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MECHANISMS OF LIGHT-INDUCED STRUCTURAL CHANGE IN CHLOROPLASTS

II. THE ROLE OF ION MOVEMENTS IN VOLUME CHANGES

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SUMMARY

1. The effect of certain anions in enhancing light-induced light-scattering increments in isolated spinach chloroplasts has been studied, and the relation of these effects to volume changes and ion movements has been investigated.

2. Changes in transmission or absorbance have been shown to provide a better indication of volume changes of chloroplasts in suspension than do changes in 90° light scattering. Absorbance measurements were therefore employed to study osmotic behavior of chloroplasts suspended in a variety of sodium and ammonium salts. Chloroplasts swell rapidly in the dark in the ammonium salts of weak acids. Since the rate of swelling is greatest with anions whose pK_a is highest, it is concluded that chloroplasts are permeable to undissociated acids and ammonia.

3. A mechanism has been proposed to explain the effects of anions in enhancing light-scattering changes in illuminated chloroplasts. It is suggested that on acidification of the chloroplast interior by light-induced hydrogen ion uptake, weakly acidic anions are lost as a result of the displacement of the equilibrium of undissociated acid across the chloroplast membrane, and that the volume changes observed are by osmotic equilibration. The kinetics and pH dependence of the light-induced light-scattering and hydrogen ion changes are consistent with this mechanism.

4. The light-induced movement of cations in suspensions of chloroplasts in acetate and chloride media has also been measured. On illumination in Tris–acetate, the small amount of cation retained by chloroplasts is irreversibly lost but a reversible uptake of cation on illumination of chloroplasts suspended in choline or Tris–chloride is observed. The extent of the light-induced cation movements is too small to account for chloroplast volume changes.

INTRODUCTION

Chloroplasts illuminated in chloride medium show an increase in light scattering which is parallel to the light-induced pH change, and the companion paper[1] has shown that these phenomena are closely associated. However, a number of workers[2,3] have

Abbreviation: PMS, *N*-methyl phenazonium methosulphate.

shown dramatic modifications of the light-scattering behavior of chloroplasts or rates of electron flow in media of more complex ionic constitution. It seemed likely that an explanation of these effects might be related to the ability of certain ions to penetrate the chloroplast membrane, and the present work was carried out to investigate this possibility.

Dark permeability

The permeability of spinach chloroplast membranes to ions has been only tentatively investigated. STOCKING AND ONGUN[4] found that potassium and sodium ions were lost from spinach chloroplasts during isolation in sucrose media. More recently, TOLBERG AND MACEY[5] on the basis of the distribution of potassium, sodium and chloride ions between pellet and supernatant in chloroplasts centrifuged down from a variety of media, have suggested that sodium and chloride ions equilibrate rapidly between all aqueous phases, whereas potassium ions may be partially retained. Indirect evidence about permeability is provided by measurements of the osmotic properties of chloroplasts[5,6] and chloroplast fragments[7] when suspended in solutions of different solutes. NISHIDA AND KOSHII[5] have shown that solutions of the chloride, nitrate and sulphate salts of a variety of monovalent cations, and sodium acetate, are able to support chloroplasts osmotically, as are solutions of sucrose, glucose, and mannitol. GROSS AND PACKER[7] reported that preparations of sonically disrupted chloroplasts, which consist largely of isolated grana stacks, have similar osmotic properties. Although the slow dark swelling[6,8] observed when chloroplasts are suspended in solutions of some inorganic salts suggests a degree of permeability to small cations and anions, in general it may be concluded from the osmotic behavior of chloroplasts that the rates of penetration of either cations or inorganic anions, or of both, are relatively slow. It may also be concluded that the grana stacks of the chloroplasts represent at least one osmotically active compartment. Osmotic effects of organic anions other than acetate have not been reported.

Light-induced changes and ionic environment

Modifications of light-induced changes by ionic environment have been more fully investigated than has dark permeability. PACKER AND SIEGENTHALER[2] have shown that phosphate, arsenate and a wide range of organic anions greatly stimulate the extent of the light-induced electron flow dependent scattering increments of chloroplasts[9,10], while pyrophosphate and amino acids have little or no effect. A parallel inhibition by these anions of the light-induced swelling of chloroplasts in NaCl solutions was also observed. GOOD[11] has also demonstrated an uncoupling effect of certain anions on the Hill reaction with ferricyanide. NOBEL AND PACKER[12] first observed a light-induced binding of sodium and calcium ions by chloroplasts; however, the kinetics and extent of the changes show no relation to either the rapid ion movements measured by other workers[13,14], or to the light-induced structural changes[2,9,10]. DILLEY[13] and DILLEY AND VERNON[14] measured light-induced movements of potassium, sodium and magnesium ions which occur relatively rapidly in an acetate containing medium, and have proposed a relation between cation transport and volume changes in chloroplasts.

The uptake of hydrogen ions induced by light has been perhaps the most thoroughly investigated aspect of ion transport by chloroplasts. JAGENDORF and

co-workers[15,16] investigated the energy relationship of the light-induced pH change, and have shown that its behavior with respect to kinetics and response to inhibitors is similar to that of the high-energy state of photophosphorylation, X_E (ref. 17). The relation between proton transport and structural changes in chloroplasts suspended in NaCl has been discussed at length in the accompanying paper[1]. It has been suggested that under these conditions, the increase in light scattering observed on illumination is due primarily to a 'precipitation' of bound protein caused by a lowering of the pH within the chloroplast.

The present paper is concerned with the mechanism whereby certain anions are able to enhance the light-scattering change, inhibit swelling and induce a decrease of chloroplast volume upon illumination. It was thought that the stimulatory action of the anions might be related to their ability to penetrate the chloroplast membrane. The osmotic properties of chloroplasts have therefore been studied by observing the rate and extent of dark swelling following suspension of the organelles in solutions of the sodium and ammonium salts of a variety of anions. It has been concluded that ammonia, and the undissociated acids of many organic and inorganic anions can freely penetrate the chloroplast membrane, but that dissociated cations pass across more slowly. Evidence is presented in support of the suggestion that conformational changes are caused by a loss of anion from within the chloroplast as a result of the displacement of the equilibrium of undissociated acid across the membrane which follows acidification of the interior of chloroplasts on uptake of H^+. Light-induced cation transport has also been studied, and the relation between hydrogen ion transfer and the movement of other cations investigated.

METHODS AND MATERIALS

Isolation of chloroplasts

Spinach was purchased from local markets, and chloroplasts prepared either the same day or after short storage at 2°. Chloroplasts were isolated by a variety of methods, as below.

Method 1. Washed spinach leaves were homogenized for 20 sec in a Waring blender, in a medium containing 0.35 M NaCl, 40 mM Tris–chloride (pH 7.5). The homogenate was filtered through 8 layers of cheese-cloth, and cell debris was spun down from the suspension by centrifugation at 270 × *g* for 1 min. The supernatant was centrifuged at 750 × *g* for 7 min, and the resulting pellets were resuspended in the same isolation medium as above. The suspension was centrifuged again at 750 × *g* for 7 min, and the pellets resuspended in a minimal volume of medium, and combined. Unless otherwise stated, chloroplasts were prepared by this method.

Method 2. Washed leaves were homogenized, centrifuged and washed as above, but in a suspending medium containing 0.35 M choline chloride, 25 mM Tris–chloride (pH 7.5).

Method 3. Washed leaves were ground in a cold mortar without sand in a medium containing 0.5 M sucrose, 10 mM Tris–chloride, after the method of SPENCER AND UNT[18]. Filtration, centrifugation and washing were as above, except that the sucrose medium was used throughout. Chlorophyll was estimated spectrophotometrically[19].

Apparatus

Simultaneous measurements of light scattering and pH were carried out as described in the accompanying paper[1].

