Chloroplast Damage Due to Enzymatic Hydrolysis of Endogenous Lipids[1, 2, 3]

Richard E. McCarty[4] and A. T. Jagendorf
Biology Department and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore

Jagendorf and Evans (15) reported that the rates of 2, 3', 6-trichlorophenolindophenol (TCPIP)[5] reduction by chloroplasts isolated from the primary leaves of red kidney bean are strongly dependent upon the pH of the buffer in which the leaves are homogenized. Grinding leaves at pH values below 8 resulted in chloroplasts whose Hill reaction was rapidly and irreversibly inhibited. In contrast, the chloroplasts of some plants, including those from spinach, are not inactivated under these conditions. Clendenning and Gorham (7) also found that homogenates of several *Phaseoli,* prepared in 0.4 M sucrose, have poor Hill reaction rates compared to those of spinach. This pH-specific inactivation of primary bean leaf chloroplasts has been reinvestigated using Black Valentine bean. The course of this work has led to a further definition of the changes in bean chloroplasts caused by isolation at low pH and to the elucidation of the probable cause of these changes.

Materials and Methods

Fully expanded primary leaves of *Phascolus vulgaris* var. Black Valentine were harvested from plants grown either in vermiculite or gravel. Illumination was provided by a bank of fluorescent and incandescent bulbs yielding about 1000 ft-c at the surface of the leaves. A 12-hour light-dark cycle was employed and the temperature maintained at roughly 23°. Spinach was purchased at the local market.

Chloroplasts were prepared in a variety of grinding media as described previously (14) and were usually used unwashed. Chlorophyll was determined by the method of Arnon (2).

DCPIP reduction was assayed by 3 different methods: First, by the spectrophotometric method of Jagendorf (13) using 23.5 for the millimolar extinction coefficient for the dye at pH 7.2 and 625 mμ. Second, by automatic titration with ferricyanide taking advantage of the fact that the redox potential of the reaction mixture falls as reduction proceeds. The ferricyanide quantitatively reoxidizes the reduced dye as it is formed and, thus, the redox potential and concentration of the oxidized dye are kept constant. These conditions are realized by using a radiometer titrator, syringe burette, and recorder assembly with platinum and calomel electrodes. The reaction mixture (10 ml) was placed in a water-jacketed cuvette maintained at 20° and consisted of: citrate (for pH 4–5) or Tris-maleate (for pH 5.5–9), 20 mM; NaCl, 14 mM; DCPIP, 0.03 to 0.1 mM and chloroplasts containing from 25 to 100 μg chlorophyll. Illumination was provided by a 500 w reflector flood lamp. The light intensity after passage through 30 cm of water was about 10,000 ft-c. The burette was filled with 5 mM potassium ferricyanide. The potential of the reaction mixture was allowed to reach equilibrium and this potential was used to set the potential of titration. The instrument was then used as an EMF-stat in direct analogy to the pH-stat. Upon illumination, the potential begins to fall and ferricyanide is added automatically to compensate for this fall. The amount of ferricyanide added is recorded against time and this amount of ferricyanide is equivalent to the amount of dye reduced. The rates of reduction determined by this method agree well with those obtained by other methods except in a special case noted in the text.

Third, the reduction of DCPIP and ferricyanide were assayed by oxygen evolution using a Clark-type oxygen electrode (6).

Ferricyanide reduction was determined by the method of Jagendorf and Smith (16). Phosphorylation coupled to ferricyanide reduction was estimated as described previously (14).

NADP reduction was followed spectrophotometrically by the method of San Pietro (30). When reduced DCPIP was used as the electron donor, 0.4 μmole DCPIP and 5 μmoles of ascorbate were added in a final volume of 3 ml.

Pyocyanine-catalyzed phosphorylation was assayed as previously described (16) using the Taussky and Shorr assay for residual Pi (35).

Total lipids and dry weights were determined gravimetrically as outlined by Mego and Jagendorf (26).

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[3] This work constitutes a part of the dissertation submitted by R. E. McCarty to The Johns Hopkins University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
[4] Present address: Department of Biochemistry, Public Health Research Institute of the City of New York, New York 9, N.Y.
[5] Abbreviations employed in this paper include: DCPIP for 2, 6-dichlorophenolindophenol; TCPIP for the tri-chloro derivative; CMU for *p*-chlorophenyl-1, 1-dimethylurea; FeCN for potassium ferricyanide (in tables).

Prior to the determination of nitrogen by nesslerization, the proteins were precipitated with 5 % trichloroacetic acid and washed twice with 1 % trichloroacetic acid.

Isooctane extracts were prepared by extracting chloroplast suspensions at pH 3 to 3.5, containing 10 to 15 mg of chlorophyll, 3 times with equal volumes of isooctane. The pooled extracts were evaporated to dryness and the residue redissolved in 5.0 ml absolute ethanol.

The concentration of linolenic acid in 1.0 ml aliquots of the ethanolic extracts was determined spectrophotometrically by the micromethod of Holman (12).

Enzymes which hydrolyze galactolipids were assayed by determining the decrease in acyl ester content (9) of chloroform extracts of the reaction mixtures as described by Sastry and Kates (31). Monogalactosyl diglyceride was isolated from spinach leaves by the methods of these workers (32).

ADP, linolenic and linoleic acids were purchased from the Sigma Biochemical Corporation and oleic acid from Nutritional Biochemical Corporation. The fatty acids were stored under liquid nitrogen and solutions were prepared in ethanol immediately before use.

Results

Chloroplasts isolated from pea or spinach leaves are not inactivated by grinding the leaves at pH 6 (table I); their ability to photoreduce either DCPIP or ferricyanide, or to catalyze ATP formation with pyocyanine as cofactor, is unimpaired compared to chloroplasts isolated from homogenates at pH 8. Bean chloroplasts, on the other hand, show a marked sensitivity to isolation at pH 6, which results in a strong inhibition of all 3 activities. Bean chloroplasts isolated at pH 8 resemble those of spinach or pea and will be designated as control chloroplasts, for the remainder of this paper. Bean chloroplasts isolated at pH 6 will be referred to as damaged.

