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Photosynthesis, Growth, and the Role of Chloride[1, 2]

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ABSTRACT

Previous studies with isolated chloroplasts have indicated that Cl^- is an essential cofactor for photosynthesis. Considerable support for the postulated Cl^- requirement in photosynthesis came from the observation that Cl^- is essential for growth. Data are presented which show that a 60% reduction in growth which occurred in Cl^--deficient sugar beet (*Beta vulgaris* L.) was not due to an effect of Cl^- on the rate of photosynthesis *in vivo* (net CO_2 uptake per unit area of attached leaves). The principal effect of Cl^- deficiency was to lower cell multiplication rates in leaves, thus slowing down their growth and ultimately decreasing their area. The absence of an effect of Cl^- on photosynthesis *in vivo* was unlikely to have been due to Cl^- retention by the chloroplasts because their Cl^- concentration (measured after nonaqueous isolation) decreased progressively with decrease in leaf Cl^-.

An effect of Cl^- with isolated chloroplasts *in vitro*, however, was confirmed. Addition of Cl^- to the reaction medium after washing chloroplasts in EDTA increased the rate of ferricyanide photoreduction 10-fold. This effect of Cl^- did not appear to be related to the Cl^- concentration of the chloroplasts since chloroplast Cl^- was not decreased further by washing in EDTA. It is concluded that Cl^- has not yet unequivocally been shown to be an essential cofactor for photosynthesis and that the response to Cl^- *in vitro* probably does not have a physiological basis.

Since Warburg and Lüttgens (25) first showed that photosynthetic O_2 evolution from isolated chloroplast fragments depended on the presence of Cl^-, there has been some question as to whether Cl^- is an essential cofactor for photosynthesis. Alternatively it has been suggested that Cl^- might be required *in vitro* to protect chloroplasts during their isolation or assay (1, 6, 16). Later work (3, 8-10) indicated that Cl^- was required for photosynthetic O_2 evolution at PSII.

Virtually all of the evidence supporting the Cl^- requirement for photosynthesis was obtained from studies carried out *in vitro*; in these experiments the photosynthetic activity of isolated chloroplasts (or chloroplast fragments) was decreased by washing to decrease the Cl^- content, and then restored by adding Cl^- to the reaction medium (1, 3, 8-10, 25). The improvement in the *in vitro* rate of photosynthesis which occurs when Cl^- is added to the reaction medium compared to the rate when no Cl^- is added is referred to elsewhere (9) and subsequently in this paper as the "Cl^- effect."

In 1949 Arnon and Whatley (1) considered it unlikely that Cl^- was an essential cofactor for photosynthesis since, up to that time, Cl^- had not been shown to be required for plant growth. When in 1954 Broyer *et al.* (4), and later others (12, 24), demonstrated the essentiality of Cl^-, this theoretical objection to the role of Cl^- in photosynthesis was apparently removed. In later reports related to the Cl^- effect, the requirement for Cl^- in plant growth is usually noted (3, 9, 15), and the assumption implicit in making such an observation is that Cl^- is required for growth because of its role in photosynthesis. However, this assumption had not been tested experimentally. The objective of the present investigation therefore was to study further the role of Cl^- in photosynthesis and growth and to determine in particular whether the effect of Cl^- on plant growth is mediated via an effect on the rate of photosynthesis *in vivo* as opposed to an effect on some other facet of growth.

Data are presented below which show that the reduction in growth which occurred in response to Cl^- deficiency was not due to an effect of Cl^- on the rate of photosynthesis *in vivo* as measured by net CO_2 uptake of attached leaves. The principal effect of Cl^- deficiency was to reduce cell multiplication rates in leaves, which in turn decreased leaf expansion, and thereby plant growth by up to 60%. Despite the absence of a "Cl^- effect" *in vivo* in sugar beet, the Cl^- effect could be readily obtained with isolated chloroplasts *in vitro* using the washing procedures described by Izawa *et al.* (10). I concluded that Cl^- has not yet been shown unequivocally to be an essential cofactor for photosynthesis and that the response of Cl^- *in vitro* may not have a physiological basis.

MATERIALS AND METHODS

Plant Culture and Growth Analysis. Sugar beets (*Beta vulgaris* L. cv. F58-554H1) were cultured hydroponically at 25 C, and illuminated at 40,000 lux over a 16-hr day. The plants were cultured for 2 weeks following planting in vermiculite with half-Hoagland solution then transplanted to culture solutions containing five different concentrations of Cl^-: 0, 0.5, 5, 50, and 500 μmol l^{-1}. The composition of the culture solution (exclusive of Cl^-) was: in mmol/l, 2 $Ca(NO_3)_2$, 1 K-phosphate, 2.5 KNO_3, and 1 $MgSO_4$, and in mg/l, 0.25 B, 0.25 Mn, 0.025 Zn, 0.01 Cu, 0.005 Mo, and 2.5 Fe (as ferric-sodium ethylenediaminetetraacetate complex).

Chloride was added as the Na salt, the Na concentrations being maintained at 0.5 mM in all treatments by adding Na_2SO_4. At 2 and 4 weeks from transplanting, concentrated stock solutions of nutrients other than Cl^- were supplied in amounts to equal the initial culture solution composition. Each week plants were removed for the determination of fresh and dry weights of blades, petioles, storage roots and fibrous roots, and for the determination of areas and numbers of living leaves as well as the numbers of dead leaves. One complete experiment consisted of 25 plants (sufficient for five Cl^- treatments to be harvested on five successive occasions) randomized within one growth chamber. This experiment was replicated five times.