Measurements of changes of absorbance with time were made in a Beckman DB spectrophotometer, adapted for recording. The rapid dark swelling was followed by the change at 546 mμ on rapid mixing of a small volume of a dense suspension of chloroplasts into solutions contained in the cuvette.

Simultaneous measurements of light scattering and transmission were made in a Brice–Phoenix photometer modified as described by PACKER[20]. Measurements of the light-scattering envelope of chloroplasts were made in the Brice–Phoenix photometer. The incident beam was restricted to a width of 2 mm by means of 1.3-mm slits 2.5 cm apart placed between the light source and the light-scattering cell. Similar slits masked the photomultiplier used to measure the reflected light. Both light source and photomultiplier were screened by Baird Atomic interference filters (Type B-1 with extra high-side blocking) with a maximum transmission at 546 mμ.

Simultaneous measurements of changes in the concentration of hydrogen and potassium ion in chloroplast suspensions were made with ion-selective glass electrodes as described by CHAPPELL AND CROFTS[21]. The potassium electrode (Type GKN 33; Electronic Instruments Limited, Richmond, Surrey, Great Britain) was shown by titration to be insensitive to changes in hydrogen ion concentrations of the magnitude involved in these experiments. The extent of both hydrogen and potassium ion changes was estimated by comparison with the deflection produced on addition of a known amount of KCl or HCl, added at the end of each experiment.

Illumination in all experiments was with broad-banded light equivalent to 1000 foot candles.

Estimations of particle size by Coulter counter, or by packed volume were as described elsewhere[22]. Electron microscopy was as described in the companion paper[1].

Materials

Simple inorganic and organic chemicals were of reagent grade where possible, or otherwise of the highest commercial purity obtainable. Solutions of the ammonium salts of organic acids were made by neutralization of weighed amounts of the acids with NH_4OH to a final pH of 7.4. Adjustment of the pH of other solutions was with the acids of the anions or bases of the cations of the solutions (*e.g.* acetic acid or NH_4OH in a solution of ammonium acetate). *N*-Methyl phenazonium methosulphate (PMS) solutions were prepared fresh daily.

RESULTS AND DISCUSSION

The osmotic behavior of chloroplasts in solutions of ammonium salts

The relation between particle volume, light scattering and absorbance, and the effect of osmotic strength on these parameters has been investigated in chloroplasts[22] and chloroplast fragments[7], and in a variety of other systems[21,23–25]. In general it has been shown that absorbance is inversely proportional to particle volume, and that volume varies inversely with changes in osmotic strength. Absorbance changes

may therefore be used as an indication of changes in volume, at least in the case of osmotically induced changes. The relation between absorbance, light scattering and molar concentration for chloroplasts suspended in NaCl or sodium acetate is shown in Figs. 1a and b. It is apparent that between 0 and 100 mM, absorbance is proportional to osmotic strength for both these solutions. However, the changes in light scattering, although proportional to osmotic strength, are relatively small compared to the changes in absorbance. This difference is discussed further below.

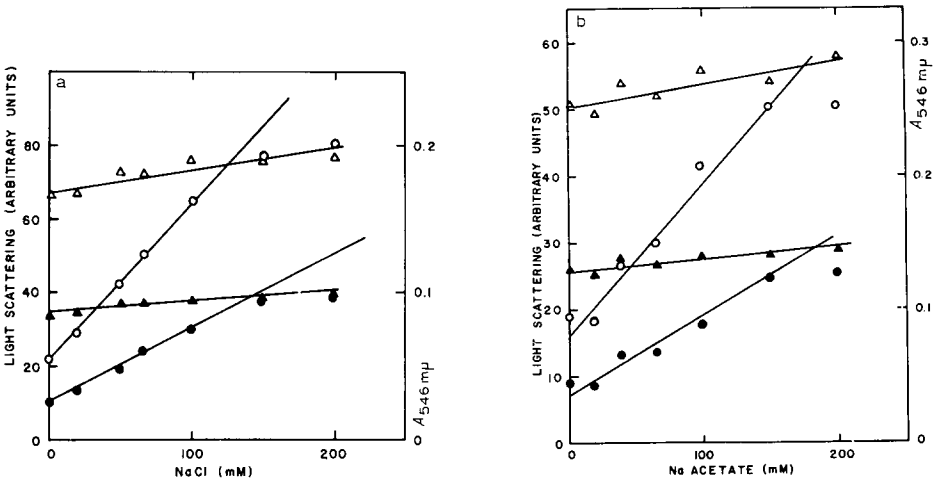


Fig. 1. Comparison of 90° light scattering and absorbance for chloroplasts suspended in NaCl or sodium acetate. Chloroplasts were added to 3 ml of the salt solution at pH 7.4, 25°, contained in the cuvette of a Brice–Phoenix photometer as in METHODS. Values were read after 5 min of equilibration. Absorbance values were calculated from % transmission. Light scattering (△, ▲) and absorbance (○, ●) were measured at 2 concentrations of chloroplasts, as below. a. 2.2 μg/ml (closed symbols) or 4.4 μg/ml (open symbols) of chloroplast chlorophyll in solutions of NaCl. b. 2.7 μg/ml (closed symbols) or 5.4 μg/ml (open symbols) of chloroplast chlorophyll in solutions of sodium acetate.

When the absorbances of suspensions of chloroplasts in solutions of NH_4Cl and ammonium acetate were compared, a marked difference in slopes was observed, indicating that chloroplasts in ammonium acetate have a considerably larger volume than those suspended in NH_4Cl at an equivalent concentration (Fig. 2). This finding was confirmed by measurement of packed volume as shown in Table I, and by electron microscopy (Fig. 3c). The rate and extent of swelling could be more readily observed by recording the changes in absorbance on addition of a small volume of a densely packed chloroplast suspension to solutions of a variety of sodium and ammonium salts of similar concentration and pH as shown in Fig. 4. The absorbance change after equilibration in sodium salts was within experimental error similar to that for NaCl, though initially, the absorbance was as much as 0.07 units lower. These traces have been omitted for clarity. It can be seen that chloroplasts swell rapidly in the dark when suspended in solutions of the ammonium salts of a variety of anions. Most pertinent to the present argument is the observation that the rate of swelling is related to the concentration of undissociated acid present, as judged by the pK_a's of the anions used.

It has been suggested for both erythrocytes[26] and mitochondria[21] that swelling

or lysis in ammonium salts is indicative of penetration of the osmotically effective membrane by both cationic and anionic species of the suspending medium. A mechanism for ammonium salt swelling of mitochondria has been proposed by CHAPPELL AND CROFTS[21] in which it is suggested that the uncharged molecules of ammonia and undissociated organic anion are freely able to penetrate the mitochondrial membrane, and that it is the movement of these molecules which accounts for the failure of the ammonium salts to provide osmotic support. The dependence of rate of swelling of chloroplasts suspended in ammonium salts upon the pK_a of the supporting anion suggests that a similar explanation applies in chloroplasts, as shown in Fig. 5.

Although these experiments indicate that ammonia and undissociated acids are freely able to penetrate the chloroplast membrane, they throw little light upon the ability of charged cations and anions to enter the osmotically effective compartment. This is because the need to maintain charge balance implies that the failure

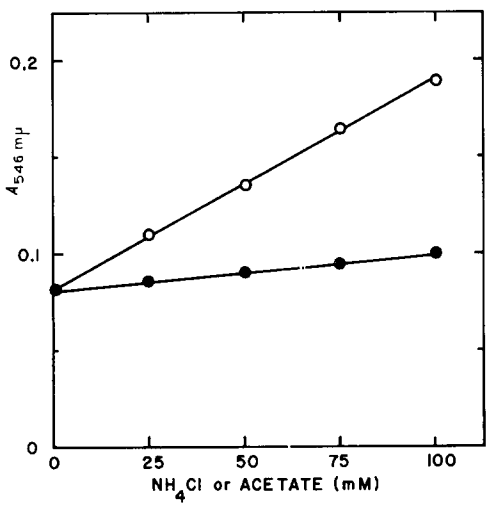


Fig. 2. Absorbance of chloroplasts suspended in ammonium salts of chloride or acetate. Conditions as in Fig. 1. Chloroplasts (5.8 μg chlorophyll/ml) were added to 3 ml of ammonium chloride (open circles) or ammonium acetate (closed circles).