Table I. *Lack of Effect of pH of Grinding on the Photosynthetic Activities of Spinach and Pea Chloroplasts*

The chloroplasts were prepared in 0.35 M NaCl containing 0.1 M Tris-maleate at pH 8 or pH 6. Assays were performed spectrophotometrically.

Preparation	Activity*		
	DCPIP reduction	FeCN reduction	Phosphorylation
Spinach pH 8	512	228	236
Spinach pH 6	521	226	238
Pea pH 6	516	232	237
Pea pH 8	486	217	241
Bean pH 8	346	160	322
Bean pH 6	52	123	0

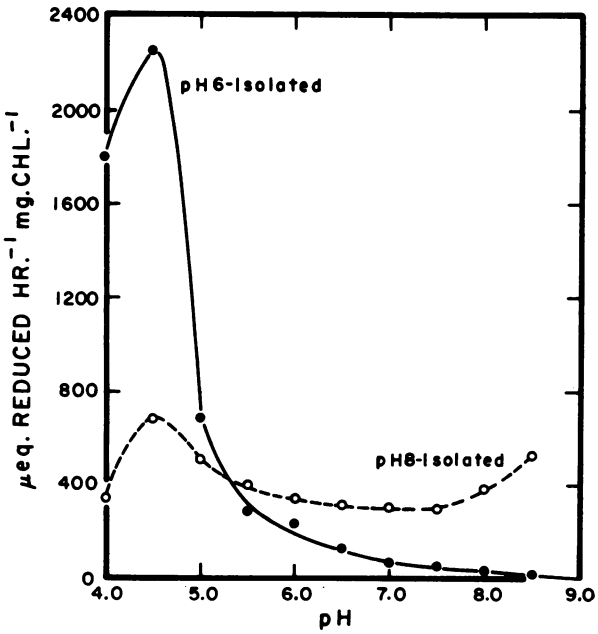
* Expressed as μeq reduced per hour per mg chlorophyll for DCPIP and FeCN reduction and μmoles Pi esterified per hour per mg chlorophyll for phosphorylation (pyocyanine as cofactor).

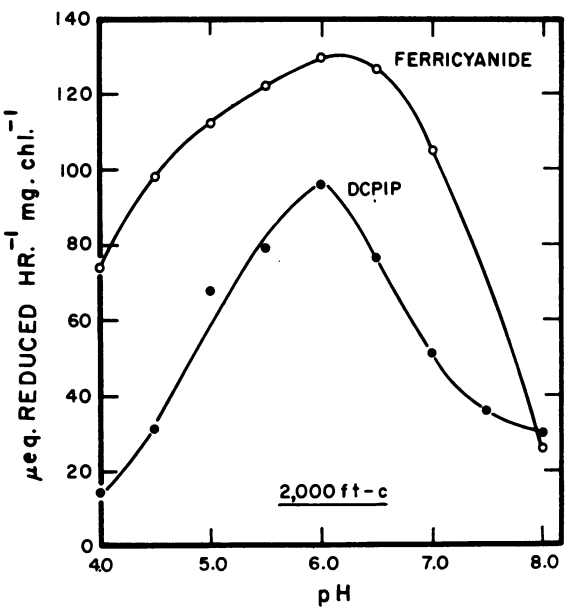
The damaged bean chloroplasts reduce DCPIP with a pronounced optimum at pH 4.5 (fig 1) especially when reduction is assayed by the radiometer EMF-Stat procedure. This reduction is inhibited by CMU (50 % at 5×10^{-7} M), and by the detergent Triton-X-100 (50 % at 8×10^{-5} M). It is accompanied stoichiometrically by oxygen evolution, and is completely abolished by heating the chloroplasts at 100° for 1 minute. Therefore, in all these respects it appears to be a genuine Hill reaction. The control bean chloroplasts (fig 1) have much less activity at this pH, and in general have far greater activity above pH 7.

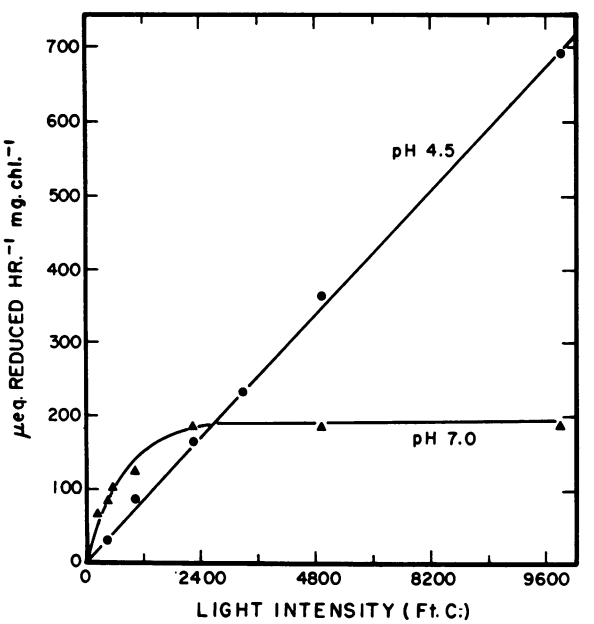
Surprisingly, reduction of ferricyanide by the damaged bean chloroplasts does not show the pH 4.5 optimum. Instead, a broad, relatively flat curve is found (fig 2) with an optimum near pH 6.0. Toluylene blue (E'_0 = + 115 mv) and xyloquinone (E'_0 = + 223 mv) show a pH dependency for photoreduction similar to that of ferricyanide (E'_0 = + 430 mv).

Figure 3 shows the light intensity dependence of DCPIP reduction by damaged bean chloroplasts using the oxygen electrode for measurement. At pH 4.5 the rate of oxygen evolution increased linearly with light intensity up to 10,000 ft-c; and in other experiments only began to slope off between 15,000 and 20,000 ft-c. At pH 7 on the other hand, using the same chloroplasts, the Hill reaction shows light saturation at about 1500 ft-c. At intensities below 2000 ft-c, DCPIP reduction is much more efficient at pH 7 than at pH 4.5. Thus when a pH curve is determined using any light intensity below 2000 ft-c, the 4.5 optimum is not seen, and the curve resembles that found for ferricyanide reduction (fig 2). It is consistent that at any pH ferricyanide reduction saturates at about 2000 ft-c.