Cell Numbers, Mean Cell Volume. An experiment was designed to test whether the reduction in leaf area which occurred with Cl^- deficiency was due to a decrease in the number of cells/leaf, or to a reduction in mean cell volume. Three plants were chosen which had been supplied with Cl^- and three which had

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[2] A brief presentation of this work was made at the Annual Meeting of the American Society of Plant Physiologists at New Orleans, La., June 3, 1976 (Plant Physiol. 57: S-95).

received no supplemental Cl^-. Leaves from different nodes were removed from plants 4 to 5 weeks after treatment began; their areas, fresh and dry weight, and numbers of cells were determined, and the average cell volume estimated, by a method described previously (19).

Leaf Gas Exchange. The net rate of photosynthetic CO_2 uptake/unit area was determined on individual attached leaves during the 5th week of treatment. The equipment used to make the gas exchange measurements was the same as that described by Terry *et al.* (22) except for the following modifications. The leaf chamber was round in shape with an i.d. of 25 cm. The leaf was placed between two water jackets to control leaf temperature. The chamber was made entirely from brass (except for two lucite windows in the upper jacket through which light was admitted) to improve the efficiency of heat transfer. A high speed fan rotated the air inside the leaf chamber itself, which had an internal depth of 3.3 cm and volume of 1,621 cm^3. Leaf temperature was measured using a Sentel model 1200 digital temperature indicator fitted with Iso-curve thermistors (Fenwall Electronics No. GB32MM2). There were two thermistors mounted on the top (shielded from direct radiation) and two on the bottom surface of the leaf. The leaf was irradiated with a water-cooled xenon-arc illumination system (Atlas Electric Devices Co., model No. RM 60) fitted with an IR inner filter (No. 12-4860-00) and a quartz outer filter (No. 20-4031-00). Water vapor pressure was determined by Cambridge Systems dewpoint hygrometer model 880 and CO_2 concentration by a Beckman IR gas analyzer model 885.

The procedure was to insert an attached leaf in the leaf chamber overnight. At 8 AM the following day the rate of CO_2 efflux in the dark was measured (dark respiration) after which the leaf was irradiated at 1990 μeinsteins m^{-2} sec^{-1} at 25 C and an ambient CO_2 concentration of 300 $\mu l\ l^{-1}$. Net CO_2 uptake was determined after 2.5 hr. The ambient CO_2 concentration was lowered to 200 $\mu l\ l^{-1}$, then 100 and 0 $\mu l\ l^{-1}$ with net CO_2 exchange being determined for 1 hr at each CO_2 concentration. The rate of efflux of CO_2 into CO_2-free air in the light was considered to be a measure of light respiration. Leaf (mainly stomatal) diffusion resistance was computed from measurements of transpiration rate and leaf temperature as described by Terry and Ulrich (21).

The effect of irradiance on photosynthetic CO_2 uptake was determined. The leaf was irradiated at 1,995 μeinsteins m^{-2} sec^{-1} for 1 hr, then for 1 hr at each of the irradiances, 310, 510, 930, 1995, and 3650 μeinsteins m^{-2} sec^{-1}. The ambient CO_2 concentration was maintained at 300 $\mu l\ l^{-1}$ and the temperature at 25 C. Different irradiances were obtained by varying the height of the lamp, the lamp wattage, and by inserting muslin cloth filters between the lamp and the leaf chamber. Irradiances were measured using a Lambda Instruments LI-COR quantum sensor. The total leaf Chl content was determined as described previously (20).

Leaf Chloride. The dried plant material (plus 6 ml of 2% K_2CO_3/1-g sample to prevent loss of Cl^- [11]) was ashed at 550 C in a muffle furnace for 60 min. The ashed material was dissolved in nitric acid to give a normality of 0.1. The Cl^- content of the sample was determined by titration with silver nitrate according to the procedure of Johnson and Ulrich (13). A "blank" Cl^- determination was made by carrying out the entire procedure except for addition of the plant sample, and the blank value subtracted from the sample value.

Nonaqueous Chloroplast Isolation. The procedure used for nonaqueous isolation of chloroplasts was essentially the same as that described by Stocking (17). Prior to chloroplast isolation plants were cultured for 4 to 5 weeks from transplanting with and without supplied Cl^-. The leaves were harvested 2 hr after illumination began so as to minimize the starch content of the chloroplasts. Contamination of leaf material with Cl^- was lessened by the use of rubber gloves. On harvesting, the leaves were transferred to a −20 C cold room. They were cut into 3-cm squares and frozen on wax paper for 5 min. The frozen leaf material was lyophilized in a freeze-dry apparatus for 9 hr or until dry.

Four 0.25-g samples of dry leaf material (*i.e.* to give a 1-g sample) were ground in a TenBroeck homogenizer with 20 ml of carbon tetrachloride-hexane mixture with a density of from 1.40 to 1.42. The homogenate was then filtered through silk to remove large cellular debris and was centrifuged for 1 min at 3000*g* in a 15-ml centrifuge tube. The chloroplasts remained at the surface of the fluid while other cellular material sedimented on centrifugation. The chloroplasts were removed by pipette and an equal volume of hexane added to them before centrifuging at 1000*g* for 1 min. The supernatant was collected and analyzed for Cl^-. The pellet and the other residues in the isolation procedure were dried under vacuum and analyzed.