TABLE I

EFFECT OF ACETATE AND CHLORIDE SALTS ON CHLOROPLAST VOLUME

Chloroplasts (0.11 mg chlorophyll/ml) were suspended in 3 ml of the solutions shown, and allowed to equilibrate for 10 min in the dark at 0° before centrifugation.

Suspending medium	*Concn. (mM)*	*Packed volume (μl/mg chlorophyll)*
Sodium chloride	200	120
Sodium chloride	50	252
Ammonium chloride	200	180
Tris–chloride	200	144
Sodium acetate	200	150
Ammonium acetate	200	540
Tris–acetate	200	168

of either ionic species to penetrate will prevent the net flow of salt across the membrane. However, from the observations of STOCKING AND ONGUN[4] and TOLBERG AND MACEY[5] that chloroplasts lose K^+ and Na^+ during isolation or suspension in salt or sucrose media, it might be supposed that small monovalent cations can penetrate the chloroplast membrane, albeit relatively slowly. SALTMAN, FORTE AND FORTE[27]

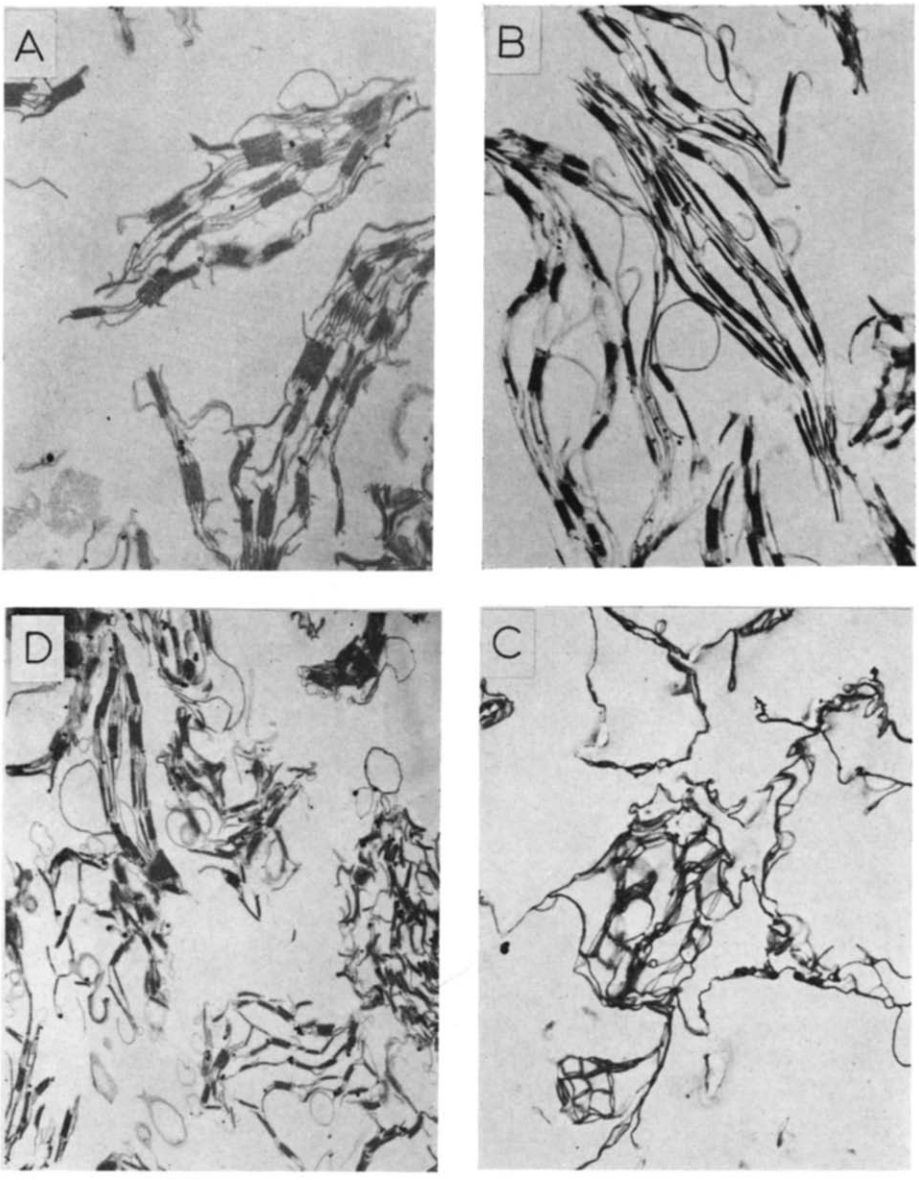


Fig. 3. Morphology of chloroplasts fixed with glutaraldehyde in various structural states, as below. A. Chloroplasts in the dark in 100 mM sodium acetate. B. Chloroplasts in the light in 100 mM sodium acetate containing 20 μM PMS. C. Chloroplasts in the dark in 100 mM ammonium acetate. D. Chloroplasts in the dark in 100 mM sodium acetate, 20 μM PMS, 75 sec after exposure to light for 3 min. In each case the pH was 7.5, temp. 25°. Magnification 5850 ×.

have studied the efflux and uptake of isotopically labelled monovalent cations and anions in Nitella chloroplasts. From the slow rates observed they have concluded that the membrane surrounding these chloroplasts is not penetrated rapidly by Na^+, K^+, Rb^+ or Br^-, and may actively exclude Na^+. B. WINOCUR (personal communication) has shown in 1966 that isotopically labelled sodium and potassium ions equilibrate between spinach chloroplasts and ionic suspending media with a half time of about 9 min. Such an equilibration must represent either a net flow of cation across the membrane or an exchange with external cation, but the evidence available does not allow a distinction to be drawn between these two possibilities. The permeability properties of the chloroplast membrane to ions are discussed more fully below.

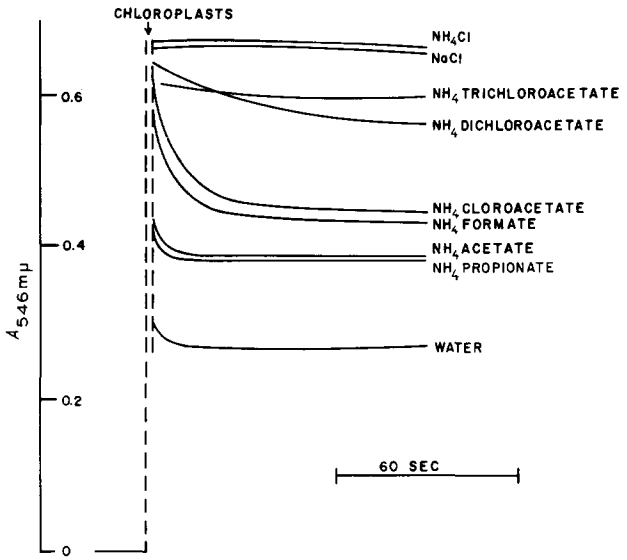


Fig. 4. Absorbance changes on suspension of chloroplasts in solutions of the ammonium salts of weak acids. Chloroplasts (20 μg chlorophyll/ml) were mixed in the dark into 3 ml of 100 mM solutions of the indicated salts at pH 7.2–7.5 and 22°. Measurements were as in METHODS.