Others (23, 34) have shown that the rates of reduction of ferricyanide are independent of the redox potential of this acceptor system. Figure 4 shows that this is the case for control bean chloroplasts. In contrast, the residual ferricyanide reducing activity of chloroplasts damaged by isolation at pH 6 depends on the ratio of ferri- to ferro-cyanide. This phenomenon cannot be explained by oxidation of ferrocyanide since none can be observed. Also, it is not simply due to limiting ferricyanide concentrations as control experiments indicated that the rate of ferricyanide reduction at the lowest concentration used was equal to that at the highest. Furthermore, it is not caused by the increasing ferrocyanide concentration per se. An experiment was performed in which the concentrations of ferri- and ferrocyanide were varied while keeping the ratio constant. The rate of reduction with a reaction mixture containing 0.25 mM ferricyanide and 0.58 mM ferrocyanide was identical to that observed with a reaction mixture containing 1.0 mM ferricyanide and 2.33 mM ferrocyanide. However, a change in the







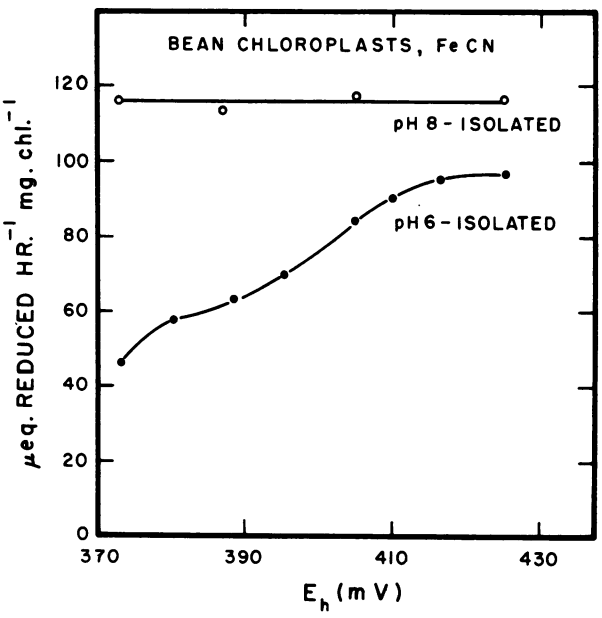


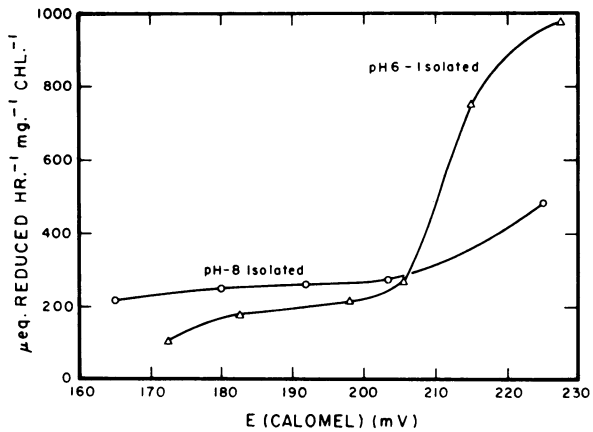
FIG. 1 (*upper left*). pH Dependence of DCPIP reduction. Bean chloroplasts were prepared either in unbuffered 0.35 M NaCl (final pH of the homogenate was 5.9; designated as pH-6 isolated) or in 0.35 M NaCl containing 0.1 M Tris pH 8. Photolysis was followed by the radiometer EMF-stat assay. Light intensity about 10,000 ft-c.

FIG. 2 (*upper right*). Reduction of DCPIP and of ferricyanide at 2000 ft-c (low light intensity). Bean chloroplasts were isolated in 0.35 M NaCl (e.g. at pH 6). Photolysis was followed by polarographic determination of oxygen evolution.

FIG. 3 (*lower left*). Light intensity kinetics of DCPIP reduction. Bean chloroplasts were isolated in 0.35 M NaCl (pH 6.0) and DCPIP reduction was assayed with the oxygen electrode at a chlorophyll concentration of 19 μg/ml. The light intensity was varied using wire screens and was measured with a Weston ft-c meter.

FIG. 4 (*lower right*). Dependence of ferricyanide reduction on the redox potential of the medium. Bean chloroplasts, isolated at either pH 6 or pH 8. The amount of ferricyanide reduced was measured spectrophotometrically in a reaction mixture at pH 7.0 containing 33 μg chlorophyll/ml and ferri-ferrocyanide at a total concentration of 1.7 mM. The redox potential was varied by changingthe rate of ferri- to ferrocyanide, while keeping the total concentration constant. Redox potentials were determined under reaction conditions with the radiometer platinum and calomel electrodes.

ratio from 5:6 to 2:8 (an increase of the ferrocyanide concentration from 1 mM to only 1.33 mM together with a decrease in the ferricyanide concentration from 0.83 mM to 0.33 mM) resulted in 50 % inhibition. Thus, the observed dependence is on the ratio of oxidized to reduced acceptor and represents redox potential dependence. If this is so, then we infer that the midpoint potential at pH 7.0 of the substance in the chloroplast which is affected by the ferri-ferrocyanide mixtures is about +400 mv, as indicated by the region of maximum change in rate per change in E_h.



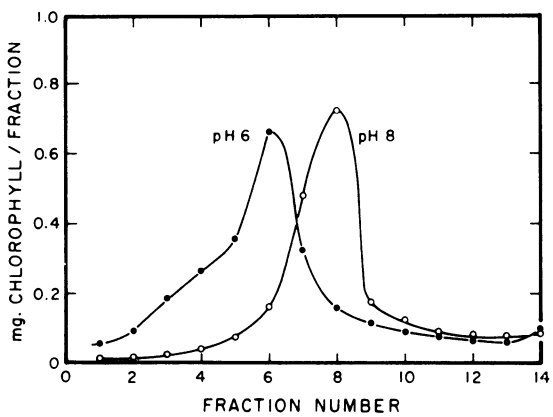


FIG. 5. Dependence of DCPIP reduction on redox potential. Bean chloroplasts were prepared either at pH 6 or pH 8. DCPIP reduction was measured by the EMF-stat assay at pH 4.5, with a final dye concentration of 0.1 mM. Redox potentials were varied by the addition of reduced dye and were maintained by setting the radiometer controls at differing levels. Other conditions as in (1). To convert from E (calomel) to E_h, add 250 mv.