Observation of the chloroplast fraction using a light microscope indicated the presence of chloroplasts with apparently very little contamination with other cell materials. Other work has indicated that contamination of chloroplasts with cytoplasmic materials in the nonaqueous isolation procedure is relatively small (2, 18, 23).

Ferricyanide Photoreduction by Isolated Chloroplasts. Leaves were removed from 4-week-old plants well supplied with nutrients. A 9-g chopped leaf sample was homogenized in a Waring Blendor for about 30 sec with 50 ml of medium A (see Table I for composition of media). The homogenate was then filtered through nylon gauze and centrifuged at 200*g* for 1 min to remove starch and large cell particles. The supernatant containing intact chloroplasts was decanted and centrifuged at 2,500*g* for 1 min. The sedimented chloroplasts were resuspended and washed in medium B. The washed chloroplasts were centrifuged at 10,000*g* for 30 sec. At this point the chloroplasts were washed twice with EDTA (medium C), and then one more time with medium D. After centrifugation, the chloroplast pellet was resuspended in medium D. The rate of photoreduction of ferricyanide was determined (see ref. 20 for method) with and without 5 mM NaCl added to medium E. In some instances Cl^- was added as the Ca, Mg, or K salt.

In one experiment, the Cl^- concentration of the chloroplasts was determined before and after the EDTA washes. The same procedure for the isolation, washing, and determination of ferricyanide photoreduction was used as described above except for the following modifications. A 50-g chopped leaf sample was homogenized with 200 ml of medium A. The homogenate was centrifuged in six 50-ml tubes. After the first sucrose wash (medium B), the six sedimented chloroplast samples were combined using 40 ml of medium B. The suspension containing the chloroplasts was divided into two tubes and centrifuged. From one tube a small amount of chloroplast pellet was resuspended in medium B and the rate of ferricyanide photoreduction determined. The remaining part of the chloroplast pellet was used for Cl^- determination. The sample in the second tube was washed

Table I. Composition of various media used for chloroplast isolation, washing or determination of ferricyanide photoreduction

Medium	Tricine buffer	TES-NaOH buffer	Na_2SO_4	Sucrose	Na_2EDTA	$MgSO_4$	$K_3Fe(CN)_6$	pH
	mM							
A	—	30	200	—	—	—	—	7.4
B	—	5	—	100	—	—	—	7.4
C	2	—	—	—	0.5	—	—	7.8
D	—	5	—	100	—	5	—	7.4
E	—	25	—	100	—	3	2	7.4

twice with EDTA (medium C), once with sucrose (medium D), and the chloroplast pellet subsampled for measurement of ferricyanide photoreduction and Cl^- content as before. The pellet used for Cl^- determination contained sucrose, which was determined colorimetrically by anthrone (5), and subtracted from the weight of the dry chloroplast pellet.

RESULTS

Effects of Cl^- Deficiency on Growth and Photosynthesis. Plants began to show signs of Cl^- deficiency about 2 to 3 weeks after treatment began. At 3 to 4 weeks, young leaves developed small (0.5-mm diameter) yellowish spots and an uneven distribution of Chl, the interveinal areas often being deeper green than those areas close to the veins. Also some "hooking" or "cupping" of the leaf blade at the midrib was apparent in severely deficient leaves which was reminiscent of symptoms of Ca deficiency. The fibrous roots were stunted and developed a "herring bone" appearance.

Chloride deficiency caused up to 60% decrease in plant dry weight (Fig. 1A). There was no decrease in growth until the concentration of Cl^- in the leaf blade decreased to about 20 μmol g^{-1} dry weight; below that concentration plant dry weight decreased linearly with decrease in leaf Cl^-. The net rate of photosynthesis did not decrease with decrease in leaf Cl^- below 20 μmol g^{-1} even though the concentration of Cl^- in the leaf blade reached as low as 1 μmol g^{-1} dry weight (about 0.1 μmol g^{-1} fresh weight) (Fig. 1B).

The photosynthesis values were mostly in the range of 40 to 50 mg CO_2 dm^{-2} hr^{-1} under conditions of saturating irradiance, 25 C, and an ambient CO_2 concentration of 300 μl l^{-1}. Rates of photosynthesis measured at lower ambient CO_2 concentrations, *i.e.* 200 or 100 μl l^{-1}, were also unchanged by Cl^- deficiency, as were rates of photosynthesis measured at different irradiances

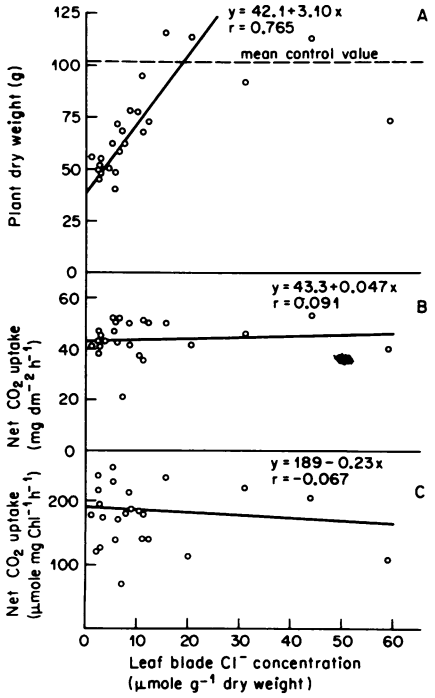

FIG. 1. Effects of Cl^- deficiency on growth and photosynthesis. Changes in total plant dry weight with decrease in leaf blade Cl^- concentration (A). Rates of net photosynthetic CO_2 uptake by single attached leaves at saturating irradiance, 300 μl CO_2 l^{-1} air and 25 C expressed per unit area (B) and per unit Chl (C). In (A) the values for the control treatment (500 μmol Cl^-/l) were excluded from the linear regression (——) and were averaged (- - -).