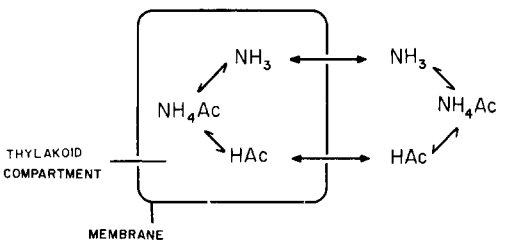


Fig. 5. Mechanism for dark swelling of chloroplasts in the ammonium salts of weak acids (acetate shown). Dissociation of the ammonium acetate has been omitted for clarity.

The relation between changes in volume and light scattering

Illumination of chloroplasts suspended in NaCl gives rise to an increase in light scattering[8] which is accompanied by an increase in volume as measured by absorbance change, packed volume and particle size[1,8], and by a structural deterio-

ration as observed by electron microscopy[1], the mechanism of which has been discussed at length in the previous paper[1]. An increase in light scattering has generally been supposed to indicate a decrease in chloroplast volume, and this has been shown to be the case when phosphate[10] or acetate[28] is present in the supporting medium. However, as shown in the accompanying paper, this is not the case when chloride is the only anion present. Furthermore, as already noted above (Figs. 1a and b) changes in light scattering of only about 10–15 % accompany osmotically induced volume changes of a much larger magnitude. These small changes are to be contrasted with increases in light scattering of 70 and 160 % observed on illumination of chloroplasts in NaCl and sodium acetate, respectively.

It is obvious from these discrepancies that changes in 90° light scattering are not a reliable indication of changes in volume of the osmotically active compartment of chloroplasts. It seems more likely, as suggested in the previous paper, that such changes are in part indicative of a modification of ionic environment leading to refractive index differences between membranes and supporting medium, and that light-scattering increments reflecting volume changes are superimposed on top of these.

In view of the discrepancies noted above, a study was made of the light-scattering properties of chloroplasts and the relation of these to volume changes and changes in transmission in chloroplast suspensions.

The light-scattering envelope of chloroplasts suspended in sodium acetate in the light, in the dark, and after treatment with Triton X-100 is shown in Fig. 6. As with mitochondria[29], light scattering is predominantly in a forward direction, the scattering at 90° being near the minimum. No dramatic modification of angular scattering was observed on illumination or following treatment with detergent.

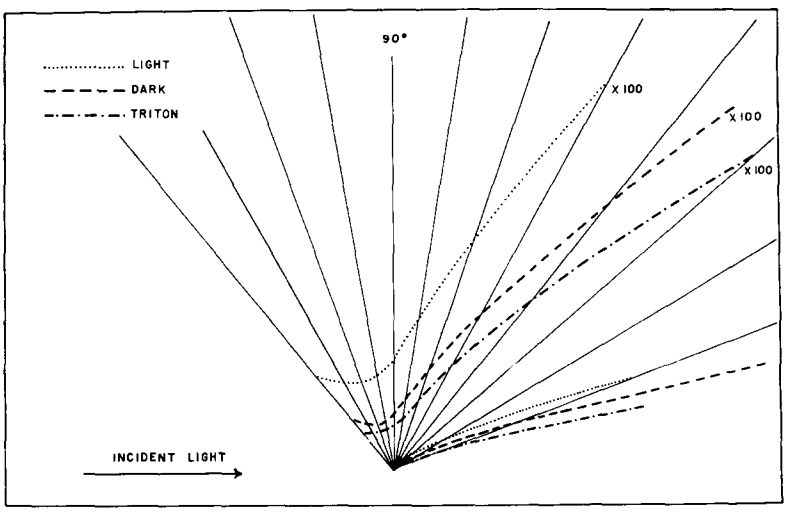


Fig. 6. Determination of the light-scattering envelope of chloroplasts. Chloroplasts (5 μg chlorophyll/ml) were suspended in 40 ml of 0.1 M sodium acetate in the light (·····), in the dark (– – – –), and after treatment with Triton X-100 (–·–·–). Curves are as indicated on the figure, the scale for the upper series being 100 × that for the lower curves. pH 7.5; temp. 22°. Illumination with broad-band red light was from above. Triton concn. 0.01 %. Light-scattering measurements were made at intervals of 5°, as described in METHODS.

A simultaneous recording of changes in light scattering and transmission during illumination and after treatment with Triton is shown in Fig. 7. A number of marked differences can be observed between the 2 traces. In particular the degree of dark reversibility shown by light scattering is considerably greater than that shown by changes in transmission. Under similar conditions, determination of particle size by Coulter counter and of chloroplast morphology by electron microscopy (Figs. 3A, B and D) have shown that changes in transmission more accurately reflect volume changes than do changes in 90° light scattering (Table II).

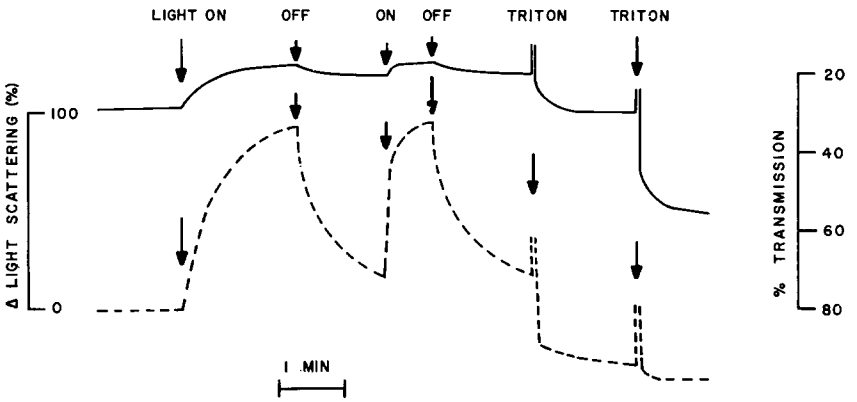


Fig. 7. Comparison of changes in 90° light scattering and % transmission of a suspension of chloroplasts in sodium acetate. Chloroplasts (15 μg chlorophyll/ml) were suspended in 0.1 M sodium acetate, 20 μM PMS at pH 7.8, and 25°. Light-scattering sensitivity was adjusted so that the dark level gave a half-scale deflection on the recorder, and changes in light scattering from this level in the light or on treatment with Triton X-100 were recorded. Scale shows the % change from the dark level. Triton was added at the arrows to a concn. of 0.0083 and 0.0167%. Measurements were made as described in METHODS. ———, transmission; – – – –, light scattering.

TABLE II

EFFECT OF ILLUMINATION ON DETERMINATION OF CHLOROPLAST VOLUME

Solutions were all at 100 mM, and contained 20 μM PMS. The particle volume was taken from the peak of the particle size distribution, as measured by Coulter counter with a 50 μ orifice. The readings were taken between 2 and 3 min after initiation of the condition described. Figures in brackets are readings after 10 min.

Suspending medium	*Condition*	*Particle volume* (μ^3)
Sodium chloride	Dark	33
Sodium chloride	Light	50 (80)
Sodium acetate	Dark	35
Sodium acetate	Light	14 (14)
Sodium acetate	3 min Light–dark	17 (22)

It seems probable that the major differences between light scattering and percent transmission found are due to the effect which lowering the pH has upon the light-scattering properties of chloroplasts[1]. Light-scattering changes of chloroplasts suspended in sodium acetate are accompanied by uptake of hydrogen ions (see below), and a consequent acidification of the interior of the chloroplasts, as well as by a

volume change. It is likely that the light-scattering increments observed are a sum of those due to a lowering of the pH within the chloroplasts, and those due to volume changes. Thus, in the second illumination cycle, although the volume change measured is small, the light scattering and pH changes (see below) are both quite large in comparison with those observed in the first illumination cycle.

