of 20 % trichloroacetic acid was added, precipitated proteins removed by centrifuging, and the optical density determined at 420 mμ to measure residual ferricyanide. This procedure is preferable to measuring the optical density at 420 mμ directly in the reaction mixture before and after illumination, as described previously (4). With the previous procedure it has been possible to detect some artifacts due to chloroplast aggregation. In particular, an increase in density at 420 mμ is seen when 10^{-3} M Mn^{++} is added to chloroplasts, and this is prevented by adding ADP, Pi, and Mg (J. S. Kahn, unpublished experiments). These optical density changes were found to be non-enzymatic, non-light dependent, and caused by chloroplast aggregation.

ADP and ATP were purchased from Pabst; phenazine methosulfate from Sigma. Pyocyanine was

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[4] Abbreviations used in this paper include: tris for tris-(hydroxymethyl)aminomethane; ADP for adenosine diphosphate; EDTA for ethylenediamine tetraacetic acid; ATP for adenosine triphosphate.
[5] A preliminary report of this work has appeared in Plant Physiol. 36 suppl: xi. 1961.

Table I

Phosphorylation by Chloroplasts Exposed to Water

Buffer used in Grinding & washing	Phosphorylation rate µmoles P/mg chlorophyll/hr	
	Whole chloroplasts	Water treated
NaCl-tris pH 8	410	750
NaCl-tris pH 7.3	450	550
Sucrose-tris-NaCl pH 8	575	745
Sucrose-tris-NaCl-EDTA pH 8	645	0

NaCl was 0.35 M, tris 0.02 M, sucrose 0.40 M; NaCl with the sucrose media was 0.01 M, and EDTA 0.005 M. The assay was run with pyocyanin as cofactor, for 5 minutes in air at room temperature; other conditions as in the standard assay described under Materials & Methods.

prepared by illumination of phenazine methosulfate followed by repeated extractions into chloroform (when alkaline) and back into acidified water.

Results

Our first attempts at breaking chloroplasts in water resulted in severe losses of the ability to phosphorylate (table I). This effect was dependent upon the prior use of EDTA in the buffers for grinding the leaves and washing the chloroplasts. Other experiments showed that EDTA was effective at concentrations as low as 1×10^{-3} M.

Table II

Damaging Chloroplasts by Exposure to Water Without Previous EDTA Treatment

g leaf/ml of grinding solution	Grinding medium	Phosphorylation rate µmoles P/mg chlorophyll/hr	
		Expt. 1	Expt. 2
1/2	Buffer	300	520
1/4	"	200	360
1/10	"	90	120
1/20	"	90	140
1/20	Homogenate supernatant	280	320
1/20	Boiled homogenate supernatant	325	380
Control, whole chloroplasts		400	500

100 ml of fluid were used in the Waring Blendor in each case, and the weight of leaves was varied from 5 to 50 g. The buffer contained 0.40 M sucrose, 0.02 M tris, and 0.01 M NaCl, but no EDTA. All chloroplasts except the controls were washed once, resuspended in water, then collected by centrifuging at 10,000 × *g*.

The homogenate supernatants were obtained from a previous batch of leaves, ground in EDTA-free buffer at a ratio of 1 g leaf/2 ml buffer.

Other factors which could be varied without effect on sensitivity to water included: grinding the leaves for 60 seconds rather than 10 in the Waring Blendor, or addition of an anti-foaming reagent during grinding.

Chloroplast preparation with EDTA is not an absolute requirement for this type of damage, however. In table II it is shown that suspension in water also leads to an inhibition of phosphorylation if the spinach leaves are originally ground in a *large* volume of EDTA-free buffer. As the ratio of buffer to leaves reaches 10:1 on a weight basis, the resulting chloroplasts are inhibited after suspension in water. It was interesting that dilution of isolated and once centrifuged chloroplasts in amounts of water up to 60 times the weight of the original leaves did not lead to inhibition if the original homogenate had been a concentrated one. When water-resistant chloroplasts were washed repeatedly in EDTA-free buffer, a partial sensitivity to water was induced (table III).