Table II. Net rates of photosynthetic CO_2 uptake at different irradiances for plants grown with varying amounts of Cl^-

Irradiance μeinstein m^{-2} sec^{-1}	Cl^- supplied μM				
	0	0.5	5.0	50	500
310	13.9	12.0	11.1	13.0	11.2
510	21.3	20.4	19.2	24.1	19.8
930	33.2	32.4	31.3	36.1	31.4
1995	46.1	48.5	43.5	50.1	40.4
3650	46.8	48.0	44.5	51.6	40.0

Values are means of data from two leaves and are expressed in units of mg CO_2 dm^{-2} leaf surface hr^{-1}.

(Table II). Rates of photosynthesis expressed/unit Chl did not change with leaf Cl^- (Fig. 1C). This was because there was no decrease in the Chl content/unit leaf area, despite the apparent uneven distribution of Chl in the blade. Thus, leaves that contained very low concentrations of Cl^- had high *in vivo* rates of photosynthesis, despite the fact that the plants to which the leaves were attached showed clear symptoms of Cl^- deficiency and up to a 60% reduction in weight.

Some other physiological attributes were also unaffected by Cl^- deficiency. Respiratory CO_2 evolution in the dark did not change with change in leaf Cl^- concentration (Fig. 2A), nor did respiratory evolution of CO_2 into CO_2-free air in the light (Fig. 2B). Stomatal diffusion resistance was not affected by Cl^- deficiency since leaf diffusion resistance did not change with decrease in leaf Cl^- concentration (Fig. 2C), except for the leaf of one plant which wilted during the experiment and caused the stomata to close.

Role of Cl^- in Plant Growth. The data (Fig. 4) show that the reduction in plant growth of Cl^- deficient plants was through an effect on the expansion of individual leaves. The total leaf area/plant changed in the same way as the total plant dry weight with decrease in leaf Cl^- (Figs. 1A and 3A). Leaves unfolded from the terminal bud at the same rate in Cl^--deficient plants as in the controls (Fig. 3B), and the number of living leaves/plant remained the same regardless of the Cl^- treatment. The effect of Cl^- deficiency on total leaf area was due to an effect on individual leaf size, not to an effect on leaf production rates.

The effect of Cl^- deficiency on individual leaf growth was studied in a separate experiment. The leaves of deficient plants attained smaller areas than the leaves of control plants at the same nodal position (Fig. 4A). This was because fewer cells were formed/leaf in Cl^--deficient plants compared to the controls (Fig. 4B); average leaf cell volume was unaffected by Cl^- deficiency (Fig. 4C).

Since leaf growth was decreased by Cl^- deficiency and the rate of photosynthetic CO_2 uptake/unit area was not, then the rate of carbohydrate production should have exceeded the capacity of the plant to utilize sugar products in leaf growth. This was apparently the case since the storage root became proportionally larger with time in Cl^--deficient plants than in the controls (Fig. 5A). The proportion of fibrous roots to leaves dry matter, however, was not affected by Cl^- deficiency (Fig. 5B), nor was the ratio of dry matter/unit area of leaves (Fig. 5C). Apparently the excess sugar products were accumulated only in the storage root.

Relation between Cl^- in the Leaf and Cl^- in the Chloroplast. The fact that plant growth did decrease substantially as leaf Cl^- decreased below 20 μmol g^{-1}, and that rates of photosynthesis did not, may be interpreted to mean that Cl^- is not required for photosynthesis. An alternative explanation is that Cl^- was retained by the chloroplasts at concentrations high enough to satisfy a postulated Cl^- requirement for photosynthesis, even

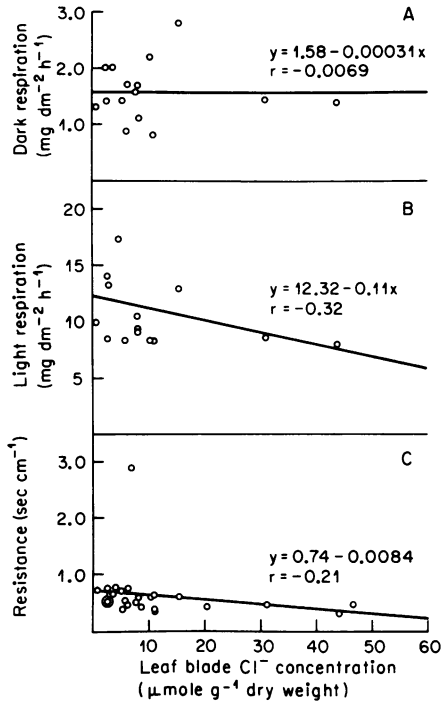

FIG. 2. Effects of Cl^- deficiency on respiration and leaf diffusion resistance. A: rates of respiratory CO_2 evolution in darkness; B: rates of respiratory CO_2 evolution into CO_2-free air in light; C: Leaf (mainly stomatal) diffusion resistance for water vapor.

