ION EXCHANGE IN CHLOROPLASTS WITH SPECIAL REFERENCE TO THE EFFECT OF LIGHT

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ABSTRACT.

The absorption of chloride by detached leaves of Elodea densa and the absorption of chloride and calcium by isolated pea chloroplasts was investigated using radioactive isotopes.

Chloride absorption by Elodea is stimulated by light and dependent upon photophosphorylation as the energy source for accumulation. Both photosystems could support uptake although photosystem II was more efficient than photosystem I. DCMU severely inhibited uptake. Accumulation in the light could proceed in the absence of oxygen and was stimulated under CO_2-free conditions, whereas uptake in the dark was dependent upon oxygen and CO_2. Bicarbonate inhibited both uptake in the light and dark.

When detached pea leaves were fed with radioactive chloride the isolated chloroplasts contained high levels of chloride. Preliminary experiments with isolated chloroplasts indicated that ATP was involved in chloride uptake. Using a faster method of separation of chloroplasts from experimental solutions, very rapid and large chloride movements occurred in the light with slower movements taking place in the dark. These results indicated that the chloroplast membrane in vitro was permeable to chloride.

The accumulation of calcium was stimulated by very low light intensities. The mechanism was very labile and showed a high degree of specificity. Uptake was not associated with phosphorylative processes but with electron flow. Inhibitor experiments indicated that the accumulatory mechanism probably lies close

to the electron flow chain but not directly upon it. Approximately 60% of the accumulated calcium was present in a water-soluble fraction, though not associated with protein. A further 30% was present associated with a component that could exchange with non-radioactive calcium. A small fraction of the accumulated calcium was present in a lipid-chlorophyll fraction. The calcium accumulation reported here shows several new features. The results are discussed in terms of a mechanism of ion exchange induced by light in chloroplasts.

Abbrevations

ADP	-	adenosine - 5' - diphosphate
ATP	-	adenosine - 5' - triphosphate
Pi	-	inorganic phosphate ion
DPIP (H_2)	-	2, 6 - dichlorophenol - indophenol (reduced)
FeCy	-	ferricyanide ion
FMN	-	flavin mononucleotide
PMS (H_2)	-	phenazine methosulphate (reduced)
CCCP	-	carbonylcyanide m-chlorophenylhydrazone
CMU	-	3-(4-chlorophenyl) -1, 1- dimethylurea
DCMU	-	3-(4-dichlorophenyl) -1, 1- dimethylurea
DNP	-	2:4 - dinitrophenol.
ATP-ase	-	the enzyme adenosine triphosphatase
NADP (H)	-	nicotinamide adenine dinucleotide (reduced)
FD	-	ferredoxin
EDTA	-	Ethylenediamine tetra acetic acid
PMA	-	phenylmercuric acetate
TMPD	-	N, N, N', N' - tetramethylphenylenediamine
TCA	-	trichloroacetic acid
O.D.	-	optical density
Ca: 2e	-	number of calcium atoms absorbed for each pair of electrons transferred
P: 2e	-	number of ATP molecules formed for each pair of electrons transferred

INTRODUCTION.

A. Ion accumulation and photosynthesis in plants.

1. General aspects of ion accumulation.

Plant cells can accumulate a variety of ions. Early investigators of this phenomenon believed that salts enter into the cells passively together with water. Later such mechanisms as diffusion, mass flow, adsorption, ion exchange and Donnan equilibria were advanced to explain ion uptake. In addition to such physical phenomena, ion absorption is now recognised to be closely related to cellular metabolism. Extensive reviews on the physiology of ion transport are to be found in the books by Sutcliffe (1), Jennings (2) and Briggs, Hope and Robertson (3).

Generalisations about ion accumulation in plant cells are difficult to make, partly because of differing sources of tissues and partly because accumulated ions themselves may have secondary effects upon the tissues.

The vacuole serves as the chief store of accumulated ions. Both cations and anions may be absorbed until their concentrations are much greater than that in external solutions (4). Ions, previously accumulated, are not lost from higher plant tissues when they are transferred from salt solutions to water (5)(6) indicating that plant cell membranes have a low permeability to free ions.

A remarkable property of plants is the selectivity which the ion-accumulating mechanism shows e.g. the preferential

uptake of potassium compared to sodium in almost all land plants (7). Most, if not all, higher plant tissues show an initial biphasic time course of uptake when transferred from water to salt solutions; namely a rapid initial ion uptake with a low temperature coefficient (approximately that of diffusion) followed by a slower rate of accumulation characterised by a temperature coefficient as high as those for metabolic reactions. This initial uptake is largely physical or passive (8) whilst the second uptake phase is mainly active or metabolic (9).

When metabolism is inhibited, ion uptake is also inhibited and among the many inhibitory conditions which have been demonstrated on ion absorption are low oxygen tensions (10), low temperatures (11) and metabolic poisons (12(13).

Early work by Lundegårdh and Burstrom (14) and others (15)(16) established that salt has a stimulatory effect on oxygen uptake in respiration. The increase in respiration, which is related to the amount of anion absorbed, was called "anion respiration", though nowadays "salt respiration" is preferred since Epstein (17) and others (18) have shown that cation exchange also induces a stimulation of respiration. Evidence for involving the respiratory chain electron flow and quantitative correlations between the rates of anion absorption and anion respiration have led to the hypothesis that there is a connection between electron flow in the respiratory chain and anion movement in the opposite direction (19)(20).

The Lundegårdh hypothesis postulates that, as electrons from respiratory intermediates flow through the cytochromes (towards cytochrome oxidase) and other components of the electron transfer sequence, so there is a corresponding counter-flow of anions. The anions are visualised as forming temporary complexes with the oxidised components of the chain and at each stage an anion takes the place of an electron as it is moved to the next member of the sequence. Cations are thought to move in passively to balance the anions absorbed. The realisation that the cytochromes are located strictly within the mitochondria has prompted Robertson (20) to extend the Lundegårdh hypothesis and suggest that the mitochondria can act as intermediate sites of ion accumulation.

Whilst there is evidence in favour of the above hypothesis several authors offer other explanations of salt respiration. When salts are added to components of the cytochrome chain oxygen uptake is stimulated (21), and this occurs clearly where salt accumulation is not involved. Further, salt respiration can continue long after the salt is removed (18)(22). The most compelling argument against the hypothesis is that DNP* greatly stimulates oxygen uptake whilst severely inhibiting salt accumulation (13)(23). Budd and Laties (24), maintaining electron flow through an abbreviated part of the cytochrome chain under anaerobic conditions, found that active uptake of chloride is sufficiently similar to aerobic uptake to suggest that the same mechanism is responsible in both cases.

Considerable evidence now links ion transport in plants not to electron flow per se but to the phosphorylations coupled to electron flow (23)(25)(26). Phosphorylated intermediates and ATP

* See list of abbreviations page 6

are implicated in the transport of inorganic ions and organic solutes in animal tissues (27), and there is ample evidence (13) (23)(28)(29) to suggest a similar relationship in plant tissues.

The discovery that ATP-ases in animal tissues are involved in sodium and potassium absorption (30) has prompted a search for the same situation in plant tissues. The ATP-ases of animal tissues are activated by sodium and potassium ions and are sensitive to ouabain (a cardiac glycoside). In Nitella (31) and Hydrodictyon (32) potassium influx and sodium efflux are sensitive to ouabain whereas rubidium absorption by barley roots is insensitive (33). This limited amount of information on ouabain-sensitive ATP-ases in plants suggests that they may play a role in the maintenance of sodium and potassium levels in algae but not, so far as is known, in roots and other tissues of higher plants. Photosynthetic ATP-ases have been implicated in the absorption of cations by isolated chloroplasts and this is discussed in a later section.

The concept of carriers has arisen to account for some of the properties of plant transport systems. Carriers are considered to be molecules within the membranes which can form reversible complexes with ions and transport them across the permeability barrier. Epstein and Hagen (34) have extended this idea by applying the classical concepts of enzyme kinetics to rubidium absorption by excised barley roots. The role of a carrier in transport is thus comparable with the enzyme-substrate complex. Evidence suggests that there are many carriers or sites with differing affinities for various ions, and many competitive pairs of ions are known e.g. calcium-strontium, bromide-chloride (7). Selectivity implies binding by specific sites and in some cases these have been recognised (35)(36).

Although a distinction is often made between Lundegårdh's scheme and other so called "carrier" theories, it can be seen that the cytochromes themselves may be regarded as carriers of anions. Several authors have suggested identities for carriers

in plant ion transport systems other than cytochromes. Thus Goldacre (37) proposed that folding and unfolding of protein chains might function as non-specific carriers to transport ions across membranes and the inhibitory effects of chloramphenicol on potassium absorption by carrot and red beet slices have been interpreted as due to its interference with protein metabolism (38)(39). However the effects are more readily explained on the basis that chloramphenicol inhibits energy-linked processes in mitochondria (40). Bennett-Clark (41) has proposed that cellular phospholipids may act as amphoteric carriers of ions across plant cell membranes. A redox-pump system in which reduced respiratory intermediates acting as carriers are responsible for ion transport has also been suggested (42).

Whilst the concept of carriers is a useful one for the transport of ions, it leaves unanswered the question of transport of neutral molecules. Further the correctness of this type of approach must be questioned on the grounds that several ion fluxes may be involved in establishing any observed uptake.

Both electron flow and phosphorylative mechanisms have been proposed to link ion transport with metabolism. Any scheme utilising electron flow is limited to the active movement of one ion (the counter-ion must move in passively in amounts to restore electrical neutrality). A phosphorylative mechanism however, provides a uniform scheme for the transport of neutral molecules as well as ions, and will apply to both aerobic and anaerobic ion movements. Such a mechanism will also include those systems where energy for transport is provided not through oxidative phosphorylation but through photophosphorylation (43)(44).

Light has been shown to have a stimulatory effect upon salt absorption in green plants. Before discussing this effect it is pertinent to consider the role of light in the partial reactions of photosynthesis, since any light effects upon absorption are presumably mediated through the chloroplasts, the sites of photosynthesis.

2. Photosynthesis.

Photosynthesis is the mechanism by which green plants (and certain bacteria) transform light energy into chemical energy. The products of photosynthesis are organic compounds and oxygen and the overall process may be represented by the empirical equation:

$$CO_2 + H_2O \xrightarrow{\text{LIGHT}} (CH_2O) + O_2 \quad \ldots\ldots\ldots\ldots\ldots\ldots 1$$

In the uptake of carbon dioxide and water to make organic compounds and evolve oxygen, energy is provided by light. The primary stable products of the light absorption reactions are molecular oxygen, ATP and $NADPH_2$ (45) - (47) and this can be summarised as:

$$H_2O + NADP + ADP + Pi \longrightarrow \tfrac{1}{2} O_2 + NADPH_2 + ATP \ldots\ldots 2$$

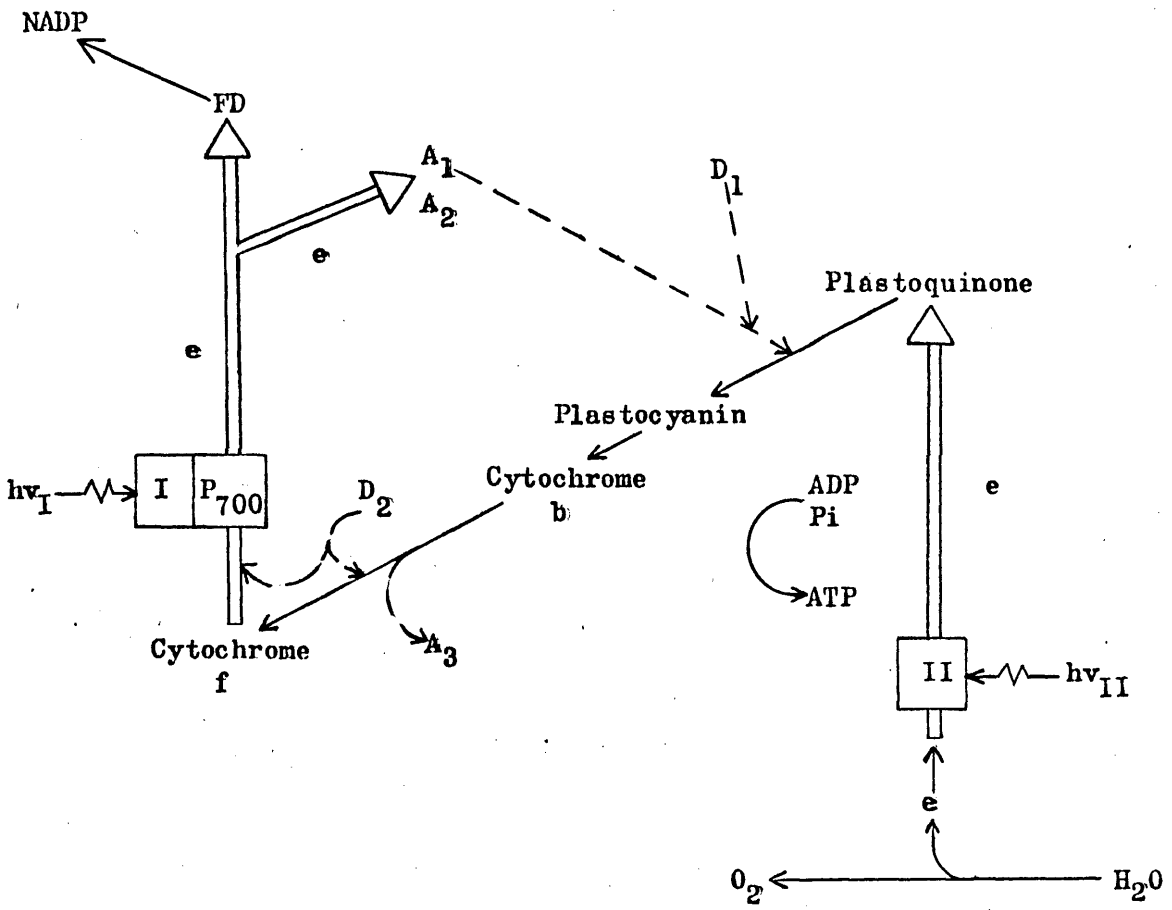
Since the work of Emerson et al (48) it has been generally accepted that two pigment systems are involved in photosynthesis. Many experiments (49) - (51) show that light absorbed in the far-red is ineffective in photosynthesis unless accompanied by light of shorter wavelengths. These observations and many others (52) - (54) support the hypothesis that efficient photosynthesis requires the interaction of two photochemical reactions that operate sequentially. The two photosystems are coupled by a set of electron carriers (55). The carriers and pigment systems have been identified primarily on the basis of difference absorption spectra (56) - (58).

Numerous schemes have been presented (54)(59)(60) to account for enhancement phenomena and other facts of photosynthesis, and an overall scheme incorporating some of the experimental data is presented in figure 1.

Photosystem I contains a special form of chlorophyll a - P700 (56) - which absorbs light at wavelengths greater than 685 mμ, causing the chlorophyll to be oxidised by loss of an electron. Photosystem II absorbs light at wavelengths less than 685 mμ and contains chlorophyll a and accessory pigments and is capable of reducing the oxidised photosystem I. On donating an

Figure 1 Electron Flow Scheme of Photosynthesis

Legend to figure overleaf



Legend to figure 1

A_1 - redox agents catalysing cyclic phosphorylation, e.g. DPIP (61), PMS (62), FMN (63), Vitamin K (64), Quinones (65) and FD (66). These agents can donate electrons (after reduction) to the intermediate electron transfer chain, prior to the site of ATP-formation, and require anaerobic conditions.

A_2 - agents accepting electrons, e.g. FeCy (47), NO_3 (67) and NO_2 (68).

A_3 - electron acceptors reduced by photosystem II and supporting phosphorylation, e.g. FeCy (69) and DPIP (70).

D_1 - electron donors supporting phosphorylation, e.g. $PMSH_2$ ($< 10^{-5}$M) (71) and $DPIPH_2$ ($> 10^{-5}$M) (72).

D_2 - electron feeders not supporting phosphorylation, e.g. $PMSH_2$ ($> 10^{-4}$M) (73) and TMPD (74).

electron to photosystem I, photosystem II is thought to accept an electron from water resulting in the evolution of oxygen.

A series of electron carriers connect the two photosystems and include the cytochromes f and b6 (55)(75). These electron-carriers are coupled to enzyme systems catalysing phosphorylation in which electron energy is converted into pyrophosphate bond energy. A decrease in the free energy of an electron in passing from photosystem II to photosystem I may result in a phosphorylation.

The number of phosphorylation sites involved with photosynthetic electron flow has long been debated and many measurements of P:2e ratios have been made. Some recent evidence (76) suggests there are at least two sites whilst earlier results point to a single site (77)(78). Part of the difficulty in establishing the number of phosphorylating sites lies in assessing how much non-phosphorylating electron flow occurs and how much electron flow occurs in a cyclic fashion giving no net measurable electron flux. Experimentally, two types of phosphorylation are recognised, cyclic and non-cyclic (62)(47)(79).

Cyclic phosphorylation involves photosytem I and results only in the production of ATP. On absorbing far-red light, this photosystem loses an electron, which has been raised to a higher energy level. On falling from this level the electron may be transferred to a series of carriers in turn, and where the free energy decrease of the electron is large enough (ΔG = 8 Kcal) a phosphorylation may occur. P:2e ratios cannot be measured for cyclic phosphorylation since the electrons cycle and are not removed from the system This cyclic flow of electrons can be catalysed by both physiological and non-physiological cofactors. Physiological cofactors are known constituents of chloroplasts and include Vitamin K, FMN and FD, whereas non-physiological agents are redox agents like PMS and DPIP. Recent evidence (80) now points to FD as being the natural catalyst of cyclic phosphorylation in chloroplasts under conditions where non-cyclic phosphorylation

is stopped.