Table III

Effect of Repeated Washes in EDTA-Free Buffer on Sensitivity to H_2O

	No. of washes	Phosphorylation rate µmoles P/mg chlorophyll/hr		% of control
		Control	H_2O treated	
Experiment 1	1	590	645	110
	3	645	470	73
	5	670	362	54
Experiment 2	1	595	535	90
	3	545	400	73
	5	655	181	38

Whole chloroplasts were washed the number of times shown in EDTA-free buffer, then suspended in water or in more buffer. Phosphorylation was assayed as under Materials & Methods.

From these effects of dilution in the homogenate vs. dilution of isolated chloroplasts it is suggested that the original homogenate contained a factor which changed chloroplasts so that they became resistant to subsequent water extraction. This was confirmed by homogenizing leaves in a large volume of supernatant fluid from a previously prepared homogenate (table II). In this case, in spite of the large dilution on homogenization the resultant chloroplasts were relatively resistant to water extraction. A boiled supernatant was equally effective (table II) and in other experiments the homogenate soluble factor was present in the ash. From this, the stabilizing factor in concentrated homogenates appears to be an inorganic salt.

There are, thus, two ways to prepare chloroplasts susceptible to damage by water extraction: grinding leaves in a large volume of EDTA-free buffer, or grinding at any dilution in a medium containing EDTA. If chloroplasts prepared in either of these two ways are suspended in a dilute salt solution instead of in water, the damage is to a large extent, or even completely, averted (see table IV for some typical experiments). The salt will be said to pro-

Table IV

Protecting Phosphorylation Activity of EDTA-Washed Chloroplasts by Salts Added to Low Ionic Strength Medium

Concentration of salt	% of Control rate			
	$MgCl_2$	$BaCl_2$	NaCl	Tris-Cl
0	6	18	16	...
10^{-6} M	...	22	...	...
10^{-5} M	6	14	...	...
10^{-4} M	84	103	...	...
10^{-3} M	85	98	0	8
10^{-2} M	...	...	92	76
5×10^{-2} M	...	...	93	107
Control rate	615	730	820	770

Rate of control chloroplasts shown in μmoles phosphate esterified per mg chlorophyll per hour. Procedure and assay as described in Materials & Methods.

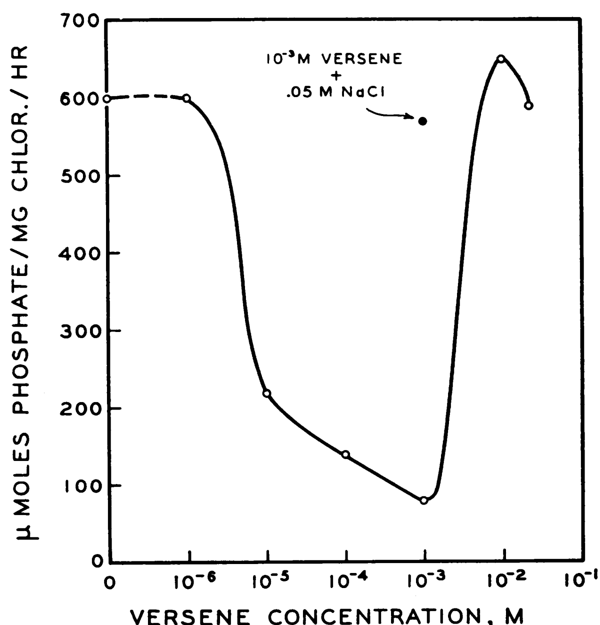

Fig. 1. Phosphorylation of washed chloroplasts pretreated with EDTA. Chloroplasts were prepared in an EDTA-free medium, then suspended in EDTA, pH 8, at the concentrations shown. Chloroplast fragments were collected and assayed in the absence of EDTA, as described in Materials & Methods. The single point represents chloroplasts suspended in a solution of both 10^{-3} M EDTA and 0.05 M NaCl.

tect the chloroplasts if the subsequent rate of phosphorylation is 75 % or more of the control rate shown by whole, untreated chloroplasts. A failure to protect will be defined by a phosphorylation rate 33 % or less than that of the control.