It is apparent from the above that considerable caution is needed when relating the extent of light-scattering changes to changes in volume. Parallel measurements of transmission indicating kinetics, or of particle size indicating the extent and direction of volume changes, have therefore been made during the course of the research reported here. Where these have shown important differences from the changes indicated by light-scattering data, this has been noted in the text. Contradictions between data have as far as possible been resolved by electron microscopic examination of chloroplasts fixed in structural states with glutaraldehyde. Some electron micrographs are shown in Fig. 3, and reference is made to these in the text where appropriate.

Mechanism of light-induced volume decrease of chloroplasts suspended in salts of organic anions

It has been demonstrated above that the undissociated acids of organic anions are able to pass freely across the chloroplast membrane. NEUMANN AND JAGENDORF[15] have demonstrated a reversible disappearance of hydrogen ions from the suspending medium on illumination of chloroplasts, and have suggested that this represents a light-dependent transport of hydrogen ions into the chloroplasts mediated by electron flow. It is apparent that a mechanism accounting for the enhancement of shrinkage and inhibition of swelling induced by certain anions[2], can be deduced from these observations. This is shown diagramatically in Fig. 8. It is suggested that when chloroplasts are suspended in solutions of the salts of organic anions, the anion equilibrates rapidly between the osmotically active space and the medium, in part because of the ability of the undissociated acid to pass freely across the chloroplast membrane.

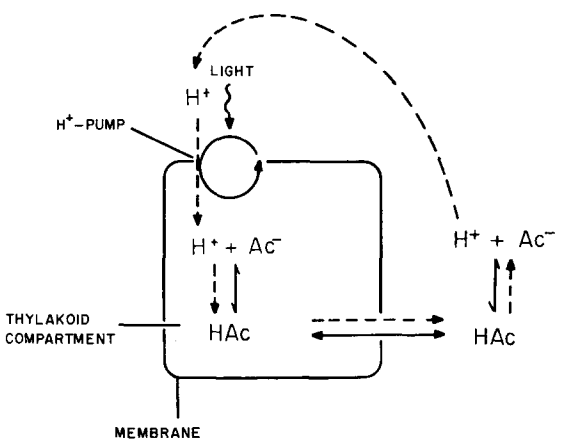


Fig. 8. Proposed mechanism for shrinkage of chloroplasts upon illumination in the presence of anions of weak acids. Possible movements of charged ions have been omitted for clarity. Solid arrows represent dark equilibration reactions, dashed lines show light-induced changes, the directions of which are indicated by the arrows.

Upon initiation of electron flow by illumination, the following course of events is envisaged:

(a) Hydrogen ions are pumped into the chloroplast, resulting in the conversion of dissociated anion into undissociated acid as the internal pH falls; (b) undissociated acid re-equilibrates across the membrane; (c) ions which are able to penetrate move to maintain charge balance; (d) the net efflux of anion causes a decrease in osmolarity within the chloroplast, resulting in loss of water, and a decrease in volume.

The hypothesis that volume changes are due to displacement of anion from within the chloroplast following acidification of the interior is open to a number of experimental tests. Some of these are given below.

(a) The kinetics and extent of the pH change in salts of weak acids should be markedly different from those in NaCl; (b) if the movement of undissociated acid across the membrane is in any circumstance rate limiting, a relation between rate of change, and concentration of undissociated acid as judged by the pK of the anion used, might be expected; (c) the rate at which anion is displaced from within the chloroplast, as judged by volume change, should not be greater than the rate at which hydrogen ions are pumped into the chloroplast.

Kinetics of pH change and light scattering in sodium chloroacetate and NaCl

It can be seen from Fig. 8 that the net change in hydrogen ion concentration to be expected during the displacement of anion from within the chloroplast will bear no relation to total movement of ions and water, since each hydrogen ion entering the chloroplast leaves again as undissociated acid. It is to be expected that after an initial rapid uptake into the chloroplast, hydrogen ions will re-appear in the medium as the internal anion is displaced, and as the internal volume decreases. It can be seen from Fig. 9, which shows the course of light-induced changes in pH and light scattering for chloroplasts suspended in sodium chloroacetate, that such polyphasic kinetics are observed for the pH change induced on the first illumination cycle, while the light-scattering change follows first-order kinetics[9] (see also ref. 14). The

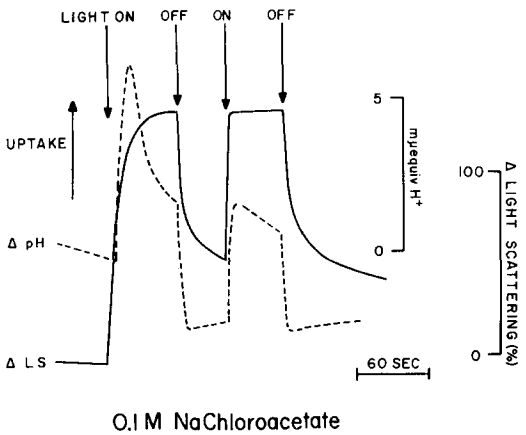
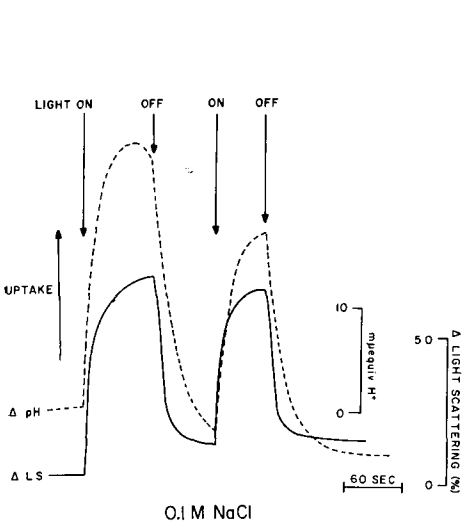


Fig. 9. Effect of illumination on changes in pH and light scattering (LS) of a suspension of chloroplasts in sodium chloroacetate. Chloroplasts (20 μg chlorophyll/ml) were suspended in 3 ml of 100 mM sodium chloroacetate, 20 μM PMS at pH 6.3 and 25°.

kinetics of these changes are similar when observed in salts of other organic anions, such as acetate and formate[9]. It is obvious that in solutions of organic anions, the initial kinetics of light-scattering and pH changes may bear no direct relation to each other. This is to be contrasted with the kinetics of similar changes observed in chloroplasts suspended in NaCl which are shown in Fig. 10. Here a close similarity in the time courses of the changes is observed, as reported elsewhere[30], and as would be expected from the mechanism suggested in the companion paper[1]. It is apparent that a mechanism involving the precipitation of membrane components as the internal pH falls, does not adequately explain the enhanced light scattering in salts of organic anions, since apart from the kinetic differences noted above, it can be seen from Figs. 9 and 10 that the increase in the magnitude of the light-scattering change in chloroacetate is associated with a decrease in the magnitude of the pH change. This is shown more clearly in Fig. 11 in which the extent of light-scattering and pH changes in NaCl and sodium chloroacetate are plotted against initial pH. It should be noted that, because of the polyphasic kinetics of the pH change on first illuminating chloroplasts in salts of organic acids, points for the pH curve have been measured from the change observed during the second cycle of illumination. It can be seen that the H^+ changes in both media have similar pH optima, and that at all pH's, the extent of the change in sodium chloroacetate is considerably less than that of the change in NaCl, being about 20 % at the optimum at pH 6.3.