Non-cyclic phosphorylation involves both photosystems and results in the production of ATP, the reduction of NADP and the evolution of oxygen (47):

$$NADP + ADP + Pi + 2H_2O \longrightarrow NADPH_2 + ATP + \tfrac{1}{2}O_2 + H_2O \quad \ldots\ldots\ldots 3$$

Chloroplasts do not react directly with NADP but with FD (81). It is envisaged that photosystem I is excited on absorbing light and transfers an electron to FD and thence to NADP. The oxidised photosystem I is reduced by electrons from water via photosystem II with a concomitant evolution of oxygen and formation of ATP.

In contrast to cyclic phosphorylation, this mechanism consumes electrons, which are derived from water, and consequently an electron flow (or oxygen evolution) may be measured. A non-physiological variation of this reaction is one where **ferricyanide** can replace NADP (47)

$$2Fe^{+++} + ADP + Pi + 2H_2O \longrightarrow 2Fe^{++} + ATP + \tfrac{1}{2}O_2 + H_2O + 2H^{+} \ldots\ldots 4$$

Non-cyclic electron flow has been experimentally separated into two component reactions (82), firstly the photolysis of water which is inhibited by DCMU, CMU or o - phenanthroline and secondly the formation of ATP and $NADPH_2$ by the oxidation of added electron donors when photosystem II is inhibited.

Pseudocyclic phosphorylation (83) is a variant of non-cyclic in which FD-catalysed phosphorylation, in the presence of oxygen, transfers electrons from water to FD and thence to molecular oxygen. This gives the appearance of a cyclic flow since the oxygen evolved balances the oxygen consumed.

Whilst there is a great deal of evidence for the electron flow scheme,,other explanations have been advanced to

explain enhancement and ATP-formation in chloroplasts (84) - (86). The most recent alternative to the scheme has been presented by Arnon et al (87)(88). Arnon now visualises photosynthesis as two individual one quantum reactions in which the two pigment systems have no direct coupling as shown in figures 2a and b.

This new scheme is based upon the facts that FD-catalysed cyclic phosphorylation only occurs when non-cyclic phosphorylation is prevented (89) and that desaspidin, an anti-helmintic agent, differentially inhibits both types of phosphorylation. This latter conclusion is open to question as there are several conflicting reports on the action of desaspidin (90) - (92).

The chlorophyll depicted in cyclic and non-cyclic reactions represents chlorophyll a and b together with the accessory pigments involved in photosynthesis. Cyclic electron flow would occur at wavelengths more than 700 mμ. Enhancement in terms of this newer concept would mean that the addition of short wavelength light to far-red light restores non-cyclic flow and makes for more efficient use of the ATP produced by cyclic flow.

3. Effects of light on ion accumulation.

From the foregoing section it is apparent that light can initiate both electron flow and ATP-formation in chloroplasts. The possibility that these mechanisms may be involved in ion transport in the systems that show a light-stimulated accumulation has been investigated. Whilst the earlier literature contains many reports of light-stimulated ion transport, insufficient

Figure 2a CYCLIC PHOTOPHOSPHORYLATION

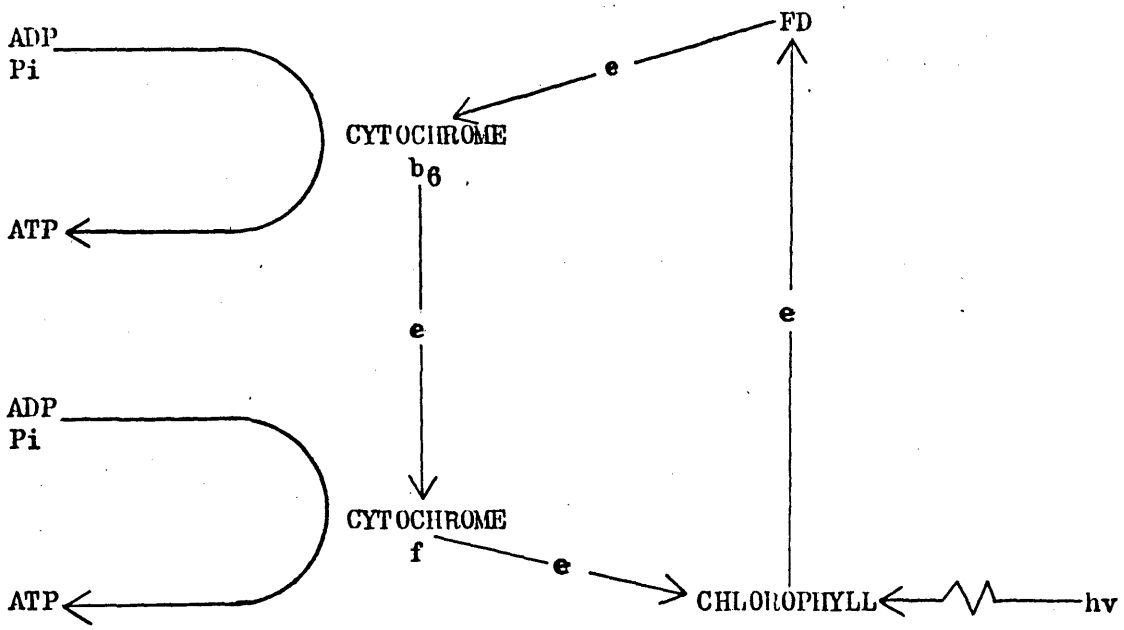
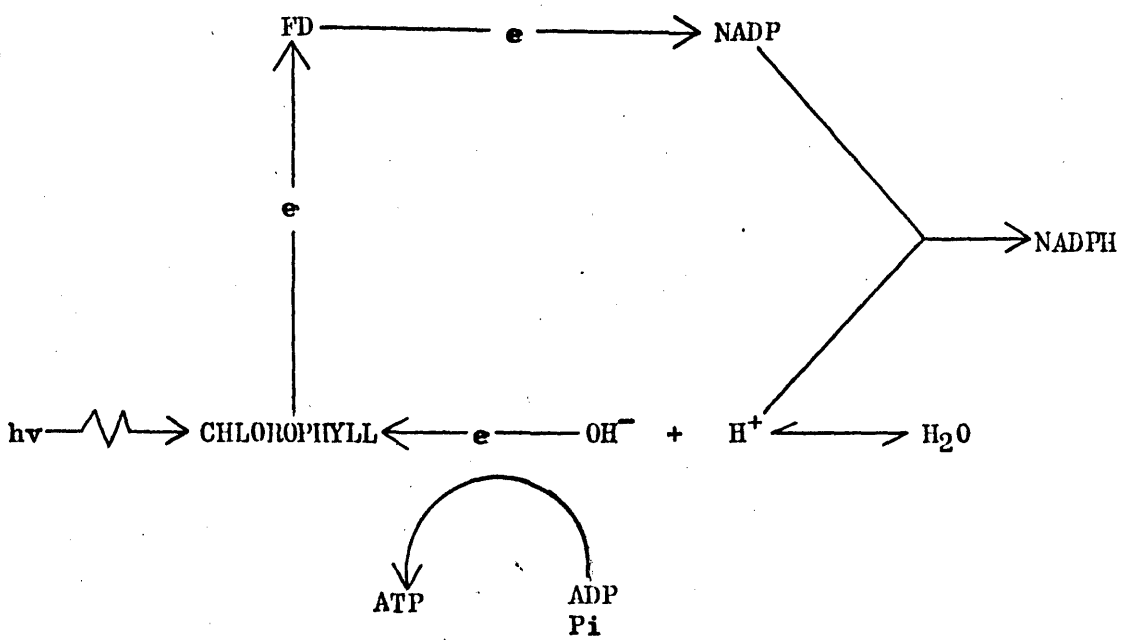


Figure 2b NON-CYCLIC PHOTOPHOSPHORYLATION



knowledge of the mechanism of photosynthesis prevented, in most cases, a detailed analysis of the stimulation beyond that of implicating the products of photosynthesis in the accumulatory process. Work by Lepeschkin (93) however did suggest that light alters the permeability of cells.

Hoagland and Davis (94) were the first to clearly demonstrate the stimulatory effect of light upon salt uptake in green plants. Using Nitella cells, they showed that light enhances the uptake of chloride, bromide and nitrate. Further work (95) led these authors to the conclusion that light is an essential factor for the uptake of these ions by Nitella.

Jacques and Osterhout (96) showed that light stimulates the rate of potassium absorption into Valonia. A similar effect was demonstrated by Ingold using shoots of Elodea (97). Both sets of authors emphasised the importance of exchange reactions involving hydrogen ions, thereby showing the interdependence of ion fluxes. A cycle of light-stimulated sodium and potassium influx was found in Ulva and Valonia by Scott and Hayward (98) and in Porphyra by Eppley (99). The latter worker suggested that sulphydryl groups might be involved in the mechanism, since arsenite and PCMB inhibit the light effect.

In 1947, Arisz (100)(101) reported a light-stimulated chloride uptake in Vallisneria leaves, an effect which is even larger in the absence of carbon dioxide. This is an important finding, since prior to this date other workers had supposed that

light had an indirect effect because it produced carbon assimilation. The result suggests that light is having a more direct effect upon absorption. Further results suggested that a substance might be produced in an illuminated region of a leaf and subsequently transported to a darkened region where enhanced chloride uptake could then be seen. A more comprehensive study of the effects of light and sucrose on chloride absorption in Vallisneria leaves was later published by Arisz and Sol (102). They showed that both sucrose and light increase uptake but in different ways. The effect of sucrose is limited to the zone of the leaf in which it is administered, whereas light effects can be exerted upon darkened regions adjacent to the illuminated zone.

van Lookeren Campagne (43) obtained an action spectrum for chloride absorption by Vallisneria leaves. This showed that the light responsible for accumulation is absorbed by the chlorophyll pigments since the action spectra for chloride uptake and photosynthesis are the same. Work by Simonis and Mechler (103) and Kylin (104) substantiate this view that light-stimulated ion transport is mediated by light absorbed through the chlorophyll system. Since light-stimulated ion absorption also occurs in carbon dioxide-free air (43)(100)(105), the stimulation of uptake does not seem to be via net photosynthesis. Hence the light stimulation of ion movements must involve some partial reaction(s) of photosynthesis and thus implicates the chloroplasts in the absorption of ions, either directly (by acting as accumulation

sites) or indirectly (by supplying an energy source to the cytoplasm for use in ion transport).

Kylin (104), in examining sulphate uptake into the leaves of aquatic and terrestial plants, concluded that the energy sources for accumulation in the light and dark are different, and that the dark mechanism must be inhibited upon illumination. This latter observation is of theoretical importance since there has been a discrepancy between the stimulatory effects of light on ion uptake observed and the inhibitory effect predicted by the Lundegårdh theory of salt respiration. According to this theory ion accumulation is dependent on the direction of the oxygen gradient in relation to the respiratory cytochrome chain. Kylin concluded that the inhibited dark process corresponds to the action of a reversed oxygen gradient upon the cytochrome chain but that the light uptake would be mediated by the electron flow system of photosynthesis. Kylin alternatively considered that photophosphorylation can remove so much ADP and Pi from a common pool into the chloroplast, that oxidative phosphorylation is blocked. The energy source for sulphate uptake would then move from one type of phosphorylation to the other, under the influence of light.

In a series of papers, MacRobbie has presented a detailed analysis of the driving force for ion movements in Nitella translucens. Based on a study of light-dependent fluxes, she first suggested that there is active chloride movement at the plasmalemma membrane (106) and this process involves the formation

and transport of vesicles in which chloride ions are absorbed in groups of approximately 50. Studies on the active uptake of chloride (44)(107) show that chloride influx requires the participation of photosystem II and is directly linked to the light-driven electron flow reactions associated with oxygen evolution; it is not dependent on photophosphorylation. By contrast active potassium influx is dependent on the use of ATP and can occur when photosystem I alone is operative. Smith (108) also found that phosphate influx could occur in Nitella under conditions where photosystem I alone is operative.

Similar results to those of MacRobbie have been obtained by Raven (32). He showed that light can stimulate both influx and efflux of potassium, sodium and chloride in Hydrodictyon africanum. Active chloride influx requires photosystem II and is relatively insensitive to CCCP but is drastically inhibited by DCMU. The active potassium influx and active sodium efflux can be supported by photosystem I, are inhibited by CCCP but not by DCMU.

The association of chloride influx with photosystem II is interesting in view of the fact that it is this portion of photosynthetic reactions which require chloride (or bromide) as an essential cofactor (109)(110).

In direct contrast to these results with Nitella, Jeschke (111) found that the light-dependent chloride uptake in Elodea is maintained by photophosphorylation and is sensitive to the uncoupling agents Atebrin and CCCP. The action of DCMU upon

chloride uptake varied according to the gas phase present. Using nitrogen or carbon dioxide-free air, DCMU has no effect, whereas under nitrogen with low carbon dioxide present, the uptake was partially inhibited by DCMU. This author also found that a small light-driven chloride uptake is supported by far-red light. The distribution of chloride indicates that the cytoplasm is the primary site of accumulation in short periods of absorption but with longer periods up to 24% of the accumulated chloride could be found in the chloroplasts.

Thus the driving force for ion transport in plant cells may be derived from respiration or photosynthesis. With either source of energy there are two possible links with ion transport - a direct coupling with the electron flow reactions (Lundegårdh scheme, chloride fluxes (44)(107)) or an indirect coupling through the use of ATP produced by oxidative or photosynthetic phosphorylation (sodium and potassium fluxes (32)(44).

The results of various workers presented in this section have shown that the effects of light upon ion absorption are mediated by the photosynthetic mechanism present in the chloroplast. This fact together with the development of theories upon the mechanism of photophosphorylation has prompted extensive research into the ion and water relationships of isolated chloroplasts. These studies are discussed in the following sections.

B. Ion and water movements in chloroplasts.

1. Structural and volume changes.

Observations of chloroplasts *in vivo* show that these organelles can alter their volumes by a mechanism that is light-activated (112)(113). The observations of Honda (114) and others (115)(116) show great alterations to chloroplasts in living cells, protuberances can be seen developing and budding away from the chloroplasts and migrating into the surrounding cytoplasm. Such *in vivo* studies suggest these phenomena are physiologically significant.

Studies with isolated chloroplasts using measurements of light scattering (117) (90° light scattering from cuvettes located in photometers), absorbancy changes (118) (at wavelengths with no appreciable absorption by chlorophyll), packed volumes (119) (obtained by centrifuging chloroplast suspensions into hematocrit tubes), volume distribution curves (120) (obtained using Coulter counters) and electron microscopy (121) show that plastids undergo reversible volume changes that are induced by light. In addition, chloroplasts respond to solutions of varying tonicity and so are osmotically sensitive (122).

When chloroplasts are suspended in either sodium chloride or certain amino salts, illumination results in swelling, which may be damaging to the organelle since it is not fully reversible in the dark (123)(124). However, in media containing salts of certain organic acids or under conditions favourable to

light triggered ATP-ase activity (i.e. the hydrolytic enzyme of chloroplasts that is activated by light and requires magnesium ions, a sulphydryl group and an electron carrier to function), illuminated chloroplasts undergo a rapid shrinkage, which is fully reversible in the dark (125). Concomitant with certain of these conformation changes, ion fluxes take place (126).

The initial observations of Packer (127), Itoh et al (128) and others (129)(130) indicated that certain uncouplers, Hill reaction inhibitors, ATP-formation and ageing, all lead to the suppression of light-induced structural changes. These observations led to the hypothesis that a high energy intermediate is responsible for volume changes by an associated formation of ATP. However Hind and Jagendorf (131) have demonstrated that phosphorylation and volume changes respond differently to a number of variables, and conclude that volume changes are not dependent on the ATP generated by photosynthesis.

Several reports (132)-(134) show that there is a light-induced pH rise in chloroplast suspensions which could result in a decreased internal pH of the plastids. Deamer et al (121) and Dilley (135) have shown that, by varying the pH of chloroplast suspensions and comparing the effects with those produced by light, decreasing the external pH giving a high proton concentration inside the chloroplasts, results in light scattering changes similar to those induced by light. Further, there is a close relationship between the kinetics of light-induced proton transport and light

scattering changes in chloroplasts.

Investigation of the chloroplast component responsible for pH-induced changes in light scattering (121) shows that using osmotically shocked chloroplasts some 10-20% of the original light scattering can be accounted for in soluble extracts from chloroplasts. A further 60% is due to the membrane fragments themselves. (Some 10-20% seems due to a modification of the integrated chloroplast structure, as osmotic shock reduces pH-induced light scattering by this amount). This result agrees with the finding that chloroplast membrane fragments undergo volume and light scattering changes in response to light (136). Electron micrographs of light - and pH-induced structurally changed plastids show that the grana membranes are the functional elements causing structural changes (121).

Light scattering changes induced by pH are not large enough to account for all the light-induced structural changes seen in the presence of organic anions (e.g. acetate). Such anions enhance light scattering with an accompanying decrease in volume. Most investigators now consider that ion penetration of the chloroplast membrane system plays a part in volume changes and two models have been advanced to explain light-induced volume changes of chloroplasts.