By this criterion the following salts have a protective action at 10^{-4} M or below: $MgCl_2$, $MnCl_2$, $BaCl_2$, $CaCl_2$, $SrCl_2$, $CdCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, and the hydrochlorides of spermine and spermidine. Protection is thus not a specific function. While most chloroplasts prepared with EDTA were protected by 10^{-4} M solutions of these salts, a few preparations appeared to be protected by 10^{-5} M as well.

Water-sensitive chloroplasts prepared by grinding leaves in a large volume of EDTA-free buffer were protected by the same series of salts and by the two polyamines. In general, however, these chloroplasts appeared to require only about a tenth the concentration of salts for protection that the EDTA-exposed chloroplasts did. This differential was noticeable with chloroplasts prepared from the same package of spinach on the same day.

Monovalent salts gave protection at 10^{-2} M but not at 10^{-3} M or below. This category included NaCl, KCl, tris-chloride (pH 8.0), ethylamine chloride, ammonium chloride, and sodium versenate (pH 8.0). Sodium chloride re-crystallized from hydrochloric acid was just as effective as the original reagent grade salt; so it is not likely that the effect of the monovalent salt is due to a 1 % contamination with a divalent cation.

The dual effect of EDTA is illustrated strikingly in figure 1. Chloroplasts were prepared in EDTA-free buffer solutions, then suspended either in water (which in this case was not damaging) or in increasing concentrations of EDTA. Phosphorylation was measured after removal of chloroplasts from the test solution. It can be seen that 10^{-6} M EDTA had no effect; 10^{-5} to 10^{-3} M EDTA caused increasing damage; but that 10^{-2} and 5×10^{-2} were no longer damaging.

Although a large number of ionic materials had a protective effect, sucrose and mannitol were ineffective even at the hypertonic concentrations of 0.6 M (table V).

Although damaged in their ability to phosphorylate, water-treated chloroplasts show no loss of ability to perform a Hill reaction. Reduction of either trichlorophenol-indophenol dye (at pH 7.2) or of ferricyanide (at pH 8.0, with no additions) is, indeed, more rapid with the water-treated particles than with those protected by $MgCl_2$ (table VI). Mg-protected particles show a typical coupling phenomenon—a more rapid rate of ferricyanide reduction when ADP, PO_4,

Table V

Failure of Hypertonic Sugar Solutions to Give Protection

Concentration	% of Control rate	
	Sucrose	Mannitol
0	19	21
0.2 M	22	21
0.4 M	21	25
0.6 M	5	26
Control rate	810	770

Experimental conditions as shown under table IV and in Materials & Methods.

and Mg are present than without, and an inhibition of the rate by ATP (4, 5, 8, 9). The water-treated particles have a high rate of reduction to begin with; this is not increased by adding the phosphorylating reagents. At the same time ATP is synthesized by properly supplemented Mg-protected particles, whereas the water-treated particles are virtually incapable of phosphorylation.

Ferricyanide reduction described above was measured at pH 8.0. The pH curves for these different types of particles are shown in figure 2. Both unbroken chloroplasts and the Mg-protected particles have a sharp optimum at about pH 8.0 in tris-PO_4 buffer (the exact position of the peak has varied from one day to another, between 7.8 & 8.3). With pyrophosphate buffer the optimum is shifted to a more alkaline range. No matter what the buffer, the pH curve for damaged particles has become much broader, and apparently shifted somewhat to the more acid range (between 6.5 & 7.75). The maximal rate with water-treated particles is a great deal higher than those of unbroken chloroplasts or of Mg-particles