FIG. 6. Sucrose density gradient centrifugations. One to 2 M sucrose gradients were prepared as in table III. Bean chloroplasts were isolated at pH 6 and pH 8 and 2 ml aliquots containing 2 mg of chlorophyll were layered on the top of each gradient. After centrifugation at 5000 rpm for 45 minutes in a Servall swinging bucket rotor, 2 ml fractions were collected and the chlorophyll content of each fraction determined.

Table II. *NADP Reduction by Bean Chloroplasts*

Chloroplasts were isolated at pH 6 or pH 8 in 0.35 M NaCl containing 0.1 M Tris-maleate. The chlorophyll concentration was 100 μg/3 ml and the illumination time was 5 minutes. Activities are expressed as μeq reduced per hour per mg chlorophyll. The CMU concentration was 3 μM.

Preparation	Electron donor		
	H_2O	H_2O + CMU	DCPIP-ascorbate + CMU
pH 6	7.2	0	29
pH 8	51	0	31

Potential dependence may also be observed with oxidized and reduced DCPIP mixtures at pH 4.5 (fig 5). In this case, even the control chloroplasts are slightly inhibited by the lower redox potentials. The inferred midpoint potential at this pH is around +400 mv. The potential effect with DCPIP is very strong. The addition of 10 μM reduced DCPIP to 90 μM oxidized DCPIP resulted in an 80 % inhibition of the rate observed with the oxidized dye alone. This result partially accounts for the fact that the rates of DCPIP reduction at pH 4.5 are lower when assayed by oxygen evolution than those obtained with the radiometer EMF-stat assay. With the EMF-stat assay, the redox potential is kept constant while with other assay methods, the potential falls and the reaction quickly runs at inhibited rates.

Damaged bean chloroplasts reduce NADP only slowly using water as the electron donor, but fairly rapidly if reduced DCPIP is this electron donor (table II). This result is usually taken to indicate that the main site of damage is close to the oxygen evolving system. Control chloroplasts use water as an electron donor at higher rates than they use reduced indophenol dye (table II).

Changes in structure and composition accompany those in activity, caused by isolation of bean chloroplasts at pH 6. The control chloroplasts have a distinct kidney shape and have no easily discernible internal structure. By contrast, the damaged chloroplasts have distinct grana when viewed either with the phase contrast or the ordinary light microscope. They are also larger than the controls, with diffuse outlines. In short, they seem to be almost identical in morphology to the salt-burst spinach chloroplasts described by Von Wettstein and Kahn (36). Damaged bean chloroplasts seem to have lost their osmotic properties, since they can be suspended either in deionized water or in 10 mM NaCl without any observable further changes in structure, or any change in the speed with which they sediment. The control chloroplasts bleb out and eventually fragment, in the same hypotonic media. The lack of osmotic properties for damaged bean chloroplasts is consistent with the electron micrographs of salt-burst spin-

ach chloroplasts, showing that they have lost their limiting membranes (36).

Damaged bean chloroplasts are more dense than the control chloroplasts, and the 2 types may be separated on sucrose density gradients (fig 6). Sixty-five percent of the damaged chloroplasts float within a density range of 1.2 to 1.175 while within this same range, only 12 % of the controls float. As seen in table III, this increase in density is correlated with the loss of ability to reduce DCPIP.

Bean chloroplasts isolated at low pH have lost 40 to 50 % of their nonchlorophyll lipid compared to control chloroplasts (table IV). Little or no loss of nitrogen is observed. This loss of lipid in damaged chloroplasts is consistent with their higher density.

As previously noted, spinach chloroplasts are not altered by isolation at low pH. However, if they are aged at pH 6, changes similar to those which occur in bean chloroplasts are observed. Figure 7 shows the change in the pH dependence of DCPIP reduction by spinach chloroplasts aging at pH 6. Within 24 hours, the pH curve closely resembles that observed for the damaged bean chloroplasts. Also, the aged spinach chloroplasts have lost their ability to carry out pyocyanine-catalyzed phosphorylation and assume the salt-burst structure noted above. Aging at pH 8 does not lead to these changes.

If the changes which occur in aging spinach chloroplasts are truly similar to those observed with bean, one would expect that chloroplasts aged at pH 6 would lose more lipid than those aged at pH 8. Table V shows that chloroplasts aged for 21 hours at pH 6 lose roughly twice as much lipid as those aged at pH 8, while losing no more protein.

Dialyzed bean homogenates greatly accelerate

Table III. *Correlation of Density Increase with Activity Decrease*

Bean chloroplasts were prepared in 0.35 M NaCl containing 0.1 M Tris-maleate at the indicated pHs. One to 2 M sucrose density gradients were prepared by layering 2.5 ml of each solution (in 0.1 M increments) and allowing them to stand overnight. Chloroplast suspensions containing about 2 mg of chlorophyll were layered on the top of the gradient and the tubes were centrifuged at 5000 g for 30 minutes in a Servall swinging bucket rotor. Fractions of 1 ml were collected and the chlorophyll content was determined. DCPIP reduction was assayed spectrophotometrically in the presence of 2 mM NH_4Cl.

pH of isolation	DCPIP Reduction μeq/hr per mg chlorophyll	Density of fraction with highest chlorophyll conc (g/cc)
6.5	39	1.213
7.0	46	1.194
7.5	189	1.181
8.3	252	1.172

Table IV. *Composition of pH 6- and pH 8- Isolated Bean Chloroplasts*

Bean chloroplasts were isolated in the NaCl-Tris-maleate medium and washed twice in this medium. The chloroplasts were suspended in 10 mM NaCl, allowed to stand 30 minutes at 0°, centrifuged at 10,000 × *g* for 15 minutes and finally resuspended in cold deionized water. After dilution to 1.5 mg/ml, 2 ml aliquots were taken for dry weight analysis, 0.1 ml for nitrogen determination and 5 ml for lipid analysis. Percentage values are expressed on a dry weight basis.