Dilley (137) has proposed a model to relate volume changes with concomitant ion fluxes. This model depicts electron flow processes resulting in the protonation of fixed negative charges within the grana. Potassium and magnesium in the plastid

act as the counter ions to the fixed negative groups. Upon protonation of these groups, the cations diffuse down the electrochemical gradient across the granal membrane followed by water to maintain osmotic equilibrium as shown in figure 3.

For such a model to be operative the plastid must have certain features:

(a) there must be an osmotically sensitive compartment.

(b) this compartment has to change volume in response to external pH (as long as external protons can exchange for internal cations).

(c) the osmotic compartment must have a greater concentration of mobile cations than anions (a Donnan-type distribution).

There are many reports to show that chloroplasts are osmotically responsive to sucrose and salts (122)(135)(138). One method of testing whether a system behaves as an osmometer is to plot the volume of the material against the reciprocal of the osmotic pressure (or solute concentration to a first approximation). This is the Boyle-van't Hoff equation (139).

$$V-b = RTn/\alpha \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 5$$

where V is the measured volume, b is the non-solvent volume (solid material), α is the osmotic pressure, n is the number of solute particles within the measured volume and R and T have their usual meanings. The slope of the plot is equal to RTn and the y-intercept gives the non solvent volume, b.

Chloroplasts obey equation 5 over a range of sucrose concentrations 0.16 - 0.8M. At concentrations less than 0.1M the

Figure 3

Model of chloroplast in regard to compartmentation and permeability of membranes to various solutes

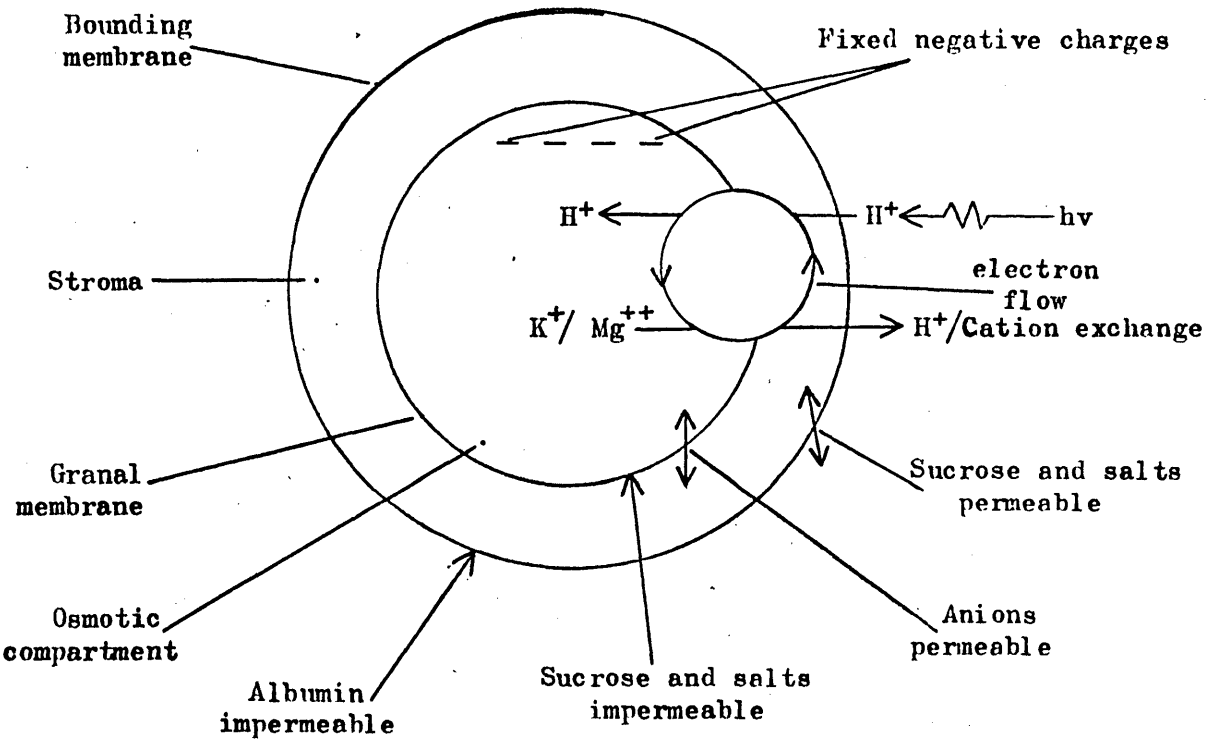
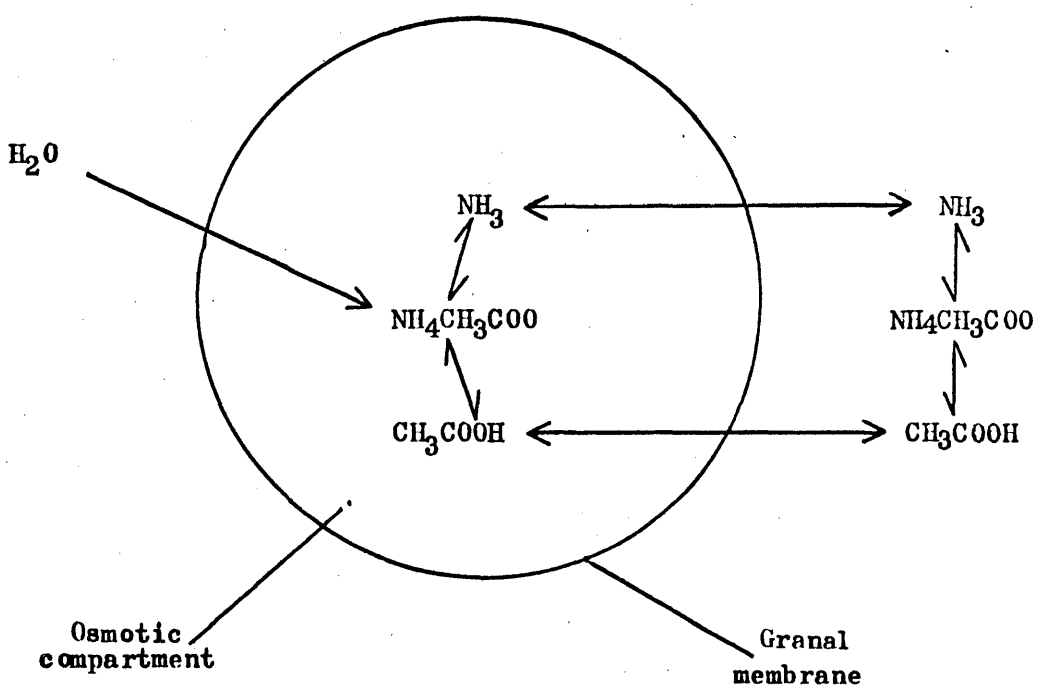


Figure 4

Dark swelling of plastids in ammonium salts of weak acids (acetic acid)



plot deviates from linearity and establishes a second linear line with a lesser slope. Thus comparison of the slopes provides a means of estimating the relative sizes of the osmotic compartments in the two regions (137).

Estimations of the sucrose and albumin permeable spaces in chloroplast pellets show that sucrose must penetrate the bounding membrane but not some inner "compartment". Dilley concludes that this compartment is most likely to wholly contain the grana and this is in agreement with electron micrograph studies and the results of Gross and Packer (121)(136). (However, very recent work by Dilley and Rothstein (135) indicates that sucrose may penetrate the inner compartment).

The grana respond by volume changes to external pH changes. The isoelectric point for chloroplasts is approximately pH 4.7 (140)(141) with swelling occurring above and below this point - a situation typical of polyelectrolytes and strong evidence for the existence of a Donnan system.

The distribution of rubidium and chloride ions between plastids and medium at various pH values supports the hypothesis of a Donnan system. The concentration ratio of rubidium in the chloroplast to rubidium in the medium increases as pH increases from 4.7 to 8.0 but the chloride ratio remains essentially at unity. The data of Dilley (137) indicate that proportionally more rubidium is absorbed than expected from the volume increase whereas chloride increases proportionally with volume. This is most likely to occur

as a result of an exchange of the rubidium for hydrogen ions as the negative groups increase with rising pH.

Packer et al (142)(118) have proposed an alternative hypothesis of light induced volume changes in chloroplasts in the presence of organic acid salts or sodium chloride. It has been suggested (143) that mitochondrial swelling and lysis in ammonium salts is due to the penetration of the osmotic compartment by the medium. Uncharged molecules of ammonia and organic acid are able to penetrate the mitochondrial membrane and this mechanism has been advanced to explain the dark swelling of plastids suspended in ammonium salts of weak acids (152). Experiments show that both the rate and extent of plastid swelling in the dark are related to the concentration of undissociated acid present (as judged by the pK_a of the anions used. This is depicted in figure 4 (on page 28).

Undissociated organic acids can pass freely across the plastid membrane (118) and, as mentioned earlier, illuminating chloroplasts results in a proton uptake by a mechanism dependent upon electron flow (light). Packer et al (142) have suggested that when chloroplasts are suspended in salts of organic acids, the anion equilibrates rapidly between the osmotic compartment (granum) and the medium. Upon illumination, electron flow begins and protons are moved into the plastid resulting in the conversion of dissociated acid anion to undissociated acid as the internal pH falls. The undissociated acid re-equilibrates across the membrane, and the consequent net efflux of anion causes a decrease in osmolarity,

resulting in water loss and hence shrinkage of the chloroplast. This is illustrated in figure 5.

As predicted by figures 5 and 6 the kinetics and extent of proton transport and volume changes of chloroplasts suspended in sodium chloride and weak acid salts are very different. In solutions of organic acids the kinetics of volume change and proton transport do not have any relationship to each other. This is to be contrasted with kinetics of similar changes seen with chloroplasts in sodium chloride solution.

If undissociated acid movement across the membrane is rate limiting then there should be a relationship between rate of volume change and concentration of undissociated acid (as measured by the pK_a of the anion used). Packer and Siegenthaler have shown that many organic anions enhance shrinkage and in general any anion capable of giving approximately 0.1μM (or more) of undissociated acid, in solution at a pH supporting proton uptake, is able to stimulate photo-shrinkage. Measurements of volume changes and proton transport induced by light, show that this mechanism, outlined above, can account for the rate and extent of the volume changes in terms of anion movements.

Packer et al (142) believe that the decrease in internal pH of the grana resulting from light-induced proton uptake into chloroplasts suspended in sodium chloride, causes swelling, possibly in conjunction with chloride movements as shown in figure 6.

Figure 5 Shrinkage of illuminated plastids in the presence of anions of weak acids

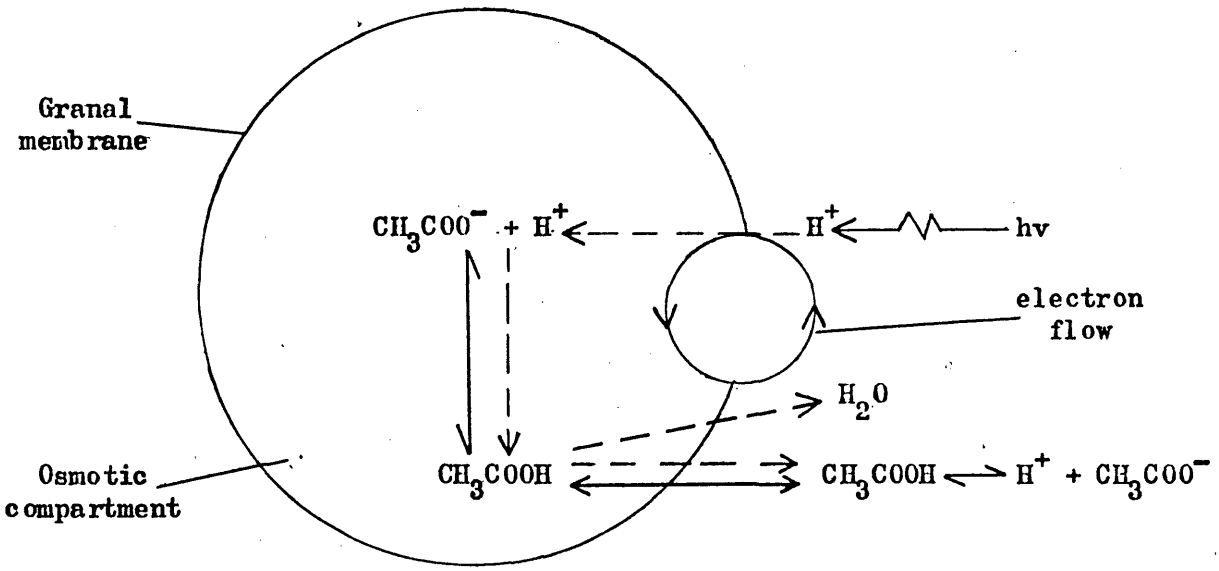
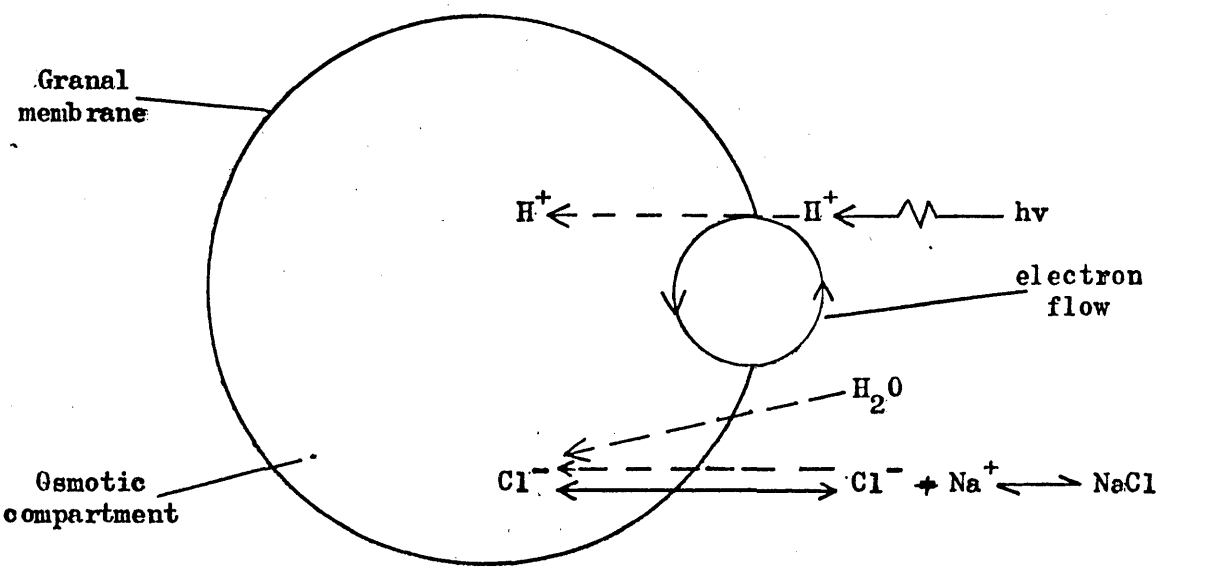


Figure 6 Light-induced swelling of plastids suspended in sodium chloride



$\longleftrightarrow$ Dark equilibration reactions

$- - - - \rightarrow$ Light-induced changes

2. Ion movements in chloroplasts (including the chemi-osmotic hypothesis).

The chloroplast volume changes described in the previous section are dependent upon solute and/or ion movements into some osmotically sensitive compartment (identified as the grana). Some of these movements are passive in the sense that they are primarily governed by the degree of dissociation of a molecule and by the existing concentration gradients. Their subsequent movement, however, may be influenced by movements of other ions, particularly protons, whose transport into and out of the chloroplast is light-dependent and associated with electron flow. Most of these volume studies have been done using naked lamellae systems (grana lacking stroma and an outer membrane). The bounding membrane, whose existence is now generally accepted on the basis of electron microscopy studies, may act as a barrier to the penetration of metabolites to the sites of photosynthesis located in the stromal and granal compartments of the plastids. The rates of photophosphorylation and Hill reaction are both considerably higher using broken chloroplasts (plastids lacking the bounding membrane) than with intact organelles (146)(147).

The permeability properties of the membrane have not been extensively investigated. This is due in part to the difficulty in obtaining preparations with a high percentage of intact organelles. Stocking and Ongun (148) found that plastids isolated in sucrose lose substantial amounts of potassium, sodium, magnesium, calcium and nitrogen. Whereas chloroplasts isolated from lyophilised material in non-aqueous solvents contain up to 40% of the total leaf content of

these elements. This finding suggests that either the membrane is damaged during aqueous isolation or that its permeability properties change on isolation.

Tolberg and Macey (138) found that at constant pH the slope of the Boyle - van't Hoff plot for chloroplasts in sodium chloride solutions is almost double that for plastids suspended in sucrose solutions. Chemical analyses, of the distribution of potassium, sodium and chloride ions between the pellets and supernatants obtained by centrifuging chloroplasts from a variety of media, show that sodium and chloride ions equilibrate rapidly between all the aqueous phases of the plastid, whereas potassium ions may be partially retained.

Osmotic studies (138)(149) indicate that solutions of chloride, sulphate and nitrate salts of a variety of monovalent cations and sodium acetate are able to support chloroplasts osmotically. Nishida and Koshii found that there is a slow dark swelling of plastids in these solutions which may indicate a slow degree of penetration of these salts. Salts of divalent cations, especially calcium, give a very rapid initial shrinkage of chloroplasts and this is followed by a gradual swelling towards the original volume, indicating some degree of penetration by these agents.