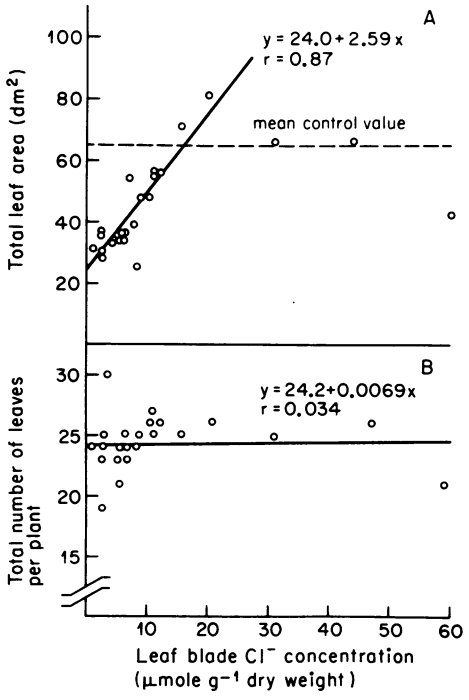

FIG. 3. Effects of Cl^- deficiency on the expansion of the leaf surface. A: total leaf area/plant; B: number of leaves produced/plant. In (A) (- - -) represents the mean of five values for the control treatment (500 μmol Cl^-/l).

though Cl^- levels in the leaf blade were sufficiently low to cause a 60% reduction in growth.

In order to determine whether the Cl^- concentration of the chloroplasts remained high as Cl^- in the leaf blade declined, chloroplasts were isolated nonaqueously (17). Nonaqueous isolation was used because aqueous isolation results in considerable losses of Cl^- and other ions through the outer chloroplast membranes (17, 18). The Cl^- concentration of the chloroplasts was found to decrease almost linearly with decrease in leaf Cl^- below 80 μmol g^{-1} (Fig. 6). Values of over 100 μmol Cl^- g^{-1} dry leaf were attained in leaves plentifully supplied with Cl^- but chloroplast Cl^- never attained values greater than 93 μmol g^{-1}. The additional Cl^- in the leaf at these high concentrations was presumably stored in the vacuoles or cytoplasm.

The Cl^- concentration in the chloroplasts decreased to about 1 μmol g^{-1} which represents a loss of Cl^- from the control leaf chloroplasts of almost 99%. The five lowest concentrations obtained for the nonaqueously isolated chloroplasts were 1.1, 2.8, 3.2, 3.5, and 3.6 μmol Cl g^{-1} dry chloroplasts. These chloroplast concentrations were associated with leaf blade Cl^- concentrations of 1.4, 2.2, 1.0, 1.6, and 2.1 μmol g^{-1}, respectively; so in four out of five instances there appeared to be slightly more Cl^- in the chloroplasts than in the parent leaf material from which they were isolated. These chloroplast Cl^- values may have been slightly higher than they should have been because there was almost certainly some contamination of the chloroplasts with extraneous Cl^- during the isolation procedure. (Chloroplasts, and the residual leaf material remaining after isolation, generally contained slightly more Cl^- than the leaf blade from which they were isolated.) Nevertheless, the Cl^- concentrations of the chloroplasts closely reflected those in the blade as a whole. It is concluded therefore that chloroplasts do not retain Cl^- to an appreciable extent since the Cl^- concentration in the chloroplasts decreased concomitantly with the Cl^- concentration of the leaf.

Effects of Adding Cl^- to Washed Chloroplasts. The evidence presented thus far indicates that although the Cl^- concentration of chloroplasts was reduced to very low levels by Cl^- deficiency, photosynthesis by sugar beets *in vivo* was not affected. If, in addition, it were not possible to obtain a Cl^- effect *in vitro*, *i.e.*

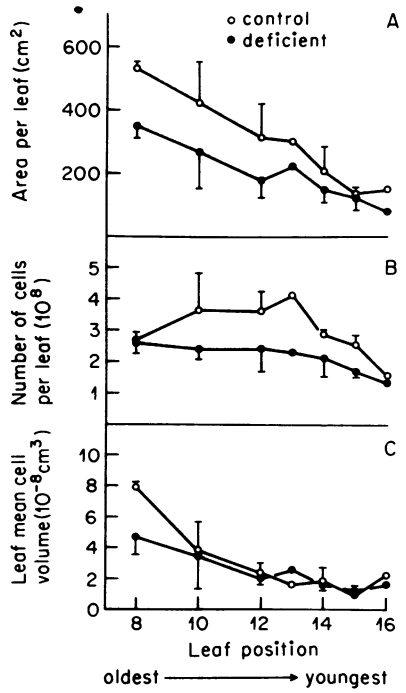

FIG. 4. Effects of Cl^- deficiency on area/leaf (A), number of cells/leaf (B), and the average leaf cell volume (C) for leaves at different nodal positions. Plants (three replicates) were cultured for 5 weeks from transplanting with 500 μmol Cl^-/l (control) or with no Cl^- supplied (deficient).

by washing experiments with isolated chloroplasts, then one might conclude that sugar beets differed from other species, perhaps in having a very low Cl^- requirement which was difficult to demonstrate *in vivo* or *in vitro*.