It seems likely that the suppression of the pH change observed is a reflection of the fact that the volume of the internal space in contracted chloroplasts (see Table II) is very much less than that in the more swollen chloroplasts produced on illumination in NaCl. Hence, the transport of fewer hydrogen ions is required to bring



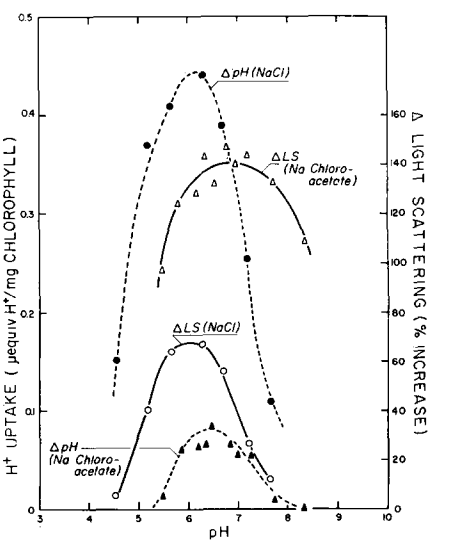


Fig. 10. Effect of illumination on changes in pH and light scattering (LS) of a suspension of chloroplasts in NaCl. Conditions as in Fig. 9, except that chloride replaced chloroacetate as the anion in the medium.

Fig. 11. Dependence on pH of light-induced light-scattering (LS) and H^+ changes of suspensions of chloroplasts in NaCl and sodium chloroacetate. Conditions as in Figs. 9 and 10, except that the pH was adjusted to the value indicated immediately after addition of chloroplasts.

about an equivalent lowering of the internal pH. The efflux of hydrogen ions as undissociated acid may also contribute to the suppression, though during the second illumination cycle, the volume change observed is very much smaller than during the initial light phase (Table II, Figs. 3, 7).

The pH dependence of the extent of the light-scattering change in NaCl shows an optimum similar to the H^+ change. However, in sodium chloroacetate the change is maximal between pH 6.5 and 7.5, with an optimum at pH 7.0. This difference is also shown in Fig. 12 in which the pH dependence of both the rate and extent of the light-scattering change are shown in relation to the pH dependence of the H^+ change for chloroplasts suspended in sodium formate. Although the pH optimum for the extent of the light-scattering change is at the higher pH, that for the rate at which the change occurs has an optimum similar to that for the extent of the pH change. A similar pH optimum for the rate of the initial light-scattering change is found for chloroplasts suspended in sodium chloroacetate and sodium acetate and in sodium phosphate (see also ref. 31).

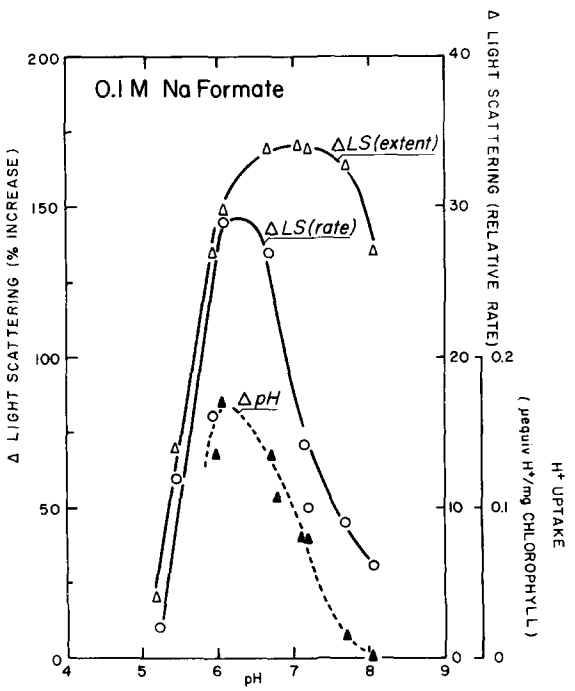


Fig. 12. Dependence on pH of light-induced light-scattering (LS) and H^+ changes of suspensions of chloroplasts in sodium formate. Conditions as in Fig. 9, except that formate replaced chloroacetate as the anion, and the chloroplasts were at a concn. of 24.2 μg chlorophyll/ml.

Relation between pK_a of anion and rate of light scattering

PACKER AND SIEGENTHALER[2] have shown that a wide variety of organic anions are able to enhance light scattering and inhibit swelling in illuminated chloroplasts, but that amino acids and pyrophosphate are not. A number of other anions have been tested during the course of the present research, and the results from these sources are summarized in Table III. In general, it can be seen that any anion which is

able to give rise to a significant level (about 10^{-7} M or more) of undissociated acid in solution at a pH within the effective range shown for hydrogen ion uptake (see Figs. 9 and 10) is able to induce an enhancement of the light-induced light-scattering change. A number of other points from this table are of particular interest, and will be discussed in greater detail below.

TABLE III

ANION EFFECTIVENESS FOR ENHANCING LIGHT-INDUCED SCATTERING INCREMENTS

Effectiveness in enhancing light-scattering changes was dependent upon pH and concentration of anion. The number of + signs is an indication of effectiveness at an anion concentration of 0.1 M, at pH 7.5 and in the presence of 20 μM PMS, with reference to sodium acetate.

Anion	*pK_a*	*Total scattering*	*Scattering rate*
Monovalent organic			
Propionate	4.87	++++	++++
Acetate	4.75	++++	++++
Formate	3.75	++++	++++
Chloroacetate	2.85	++++	++++
Dichloroacetate	1.48	+++	++
Trichloroacetate	0.7	+++	+
Divalent organic			
Succinate	4.16	+++	+++
Malate	3.40	++	++
Oxalate	1.23	+	+
Zwitterions			
Glycine	—	o	o
Alanine	—	o	o
Serine	—	o	o
Aspartate	—	o	o
Weak acid inorganic			
Fluoride	3.45	+++	+++
Arsenate	2.25	++	+
Phosphate	2.12	++	+
Strong acid inorganic			
Chloride	—	o	o
Sulphate	—	o	o
Nitrate	—	o	o
Perchlorate	—	o	o

(a) Although NaCl is not able to support a full light-scattering response, NaF (with a pK of 3.45 for hydrofluoric acid) is as effective as organic anions of similar pK.

(b) A number of bivalent organic anions (succinate, malate and oxalate in order of effectiveness) stimulate the light-scattering response. However, the rate of change of increments in bivalent anions is considerably slower than rates in monovalent anions of equivalent pK. In particular, malate and oxalate are relatively ineffective above pH 7, though they show considerable enhancement at lower pH.

(c) In no case was an enhancement observed when chloroplasts were suspended in solutions of the salts of strong acids (Cl^-, NO_3^-, SO_4^{2-} or ClO_4^-).

Although trichloroacetate was able to stimulate light-scattering increments, the

rate of change was slow. It is worth pointing out that even at pH 7, a 0.1 M solution of sodium trichloroacetate contains undissociated acid at much the same concentration as it does hydrogen ions, and that lowering the pH within the chloroplasts to 3.6 (ref. 1) will give rise to a concentration difference of more than 10^3 across the chloroplasts membrane if equilibration had occurred as suggested above.

(d) Zwitterionic organic anions were unable to give rise to an enhancement of the light-scattering change.

As will be discussed below, the rate of change of light scattering of chloroplasts suspended in salts of weak acids during the initial illumination cycle appears to be limited by the rate at which hydrogen ions are taken up by the chloroplasts. However, as can be seen from Fig. 9, the kinetics of the light-scattering change in the second and subsequent illumination cycles are very much faster. Although it has been shown that the light-scattering changes in the second cycle do not reflect volume changes of a similar magnitude, it seemed possible that the faster rate might reveal a dependence on concentration of undissociated acid as judged by the pK of the anion used. As shown in Fig. 13 such a relation is observed for a series of monovalent organic anions, and fluoride. The points for bivalent organic anions fall on a different curve, and have been omitted from the figure.