	pH 6–chloroplasts		pH 8–chloroplasts	
	Expt 1	Expt 2	Expt 1	Expt 2
Chlorophyll–%	9.1	9.3	7.4	7.5
N–%	8.1	...	7.0	...
Lipid–% (non-chlor)	14.2	11.5	22.5	21.2
N/chlor	0.91	...	0.95	...
Lipid/chlor	1.56	1.24	3.04	2.83

Table V. *Lipid Loss on Aging Spinach Chloroplasts*

Spinach chloroplasts were prepared either unbuffered 0.35 M NaCl (final pH of the homogenate 6.0) or 0.4 M sucrose, 20 mM Tris pH 8, 10 mM NaCl, and aged 22 hours in these media. After aging, the chloroplasts were centrifuged at 10,000 × *g* for 20 minutes. The supernatant fractions (15 ml), after removing an aliquot for protein determination by the Lowry method (24), were lyophilized to dryness and the lipids of the residue extracted and determined gravimetrically.

pH during aging	Chlorophyll conc during aging	Mg lipid lost per mg chlor	Mg protein lost per mg chlor
	(mg/ml)		
6	0.91	0.93	0.18
8	0.99	0.55	0.17

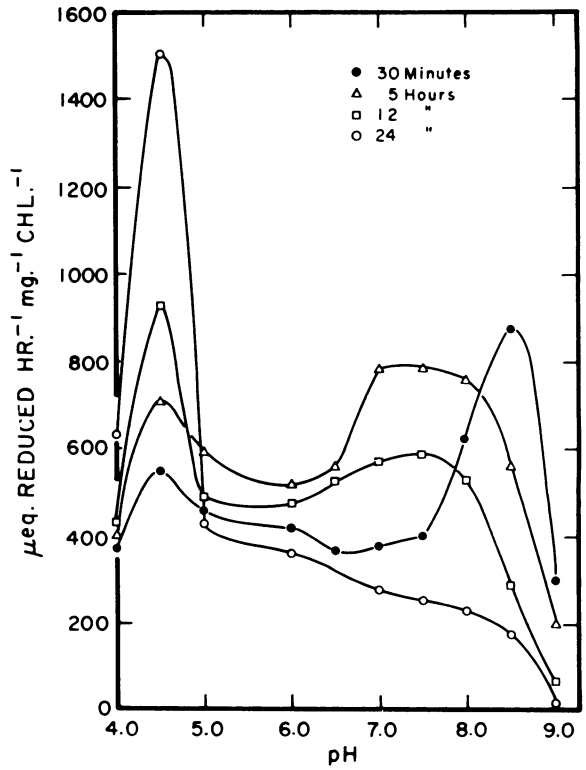
the changes which occur in aging spinach chloroplasts (fig 8). This activity is precipitated by ammonium sulfate and is heat labile. This observation, coupled with the pH specificity of inactivation, suggested the possibility that primary bean leaves may contain lipases, which are active only at pH 6.

Sastry and Kates (31) demonstrated the presence of enzymes in runnerbean and other primary leaves of *Phaseoli* which hydrolyze galactolipids, the major lipid components of chloroplasts. Extracts of primary leaves of Black Valentine are capable of hydrolyzing monogalactosyl diglyceride isolated from spinach leaves (table VI). It is seen that there is little activity at pH 8 and that spinach leaf extracts prepared in the same manner as bean extracts have less than 10 % of the activity that bean extracts have.

Of the esterified fatty acid of the galactolipids of chloroplasts 94 to 96 % is linolenic acid (32). Thus, if galactolipid hydrolysis to free fatty acid is responsible for the changes due to low pH isolation or aging, one should be able to detect an increase of free linolenic acid in the chloroplast suspensions. As seen below, this is the case. Free fatty acids have pronounced effects on mitochondria. These effects include uncoupling (28), inducing swelling (22) and inhibiting respiration (5). Fatty acids are also known to inhibit the Hill reaction (21). Possibly, then, some of the activity changes caused by low pH isolation and aging might be due to the linolenic acid released.

Linolenate at 5×10^{-5} M will induce the pH 4.5 optimum of DCPIP reduction by freshly prepared spinach chloroplasts (fig 9). Higher concentrations inhibit virtually all activity above pH 5.5 and also reduce activity at pH 4.5. This activity of linolenate is shared by linoleic acid, but not, apparently, by oleic acid even though it inhibits activity in the higher pH ranges as severely as the other fatty acids in this series.

Linolenate inhibits pyocyanine-catalyzed phosphorylation 50 % at about 10^{-6} M and acts as an



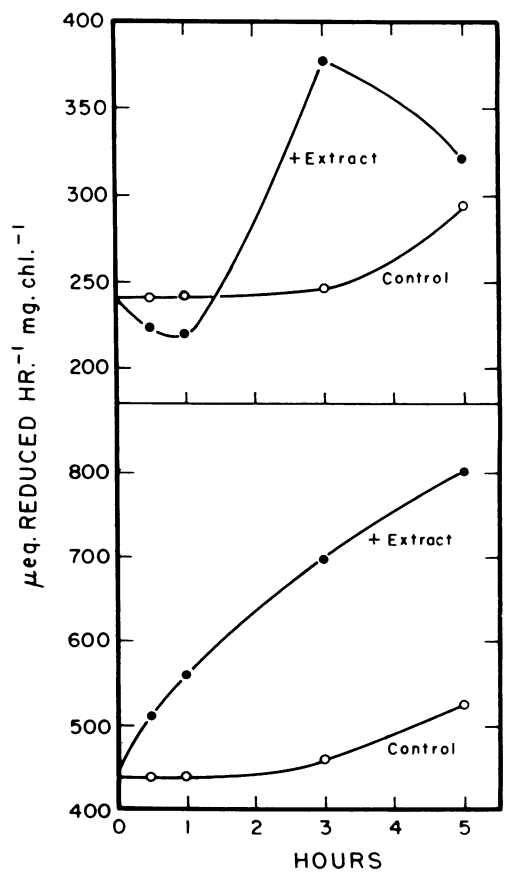


FIG. 7. pH Dependence of DCPIP reduction in aging spinach chloroplasts. Isolation of the chloroplasts was in 0.35 M NaCl, and aging occurred in the same medium (at pH 6) at 0° at a chlorophyll concentration of 1 mg/ml. DCPIP reduction was measured by the radiometer EMF-stat assay.