The possibility that chloroplasts isolated from sugar beets would not show a Cl^- effect was investigated. The procedure adopted was essentially that of Izawa *et al.* (10) who obtained a 10-fold increase in the rates of photoreduction of ferricyanide when Cl^- was added to the reaction medium by first washing isolated spinach chloroplasts with EDTA. In five separate experiments, the rate of photoreduction of ferricyanide by sugar beet chloroplasts after washing with EDTA was 95.3 ± 23.5 μmol Fe^{3+} mg^{-1} Chl hr^{-1} with no Cl^- added to the reaction medium, and 945 ± 148 μmol Fe^{3+} mg^{-1} Chl hr^{-1} in the presence of Cl^- (5 mM NaCl). The effect was solely due to the addition of Cl^- since supplying Cl^- as the salt of Ca^{2+}, Mg^{2+}, or K^+ also gave a 10-fold increase while the addition of Na^+ as Na_2SO_4 did not significantly increase the rate of ferricyanide photoreduction. Thus, the observation made by Izawa *et al.* (10) that Cl^- addition to EDTA-washed chloroplasts of spinach accelerated the Hill reaction was confirmed for sugar beets also.

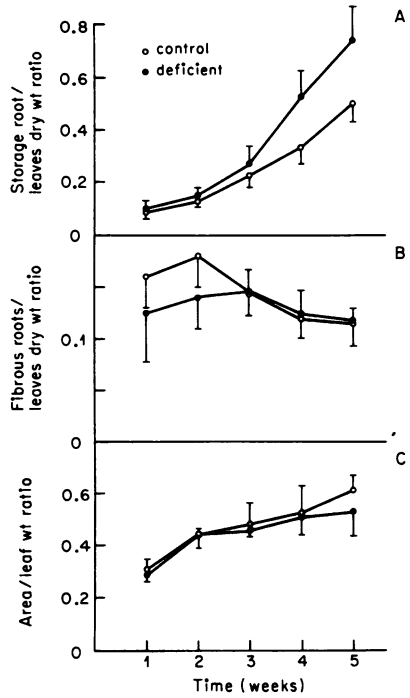

FIG. 5. Effects of Cl^- deficiency on the partitioning of dry matter with time. A: storage root/leaves dry matter ratio; B: fibrous root/leaves dry matter ratio; C: dry matter/unit area of leaf blades ratio (g dm^{-2}). Plants were cultured for 5 weeks from transplanting with 500 μmol Cl^-/l (control) or with no Cl^- supplied (deficient).

Izawa *et al.* (10) concluded that the effect of washing with EDTA was to make the thylakoid membranes more permeable so that Cl^- might be more easily washed from the "internal space" of the thylakoids, *i.e.* they attributed their results to a removal of Cl^- by the EDTA. This theory was tested by measuring the Cl^- content of the chloroplasts before and after washing with EDTA. Five experiments were carried out: three with Cl^--deficient plants and two with Cl^--sufficient (control) plants. The rates of ferricyanide photoreduction with and without Cl^- in the reaction medium were also determined before and after washing with EDTA.

The results are summarized in Table III. They show that

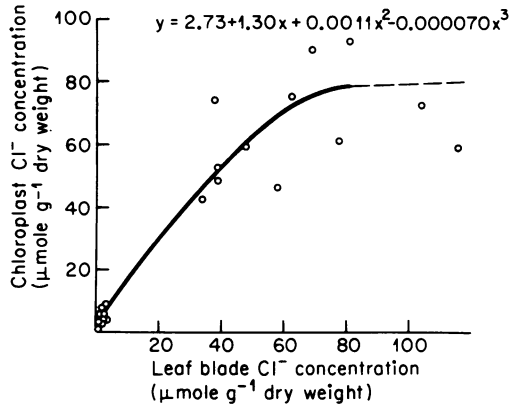

FIG. 6. Changes in chloroplast Cl^- concentration with leaf blade Cl^- concentration. (——) represents curve fitted by regression analysis.

Table III. Effect of washing on the Cl^- concentration and photosynthetic activity of isolated chloroplasts

Chloroplasts were isolated and washed once with medium B ("sucrose washed"); then twice with EDTA (medium C) and once with sucrose (medium D) ("EDTA washed"). The rates of ferricyanide photoreduction and the Cl^- concentrations were determined for both the sucrose washed and EDTA washed chloroplasts.

Treatment		Chloride concentration*		Ferricyanide reduction**	
Plants	Chloroplasts	Leaf blade	Chloroplasts	Basal rate	5mM NaCl added
Control	Sucrose washed	77·6 ± 33·2	2.45 ± 0·35	115 ± 38·1	242 ± 43.1
			20·6 ± 3.1		
	EDTA washed	——	4·10 ± 2.26	63.5 ± 7.78	559 ± 236
			29·0 ± 9·9		
Cl-deficient	Sucrose washed	1·22 ± 0·028	0·75 ± 0.35	109 ± 35·7	298 ± 116
			6·22 ± 1.48		
	EDTA washed	——	1·25 ± 0.21	61·7 ± 9·07	271 ± 54·8
			9·37 ± 0·64		

*Cl^- concentration is expressed as μmol Cl^- per gram dry weight and as μmol Cl^- per mg Chl (italics).

**μmol Fe^{3+} mg Chl^{-1} hr^{-1}.

chloroplasts isolated from control plant leaves with an average Cl^- concentration of 78 μmol Cl^- g^{-1} contained 2.5 μmol Cl g^{-1} after only one wash in sucrose (medium B). Chloroplasts isolated from leaves with about 80 μmol g^{-1} concentration usually contained about the same concentration of Cl^- as the leaf blade (Fig. 6). Making the assumption that chloroplasts from leaves with Cl^- at 78 μmol g^{-1} contained Cl^- at about the same concentration, then the effect of isolation and one sucrose washing of the chloroplasts was to decrease their Cl^- concentration by about 97%.