Investigations of ion movements in chloroplasts have received fresh impetus from the publication of the chemi-osmotic hypothesis by Mitchell (150) and perhaps the best documented example of ion movement into plastids is that of protons. Mitchell in an

attempt to resolve the problem of coupling (simultaneous reaction of electron flow and phosphorylation) has proposed that the transport of protons across a membrane which completely encloses an inner space is the site of the coupling mechanism.

The Mitchell hypothesis is based upon five postulates namely:-

(1) The phosphorylating particle has a membrane impermeable (or nearly so) to protons. Since broken chloroplasts phosphorylate, the relevant particle must be the grana of plastids.

(2) The bounding membrane contains electron carriers. The flow of electrons through the carriers is coupled to a directional transport of protons. For chloroplasts the direction of hydrogen ion movement is from the medium into the grana. This movement could occur each time electrons move from a metal-atom carrier (e.g. cytochrome) to a carrier with one or two hydrogen atoms bound when reduced. This is illustrated in figure 7a.

(3) As electron flow continues the electrochemical activity of protons in the inner enclosed space will increase and be greater than that in the outside medium. This separation of charge and proton thus represents a store of potential energy and is in fact the high energy compound of phosphorylation.

(4) To compensate for this charge accumulation, there is a cation efflux, but the potential energy of the system is not neutralised by cation efflux (or anion uptake) and is the sum of the proton concentration difference across the membrane and the membrane potential.

Figure 7a

Possible mechanism for proton movement across the granal membrane

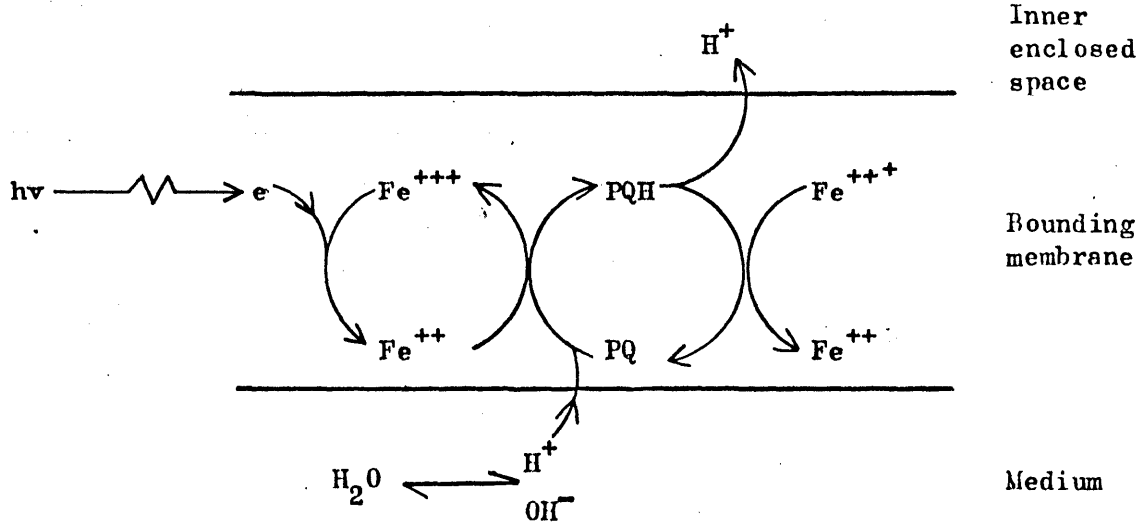
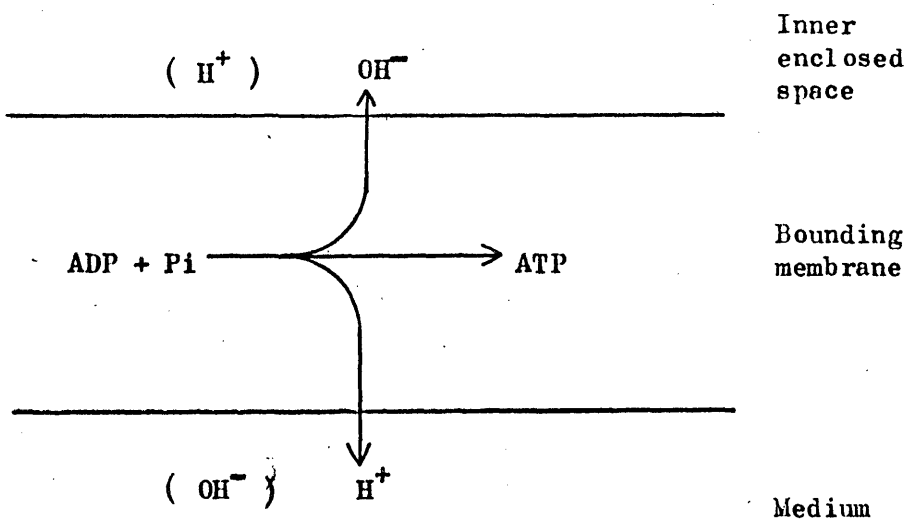


Figure 7b

Possible phosphorylation mechanism



(5) An enzyme system is present in the bounding membrane of the grana to utilise the potential energy of the proton gradient. This enzyme is a reversible ATP-ase oriented in such a way that the elements of water - a proton and hydroxyl ion - when removed from ADP and Pi are released in opposite directions. This is shown in figure 7b. The reaction between protons and hydroxyl ions on each side of the bounding membrane, lowers the concentration of water at the enzyme and makes the synthesis of ATP possible. The proton gradient established by electron flow thus acts as a dehydrating device.

Attempts to demonstrate the existence of high energy intermediates prior to ATP (192)(193) show that chloroplasts illuminated in the absence of ADP and Pi, can make ATP from ADP and Pi in a succeeding post-illumination dark period. The reaction in the light is stimulated by redox dyes enhancing electron flow and the existence of a non-phosphorylated high energy intermediate, designated X_E, was inferred by the authors.

Photophosphorylation is thus composed of two stages. Whilst inhibitor and uncoupling action upon the light and dark stages and kinetic studies of the build-up and decay of X_E reveal something of its role, no information is given as to the nature of X_E. Under optimal conditions, chloroplasts can yield approximately 270 ATP molecules for every 2500 chlorophylls. This rules out any possibility that X_E is a stoichiometric compound involving any components of the electron flow chain.

With the Mitchell hypothesis in mind, Jagendorf and collaborators considered that X_E might be a proton gradient established in the light and subsequently utilised in the dark to form ATP. Many reports (121)(132)(133) show that unbuffered or weakly buffered chloroplast suspensions exhibit a light-induced pH rise in the medium. This has been interpreted as being due to the uptake of protons into the plastids via the electron flow chain. This is confirmed by the observations of Avron and Chance (151) using bromothymol blue as an internal pH indicator of chloroplasts.

The light-induced pH rise observed has the same pH and cofactor requirements as X_E (133) and phosphorylation uncouplers depress the rate and extent of the rise (134). The amounts of protons absorbed are large - 1,800 protons per 2,500 chlorophyll molecules and may be higher. Estimates of the internal pH of the plastid suggest that it could be as low as 2.5 (121)(134), even if this were buffered 90% by groups inside the grana, a pH of 3.5 would exist. If such chloroplasts were used in an "X_E experiment" they would be incubated in a phosphorylation mixture at pH 8 giving a pH differential of 4.5. This represents an energy level of 5.6 K cal. per equivalent of protons, enough to form considerable ATP under these conditions.

Most of the available data suggest that X_E is in fact a gradient of electrochemical activity induced by light. However, the possibility remains that electron flow causes the formation of X_E, a high energy compound and proton and cation fluxes are on an

unrelated pathway. The strongest case for equating X_E to the light-induced proton gradient are the experiments (190) in which ATP is synthesised by chloroplasts in the dark upon changing the external pH. If plastids are first suspended in acid. media and then exposed to a basic phosphorylation mixture, ATP is synthesised. These experiments demonstrate that a gradient of hydrogen ions, between the inside and outside of the grana, is responsible for ATP-formation. It was also found that the yield of ATP could be considerably increased by using organic acids in the acid phase of such experiments.

Organic acids are believed to provide an inner reservoir of protons, by penetrating into the grana. There is a good correlation between ATP-synthesis and the pK of the organic acid used. The best yields are obtained using dicarboxylic acids in which 40-60% of the molecules are undissociated (194). Experiments using C^{14}-labelled succinic acid show that it penetrates into the grana only at acid pH values and does not leak out when the plastids are transferred to basic media.

Examination of the yield of ATP as a function of pH in the basic phosphorylating stage shows that a definite pH difference is required between the acid and base phases. This substantiates the view that it is the pH gradient which is supplying the energy for ATP-formation (198).

With such a large and rapid movement of protons into the plastid it is to be expected that an efflux of cations (or an

influx of anions) would occur to maintain electrical neutrality. Dilley and Vernon (126) found that under conditions of light-induced proton uptake, cations (magnesium, sodium and potassium) were excreted from the chloroplast into the medium.

The question raised by these observations is which of the two processes (proton uptake or cation excretion) is the primary process accompanying electron flow. Kinetic data (126) indicate a faster half-life for proton uptake than for cation excretion. Since the nature of the excreted cation may vary and the uptake of protons is greater than the amount of any one cation excreted, it is proposed that proton transport is the primary effect and cation efflux is dependent upon this, as illustrated in figure 8a. Alternatives to the scheme shown in figure 8a may exist, and these are depicted in figures 8b and 8c. In these schemes X_E is not equivalent to the proton gradient and proton and cations fluxes are dependent upon the presence of X_E.

As mentioned earlier, the results of Packer et al suggest that the plastid membrane is freely permeable to ammonia. Crofts (152)(153) has investigated the uptake of ammonium by chloroplasts and its relationship to phosphorylation. Simultaneous measurements of light-scattering changes, cation and pH concentrations in a suspension of chloroplasts show that on illumination, protons are taken up from the medium and a parallel light-scattering change is seen. On adding ammonium chloride and subsequent illumination, both the pH and light-scattering changes are inhibited, but cation

Possible relationships between electron flow and proton and cation fluxes

Figure 8a

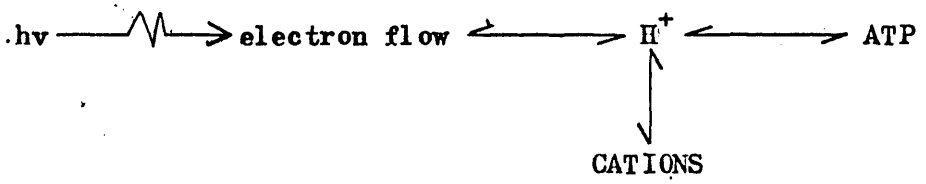


Figure 8b

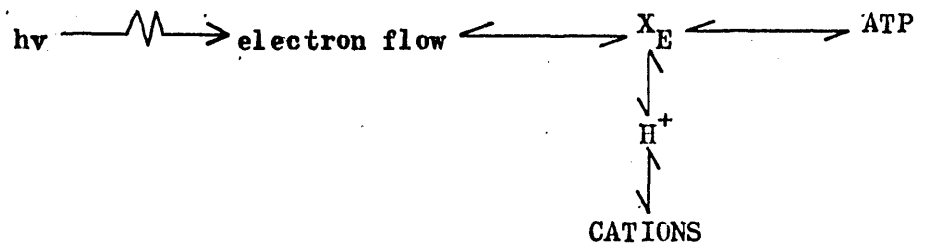


Figure 8c

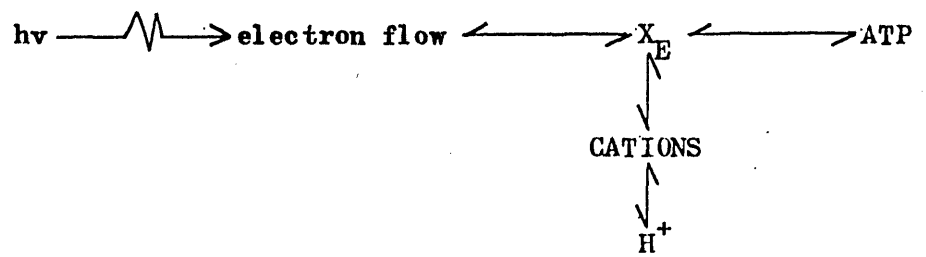
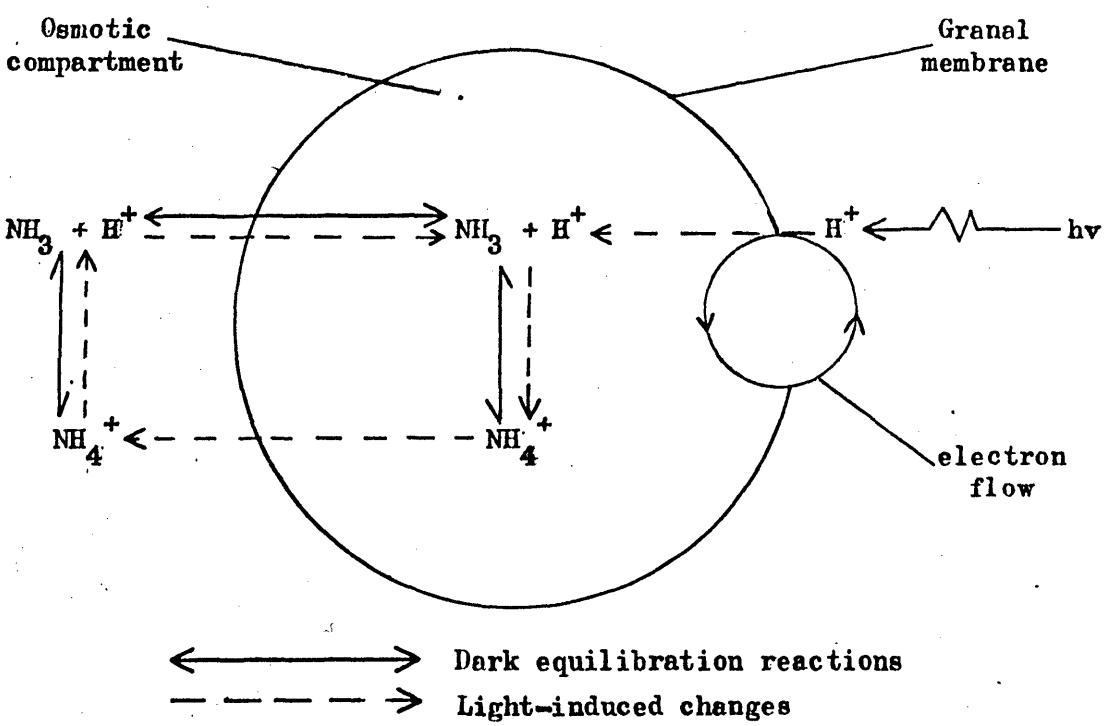


Figure 9 Equilibration of ammonia in illuminated plastids



uptake is enhanced. Ammonium uptake increases with increasing ammonium concentration and is associated with a parallell suppression of the proton uptake coupled to electron flow. The following scheme is envisaged in figure 9, proton uptake on illumination, following the equilibration of ammonia across the membrane, would displace equilibration to replace the ammonia lost by association with protons, thus increasing ammonium concentration inside the plastid. Entry of ammonia continues until ammonium ion diffuses out at the same rate as protons enter.

The kinetics of ammonium uptake are consistent with this mechanism. CCCP competitively inhibits ammonium movement suggesting that ammonium ion and CCCP compete for a common site at a level closer to electron flow than to the terminal steps of phosphate transfer. Uptake of ammonium ion can be driven in the dark by ATP hydrolysis and is associated with a stimulation of ATP-ase activity.

Packer and Nobel (154) and Nobel (155) have demonstrated another type of ion movement in chloroplasts. The kinetics and extent of changes show no relation to the very rapid ion movements and volume changes outlined in the earlier part of this section.

The accumulation of certain cations in plastids has been found, under conditions associated with the hydrolysis of ATP in the light. Uptake of calcium, strontium, sodium and phosphate is greatest in the presence of ATP, magnesium, PMS and a sulphydryl group. The process has a pH optimum of 7.9 and reaches a maximum

in 30 mins. Uncouplers and ADP inhibit the uptake of these ions whilst thiol groups elevate uptake. Nobel (155) found that light is not required whilst calcium uptake is proceeding but is necessary to trigger the ATP-ase system. Eliminating any of the cofactor requirements of the hydrolytic reaction resulted in an inhibition of ion uptake (although magnesium ions are not required for sodium movement).

Light-induced phosphate uptake requires the presence of calcium, but calcium or strontium absorption can occur in the absence of phosphate (although the hydrolysis of the added ATP probably provides sufficient phosphate for cation uptake).

Electron microscopy (156) shows the location of barium, strontium and calcium deposits to be on the lamallae system of chloroplasts. Electron-dense spots are seen on the lamellae and these are greatly increased by light under conditions of ATP-hydrolysis.

The relationship between light-induced swelling and cation uptake has also been investigated (157). Light-induced swelling of chloroplasts is required for sodium uptake and enhances strontium uptake. Sodium uptake could be accounted for by a potassium exchange as measurements of potassium efflux indicate. A rapid light-stimulated magnesium efflux was also noted in these experiments.

The relationship of the energy-transfer pathway to light-induced strontium uptake was also investigated by using various inhibitors and the relation of a coupling factor (158) to these two processes. Dicumarol, FCCP, and desaspidin decrease strontium

accumulation primarily by inhibiting light-induced swellings. Pentachlorophenol and Gramicidin J inhibit cation uptake by inhibiting swelling and by uncoupling, however octylguanidine decreased phosphorylation and cation absorption whilst enhancing swelling Cetylpyridinium bromide, phlorizin, chlorpromazine and quinacrine appear to uncouple chloroplasts at a site between that involved with ATP formation and the site involved with strontium uptake. A heat-labile coupling fraction also appears to act at this site.