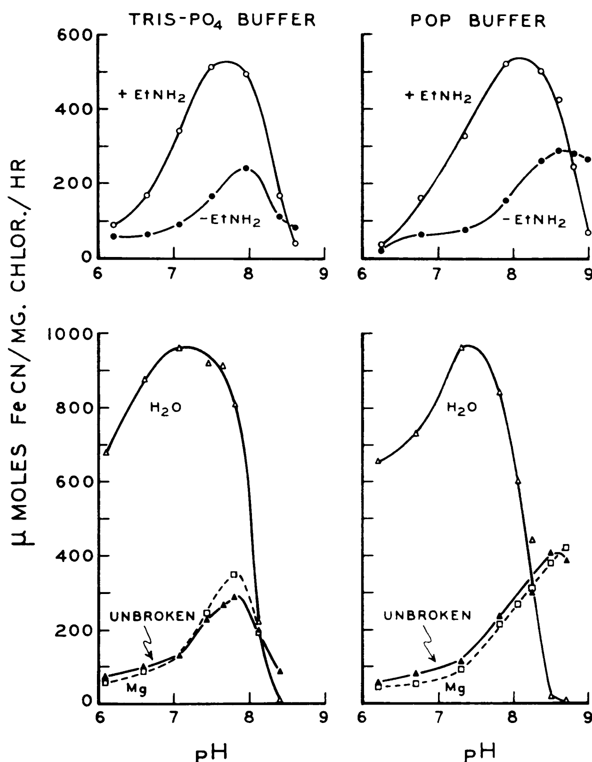

Fig. 2. pH Response of ferricyanide reduction. Bottom set of curves shows the response using either whole chloroplasts or fragments broken in H_2O or in 3 × 10^{-4} M $MgCl_2$. Curves on top are for whole chloroplasts, in the presence or absence of 3 × 10^{-3} M ethylamine. Curves to the right were run in pyrophosphate buffer, 0.033 M final concentration; those to the left in 0.033 M tris—0.033 M phosphate. pH's Were measured in the reaction mixture. Other conditions described in Materials & Methods.

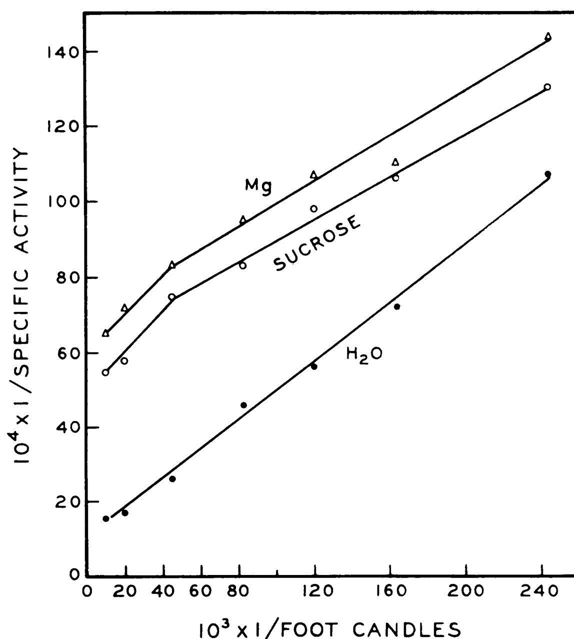

Fig. 3. Rate of ferricyanide reduction as a function of light intensity. Light intensity is shown as reciprocal of the percentage transmission of various filters used. The intensity with no filter (100 % transmission) was approximately 5,000 ft-c, the light source being a beam from a tungsten lamp passed through 5 cm of water.

(the maximal rate actually observed here is equivalent to a Qo_2^{Chl} of about 5,500). It is also of interest that ferricyanide reduction by water damaged particles is actually inhibited above pH 8.3, compared to the controls.

For comparison, figure 2 includes pH curves of normal chloroplasts uncoupled by 3 × 10^{-3} M ethylamine. This reagent also activates ferricyanide reduction in the absence of phosphorylation (8). The shape of the pH curves appears not to be altered by ethylamine in tris-PO_4 buffer, but is different from the control in pyrophosphate buffer.

The increased rate of ferricyanide reduction is much more pronounced at high than at low light intensities. The double reciprocal plot (1/velocity vs. 1/light intensity) shows a 5-fold increase in the intercept with relatively little change in the slope of the curve (fig 3). These results indicate that the most pronounced effect is on a limiting dark reaction (9). The complex kinetics of control chloroplasts, showing a break in the curve at about 1,000 ft-c (fig 3) has been observed repeatedly under our present measuring conditions.