Washing with EDTA did not decrease the Cl^- content of the chloroplasts further in control or Cl^--deficient plants. The apparent increases in chloroplast Cl^- following EDTA washing were in part due to losses of chloroplast dry matter since EDTA removes cations and other materials including proteins; the ratio of chloroplast dry matter to Chl decreased from about 8.2 to 7.4 mg chloroplast/mg Chl after washing with EDTA. In any event, washing with EDTA did not reduce the Cl^- concentration of the chloroplasts, but it did increase the extent of the response to Cl^- added to the reaction medium. After only one washing with sucrose (medium B), addition of Cl^- to the reaction medium gave a 2- to 3-fold increase in ferricyanide photoreduction (Table III). After further washing with EDTA and sucrose, the increase in the rate of ferricyanide photoreduction on Cl^- addition was 4- to 9-fold. Part of the enhanced response to Cl^- addition after EDTA washing was due to a decrease in the basal rate, *i.e.* the rate when no Cl^- was added to the reaction medium. Thus, washing chloroplasts with EDTA accelerated the rate of ferricyanide photoreduction on addition of Cl^- to the reaction medium *without* decreasing the Cl^- concentration of the chloroplasts.

DISCUSSION

The visible symptoms of Cl^- deficiency obtained in the present work were as described for sugar beet by Ulrich and Ohki (24). These authors observed that sugar beet growth was reduced when the concentration of Cl^- in the leaf blade decreased to about 25 to 50 μmol g^{-1} dry weight, and that severe deficiency occurred at 3 to 5 μmol g^{-1} dry weight; both of these observations were consistent with the present findings. Furthermore, the deficiency condition was corrected by supplying Cl^- to Cl^--deficient plants, the newly developing leaves and roots exhibiting normal healthy growth. For these reasons the treated plants were considered to be truly Cl^--deficient and not suffering from some other nutritional or pathological condition.

Despite the visible symptoms of Cl^- deficiency and the 60% reduction in growth, leaves of Cl^--deficient plants attained rates of photosynthetic CO_2 uptake of 40 to 50 mg CO_2 dm^{-2} hr^{-1} at saturating irradiance, an ambient CO_2 concentration of 300 μl l^{-1} and 25 C. These high rates were maintained even as the Cl^- concentration in the leaf blade decreased to as low as 1 μmol g^{-1} and are as high as any rates previously obtained for sugar beet (*e.g.* Hall [7]). Under saturating conditions of irradiance and CO_2 concentration (*i.e.* 3,000 μeinsteins m^{-2} sec^{-1} and 1,000 μl l^{-1} ambient CO_2) at 30 C, the rate of photosynthetic CO_2 uptake in severely Cl^--deficient plants reached 350 μmol CO_2 mg Chl^{-1} hr^{-1}. This is equivalent to an *in vivo* rate of $NADP^+$ reduction of 700 μmol mg Chl^{-1} hr^{-1}, a value which is considerably greater than those usually obtained *in vitro*.

The fact that the Cl^- concentration of nonaqueously isolated chloroplasts decreased progressively with decrease in leaf Cl^- suggests that Cl^- was not retained by chloroplasts as the plants became progressively more Cl^--deficient. Larkum (14) estimated that the dry weight of *Tolypella intricata* chloroplasts constituted about one-third of their total weight and that the solute available space was about two-thirds of the chloroplast volume. Assuming the same is approximately true for sugar beet, then chloroplasts which contained 1 μmol Cl^{-1} g^{-1} dry chloroplasts would have had a concentration of Cl^- in the solute available space of about 0.5 mM. For comparison the *in vitro* Cl^- requirement for photosynthesis by isolated chloroplasts of sugar beets was satisfied by an external concentration of 5 mM. Thus, very high rates of photosynthesis were obtained *in vivo* (about 1,050 μmol ATP mg Chl^{-1} hr^{-1}) even though the Cl^- concentration in the chloroplasts decreased to about one-tenth of that needed to satisfy the *in vitro* requirement.

Since the effect of Cl^- deficiency on plant growth is mediated via processes other than photosynthesis, the only evidence in support of the postulated Cl^- requirement for photosynthesis is from the *in vitro* experiments with isolated chloroplasts. Sugar beet chloroplasts clearly exhibited an *in vitro* Cl^- effect, there being up to a 10-fold stimulation of photosynthetic activity when Cl^- was added to the reaction medium after washing the chloroplasts with EDTA. But the Cl^- effect did not appear to be related to the Cl^- concentration of the chloroplasts since most of the Cl^- was easily removed after isolation and only one wash in sucrose (medium B; Table I). Two subsequent washes in EDTA (medium C) and one wash in sucrose (medium D) did not decrease the Cl^- concentration of the chloroplast fragments further.

Since Cl^- was easily removed from the isolated chloroplasts without the use of EDTA it seems unlikely that EDTA facilitated removal of Cl^- from the internal space of the thylakoids as Izawa *et al.* (10) suggest. Winocur *et al.* (26) also observed that Cl^- readily diffused out the "inner compartments" of isolated spinach chloroplasts. Since the extent of the Cl^- effect was increased after washing with EDTA, while the Cl^- concentration of the chloroplasts was unchanged, the response to added Cl^- did not appear to be related to the restoration of Cl^- as a cofactor. Presumably, washing with EDTA damaged or altered the chloroplasts in such a way that they were no longer representative of the normal physiological condition. It is assumed that the addition of Cl^- to these "altered" chloroplasts acted in some unknown way to ameliorate the damage sustained during washing. A similar conclusion was reached by Gorham and Clendenning (6) who showed that repeated washing of intact or fragmented chloroplasts improved the extent of the response to added Cl^- in a way that was unrelated to the Cl^- concentration of the chloroplasts.