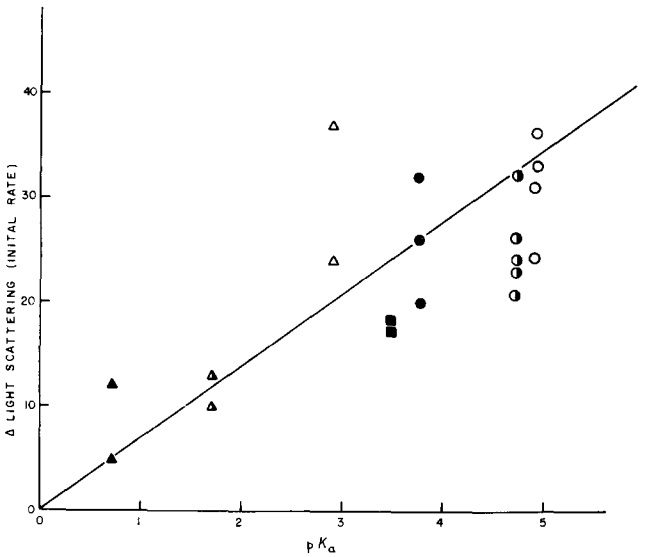


Fig. 13. Correlation between pK_a of anions and the rate of change of light scattering. Chloroplasts (10 μg chlorophyll/ml) were suspended in 100 mM solutions of certain anions listed in Table IV, in the presence of 20 μM PMS. pH 8.0; temp. 25°. Calculations for the points are from the rate of change of light scattering during the second illumination cycle. Symbols correspond to monovalent weak acid anions given in Table III.

Stoichiometry of volume changes and ion movement

It is apparent from the mechanism shown in Fig. 9 that the rate at which volume changes occur might be limited by either the rate of inward transport of H^+ or by the rate of diffusion of undissociated acid outwards across the chloroplast membrane. For the polyvalent and strongest monovalent organic acid anions a lag phase is observed before the light-scattering rise starts (see also ref. 32), and the rate

of change is slow. However, variations in the kinetics of the initial light-scattering response are not great in suspensions of chloroplasts in salts of anions with higher pK's. It should be possible therefore to show a relation between the initial rate of H^+ uptake and the time of completion of the volume change. From Table II it can be seen that the volume change observed on illumination of chloroplasts in sodium acetate is about 25 μl/mg chloroplast chlorophyll. If it is assumed that similar volume changes occur in the salts of other organic anions, as is indicated by the extent of the light-scattering change, and the osmotic equilibrium is maintained during the change, this represents (at 0.1 M) the movement of 2.5 μmoles of salt/mg chlorophyll from chloroplasts on illumination in solution of the salts of weak acids. The initial rate of the hydrogen ion uptake shown in Figs. 9 and 10 is approx. 0.06 μequiv/mg chlorophyll per sec (a conservative estimate, since rates of twice this value can be observed), allowing 42 sec for the transport of 2.5 μequiv/mg chlorophyll. It can be seen from Fig. 9 that the light-scattering change has reached completion in 60 sec, showing that within the error of the approximations used, the mechanism outlined above is easily able to account for the rate and extent of the volume change in terms of anion movements.

Cation movements accompanying volume changes

A mechanism for chloroplast volume changes in which the movement of anions in response to uptake of H^+ is the major contributing factor, is in marked contrast to the mechanism involving cation movements proposed by other workers. DILLEY[13] and DILLEY AND VERNON[14] have shown that when chloroplasts are suspended in Tris–acetate, a release of potassium ion occurs on illumination, the time course of which approximates first order kinetics, being similar in this respect to the light induced light-scattering change[9]. The extent of the potassium ion release was 0.2 μmole/mg chlorophyll. Movements of magnesium and sodium ion were also observed, the latter ion moving into the chloroplast on illumination. DILLEY AND VERNON[14] have concluded that ions are lost from the chloroplasts to compensate for the inward movement of charge accompanying H^+ uptake, and have suggested that the volume change observed on illumination is consequent upon the movement of magnesium and potassium ions from the chloroplast. These authors have also shown that the movements of ions, as measured by atomic absorption spectrometry, are reversible.

While we have been able to confirm that chloroplasts suspended in Tris–acetate lose 0.2–0.3 μmole/mg chlorophyll of potassium ion on illumination, we have been unable to find any reversal of these movements as measured by a cation-sensitive electrode. Three traces demonstrating these effects are shown in Fig. 14. On illumination, practically all the K^+ contained in the chloroplasts moves out into the medium and no further change in rate of K^+ movement is observed on a subsequent dark–light cycle. After illumination, no more K^+ is released on treatment with Triton X-100, and addition of Triton prior to illumination releases an equivalent amount of ion.

We have found that the amount of ion contained within the chloroplast (as indicated by release on lysis with Triton) varies with a number of parameters. Important among these are: (a) number of washings during isolation, (b) time after isolation, and (c) proportion in the stock chloroplast suspension between chloroplast–water volume, and volume of suspending medium. Variation of potassium ion with these parameters suggests that the ion is fairly readily lost from chloroplasts to the

suspending medium, although even after several washings or a number of hours, a small amount may be retained (see also ref. 5).

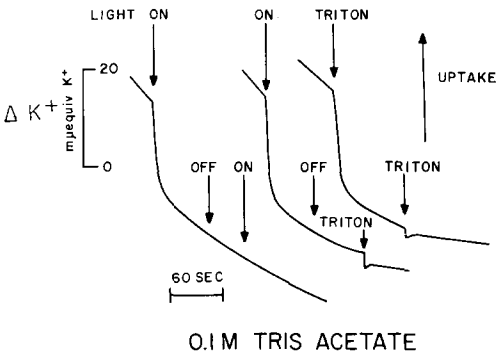


Fig. 14. Changes in K^+ concentration in a suspension of chloroplasts in Tris–acetate. Chloroplasts (9.6 μg chlorophyll/ml) were suspended in 100 mM Tris–acetate containing 20 μM PMS at pH 7.4 and 30°. Chloroplasts for this experiment were isolated in sucrose.

Although it is probable that the need to compensate for the inward movement of charge accompanying hydrogen ion uptake may contribute towards the loss of potassium ions[14], a number of observations lead us to conclude that the net movement of potassium ion seen on illumination in Tris–acetate is largely a result of the fact that a loss of anions as undissociated acid is also able to occur leading to the volume changes observed.

(a) The kinetics of potassium ion movement, while they are similar to those of the light-scattering change in acetate bear no relation to the kinetics of the hydrogen ion change in acetate.

(b) The amount of potassium ion lost is smaller by a factor of 10 than the ion movement needed to account for the water movement by an osmotic mechanism, as calculated from the volume change (see above).

(c) Volume changes and hydrogen ion changes do not vary with extent of washing or storage, as do potassium ion changes.

(d) No reversibility of potassium ion movement in the dark is seen in Tris–acetate, reflecting the small extent of reversal of the volume change observed.

(e) Potassium ion movements in chloride media can be opposite in direction to those in acetate media and are reversible (see below).

Potassium ion movements and the uptake of hydrogen ion may be measured simultaneously when chloroplasts are suspended in choline chloride media. Under these conditions, the extent and kinetics of both the light-scattering change and hydrogen ion uptake are essentially the same as in sodium chloride. Potassium ion movements may also be followed in Tris–chloride media, in which case hydrogen ion changes are difficult to measure except at low pH. However, in general, in chloride media light-scattering changes reflect the hydrogen ion changes[1] (see also Fig. 10) so that changes in light scattering may be taken as indicative of the kinetics of the hydrogen ion change. It has been found that potassium ion movements and light scattering observed for chloroplasts suspended in choline chloride or Tris–chloride are essentially the same.

The kinetics of hydrogen ion uptake and potassium ion movements for chloroplasts suspended in choline chloride are shown in Fig. 15. It can be seen that during the first cycle of illumination, the kinetics of the potassium ion changes are complex. An initial small uptake of potassium ion is followed by a rapid loss which is similar to that seen in Tris–acetate. However, on switching off the light, a further rapid efflux of potassium ion occurs. In subsequent illumination cycles, a larger uptake of potassium ion is observed on illumination, which is rapidly and completely reversible in the dark. The total loss of potassium ion during the initial illumination cycle is similar to that in Tris–acetate. It seems possible that the complex kinetics of the first cycle represent a superposition of the changes in the subsequent cycles on a loss of potassium ion such as occurs in Tris–acetate. In this case, the loss of ion may well be consequent upon an inward transport of hydrogen ions and displacement of internal potassium ions, since in well washed chloroplasts, the kinetics of the first cycle approximate more closely to those of subsequent cycles.