Work by Nobel (155) also shows that the following ions are not accumulated under ATP-ase conditions: potassium, rubidium, manganese, zinc, sulphate, iodide, bromide and chloride.

Recently, Nobel (159) has examined the relationship between calcium absorption, photophosphorylation and ATP-ase activities in isolated chloroplasts. The light-induced calcium absorption is competitive with photophosphorylation. Incubating plastids under ATP-ase conditions with ATP labelled in the terminal phosphate position and comparing the incorporation of labelled phosphate with incubations in which labelled Pi was supplied showed that a greater incorporation occurs from suspensions with ATP present than from those with labelled Pi present. ATP itself is not accumulated. Nobel suggests that the ATP-ase functions to relase phosphate into a special location in the chloroplast and this acts as a sink for calcium accumulation. A comparison between ATP-ase activity and proton influx in the light shows the two processes to have similar kinetics with similar half-times of decay and it is suggested that

the ATP-ase is activated by a proton gradient. The inhibition to calcium uptake in the light by certain uncoupling agents is thus ascribed to the inhibition of proton influx thereby preventing ATP-ase activity.

C. SUMMARY.

An inter-relationship of proton and other ion movements with phosphorylative and electron flow processes is seen to exist in isolated plastids and certain algal and higher plant cells. This inter-relationship is placed in perspective by the chemiosmotic hypothesis. Plant cells can accumulate a variety of ions and molecules and whilst the concept of accumulation is unambiguous for non-electrolytes, transport of ions is complicated by ionic charge. In considering the transport of any ion, ion movement will create an electrical potential which will influence the movement of other ions.

The effects of light upon absorption by green plants may be interpreted from four viewpoints, namely:

(1) Light causes proton movement into the chloroplast from the cytoplasm (and medium). This will create a charge and ion difference between the plastid, cytoplasm and medium. This difference may result in ion movements into the cytoplasm and thence possibly into the plastids.

(2) Light acting on the chloroplasts may cause the production of ATP which may be subsequently transported to the cytoplasm where it is utilised to facilitate the entrance of ions into the cytoplasm and thence possibly into the chloroplasts.

(3) Light may activate the photosynthetic apparatus resulting in the absorption of ions into the plastids from the cytoplasm. Depletion of the ion content of the cytoplasm by the plastids may result in the entrance of ions into the cytoplasm from the medium. (4) Light may in some way alter the permeability of the plasmalemma bounding the cytoplasm. The alteration to permeability then results in an increased passage of ions through the membrane.

If selective or specific inhibitors of photosynthetic processes are known to inhibit the ion movement under study then the proposition that permeability changes are involved can be eliminated. The use of isolated chloroplasts allows the measurement of ion movements into the plastids. In the absence of any such movements, the hypothesis that light causes the production of ATP which is then utilised by the cytoplasm would have to be considered (although the isolated plastids may have been damaged during isolation must also be considered). To distinguish between these various possibilities fractionation and assay of the individual cellular components for the ion in question would offer the better method.

The work reported here represents an attempt to investigate the accumulation of chloride and calcium into chloroplasts with regard to the effect of light upon these processes. The analysis of such movements using intact tissues is hampered to a certain extent by the simultaneous occurrence of respiratory and photosynthetic processes. Isolated chloroplasts offer a system in which respiratory processes are absent (180) and thus allows the effects of light to be interpreted in terms of the photosynthetic reactions.

EXPERIMENTAL METHODS.

1. Reagents.

Analytical grade reagents were used without further purification. Glass distilled water was passed through an Amberlite MB-3 mixed bed resin column and the "deionised water" used for the preparation of all solutions ADP and ATP were purchased from Sigma Chemical Co. Ltd. and radioactive isotopes were obtained from the Radio Centre, Amersham. Other reagents were prepared as follows:-

Potassium ferricyanide crystals of AR grade material were washed free of yellow powder. Solutions were prepared from the red crystals after drying at room temperature.

Tricine (N - Tris - (Hydroxymethyl) - Methylglycine) was initially prepared according to the method of Good (160) and twice recrystallised from aqueous ethanol. Later this buffer was purchased from B.D.H. Ltd., as was the buffer Tris (Tris - (Hydroxymethyl) - amino-methane.

PMS (N - methyl phenazonium methosulphate) was prepared immediately before use and kept in the dark until required.

Antimycin A, DCMU and CMU were prepared as 95% alcoholic stock solutions such that the final alcohol concentration in reaction mixtures never exceeded 1%. An equivalent alcohol concentration was added to the corresponding control - CCCP was prepared as a 10^{-3} M solution in 10^{-2} M NaOH, and diluted to the required strength.

2. Chloroplast preparation.

Peas (Pisum sativum L. var. Laxton Superb) were grown in

moist vermiculite for 14 - 16 days in an insulated box. Illumination was provided for 11 hours by five 40-W fluorescent tubes (Ecko daylight) at a distance of 30 inches alternating with 13 hours dark. This gave a light intensity of approximately 200 foot candles. Temperature was maintained at 18°C ± 1. Taken after a dark period the leaves contained little or no starch.

Shoots of Elodea densa were purchased commercially and maintained in an aquarium tank in tap water.

Aqueous suspensions of whole chloroplasts were prepared by a method similar to that described by Walker (161). In this method, 50 gm. of leaf material (lamina with short petiole and part of the stem) were homogenised with 120 ml. of isolating medium at approximately 1°C in a Waring blendor (M.S.E. Atomix) previously chilled in a deep freezer. Leaf material was added to the isolating medium with the blades of the blendor revolving at low speed and then after 10 seconds the speed was increased to full speed for 5 seconds only. As Walker points out, this is an inefficient homogenisation, but a large proportion of the chloroplasts survive in an intact state. The ice-cold homogenate was then filtered through two layers of muslin to remove unbroken cells and large pieces of tissue, the filtrate was then passed through eight layers of muslin to complete this process. The filtered homogenate was then poured into four 50 ml. tubes, and placed in a high-speed angle head centrifuge (M.S.E. 13), previously cooled to 0°C, and the chloroplasts separated by rapidly accelerating the rotor to 6000 r.p.m. (5000 x g) and then quickly bringing it to rest by manual braking. The time for

centrifugation was approximately 90 seconds and the total time from cutting and weighing the leaf material to collecting the chloroplast pellets was less than 6 minutes.

The chloroplast pellets in the tubes were rinsed with resuspending medium (when this was different from the isolation medium) and finally resuspended in a total of 5 ml. using a fine camel hair paint brush so that frothing and aeration were avoided. The chlorophyll content of the suspension was determined using the method of Arnon (162).

Chloroplasts were always osmotically balanced during the isolation phase. If broken chloroplasts were required, the pellets were resuspended with water to remove the membrane and then sufficient osmoticum and/or buffer was immediately added until the final osmolarity was the same as chloroplasts resuspended with buffer and/or osmoticum to maintain the outer membrane.

Chloroplasts were prepared immediately before use and where different incubation periods were necessary, the reactions which were to last the longest were started first to minimise any effect of ageing. The suspensions were examined microscopically with a Gilbert-Sibert photomicroscope which gave a magnification of 2000 diameters. Electron micrographs were prepared by D.A. Greenwood of Imperial College.

Non-aqueous preparations of chloroplasts were made using a discontinuous gradient of hexane and CCl_4 mixtures as described by Bird et al (163). Intact pea leaves were first dropped into liquid nitrogen and broken into small fragments by crushing the brittle leaves with a glass rod. The fragments were then lyophilised for 72 hours at -20°C.

over calciumchloride. After 72 hours the lyophilised material was then stored under vacuum in a desiccator over P_2O_5 and aliquots removed as required.

One hundred milligram portions of the lyophilised material were homogenised with about 30 ml. of a mixture of hexane and $C Cl_4$ of density 1.38 in a Potter hand-homogeniser. The homogenate was strained through a glass-wool pad and 15 ml. aliquots run into two centrifuge tubes. 15 ml. of solvent mixture, density 1.36, was carefully layered on top of the homogenate, followed by 2 ml. of solvent mixture, density 1.34. The tubes were slowly accelerated (to maintain the gradient) to a speed of 10,000 r.p.m. (12,000 x g) in a refrigerated centrifuge (M.S.E.13) and held at that speed for 30 minutes. After allowing the rotor to come to rest, the chloroplasts were located as a dark green discrete band at the top of the tubes in the lightest part of the discontinuous gradient.

The chloroplasts were removed from the discrete band with a Pasteur pipette, diluted with an equal volume of hexane (to decrease the density of the suspension) and again centrifuged at 10,000 r.p.m. (12,000 x g) in the M.S.E.13 for 15 minutes. The chloroplast pellets thus obtained were placed in a desiccator and evacuated for 5 minutes to remove traces of the organic solvents.

3. Isolation media.

Three isolation media were used for chloroplast preparations, all at 25 mM concentration. Tris-acetate and Tricine-NaOH were the buffered solutions and these contained osmoticum. The osmoticum

was either sucrose or sodium chloride. Tris-acetate was used with sucrose only, but Tricine - NaOH was used with both sucrose ("Tricine-sucrose") and sodium chloride ("Tricine-chloride"). The pH of the solutions was measured using a Pye expanded scale pH meter.

4. Determination of phosphorylation.

Phosphorylation was determined by following the uptake of inorganic phosphate using the method of Allen (164) as modified by Hill and Walker (165).

Reactions were run in round-bottomed test tubes. Samples (0.25 ml.) were removed from the reaction mixtures for estimation procedures. The reaction mixtures contained the following components in a total volume of 2.5 ml.:-

	Cyclic	Non-cyclic
ADP	15 µmole	5 µmole
KH_2PO4	15 µmole	10 µmole
PMS	0.3 µmole	0
FeCy	0	1.5 µmole
Chloroplasts	1.8 ml.	1.8 ml.
Buffer or other additive	0.2 ml.	0.2 ml.

The pH of all additives was adjusted to that required (usually pH8) and the additives placed in a darkened tube. The chloroplast suspension was pipetted into the darkened tube, the contents mixed and a sample removed. The tube was then illuminated and further samples removed as required. Appropriate dark controls were also run.

Each 0.25 ml. sample was run into a tube containing

9.6 ml. of a solution containing:-

0.15 ml. perchloric acid 60%.

0.15 ml. reducing agent solution.

The reducing agent solution had the following composition:-

2:4 - diaminophenol (amidol) 1 gm.

Sodium metabisulphite ($Na_2S_2O_5$) 20 gm.

dissolved in 100 ml. water and filtered.

The total volume in the tubes was brought to 10 ml. on adding 0.2 ml. of ammonium molybdate 8.3 gm. dissolved in 100 ml. water.

The contents of the tubes were mixed and development timed from this point. The tubes were centrifuged free of denatured chloroplasts and the absorption determined at 660 mμ after 10 minutes.

The difference between the O.D. of the dark control and that of the samples gave, by reference to a graphical relationship obtained with standard phosphate solutions, the uptake of inorganic phosphate in μ moles.

5. Estimation of Ferricyanide reduction.

Coupled and basal rates of ferricyanide reduction were studied using the following mixtures:-

	Coupled	Basal
ADP	5 μmole	0
KH_2PO_4	10 μmole	0
Fe Cy	1.5 μmole	1 - 2.5 μmole
Chloroplasts	1.8 ml.	1.8 ml.
Buffer or other additive	0.2 ml.	0.2 ml.
Total volume	2.5 ml.	2.5 ml.

The pH of the mixture was adjusted to pH 8.

The same experimental methods were used as for phosphorylation, and 0.25 ml. samples were added to 0.25 ml. of 10% TCA to stop the reaction.

The reaction mixtures were centrifuged free of denatured chloroplasts and a 0.1 ml. sample was taken of the clear supernatant. This sample was then made up to 2.1 ml. by the addition of water, and the following amounts of reagents added:-

0.3 ml. of reagents A and B

0.15 ml. of reagents C and D

A : 3M sodium acetate, pH adjusted to 6.0 - 6.5

B : 0.2 M citric acid

C : 0.0033M ferric chloride, dissolved in 1 M acetic acid.

D : 300 mg of 1:10 phenanthroline dissolved in 30 ml of ethanol.

Development of colour was timed from the last addition and the orange colour estimated at 510 mμ after 5 minutes in the dark. The amount of ferrocyanide present in each sample was found by difference between the O.D. of a zero time blank and that of the samples, and multiplying this difference by 0.145, giving μmoles of ferrocyanide (166).

Ferricyanide reduction was also estimated directly by the decrease in O.D. at 420 mμ on reduction.

6. Estimation of Adenosine Triphosphatase activity.

Under certain conditions chloroplasts can hydrolyse ATP. Several ATP-ase systems are known (158) (167) (168). Pea chloroplasts contain a magnesium - dependent ATP-ase (Nobel, personal communication) which is activated by light. The enzyme is also dependent on the

presence of sulphydryl compounds and the ATP - hydrolysis rate is comparable to that reported for the same system in spinach plastids (159).

An estimation of ATP-ase activity was made by incubating the following reaction mixture in the light, with chloroplasts and assaying for free phosphate in the way described for photophosphorylation.

ATP-ase reaction mixture.

Component	Concentration
ATP	1 mM
PMS	3×10^{-7} M
Magnesium chloride	1 mM
Cysteine	5 mM
Chloroplasts	0.200 mg chlorophyll

All additions were buffered with 25 mM Tricine-sucrose at pH 8. This mixture was in a final volume of 2.5 ml. and 0.25 ml. samples were removed for assay. Control tubes containing chloroplasts without ATP, and ATP without chloroplasts were used.

7. Assay of Fumarase activity.

The enzyme fumarase is present in mitochondria and the presence of enzyme activity in a chloroplast preparation can be used as a measure of cytoplasmic contamination. The enzyme was assayed by the method of Pierpoint (169). The reaction mixture contained in a final volume of 2.45 ml : 0.15 mM phosphate buffer pH 7.8, 2μ M cysteine, 0.1 ml. enzyme and water. The reaction was started by the addition of 150μM malate and the increase in optical density at 240 mμ was measured. In the control an equal volume of water was substituted for Malate.

8. Measurement of protein concentration.

Protein concentration was measured by the method of Lowry et al (170). Only soluble protein from chloroplasts was assayed. Samples containing from 5 - 100µg protein in 0.2 ml. or less were mixed with 1 ml. of solution C. After 10 minutes at room temperature 0.1 ml. D was added rapidly and the solution was mixed immediately on a Vortex mixer. After 30 minutes the intensity of the purple colour was read at 750 mµ.

Reagents. A. 2% Na_2CO_3 in 0.1.N. NaOH

B. 0.5% $CuSO_4 . 5H_2O$ in 1% Na tartrate freshly prepared.

C. 50 ml. A + 1 ml. B. - also freshly prepared.

D. Diluted Folin reagent.

The standard protein used was bovine serum albumin containing 97µg protein per 100µg dry weight.

9. Measurement of ion uptake.

There are several possible approaches to measuring ion uptake by isolated chloroplasts. The plastids may be separated from their suspending medium either by filtration or by centrifugation and the ion content of the collected particles or supernatant determined. Alternatively the ion concentration of chloroplast suspensions may be continuously monitored by the use of ion-selective electrodes which have recently become commercially available. In this study uptake of chloride and calcium were determined with the use of radio-active isotopes using centrifugation and filtration techniques.

Centrifugation of plastids is a relatively slow process and the sedimented particles always contain some supernatant trapped between the particles. This extra-chloroplast ion fraction may be lessened by centrifuging the chloroplasts through a layer of medium lacking the ion in question. As an alternative to assaying the chloroplast pellet obtained on centrifugation, the ion content of the supernatant may be measured and uptake is then defined as the difference in activity at zero time and the activity at subsequent time intervals. Chloroplasts were centrifuged from the experimental solutions using an M.S.E. Minor bench centrifuge. The centrifugation time was five minutes and the force was 1500 x g.

Supernatants were assayed for radio-active isotope by removing 0.1 ml. aliquots for scintillation counting in a Nuclear Chicago scintillation counter Model 720. A serious problem in this type of counting is "quenching", a term which covers several effects, all of which result in a decrease in counting efficiency. Biological materials contain many quencing agents but chlorophyll was the main one which was encountered in this study. The efficiency of each sample was therefore determined by the "channels ratio" method (171) and all counts were corrected to the efficiency of an unquenched standard (approximately 65%).

Chloroplast pellets obtained from the experimental solutions were resuspended in a small volume of 80% acetone, and aliquots were removed for chlorophyll estimation and counting using a Nuclear Chicago gas-flow counter.

Filtration of chloroplasts is a faster process than centrifugation, but the ion fraction in the plastids must be regarded as a relatively stable one, since breakage may occur and the forces applied during filtration may lead to the collapse of membranes. Further, as with the centrifugation technique, the collected plastids include an extra-particulate ion fraction.

Filtration of the plastids from the experimental medium was accomplished by membrane filtration under moderate vacuum using Millipore filters RAWP of pore size 1.2 μ supported on a sintered-glass disc. Retention of the radio-active isotopes by the filter material was substantially lowered by washing the filter with a 5 mM solution of inactive ion under investigation immediately before filtering the experimental solutions with chloroplasts. In some cases the deposited chloroplasts were washed with 0.35 M NaCl. The filters were then dried for 1 hour at 100°C and then glued with rubber solution to planchettes and counted with the Nuclear Chicago gas-flow counter using a gas mixture of argon with methane (90:10).