FIG. 8. Effect of bean leaf homogenate on aging spinach chloroplasts. Spinach chloroplasts were isolated in 0.35 M NaCl and one-half resuspended in 0.35 M NaCl (control) and the other half in a bean leaf homogenate prepared as follows: 20 g of leaves were ground in 60 ml 0.35 M NaCl and, after filtering through cheesecloth, the homogenate was centrifuged at 17,000 × *g* for 20 minutes and the supernatant dialyzed against 0.35 M NaCl overnight. DCPIP reduction at pH 4.5 (lower curve) and at pH 7.2 (upper curve) was determined with the radiometer assay.

Table VI. *Galactolipid Hydrolyzing Enzymes in Extracts of Bean and Spinach Leaves*

Extracts of bean and spinach leaves were prepared by grinding deveined leaves with equal weights of cold deionized water. The substrate was monogalactosyl diglyceride isolated from spinach leaves.

Preparation	pH of assay	μeq acyl ester hydrolyzed per hr per 0.2 ml extract
Bean	5.6	0.43
Bean	6.3	0.71
Bean	7.0	1.21
Bean	8.0	0.1
Bean (heated at 100°, 3 min)	7.0	0
Spinach	7.0	0.1

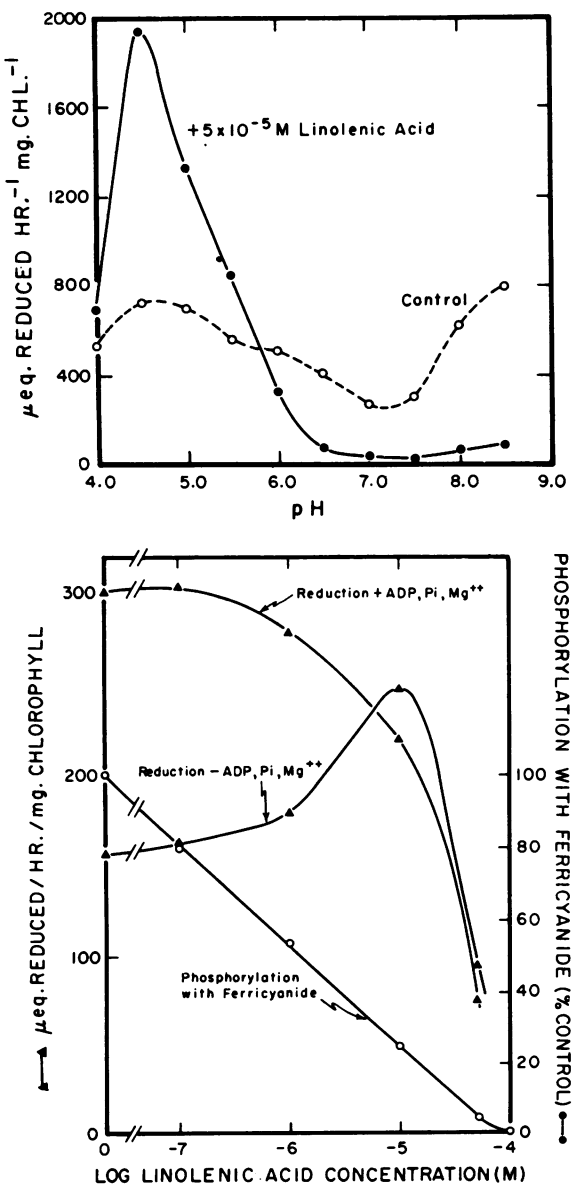


FIG. 9. Effect of linolenic acid on DCPIP reduction by spinach chloroplasts. Isolation of chloroplasts and measurement of the Hill reaction as in (13), with a final ethanol concentration in all cases of 0.5 %.

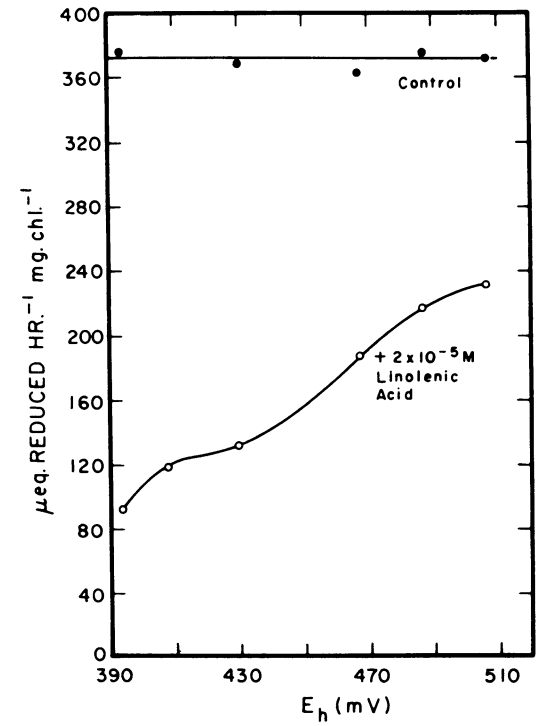
uncoupler of electron flow (ferricyanide reduction) from phosphorylation (fig 10). At higher concentrations, linolenate is a potent inhibitor of electron flow. In the presence of 5×10^{-5} M linolenate the pH curve for ferricyanide reduction by spinach chloroplasts has an optimum around pH 6.0 and closely resembles that observed for the altered bean chloroplasts (see fig 2). Details of the uncoupling action of fatty acids on chloroplasts will be published elsewhere.

At inhibitory concentrations, linolenate induces potential dependence for ferricyanide reduction by spinach chloroplasts (fig 11). Ammonia does not have this activity at any concentration tested. The plateau region (pH 8.0) is about 0.45 v in fair agreement with that found for the low pH-isolated bean chloroplasts.

Isooctane extracts of spinach chloroplast suspensions aged at pH 6 contain substances which inhibit ferricyanide reduction. Extracts of preparations aged at pH 8 have no effect on reduction even at 6 times the concentration of the pH 6 extract which gives 50 % inhibition. Inhibition by the extract is prevented by the addition of bovine serum albumin (0.2 mg/ml final conc) before the addition of the chloroplasts. The inhibitory substance is not extractable by isooctane at pH 11. Thus, this substance has the properties of a fatty acid.