Phosphorylation in air is inhibited more severely than that with nitrogen (table VI). This was observed consistently in a number of experiments with both phenazine methosulfate and ferricyanide, but has not been investigated further.

The residual PMS-supported anaerobic phosphoryl-

Table VI

Effect of Water Treatment on Hill Reactions & Phosphorylation Reactions

	Reaction	Chloroplasts pre-treated with Expt. 1		Expt. 2	
		Mg*	H_2O*	Mg*	H_2O*
Hill reaction:	TCPIP	366	420	300	590
	FeCN	313	685	263	730
	FeCN+ATP,P,Mg	257	723	168	560
	FeCN+ADP,P,Mg	725	845	630	700
ATP formation:	FeCN reduction	305	31	295	0
	Pyocyanin (air)	840	80	825	73
	Phenazine methosulfate (N_2)	850	240	890	220

Hill reaction rates are listed as μmoles of dye reduced/mg chlorophyll/hour.
ATP formation is in μmoles of phosphate esterified/mg chlorophyll/hour.
Phosphorylation in line 5 is that accompanying the reduction of ferricyanide, the amount of such reduction being shown on line 4. Lines 6 and 7 represent phosphorylation with phenazine methosulfate and with pyocyanin, respectively. Phosphorylation with ferricyanide was measured using P^{32}; that with phenazine methosulfate and pyocyanin was measured colorimetrically.
* Chloroplasts in either 5×10^{-4} M $MgCl_2$ or H_2O, following preparation in EDTA containing medium.

ation was found, surprisingly, to be quite dependent on high light intensities (fig 4). There is a distinct knee in the rate vs. light intensity curve for water-damaged particles, with no measurable phosphorylation at the lowest intensity used. Alternatively, we can point out that the inhibition of phosphorylation is more severe, the lower the light intensity, varying from only 51 % at 5,000 ft-c to 100 % at 15 ft-c.

Again, for comparison, figure 4 includes the light intensity curves for chloroplasts uncoupled by 2×10^{-3} M ethylamine. Here the percentage inhibition is similar at all light intensities, varying only from 54 % at 5,000 ft-c to 58 % at 15 ft-c.

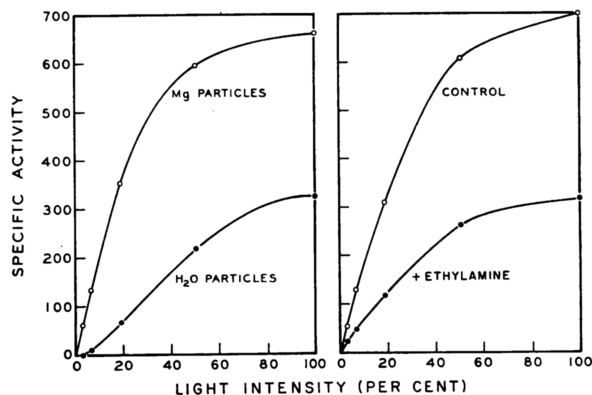

Fig. 4. Rate of phosphorylation with phenazine methosulfate as cofactor, as a function of light intensity. Reactions were performed anaerobically, and measured with the aid of P^{32}. Curves on the left show normal vs. water-treated chloroplast fragments; those on the right show whole chloroplasts in the presence or absence of 3×10^{-3} M ethylamine.

The inhibition of phosphorylation appears not to be due to the development of adenosine triphosphatase activity. Water-treated particles showed no increase in the very low rate of ATP hydrolysis characteristic of whole chloroplasts or of Mg particles.

Although damage to the phosphorylation mechanism may be accompanied by physical disruption of the chloroplasts, there is no necessary correlation between the two events. On suspending chloroplasts in water 29 % of the original protein is solubilized (table VII); but if suspended in 10^{-3} M $MgCl_2$ almost as much (25 %) is solubilized. In the first case the disrupted chloroplasts have lost most of their phosphorylation activity; in the second case they have not. Vice versa, neither sucrose 0.40 M by itself nor sucrose 0.40 M, tris 0.02 M, NaCl 0.01 M causes disruption of plastids and loss of major amounts of soluble proteins; but in the first case phosphorylating ability is lost, in the second case it is retained. No major losses of perchloric acid-soluble compounds absorbing at 260 mμ were seen under any circumstances.