Membrane blanks were prepared by filtering the incubation medium without chloroplasts and treating the filter as though it were a chloroplast sample. Blanks were prepared for every change in experimental medium and were subtracted from the sample values to correct for isotope retention by the filter. In some cases this correction was obtained by subtracting dark sample values from light sample value to give a "light - activated" value, thus eliminating the background and filter correction.

With all counting procedures, counting was continued for sufficient time to give a maximum error of 2.5 per cent (172) and

results for chloride were subjected to the "t" test (173) to ascertain their significance.

Chloride absorption in Elodea was measured by removing small (0.3 ml) samples of the solution and 0.1 ml. aliquots were assayed for radio-chloride in the scintillation counter. Occasionally the leaves were removed from the solution and washed free of adhering solution and homogenised in a Potter hand-homogeniser and aliquots removed for gas-flow counting. This method removes the fraction of chloride present in the free space of the tissue.

Simultaneous measurements of labelled carbon dioxide fixation and chloride-36 uptake were made using leaves of Elodea. For these experiments the incubation period was decreased from 60 to 30 minutes and the experimental solution was buffered with 5 mM phosphate buffer at pH 7.8 Radio-active sodium bicarbonate (1 μc) was supplied with carrier to a final concentration of 10^{-5}M and chloride-36 as a 0.5 mM solution of potassium chloride.

Aliquots of the experimental solution were removed at the beginning and end of the experiment. These were acidified with dilute hydrochloric acid (to remove any radio-active bicarbonate) and aliquots were counted in the scintillation counter for chloride-36 activities. The leaves were removed from the experimental solution at the end of the experiment and washed. They were then dried in vacuo and combusted. The combustion method was basically that of Van Slyke et al (174) and modified by Porter et al (175).

The combustion vessel was a rimless 100 ml. Pyrex flask

into the bottom of which was sealed excentrically, a glass cup. The top of the cup was concave so that it could be closed with a glass marble. A removable centre well, 1.0 x 1.5 cm., fitted with a stirrup was placed inside the cup, using a wire hook. The leaf material was placed in the flask. About 0.5 g $K_2Cr_2O_7$ was added followed by 5 ml. of the liquid reagent (1 .5g KIO_3 in a mixture of 50 ml. H_2SO_4 and 50 ml. H_3PO_4). Into the centre well was pipetted 0.5 ml. of 4N NaOH. The marble was then placed so that it rested against the side of the flask and the rim of the cup. The flask was stoppered with a serum cap, held in place with elastic bands, and oxygen flushed through for about 1 minute using two hypodermic needles in the cap. The centre well was closed by tipping the marble on to the cup, and the flask heated at 110°C for 1.5 hours. The flask was cooled and allowed to stand overnight to ensure absorption of the CO_2. Aliquots were removed from the centre well and their activity determined using the scintillation counter.

With radioactive solutions, the amount of ion absorbed was calculated by multiplying the percentage of radio-isotope absorbed by the amount of ion present initially.

Chloride was also assayed chemically using the method of electrometric titration (144).

10. Volume change and measurement.

Volume changes of chloroplasts were examined using the absorbancy technique of Shibata et al (176)(177) based on earlier work (178). The light-scattering cross-sectional area of a particle, should increase

when that particle swells, thus decreasing the absorbancy. This process may be followed by measuring the optical density at a wavelength suffering no perceptible absorption by the chloroplast pigments (540 or 780 mμ.).

Packed volume measurements of chloroplasts were made by centrifuging chloroplast suspensions into calibrated capillary tubes using the M.S.E. minor bench centrifuge. The tubes were centrifuged for 15 minutes at 1500 x g.

11. Illumination of reaction mixtures.

Experiments were conducted with the chloroplasts contained in 2 or 5 ml. centrifuge tubes or in 25 ml. conical flasks which were immersed in a circular glass water bath. Illumination was provided by a circular bank of twelve tungsten lamps situated beneath the glass bath and intensity was varied by use of neutral filters or different power tungsten lamps. A relay thermometer and pump maintained temperature, to within 0.25°C, in the range 5 - 25°C, by circulating a cooling mixture through a copper coil in the water bath. This apparatus is a more sophisticated version of one described by Hill and Walker (165).

The centrifuge tubes or conical flasks were clipped to a perspex disc which rotates at about 50 r.p.m. in the water bath. The plane of rotation of the disc is inclined to prevent centrifugal sedimentation and to promote an even distribution of the chloroplasts.

Where it was found necessary to have different temperatures or light intensities in the same experiment, use was made of the constant temperature water bath described by Delieu and Walker (179).

This apparatus can provide four differing temperatures and eight differing light intensities simultaneously within a single unit. Here the centrifuge tubes or conical flasks were clipped at an angle to a perspex holder and shaken with a "to and fro" motion. Light intensities were measured with an "EEL" light meter (selenium cell).

When selected wavelengths of visible light were required, Baltzer narrow band ($\pm$10 mμ) interference filters were used in conjunction with a slide projector. The projector was adjusted to give a parallel beam of light incident upon the filters. Chloroplasts or leaves of Elodea were placed in the required solutions in tubes of 2 ml. volume or in flattened obovate bottles of 10 ml. capacity supported on perspex frames which held the filter(s). When only one wavelength was used the back of the experimental vessel was blacked out with aluminium foil. Samples were withdrawn through a syringe needle in a serum cap sealing the experimental vessel. The serum cap also carried lines for the entrance and exit of gas mixtures used.

Chloride uptake in Elodea was investigated using small conical flasks to hold the solutions and leaves. Illumination was from beneath the flasks with a 250-W spotlight. A water trough acted as a heat filter.

12. Labelling of detached pea leaves.

Pea leaves were detached and immersed in a feeding trough containing potassium chloride - 36 solution at a concentration of 10^{-3}M. The feeding trough consisted of a perspex square containing

a circular trough divided into 4 quadrants. Each compartment could hold about 5 - 6 leaves. The whole apparatus was illuminated from above by a 250-W spotlight. A water trough acted as a heat filter and a fan ensured an even temperature as well as aiding transpiration. The level of solution in each quadrant was maintained throughout the long feeding periods used. After feeding chloride - 36 the leaves were removed and prepared for non-aqueous separation of the chloroplasts.

RESULTS

(1) Chloride uptake in Elodea

Preliminary studies were conducted of the uptake of chloride-36 by detached leaves of Elodea. These experiments were directed towards investigating whether uptake was light-activated and the nature of the relationship to the partial reactions of photosynthesis and/or overall photosynthesis.

Leaves of Elodea show an uptake of chloride from potassium chloride solutions, that is light-activated. Using non-saturating white light, figure 10 illustrates that the leaves exhibited an initial rapid uptake followed by a slower continued uptake. The light/dark ratio of chloride absorbed is approximately 4 at the end of the 90 minute period, but higher ratios were obtained with higher light intensities.

The effect of light intensity upon chloride uptake was investigated for leaves of Elodea. The process was saturated at approximately 20,000 Lux. Higher intensities were inhibitory to uptake. At saturating light intensities the Km for chloride uptake was calculated to be 7×10^{-4}M potassium chloride and the value for the dark uptake 1.9×10^{-4}M. These values were derived, using the double reciprocal plot of the Lineweaver-Burke equation (200), from the rates of uptake between 20 and 30 minutes of time course studies using various concentrations of potassium chloride. The uptake rates were linear during the period 15-40 minutes and the assumption was made that passive uptake into the free space of the tissues was complete prior to this period. These Km values were used as a guide to selection of the chloride concentration in the experiments.

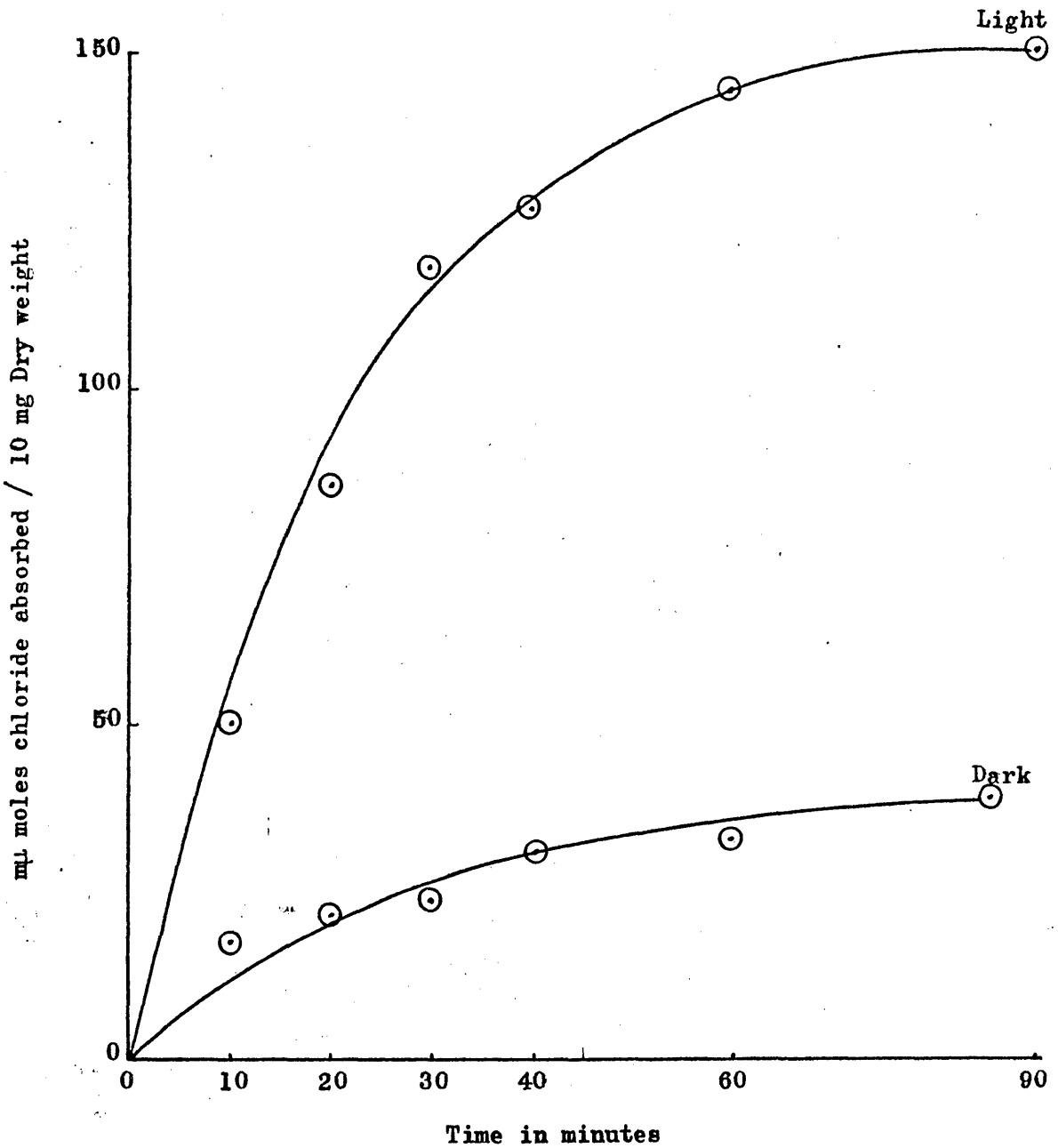
Figure 10 Time course of chloride uptake by Elodea leaves

40 leaves incubated in 10 ml. of 10^{-3} M KCl

Light intensity : 10,000 Lux

Temperature : 21 °C

Uptake measured by the change in the activity of the solution



The extent to which respiration might influence the observed chloride uptake that was light-activated is unknown. To investigate this point the effect of anaerobic conditions upon uptake was studied. Leaves were placed in distilled water in the dark and the suspension bubbled with CO_2-free nitrogen for one hour prior to the start of the experiment. After such pretreatment radioactive chloride was added and saturating illumination started while nitrogen was slowly bubbled through the illuminated and dark suspensions. A similar experiment to investigate the effect of CO_2 upon chloride uptake was done by bubbling CO_2-free air through the suspensions for 1 hour in the light prior to the start of the experiment. The results of four such experiments for each treatment are presented in table 1. Results are expressed as the average with the standard error of the mean.

Table 1 Effects of various gas mixtures upon chloride uptake in the light and dark

mμ moles chloride per 100 mg fresh weight

	AIR	AIR minus CO_2	Nitrogen
LIGHT	147 ± 10	196 ± 15	124 ± 6
DARK	33 ± 3	21 ± 8	26 ± 11

Light intensity: 20,000 Lux. Temperature: 23°C. Incubation time: 1 hour with 10^{-3}M potassium chloride-36.

Nitrogen thus inhibited the light uptake by 16% and the dark by 21%. By contrast in the light CO_2-free air bubbled through the experimental solutions resulted in a 33% stimulation to the uptake of chloride, but a 36% inhibition to the dark uptake.

The effects of selected wavelengths of visible light upon chloride uptake was examined using narrow waveband interference filters. Firstly, the light intensity curves of chloride uptake at three wavelengths (650, 707 and 725 mμ) were established. Uptake was plotted as a function of light intensity incident upon the filters. Using saturating light intensities the effects of wavelength are expressed in table 2, as ratios to the dark uptake observed.

Table 2 Effects of wavelength upon chloride uptake

Wavelength mμ	650	707	725
Experiment			
1	4.0	2.0	-
2	4.1	1.9	1.6
3	4.6	-	1.5
4	3.6	-	1.5

Light intensity: saturating. Temperature: 21-23°C.
Incubation time: 1 hour with 10^{-3}M potassium chloride.

The results show that at saturating intensities, light of 650 mμ is most effective in promoting chloride uptake. Uptake in light of 707 and 725 mμ was greater than in the dark.

Experiments to investigate possible "enhancement" of chloride uptake by giving 650 mμ light to leaves simultaneously illuminated with 707 or 725 mμ, were performed using non-saturating intensities of light at all three wavelengths. The results of four such experiments are shown, in table 3, as ratios to the dark uptake observed.

Table 3 Effects of wavelengths, given singly or simultaneously, upon chloride uptake

Wavelength mμ	650	707	725	650 + 707	650 +725
Experiment					
1	2.0	-	1.3	-	2.6
2	2.4	-	1.4	-	2.3
3	2.9	1.5	-	2.1	-
4	3.6	1.9	-	2.2	-

Light intensity: non-saturating. Temperature 21-23°C.

Incubation time: 1 hour with 10^{-3}M potassium chloride-36.

(By comparison with table 2, it can be seen that non-saturating light of 725 mμ promotes more chloride uptake by proportion to 650 mμ light. This reflects that the light intensity used at 650 mμ was approximately 50% saturating, whilst the intensity used at 725 mμ was approximately 80% saturating).

From the table it can be seen that enhancement was not observed.

The effects of 10^{-6}M DCMU on chloride uptake at 650 and 707 mμ were examined. Leaves were preincubated in white light for 30 minutes with DCMU (10^{-6}M) prior to incubation with radioactive chloride (and DCMU) using saturating intensities of the selected wavelengths. The results of three experiments are shown in table 4 as the average with the standard error of the mean.

Table 4. Effect of DCMU upon chloride uptake at various wavelengths

mμ moles chloride per 100 mg fresh weight

Wavelength mμ	650	707
-DCMU	119 ± 17	62 ± 31
+DCMU	14 ± 9	10 ± 2

Light intensity: saturating. Temperature: 21°C. Incubation time: 1 hour with 10^{-3}M potassium chloride-36.

Thus DCMU was inhibitory to chloride uptake at both wavelengths to approximately the same degree.

Simultaneous measurements of $C^{14}O_2$ fixation and chloride-36 uptake at two wavelengths showed that with saturating white light for chloride uptake, the ratio of carbon fixed to chloride absorbed (C^{14}/Cl^{36}) was approximately the same. For these experiments, the amount of radioactive-bicarbonate was less than 10^{-5}M and the uptake period was shortened to 30 minutes. Table 5 show the results of three experiments. The rate of CO_2-fixation was not the rate of maximum photosynthetic activity.

Table 5 C^{14}/Cl^{36} ratios at various wavelengths (and dark)

Wavelength mμ	650	707	Dark
Experiment			
1	14.5	14.5	11.7
2	14.8	15.4	11.2
3	16.7	16.3	12.0

Light intensity: 20,000 Lux. Temperature: 23°C. Incubation time: 30 minutes.

The effects of bicarbonate concentration upon chloride uptake were also investigated and it was found that concentrations greater than 10^{-5}M sodium bicarbonate inhibited the uptake of chloride in both the light and dark.

These last two sets of results are not strictly comparable with the previous ones since it was necessary to have buffers present.

The results described were obtained using summer plant material (April-October) and indicated that the photosynthetic light reactions were involved in the uptake of chloride by leaves of Elodea. Further it appeared that the carbon-fixing system of photosynthesis was in competition with the uptake mechanism. Using an intact tissue in this kind of study, it is difficult to separate the effects of respiration and of photosynthesis upon uptake. Since these preliminary experiments indicated that photosynthetic processes were involved in the absorption mechanism in the light, a study was begun of the response of isolated chloroplasts to chloride. Isolated plastids present a system in which the normal respiratory processes are absent (180).

Isolation of chloroplasts from leaves of Elodea proved to be difficult, partly because of the difficulty in obtaining sufficient material to give a good chloroplast yield - 100mg of leaf tissue (approximately 40 leaves) yielded less than 0.20mg of chlorophyll - and partly because of the fragility of the leaves. Further, the chloroplasts obtained proved to be inactive with respect to the Hill reaction and photophosphorylation. In view of this a study was made using chloroplasts isolated from pea seedlings.