Linolenic acid may be detected in these extracts by the alkaline isomerization method. Figure 12 shows clearly that there is very little or no linolenic acid in the extracts of spinach chloroplasts aged at pH 8. In contrast, extracts of chloroplast sus-

FIG. 10. Effects of linolenic acid on ferricyanide reduction. Spinach chloroplasts were prepared in 0.4 M sucrose, 0.02 M NaCl and 0.1 M Tris pH 8. Ferricyanide reduction was determined by the spectrophotometric assay in the presence and absence of phosphorylating reagents. Dark controls were run for each tube and the final ethanol concentration was 3 %. Phosphorylation coupled to ferricyanide reduction was assayed in a separate experiment. 100 % of phosphorylation correspounds to 79.6 μmoles phosphate esterified hr^{-1} mg $chlorophyll^{-1}$ and a P/2e ratio of 0.54.



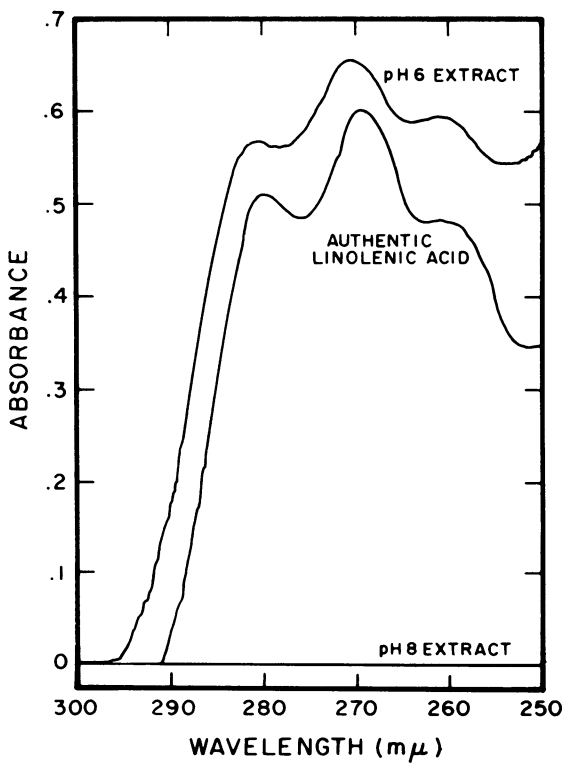


FIG. 11. Potential dependence induced by linolenic acid. Spinach chloroplasts were prepared in 0.35 M NaCl; Hill reaction was assayed polarographically at pH 8.0 in a reaction mixture containing 17 μg chlorophyll per ml, with a total concentration of ferri- and ferrocyanide of 1.67 mM. Redox potentials were varied as in figure 4.

FIG. 12. Identification of linolenic acid in extracts. One ml aliquots of isooctane extracts of pH 6 and pH 8-aged spinach chloroplasts in ethanol and a sample of authentic linolenic acid in ethanol were treated with alkaline ethylene glycol according to Holman and Hayes (12). Absorption spectra after dilution in methanol were recorded with a Bausch and Lomb Spectronic 505 spectrophotometer.

pensions aged at pH 6 contain this fatty acid, and the concentration of linolenate referred back to the original chloroplast suspension was about 2×10^{-4} M. In similar experiments, bean leaf homogenates prepared at pH 8 were found to contain only 7 % of the linolenate detected in pH 6 homogenates.

The presence of linolenic acid in the extracts was confirmed by reversed phase paper chromatography by the methods of Mangold (25). Authentic linolenate and the substance in the pH 6 extracts co-chromatographed when applied to the paper as a mixture. Little, if any, linolenate could be detected in extracts of pH 8-aged spinach suspensions. No other unsaturated fatty acid could be detected on the chromatograms.

Discussion

The results reported here show that either bean chloroplasts isolated at pH 6 or spinach chloroplasts aged at pH 6 have several new characteristics. In contrast, chloroplasts isolated or aged at pH 8 appear normal. At the low pH's, the galactolipid lipases are active (table VI) and it is these enzymes which are responsible for the loss of lipid (table IV, V) and the increased density (table III, fig 6) of the low pH isolated chloroplasts. These enzymes release linolenic acid (fig 12) which in turn causes the activity changes (fig 9–11). At pH 8, the galactolipid lipases are less active (table VI) and, thus, the biochemical and morphological changes caused by these enzymes do not occur. Spinach leaf homogenates have low lipase activity and, therefore, aging at pH 6 is required to liberate linolenate and thereby elicit the changes.

The pH 4.5 optimum for DCPIP reduction by pH damaged bean chloroplasts is different from any previously reported curve. Although the pH 4.5 optimum may be observed with the spectrophotometric and polarographic assays, maximal activity at this pH requires that the reaction be run at a constant redox potential and at high light intensity. It is possible that the observed pH curve is due at least in part to the fact that the redox potential of DCPIP changes sharply with pH. At pH 4.5 the potential of the reaction mixture was +66 mv, while at pH 5.0, this value falls to +24 mv. As seen in figure 5, this potential drop is large enough to explain the lower

activities observed at pH 5.0 compared to those at pH 4.5. The shape of the curves for rate vs. light intensity (fig 3) indicate an entirely different kinetics, and a much less efficient use of quanta for DCPIP reduction at pH 4.5. No other electron acceptor studied showed either the pH 4.5 optimum or the peculiar light intensity kinetics at this pH.

It is not surprising that the control chloroplasts show no dependence on the redox potential in the range employed here. Chloroplasts can reduce methyl or benzyl viologens (3) which have midpoint potentials on the order of −450 mv. Thus, the terminal electron donor of normal chloroplasts is extremely reducing and potential dependence in the range of +360 to 500 mv would not be expected. The data presented here can be explained if linolenic acid causes a block in the electron transport chain which interferes with the transfer of electrons to the more reducing end. Now, instead of a very low potential enzyme reducing the added acceptors, a component with a midpoint potential of around 400 mv might reduce these compounds. There are a variety of substances known to exist in chloroplasts with midpoint potentials in the range of +400 mv. These include: plastocyanin, 370 mv (17); P700, 430 mv (19); cytochrome f, 365 mv (11); and an electron spin resonance signal of chloroplasts, 440 mv (1, 23).