Table VII

Lack of Correlation Between Uncoupling & Loss of Protein Upon Fragmentation

Chloroplasts suspended in	Phosphorylation activity μmoles P/mg chlorophyll/hr	Solubilized protein μmoles N/ml	Solubilized protein % of Total	O.D. at 260 mμ
Sucrose-tris-NaCl	665	0.7	3	0.27
3×10^{-4} M $MgCl_2$	710	6.0	25	0.34
0.40 M Sucrose	185	1.2	6	0.28
H_2O	185	7.0	29	0.32

Phosphorylation was measured with phenazine methosulfate anaerobically, and estimated colorimetrically. After suspension in the media shown, both whole and broken chloroplasts were centrifuged at $10{,}000 \times g$ for 20 minutes, the supernatants withdrawn by syringe, and the pellets resuspended in sucrose 0.40 M-tris 0.02 M-NaCl 0.01 M to the same volume as before centrifuging. Aliquots of the supernatant solution and of original whole chloroplasts were made to 5 % trichloroacetic acid, and frozen and thawed. The protein precipitate was collected by centrifuging, washed once, digested with sulfuric acid and selenium catalyst, and Nesslerized. Other aliquots were precipitated with perchloric acid, and the optical density of the supernatant determined at 260 mμ.

It does appear as if 3 to 4 % of the chloroplast proteins may be solubilized only when loss of phosphorylation occurs. This is seen more clearly in table VIII, where fragments prepared in the presence of magnesium lose some additional protein when exposed to EDTA subsequently. Specific properties of this extra protein fraction have not yet been determined.

Table VIII
Protein Extraction Associated With Uncoupling

	Solubilized protein		Phosphorylation activity
	μmoles N/ml	% of Original	
Chloroplasts	...	...	555
First extraction (5×10^{-4} M $MgCl_2$)	8.65	28	490
Second extraction (5×10^{-4} M $MgCl_2$)	0.34	1.1	530
Second extraction (1×10^{-4} M EDTA)	1.39	4.5	185

Chloroplasts were suspended in 5×10^{-4} M $MgCl_2$, centrifuged as in table VII, and the supernatant analyzed for protein N. The pellet of chloroplast fragments was resuspended either in more $MgCl_2$, or in EDTA to the same volume as before centrifuging. These two samples were centrifuged a second time; the pellets were used for phosphorylation, and the supernatants analyzed for TCA-precipitable N as in table VII.

The damage occurs rapidly when chloroplasts are exposed to EDTA, and so far has been irreversible. In table IX it is shown that in 3×10^{-4} M EDTA the loss of phosphorylation is complete within 5 minutes. Pipetting the chloroplasts from the EDTA solution into 10^{-3} M $MgCl_2$ in this experiment served to stop the occurrence of damage, but not to reverse damage that had already occurred. Similarly a Mg concentration of 3×10^{-3} M in the phosphorylation medium is not sufficient to restore activity to these chloroplasts. Various attempts at adding back supernatant solutions with or without metal ions, RNA, etc., have so far not produced any signs of reactivation.

Table IX
Time Course for Uncoupling by EDTA

Chloroplasts suspended in	Time of suspension min	Phosphorylation rate μmoles P/mg chlorophyll/hr
H_2O	0	340
	11	330
EDTA, 3×10^{-4} M	0	340
	2	192
	5	64
	10	66

Chloroplasts were prepared without EDTA, then suspended in the test media shown. Aliquots were removed at the indicated times and pipetted into $MgCl_2$ solution so that the final concentration was 1×10^{-3} M. All chloroplast fragments were collected as described in Materials & Methods, and their rate of phosphorylation determined anaerobically with phenazine methosulfate as cofactor.

Discussion

All of the evidence presented here is consistent with the concept that removal of cations is the cause of loss of chloroplasts' ability to phosphorylate. Since the inhibition of ATP formation is accompanied by an increase in the ability to reduce ferricyanide (table VI, figs 2 & 3), the procedure may be called one which uncouples chloroplasts.