(2) Chloride uptake by pea chloroplasts

Pea chloroplasts were first examined for their photosynthetic activities. The rates of cyclic (PMS) and non-cyclic (FeCy) photophosphorylation were assayed in a variety of buffers at pH 7.8. Cyclic rates with intact chloroplasts varied in the range 60-200 μ mole Pi esterified /mg chlorophyll/hour. Breakage of the plastids (with distilled water) resulted in much greater rates which could be as high as 650 μ mole Pi esterified /mg chlorophyll /hour. Non-cyclic photophosphorylation rates were very much lower than cyclic rates and were in the range 20-130 μ mole Pi esterified /mg chlorophyll /hour for intact organelles; for broken chloroplasts the rates were usually double the rate of the intact plastids. Basal electron flow rates were measured for both intact and broken chloroplasts and were in the ranges of 10-20 and 20-50 μ mole FeCy reduced /mg chlorophyll /hour respectively. All the photosynthetic rates quoted were obtained using non-saturating white light of approximately 10,000 Lux.

Having established that the isolated pea chloroplasts were active in photophosphorylation and Hill reaction, the chloride content of several preparations was assayed. Three preparations of chloroplasts yielded values of 10.3, 10.1, 9.8 ppm of chloride in suspensions containing 3 mg chlorophyll. Packed volume measurements indicated that a 1 mg chlorophyll suspension in 0.4M sucrose occupied 0.1 ml. If 50% of the packed volume is assumed to be internal volume and if all the chloride in the suspension were associated with this volume, then the chloride concentration was approximatedly 1.9×10^{-2}M.

It must be emphasised that the aqueous chloroplast preparations are

contaminated with cell wall and cytoplasm as well as mitochondria and nuclei. In an attempt to obtain purer chloroplast preparations and a better estimate of plastid chloride content, the non-aqueous method of chloroplast extraction was tried.

Detached pea seedling leaves were fed potassium chloride -36 at 10^{-3}M for 12 hours. The leaves were illuminated (10,000 Lux) with a forced current of air passing over them to aid transpiration. A reservoir of potassium chloride -36 maintained the level of solution in the feeding apparatus. A long feeding period was used to allow the specific activity of the chloride inside the plastids to reach that of the fed solution.

Chloroplasts obtained by the non-aqueous method were assayed for chloride-36 and chlorophyll contents and for fumarase activity. The chloroplasts contained no detectable furmarase activity, (although all the other fractions did have activity), and this was taken to indicate a very low level or complete absence of cytoplasmic contaminants with the chloroplasts. The chloride concentration in the isolated plastids was calculated on the basis that (1) the specific activity of the chloride-36 was the same as that fed and (2) the non-aqueous chloroplast volume per mg. chlorophyll was the same as that for aqueous chloroplasts. This calculation showed that the chloride content was in the range $2 - 2.4 \times 10^{-2}$M chloride. In view of the contaminants present in the aqueous chloroplast preparations, it is considered that the amount of chloride present in the purer non-aqueous preparation is significantly larger.

With such an apparently high concentration of chloride in the plastids, the possibility of not only absorption, but also exchange reactions must be considered.

Aqueous chloroplastswere prepared by isolation in 25 mM Tris-acetate buffer at pH 7 with 0.4M sucrose present and used at a final concentration of 0.10mg /0.5 ml. reaction mixture. Uptake or exchange was, in some 20% of the experiments, significant and could be up to 3% of the supplied radioactivity in the range 10^{-3}M - 5 x 10^{-3}M potassium chloride. Lower concentrations did not show uptake of chloride. The cause of the variation in uptake was not apparent since growth and isolation techniques were made as standard as possible. Table 6 illustrates the results of one experiment in which a light-induced change in the activity of the supernatant activity was observed.

Table 6 Effect of light upon supernatant activity

	Supernatant activity (cpm)	
Time in minutes	Light	Dark
0	4126 ± 43	4180 ± 51
10	4002 ± 21	4186 ± 44
Difference	124 ± 11	—

Light intensity: 15,000 Lux. Temperature: 10°C.
Concentration: 10^{-3}M potassium chloride.

The table shows that the fraction of activity absorbed in the light was 3% but no movement of chloride was seen in the dark.

The variation in chloride-36 movement into pea chloroplasts could

result from a differential injury to the bounding membrane of the plastids which might be impermeable to chloride. A comparison was therefore made of the behaviour of intact and broken chloroplasts towards chloride-36 solutions. Significant movement of chloride-36 was seen in only a few cases. The results, in table 7, of one experiment show that broken chloroplasts exhibited more chloride-36 uptake than intact plastids and further a dark uptake was apparent, although at 5 minutes, light and dark values are not significantly different ("t test").

Table 7 Effect of membrane integrity upon chloride-36 uptake

	Difference in supernatant activity (cpm)			
	WHOLE		BROKEN	
Time in minutes	LIGHT	DARK	LIGHT	DARK
2.5	0	0	88	47
5.0	96	0	164	136

Light intensity: 15,000 Lux. Temperature: $10^{\circ}C$.

Concentration: $10^{-3}M$ potassium chloride.

As such a small movement of chloride-36 was seen, the chlorophyll concentration was increased ten-fold in an attempt to increase the size of the movement. Experimental solutions were first centrifuged and then the supernatant (which still contained some chloroplasts) was filtered through a Millipore filter of pore size 1.2 μ to complete the separation of plastids and solution. The filtrate was collected and analysed for chloride-36 content. Using such a technique and incubating chloroplast suspensions containing 6 mg. chlorophyll per 3 ml. reaction mixture

with 10^{-3}M potassium chloride did not result in any significantly larger movement of chloride. Table 8 shows the results of one such experiment.

Table 8 Effect of increased chlorophyll concentration upon chloride-36 uptake

	Supernatant activity (cpm)			
Time in minutes	Light	Difference	Dark	Difference
0	4568 ± 31	0	4718 ± 22	0
10	4442 ± 23	126	4754 ± 25	0
20	4439 ± 48	129	4743 ± 37	0

Light intensity: 25,000 Lux. Temperature: 15°C.

In an attempt to stabilise the behaviour of the chloroplasts and to increase the size of the chloride movement observed with whole chloroplasts in the light, the effects of various additives to the reaction mixtures were studied. These incubations are summarised in table 9.

Table 9

Additive	Concentration
Cyclic phosphorylation system	As described in
Non-cyclic phosphorylation system	experimental
ATP-ase system	methods
ATP	1 mM
* PMS	3×10^{-7}M
$CaSO_4$	10^{-4}M
$MgSO_4$	10^{-4}M
Ascorbic acid	10^{-4}M
$MgSO_4$ and Ascorbic acid	10^{-4}M

Of all the additives listed in the table, only ATP was effective in promoting a chloride movement. This effect was seen in both the light and the dark. The incubation marked (*) sometimes showed an increase in the activity of the supernatant i.e. the external solution was concentrated by the chloroplasts to a significant degree (> 100 cpm). This effect was also noted with some incubations containing just sucrose and potassium chloride-36.

Since water influx into the chloroplast apparently occurred without concomitant chloride absorption the effect of osmolarity upon chloride absorption was tested. Aliquots of the chloroplast suspension were incubated in the light and dark for 10 minutes in various molarities of sucrose with 10^{-3}M potassium chloride-36 present. The result of one set of such incubations is shown in table 10.

Table 10 Effect of sucrose molarity upon chloride-36 movement

	% change in supernatant activity			
Sucrose molarity	0.1	0.2	0.4	0.8
Light	+5.0	+2.1	-2.5	0
Dark	+1.3	+0.6	0	0

Light intensity: 15,000 Lux. Temperature: 10^{o}C.

The activity of the supernatant was thus increased at molarities less than 0.4M sucrose, and this effect was more pronounced in the light than in the dark. At 0.8M, where dilution of the external solution (by water from the chloroplasts) should result in a lowering of activity, no change was observed.

Addition of ATP can result in plastid shrinkage (128) and the extruded water could thus dilute the activity of the supernatant. The effect of ATP concentration was therefore examined and the results are shown in table 11.

Table 11 Effect of ATP concentration on chloride-36 movement

		Difference in supernatant activity (cpm)			
ATP concentration (mM)		0	1	2	4
Light	10 min.	104 ± 12	99 ± 16	44 ± 7	114 ± 8
	25 min.	93 ± 14	154 ± 21	102 ± 9	164 ± 22
Dark	10 min.	0	171 ± 25	124 ± 11	200 ± 9
	25 min.	0	77 ± 7	122 ± 16	82 ± 9

Light intensity: 10,000 Lux. Temperature: 10°C.

Concentration: 10^{-3}M potassium chloride.

The results indicate that after 10 minutes, the effect of ATP is greatest in the dark whereas in the light the maximal effect is not observed until 25 minutes. Whether these results (and the others found by supernatant anlysis) represent an uptake of chloride-36 into the chloroplasts or a dilution of the external solution by water extruded from the chloroplasts when they shrink, cannot be decided unless (1) the pellets are analysed for chloride-36 content or (2) the magnitude of water movement is known.

Packed volume measurements of chloroplasts suspended in 0.4M sucrose indicated 1 mg chlorophyll was contained within 0.1 ml. In the experiments described so far, 0.1 mg suspension per 0.5 ml. reaction mixture was used, and these had a packed volume of 0.01 ml. If 50% of the packed volume were internal volume then at complete shrinkage 0.005 ml. water would be extruded from the chloroplasts into the solution. Since 0.5 ml. of reaction mixture contained 0.1 mg chlorophyll, the maximum dilution would be 1% or 40 cpm per 4000 cpm present in the 0.1 ml. samples used for measuring supernatant activities. Thus the dilution of the supernatant due to

water loss by the chloroplasts may contribute a little to the observed uptake measured by supernatant analysis, but it cannot account for all the uptake.

Since the supernatant analysis method tends to overestimate the size of the chloride uptake, attempts were made to measure the activities of the chloroplast pellets obtained on centrifugation of the reaction mixtures. In analysing the pellets for activity a correction must be made for the amount of supernatant that is trapped between the particles in the pellet.

Chloroplasts were isolated and aliquots (0.9 ml.) of the suspension equivalent to 0.250 mg chlorophyll were added to 0.1 ml. potassium chloride-36 (in 0.4 M sucrose) and either (1) centrifuged immediately or (2) layered on top of 1 ml. of 0.4 M sucrose and then centrifuged. After centrifugation with either method the supernatant was drawn off with a Pasteur pipette and the tube rinsed three times with 0.4 M sucrose, avoiding as far as possible disturbance of the pellet. Finally the tubes were blotted dry with lens tissue and the pellets resuspended with 80% acetone. Aliquots of the acetone suspension were then removed for chlorophyll and chloride estimations. Comparison of the two methods of obtaining pellets is shown in table 12.

Table 12 Comparison of methods in obtaining pellets from experimental solutions

Method		Pellet cpm	Pellet chlorophyll mg	cpm/mg
(1)	i	62 ± 5	0.190	326
	ii	65 ± 3	0.192	339
(2)	i	16 ± 2	0.236	67
	ii	14 ± 1	0.236	61

The results indicate that centrifugation through a layer of sucrose substantially lowers the amount of chloride-36 in the pellet and further gives a higher yield of chloroplasts (as judged by the chlorophyll content fo the pellet). This method was then used to study the time course of chloride-36 absorption.

Chloroplasts suspended in 0.4 M sucrose were incubated with potassium chloride-36 at a final concentration of 10^{-3}M and the chloroplast pellets obtained on centrifugation were assayed for chlorophyll and chloride-36 content. The results of one such experiment are shown in table 13.

Table 13 Time course of chloride-36 uptake using pellet analysis

	Incubation time (mins.)	Pellet cpm	Pellet chlorophyll mg	cpm/mg
Light	0	13 ± 1	0.200	65
	5	16 ± 2	0.248	65
	10	14 ± 5	0.200	70
	15	15 ± 1	0.228	66
Dark	0	12 ± 4	0.198	63
	5	17 ± 2	0.232	73
	10	15 ± 2	0.210	70
	15	12 ± 3	0.210	59

Light intensity: 15,000 Lux. Temperature: 10°C.

Whilst the method gave reproducible ratios it failed to detect any chloride-36 movements into the chloroplasts. The discrepancy between the results obtained by supernatant and pellet analyses may be explained on the basis that centrifugation of the plastids through a layer of sucrose may in fact lead to the "leaching out" of chloride (and other ions) as noted

by Stocking and Ongun (148) using spinach chloroplasts. Accordingly the centrifugation technique was abandoned and the faster technique of membrane filtration was tried.

Millipore filters, RAWP, of pore size 1.2 μ were used to separate the plastids from the experimental solutions and the chloride-36 content was determined. Chloroplasts, isolated in 25 mM Tricine - NaOH buffer pH 7 with 0.35 M sucrose present, were resuspended in 0.35 M sucrose and incubated at a chlorophyll concentration of 0.1 mg. per 2 ml. with 5×10^{-3} M potassium chloride-36. The larger volume and lower sucrose molarity were found to give a faster filtration. Samples of 2 ml. volume were filtered. Table 14 shows the result of such an incubation carried out in the light and dark.

Table 14 Time course of chloride-36 uptake measured by filtration technique

	Chloride-36 activity (cpm)			
Time (seconds)	Light	(Light-Blank)	Dark	(Dark-Blank)
10	5084 ± 52	158	5066 ± 39	140
60	5229 ± 41	303	—	—
120	5691 ± 17	765	5262 ± 23	336
300	5101 ± 22	175	5501 ± 28	575

The table shows that both light and dark chloride movements are present. The direction of the light-stimulated uptake is reversed after 2 minutes illumination whereas uptake in the dark continues throughout the incubation.

Further experiments using the Millipore technique indicated that the pattern of initial net influx followed by net efflux was variable. The time period for net influx of chloride often being as short as 10 seconds as indicated in figure 11. Although chloride movements were not always seen with every preparation, this type of movement was seen with the majority of "successful" preparations and was investigated further.

Chloroplasts were incubated in the dark with 10^{-2}M potassium chloride-36 for 5 minutes and then subjected to illumination (10,000 Lux) for a further 5 minutes. Figure 12 shows the results of such an experiment and indicates that the dark net influx of chloride was apparent for 6 minutes and then a slow net efflux started. In the light net influx increased sharply for 1 minute and then a very rapid net efflux occurred.

A further experiment in which a dark-light-dark treatment was given to the plastids with chloride-36 present throughout is shown in figure 13. This preparation of chloroplasts showed a small light-activated net influx of chloride which was not effluxed until a period of 90 seconds dark treatment following illumination had elapsed.

The complexity of chloride movements seen in both the light and dark indicated that the relatively slow methods of centrifugation and filtration are not suitable to measure in detail the size and extent of chloride movements. Continuous monitoring of the external solution with chloride-sensitive electrodes would appear to offer the better method of analysis. Some explanation of these net movements of chloride may be found in the similar type of kinetics of proton and cation movements as described in relation to the chemi-osmotic theory of Mitchell. This is discussed later.