Evidence from chloroplast-washing experiments has been criticized elsewhere on the grounds that Cl^- may be an *in vitro* rather than an *in vivo* requirement (1, 6, 16). It is clear that Cl^- does afford some protection against photoinactivation (1, 6, 9). Furthermore, the Cl^- effect requires special treatment of the chloroplasts for it to be demonstrated. Hind *et al.* (9) showed that freshly isolated spinach chloroplasts show little or no Cl^- effect until they are "induced"; the induction process required aging, or heating, or raising the pH of the chloroplasts. Chloroplasts have been treated with uncouplers (9), and EDTA (10) to induce a significant Cl^- effect. Also, no matter how vigorous the washing procedure, a basal rate of electron flow often persists (which may be 50% of the Cl^- added rate) which is quite independent of Cl^- (6, 9, 10). Thus, it is concluded that Cl^- has not yet unequivocally been shown to be required for photosynthesis.

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LITERATURE CITED

1. Arnon DI, FR Whatley 1949 Is chloride a coenzyme of photosynthesis? Science 110: 554-556
2. Bird IF, HK Porter, CR Stocking 1965 Intracellular localisation of enzymes associated with sucrose synthesis in leaves. Biochim Biophys Acta 100: 366-375
3. Bové, JM, C Bové, FR Whatley, DI Arnon 1963 Chloride requirement for oxygen evolution in photosynthesis. Naturforschung 18b: 683-688
4. Broyer TC, AB Carlton, CM Johnson, PR Stout 1954 Chlorine—a micronutrient

element for higher plants. Plant Physiol 29: 526–532
5. Dische Z 1954 New color reactions for determination of sugars in polysaccharides. *In* D Glick, ed, Methods of Biochemical Analysis Vol 2. Wiley-Interscience, New York pp 313–331
6. Gorham PR, KA Clendenning 1952 Anionic stimulation of the Hill reaction in isolated chloroplasts. Arch Biochem Biophys 37: 199–223
7. Hall AE 1970 Photosynthetic capabilities of healthy and beet yellows infected sugar beets (*Beta vulgaris* L.). PhD thesis. Univ California, Davis
8. Heath RL, G Hind 1969 The role of Cl^- in photosynthesis. II. The effect of Cl^- upon fluorescence. Biochim Biophys Acta 172: 290–299
9. Hind G, HY Nakatani, S Izawa 1969 The role of Cl^- in photosynthesis. I. The Cl^- requirement of electron transport. Biochim Biophys Acta 172: 277–280
10. Izawa S, RL Heath, G Hind 1969 The role of chloride ion in photosynthesis. III. The effect of artificial electron donors upon electron transport. Biochim Biophys Acta 180: 388–398
11. Johnson CM, RP Huston, PG Ozanne 1958 Measurement of microgram amounts of chlorine in plant materials. Agric Food Chem 6: 114–118
12. Johnson CM, PR Stout, TC Broyer, AB Carlton 1957 Comparative chlorine requirements of different plant species. Plant Soil 8: 337–353
13. Johnson CM, A Ulrich 1959 Analytical methods for use in plant analysis. Calif Agric Exp Sta Bull 766:26–78
14. Larkum AWD 1968 Ionic relations of chloroplasts *in vivo*. Nature 218: 447–449
15. McSwain BD, HY Tsujimoto, DI Arnon 1976 Effects of magnesium and chloride ions on light-induced electron transport in membrane fragments from a blue-green alga. Biochim Biophys Acta 423: 313–322
16. Punnett T 1959 Stability of isolated chloroplast preparations and its effect on Hill reaction measurements. Plant Physiol 34: 283–288
17. Stocking CR 1959 Chloroplast isolation in nonaqueous media. Plant Physiol 34: 56–61
18. Stocking CR, A Ongun 1962 The intracellular distribution of some metallic elements in leaves. Am J Bot 49(3): 283–289
19. Terry N 1970 Developmental physiology of sugar-beet. II. Effects of temperature and nitrogen supply on the growth, soluble carbohydrate content and nitrogen content of leaves and roots. J Exp Bot 21: 477–496
20. Terry N, RP Huston 1975 Effects of calcium on the photosynthesis of intact leaves and isolated chloroplasts of sugar beets. Plant Physiol 55: 923–927
21. Terry N, A Ulrich 1972 Effects of phosphorus deficiency on the photosynthesis and respiration of leaves of sugar beet. Plant Physiol 51: 43–47
22. Terry N, LJ Waldron, A Ulrich 1971 An apparatus for the measurement of carbon dioxide and water vapor exchange of attached sugarbeet leaves. J Am Soc Sugar Beet Technol 16: 471–478
23. Thompson CM, CP Whittingham 1967 Intracellular localisation of phosphoglycollate phosphatase and glyoxalate reductase. Biochim Biophys Acta 143: 642–644
24. Ulrich A, K Ohki 1956 Chlorine, bromine, and sodium as nutrients for sugar beet plants. Plant Physiol 31: 171–181
25. Warburg O, W Lüttgens 1946 Photochemische Reduktion des Chinons in grünen Zellen und Granula. Biochimia 11: 321–322
26. Winocur BA, RI Macey, AB Tolberg 1968 Electrolyte exchange in isolated spinach chloroplasts. Biochim Biophys Acta 150: 32–40