Uncoupling of chloroplasts prepared in EDTA may be prevented by any one of a large variety of salts (table IV). Since the concentration of salt needed for protection shows a 100-fold difference between monovalent and divalent cations, and the anion (chloride) is the same in both cases, it is quite likely that protection is provided by the cation, at least for those effective at 10^{-4} M. This is entirely consistent with the function of EDTA in causing uncoupling in the first place. The effects of monovalent salts at 10^{-2} M could either represent protection by the cation again, or a salt effect in which the anions also participate. The protective function is extremely nonspecific, as emphasized by the fact that even compounds which uncouple when present in the reaction mixture (ammonium salts, ethylamine) are protective in the pretreatment. The dual nature of the EDTA curve (fig 1) can be explained by removal of bound divalent cations in the range from 10^{-5} to 10^{-3} M causing uncoupling, but the Na^+ ions in the Na-EDTA providing protection at 10^{-2} M.

Mitochondria (15) bacteriophages (1, 13, 16) and bacterial protoplasts (11, 12, 15) are damaged in the absence of cations, and protected from damage when cations are present. In these other systems monovalent ions do not seem to be effective, and often spermine and sperimidine are unusually effective protective agents. The requirement for cations in chloroplast membranes, therefore, seems to be even less specific than in these other structures.

From the ability to uncouple chloroplasts prepared from leaves ground without EDTA (tables II & III) it is suggested that chloroplasts in the cell may not have tightly bound divalent cations, sufficient to protect them from later washing in buffered sucrose or in water. During homogenization they would acquire such ions, possibly arising from the liberated vacuolar salts. On the other hand, when grinding in a large volume of buffer the concentration of vacuolar salts would not be high enough to cause a sufficient level of adsorption. Any monovalent cations present in these latter chloroplasts must be easily washed out in water, and, therefore, they would be uncoupled when suspended in plain water. Chloroplasts with the more tightly bound divalent cations would only be uncoupled after the bound cations had been removed by EDTA. This suggestion, of course, requires direct measurements of adsorption of cations by chloroplasts to be fully established.

The mechanism for the uncoupling described here is entirely unknown. Since it is rapid and irreversible (table IX) it suggests a change in structure of some part of the chloroplast electron transport mecha-

nism or substratum. The change in shape of the pH curve (fig 2) and the different kinetics with respect to light intensity (fig 3) suggest that ferricyanide is reduced at a step by-passing the phosphorylation site, after uncoupling. There is no direct evidence to rule out the alternative, however, of an internal breakdown of a high energy intermediate prior to ATP (4).

The kinetics of ferricyanide reduction with respect to light intensity have been studied intensively, and have usually shown a simple hyperbolic relationship (10, 14). In the present instance this simple relationship appeared only after uncoupling by treatments removing cations (fig 3). It is difficult to compare all the differences in procedure and materials between these experiments and the more extensive earlier work (14), thus it would not be at all safe to assume that the apparently complex kinetics of untreated chloroplasts is in general a more native condition.

Uncoupling by treatments removing cations differs from that due to addition of ammonium ions or amines (5, 9) in the fact of irreversibility, in the shape of the pH curve for ferricyanide reduction (fig 2), and in the response of residual phosphorylation to different light intensities (fig 4). Uncoupling by very high concentrations of anions, recently discovered by Good (6) is also easily reversible. Uncoupling by cation removal resembles that due to dilution in 0.35 M NaCl at pH 6.0 (8) with respect to irreversibility, and the shape of the resultant pH curve for ferricyanide reduction. However, the dilution in NaCl is slower to cause uncoupling (unpublished observation) and is not prevented by adding Mg or Ca salts.

Summary

► Treatments that tend to remove cations from chloroplasts lead to a decreased ability to phosphorylate and an increased rate of reduction of ferricyanide. This type of uncoupling can be prevented by a wide variety of divalent cations at 10^{-4} M and by salts containing monovalent cations at 10^{-2} M. Other characteristics of chloroplasts uncoupled by this procedure are described.

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