The NADP data (table II) indicate that the block caused by the linolenate is at some point before that which accepts electrons from reduced indophenols. However, according to the currently accepted schemes, the electron carriers with midpoint potentials in the range of + 0.4 v, with the possible exception of plastocyanin (8), are grouped in a position after this point. It is therefore tempting to speculate that part of the residual DCPIP and ferricyanide activity of damaged bean chloroplasts is proceeding only through system II (the oxygen evolving system) and the direct reductant is plastocyanine. No convincing evidence is as yet available; but the hypothesis might be tested more directly by observation of light-induced spectral changes.

An alternative possibility for our data is that some component other than the terminal electron donor is affected by the potential of the external solution. This component might have to be maintained in a largely oxidized condition for the entire photosynthetic apparatus to function properly. We would then have to postulate, further, that in control chloroplasts some internal mechanism is available to keep this component relatively oxidized; but when linolenate at inhibiting concentrations is added the internal mechanism fails, and an oxidizing condition in the surrounding medium must take its place. Given these conditions, the terminal electron donor might still be the same in control and linolenate-treated (damaged) chloroplasts.

The distribution of damaged bean chloroplasts on the sucrose density gradients (fig 6) indicates that they are very heterogeneous. Some of the chloroplasts have undoubtedly lost more than the average 40 to 50 % of their nonchlorophyll lipid and are, accordingly, more dense. It is apparent that chloroplasts may lose a major proportion of their lipid and still be intact. Albertson and Baltscheffsky (4) has reported the separation of coupled from uncoupled chloroplasts by counter-current distribution.

The aging of spinach chloroplasts at pH 6 leads to a 2-fold stimulation of the rate of DCPIP reduction at pH 7 to 7.5 (fig 7, 8). This observation is in apparent conflict with the data of Krogmann and Jagendorf (20) who found that aging spinach chloroplasts under these conditions resulted in no stimulation of TCPIP reduction, but a 3-fold increase in ferricyanide reduction. The activation of ferricyanide reduction was accompanied by loss of phosphorylation and was therefore an uncoupling. This conflict can be explained by the fact that Krogmann and Jagendorf used 23 μM TCPIP which has been shown to cause complete uncoupling of reduction from phosphorylation (18, 10, 33). Thus, no further stimulation of the rate by the addition of a second uncoupler (in this case, the linolenic acid released during aging) would be seen. In the present study, DCPIP rather than TCPIP was used at concentrations which uncouple only about 50 %; hence, linolenic acid could cause the rate to increase as uncoupling becomes more severe.

Linolenic acid can cause changes in spinach chloroplasts, which have a full complement of lipid, that are very similar to those which arise on isolating bean chloroplasts at pH 6 (figs 9–11). It is possible, therefore, that the released linolenic acid is causing most of the damage in bean rather than the loss of lipid per se. One might then expect that these changes would be reversible by washing the chloroplasts in media containing bovine serum albumin. However, a control experiment indicated that chloroplasts bind fatty acids too tightly to allow this reversal. In this experiment, linolenic acid was added to spinach chloroplasts at a concentration needed to give 36 % inhibition of ferricyanide reduction. Control and treated chloroplasts were washed 5 times in 0.35 M NaCl, 0.02 M maleate pH 6.5 containing 5 mg/ml lipid-free bovine serum albumin. The treated chloroplasts still showed 33 % inhibition of ferricyanide reduction at the completion of the washes indicating little or no reversal.

Sastry and Kates (31) demonstrated that runner-bean chloroplasts isolated in unbuffered 0.35 M NaCl have lost most of their mono- and digalactosyldiglycerides and some phospholipid compared either to whole leaf extracts or to spinach chloroplasts prepared in the same solution. Thin layer chromatograms of lipid extracts from pH 6- and pH 8-isolated bean chloroplasts according to the methods of Nichols (27), showed that the pH 6 chloroplasts have lost most of their monogalactosyl diglycerides in addition to some digalactosyl diglyceride and phospholipid.

Mitochondria have also been shown to be altered by fatty acids released by enzymes. Lehninger and

Remmerts showed that U factor, which is produced by a Ca^{++}-stimulated mitochondrial enzyme and causes uncoupling and swelling, consists of fatty acids (22). Also, Pressman and Lardy (28) demonstrated that mitochrome, a protein isolated from mitochondria, has uncoupling activity and contains free fatty acids.

Although we have shown the deleterious effects of low pH during grinding, a pH too high is also equally bad, but the mechanism of inactivation is probably different from that which occurs at low pH. The inactivation of chloroplasts at alkaline pH's has been reported by Punnett (29). Bean chloroplasts isolated at pH 8.5 are apparently normal with respect to the reduction of DCPIP and ferricyanide if assayed within 30 minutes after grinding. Large rate stimulations by ADP, Pi and magnesium are observed. However, the reductive activities of these chloroplasts are very unstable with a half-life of less than 1 hour, compared to chloroplasts at pH 8.0 which have a half-life of 6 to 10 hours.

Summary

Bean chloroplasts (*Phaseolus vulgaris*), in contrast to those of spinach are irreversibly altered by isolation at pH values below 8. Bean chloroplasts isolated at pH show several new characteristics: A) dichlorophenolindophenol is reduced with a pronounced optimum at pH 4.5 and at this pH, the reduction is not saturated at 15,000 foot candles. B) They are entirely uncoupled. C) They have lost 40 % of their nonchlorophyll lipid, and are more dense. D) The rates of reduction depend on the ratio of oxidized to reduced electron acceptor.

Many of these characteristics may be induced in spinach chloroplasts by aging them at pH 6. Homogenates of bean leaves accelerate the aging process.

Bean leaves contain enzymes which hydrolyze galactolipids and which are active below pH 8. These enzymes release free linolenic acid. Linolenate has been found to cause effects in freshly isolated spinach chloroplasts similar to those exhibited in bean chloroplasts isolated at pH 6. Large increases in linolenic acid were demonstrated in homogenates of bean chloroplasts ground at pH 6 and in spinach chloroplasts aged 21 hours at pH 6.

It is concluded that the changes in biochemical characteristics of chloroplasts due to isolation at low pH or to aging at pH 6 can be accounted for by the linolenic acid released by the action of galactolipid lipases under those conditions.

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