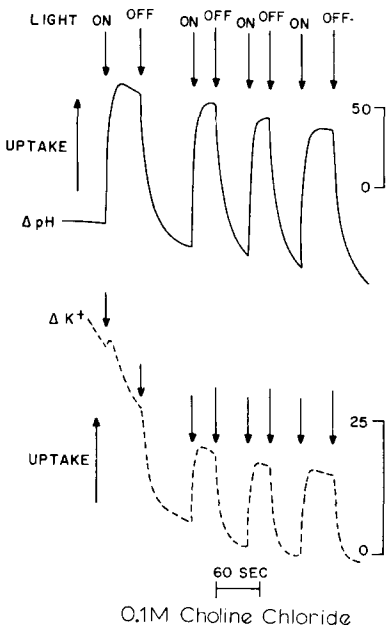


Fig. 15. Effect of illumination of changes in K^+ and H^+ concentrations in a suspension of chloroplasts in choline chloride. Chloroplasts (57.5 μg chlorophyll/ml) were suspended in 5 ml of 100 mM choline chloride containing 0.5 mM Tris–chloride and 20 μM PMS, at pH 6.95 and 25°. Cation (0.3 mM) from the chloroplast suspension was present initially. Measurements were made as described in METHODS. Chloroplasts for this experiment were isolated in choline chloride.

CONCLUSIONS

It has been suggested above that the light-dependent change in volume of chloroplasts suspended in salts of weak acids might be explained by a loss of anion from within the chloroplast due to acidification of the interior upon hydrogen ion uptake, and a subsequent re-equilibration of undissociated acid across the membrane. Such a mechanism requires that the chloroplasts should be: (a) permeable to undis-

sociated acids; (b) relatively impermeable to either cations or anions; (c) able to maintain a difference in the activity of H^+ across the osmotically effective membrane.

The failure of the ammonium salts of weak acids to support chloroplasts osmotically indicates that both cationic and anionic species of such solutions are able to penetrate the chloroplast membrane freely. The exchange data[27,*] show that sodium and potassium ions equilibrate between chloroplasts and suspending medium relatively slowly, and the ammonium ion might be expected to show a similar rate of exchange. However, the half time of dark swelling in ammonium propionate is in the order of a few seconds (Fig. 4), indicating that the ammonium ion must be entering the chloroplast in another form, probably as molecular ammonia. The dependence of the rate of swelling upon the pK_a of the anion used suggested that the other penetrating species is the undissociated acid of the anionic component of the suspending medium. A mechanism whereby dark swelling of chloroplasts might occur on penetration of the membrane by ammonia and undissociated acid has therefore been proposed above (Fig. 5). It might be expected from such a mechanism that the eventual extent of swelling would be the same in the ammonium salts of all weak acids, and this is not observed (Fig. 4). However, it is possible that if ammonia and undissociated acid are the only penetrating species, the production of ammonium salts of the stronger acids within the chloroplast would lead to a fall in pH consequent upon the dissociation and association of the ions on reaction with water, discouraging the further entry of undissociated acid. The opposite effect would be expected with the salts of the weaker acids.

As has already been pointed out, the observation of swelling in ammonium salts throws little light upon the ability of charged ions to penetrate into the chloroplast. Furthermore, neither the equilibration of sodium and potassium ions on suspension in ionic media[5], nor the exchange data[27,*] differentiate between loss by net efflux, and loss by exchange-diffusion with other cations. STOCKING AND ONGUN[4], and TOLBERG AND MACEY[5] have reported a loss of sodium and potassium ions from chloroplasts during isolation in sucrose; however, the time factor in these experiments precludes meaningful kinetic deductions. The fact that chloroplasts are able to maintain internal hydrogen[15], potassium[5] and ammonium ions[34] at concentrations considerably higher than those in the suspending medium, suggests that the movement of cations across the membrane reaches a significant rate with respect to the activity of the hydrogen ion pump only when a large concentration difference is produced across the membrane. This difference has been estimated to be about 10^3 hydrogen ions[1], and is about one-tenth of this magnitude for ammonium ions[33]. It should be noted that so long as charged anions and cations are not both able to pass into the chloroplast freely (as in indicated by the osmotic data), the mechanism proposed above to explain the shrinkage of chloroplasts on illumination in solutions of the salts of weak acids, is independent of the passage of either one of the ionic species, so long as the relative rate of penetration by the undissociated acid is high in relation to the rate of net movement of the penetrating ion.

If anions are able to penetrate at a significant rate in relation to the activity of the hydrogen ion pump, and the difference in activity of hydrogen ions across the chloroplast membrane is equivalent to the high energy state as suggested by NEUMANN AND JAGENDORF[15], then the mechanism proposed above would lead to an uncoupling

* B. WINOCUR, personal communication (1966).

of photophosphorylation from electron flow. Such an effect has been observed by GOOD[11], who showed that certain anions, notably lactate, citrate, malate, malonate, phosphate and arsenate, were able to stimulate the rate of the Hill reaction with ferricyanide, whereas chloride, sulphate and amino acids were not. The parallel between effectiveness in this activity, and in the enhancement of light scattering reported above is striking, suggesting that anions are able to cross the membrane at a rate comparable to the rate of H^+ uptake.

The movement of ions during illumination of chloroplasts suspended in choline chloride may throw some further light on the problem of ion permeability. Thus, it is obvious that the movement of potassium ion into chloroplasts accompanying hydrogen ion uptake on illumination, cannot be explained simply in terms of cation movements and charge equilibration. Furthermore, the amount of potassium ion taken up is small, and increases linearly with concentration[33] rather than in conformity with classical Michaelis–Menten kinetics as would be expected of an enzymic mechanism. It seems likely that chloride, the only anion present in these experiments must be moving into the chloroplast together with the hydrogen ion, and that a small amount of cation follows. Penetration of the chloroplast membrane by chloride is also indicated by the extensive swelling observed on illumination of chloroplasts suspended in chloride media[1], especially in the presence of ammonium or methylamine chloride at uncoupling concentrations[3]. Ammonium uncoupling has been shown to be associated with a marked uptake of ammonium from the medium[33], and an equivalent movement of anion to compensate for the charge displacement would be expected. If chloride and other anions are able to penetrate the membrane relatively easily, light-induced conformational changes of chloroplasts in the different media are more satisfactorily explained. An exchange of internal chloride for weak acid anion would lead to a more effective displacement of anion from within the chloroplast, since the weak acid anion replacing the chloride would still be lost as undissociated acid on acidification of the chloroplast interior by the mechanism discussed above.

The relation of conformational changes to energy transfer

The mechanism proposed above, and that suggested by CROFTS[33] for amine uncoupling and associated swelling phenomena, rest upon the assumption that a considerable difference in concentration of hydrogen ion across the chloroplast membrane can be maintained by coupled light-induced electron flow. Adequate evidence that this is so has been advanced by NEUMANN AND JAGENDORF[15], and in this and the companion paper[1].

The relation between the trans-membrane potential of hydrogen ion activity and energy transfer has been discussed at length elsewhere[34,35] and in relation both to photophosphorylation[15] and conformational changes[1]. The ionic mechanism for chloroplast shrinkage proposed above is consistent with hypotheses of energy transfer involving either intermediates, or direct coupling to electron flow as mechanisms leading to the production of a difference in activity of hydrogen ions across the chloroplast membrane. It is, however, in contrast to mechanisms of conformational change involving interaction between high-energy intermediates and contractile molecules, and provides an alternative explanation for certain phenomena associated with chloroplast shrinkage.

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