Figure 11 Time course of chloride uptake by chloroplasts

Plastids (0.1 mg chlorophyll in 2 ml.)
incubated with 10^{-2} M KCl
Light intensity : 10,000 Lux
Temperature : 10^{0}C
Uptake measured by filtering 2 ml. samples and counting the filters for chloride-36 activity

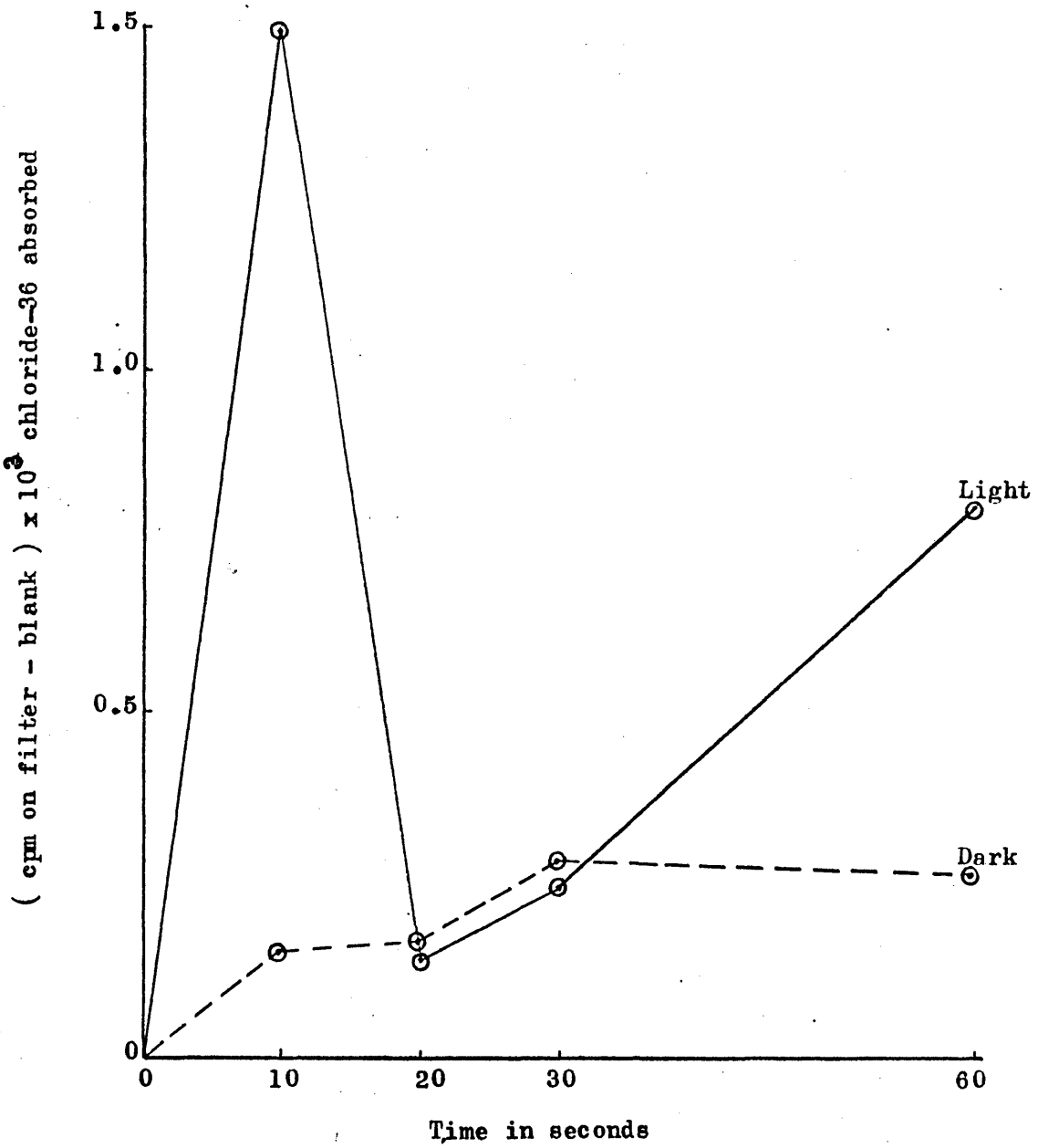


Figure 12

Effect of subsequent illumination on plastids pre-incubated with KCl in the dark

Plastids (0.1 mg chlorophyll in 2 ml.)
incubated with 10^{-2} M KCl
Light intensity : 10,000 Lux
Temperature : 10^{o}C
Uptake measured by filtering 2 ml. samples and counting the filters for chloride-36 activity

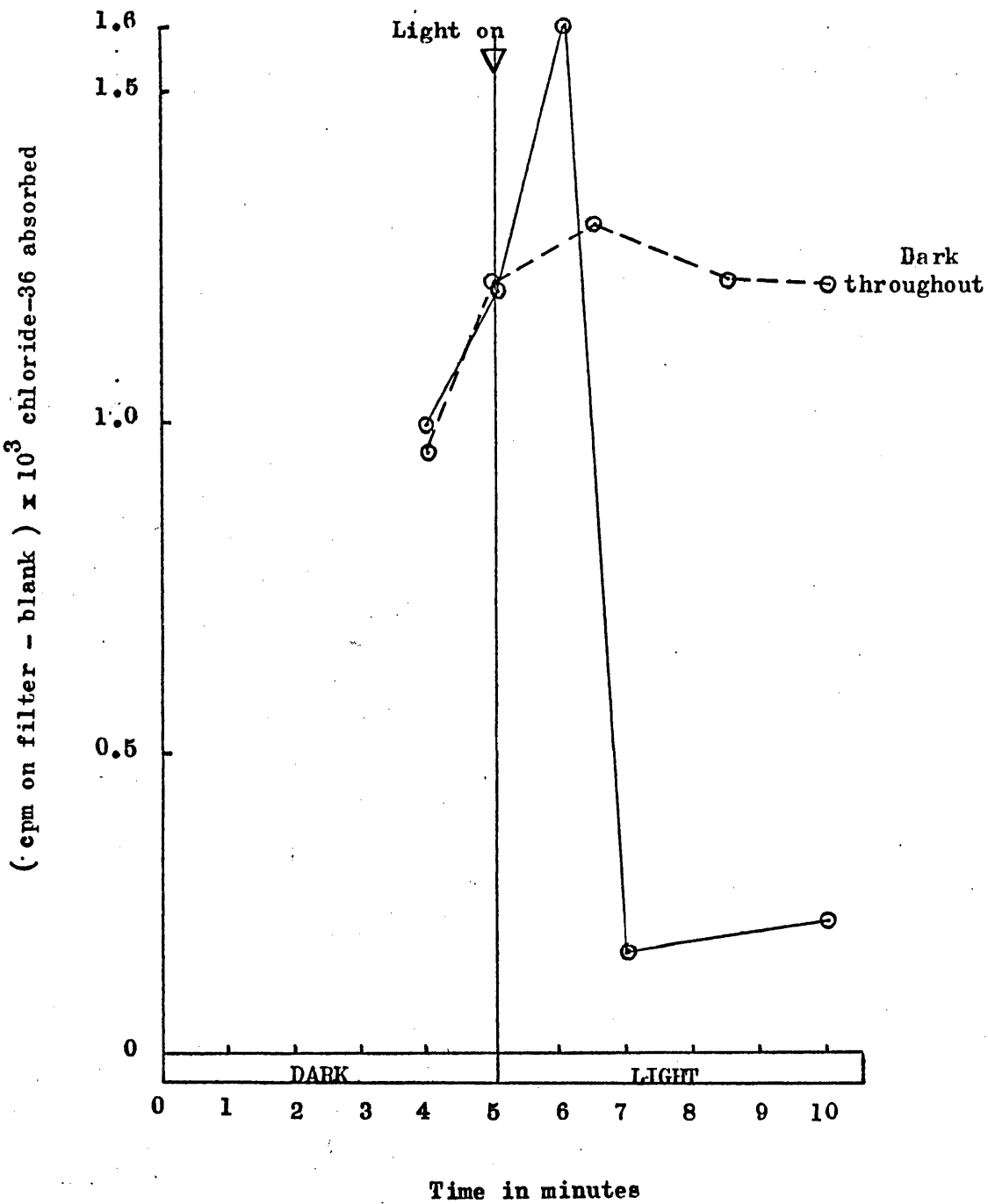
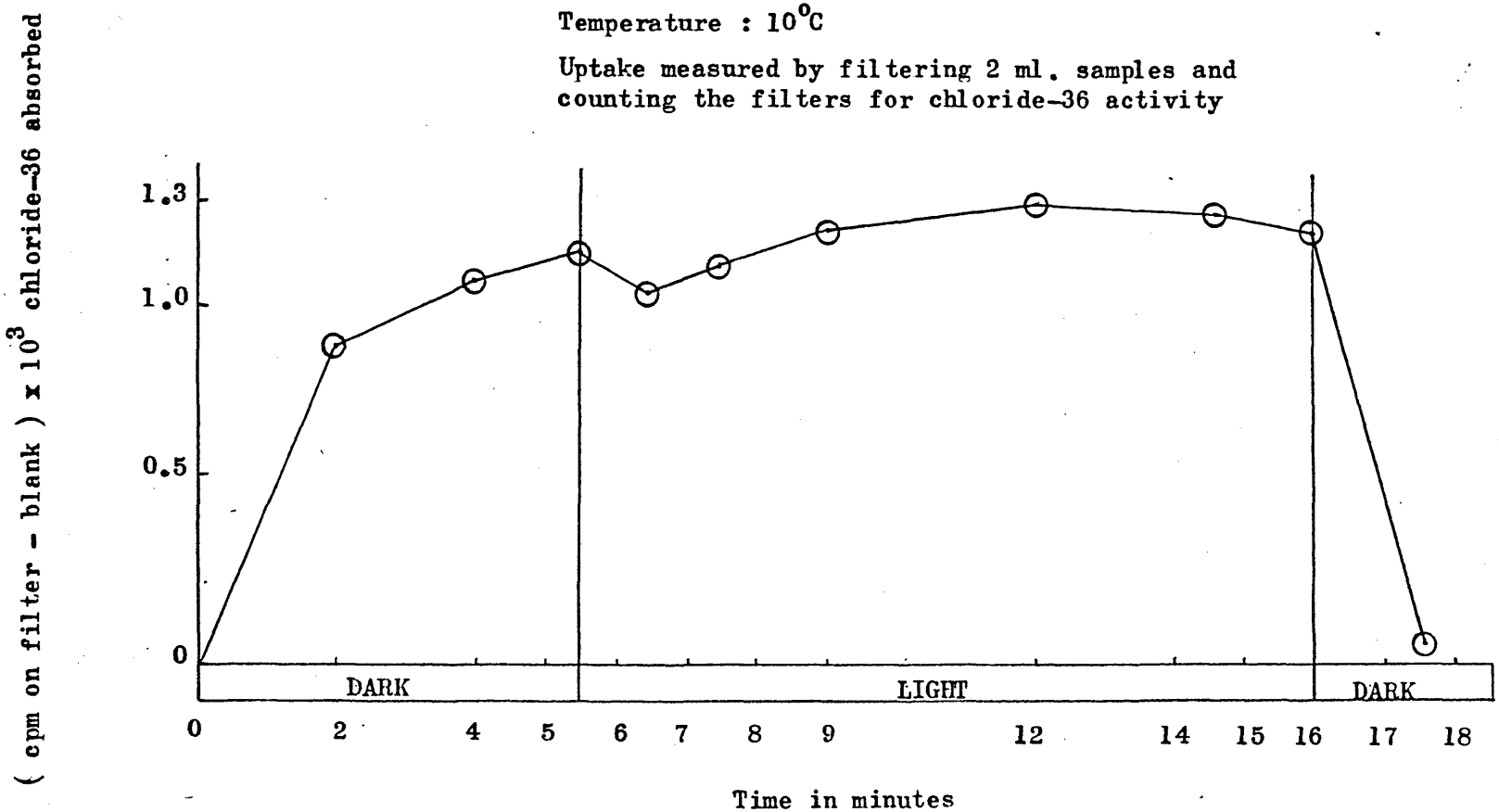


Figure 13

Effect of dark to light to dark transitions on chloride uptake by chloroplasts

Plastids (0.1 mg chlorophyll in 2 ml.)
incubated with 10^{-2} M KCl
Light intensity : 10,000 Lux
Temperature : 10^{0}C
Uptake measured by filtering 2 ml. samples and counting the filters for chloride-36 activity



In view of these experimental difficulties, further study of chloride movements in chloroplasts was abandoned. Whilst the results presented are probably significant, their magnitude makes a detailed analysis impossible due to the lack of sensitivity in the estimation of chloride. The movement of calcium-45 into chloroplasts was studied as this cation could be assayed with a good degree of sensitivity, due to its greater specific activity compared to chloride-36 (approximately 800 μc/gm for chloride and 3 μc/μg for calcium).

Calcium uptake

Calcium uptake was investigated using the Millipore filtration technique. Except as indicated otherwise the chloroplasts were isolated in Tricine-chloride media pH 7 and resuspended in Tricine-sucrose media pH 8. Sample volume was 2.5 ml. and this contained 0.120mg chlorophyll with 2.5 μc of carrier-free calcium-45. The light intensity used was 1,500 Lux and the incubation temperature was 15°C. After deposition, the chloroplasts on the filter were washed with 2.5 ml. of 0.35M sodium chloride.

(1) General features of calcium uptake

A time course study for calcium uptake, in which light and dark treatments were compared, is shown in figure 14. It is seen that uptake is approximately linear in the light for 3 minutes and in the dark for 4 minutes.

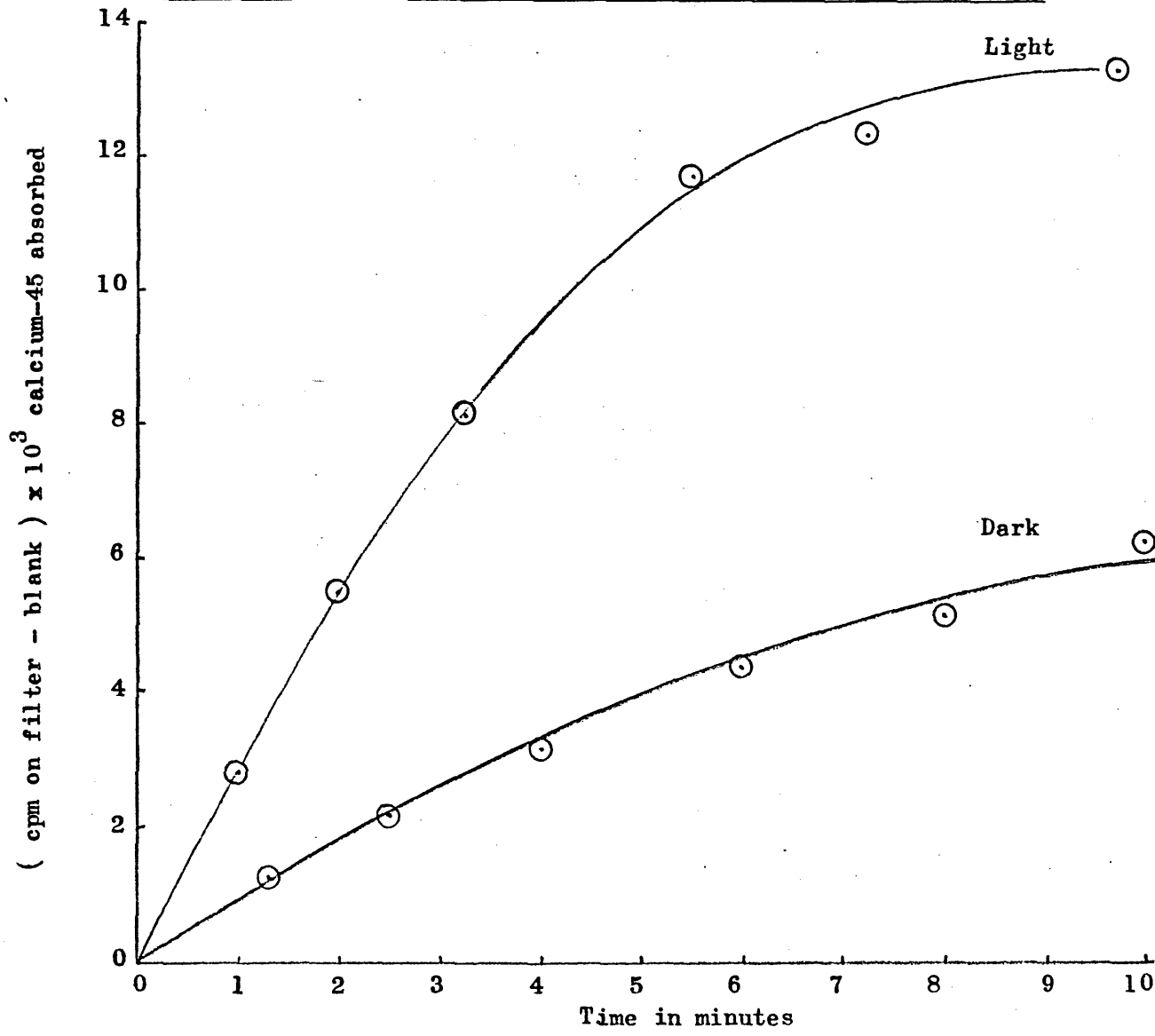
Uptake periods were limited to 2 minutes (except as indicated) from which rates were calculated. Table 15 shows the results of 3 replicates, using this short uptake period, to illustrate the effect of light upon calcium uptake.

Table 15 Effect of light upon calcium uptake

	cpm on filter - blank	
Replicate	Light	Dark
1	6979 ± 381	3821 ± 149
2	7095 ± 276	3729 ± 241
3	7122 ± 310	3733 ± 207

The table shows that the light-stimulated uptake is almost double

Figure 14 Time course of calcium uptake by chloroplasts



Plastids (0.120 mg chloro-phyll in 2.5 ml.) incubated with 2.5 μc calcium-45

Light intensity : 1500 Lux

Temperature : 15°C

Uptake measured by filtering 2.5 ml. samples, washing and counting the filters for calcium-45 activity

that seen in the dark. Further the results demonstrate that the filtration technique is capable of giving reproducible results to within 5%.

The influence of pH was determined for the uptake of calcium in the light and dark in the range pH6-9 . For these determinations, clorophyll concentration was decreased to 0.06mg/2.5 ml. sample to minimise any buffering capacity of the plastids themselves. Figure 15 shows that the pH optima for calcium uptake in the light and dark were distinctly different and the values were pH 8.5 for the light and pH 8.0 for the dark.

Since there were two distinct pH optima it was necessary to choose a single pH value at which to conduct experiments. If the dark uptake continues unabated in the light it is necessary to subtract the dark uptake value from the light uptake in order to calculate the effects of light upon uptake. Figure 15 shows that for the dark uptake the effect of pH is least in the range pH 7.5 to 8, further these initial experiments indicated that sufficient calcium was accumulated in the light atpH 8 to show a difference to the dark uptake. Hence all experiments were conducted at pH 8 (except as indicated otherwise).

Figure 16 illustrates the relationship between calcium concentration and the rates of calcium uptake in the light and dark. The uptake in the light is saturated between 1 and 2.5 mM with a K_m value of 5×10^{-4}M. The uptake in the dark was saturated between 1 and 2 mM with a K_m value of 1.2×10^{-4}M.

As the greater percentage of calcium removed occurred at lower concentrations of calcium, most experiments with carrier calcium present were done at a concentration of 0.1 mM calcium chloride. In the highest

Figure 15 Effect of pH on calcium uptake by chloroplasts

Plastids (0.06 mg chlorophyll in 2.5 ml.) incubated with 2.5 μc calcium-45

Light intensity : 1500 Lux

Temperature : 15°C

Uptake measured by filtering 2.5 ml. samples, washing and counting the filters for calcium-45 activity

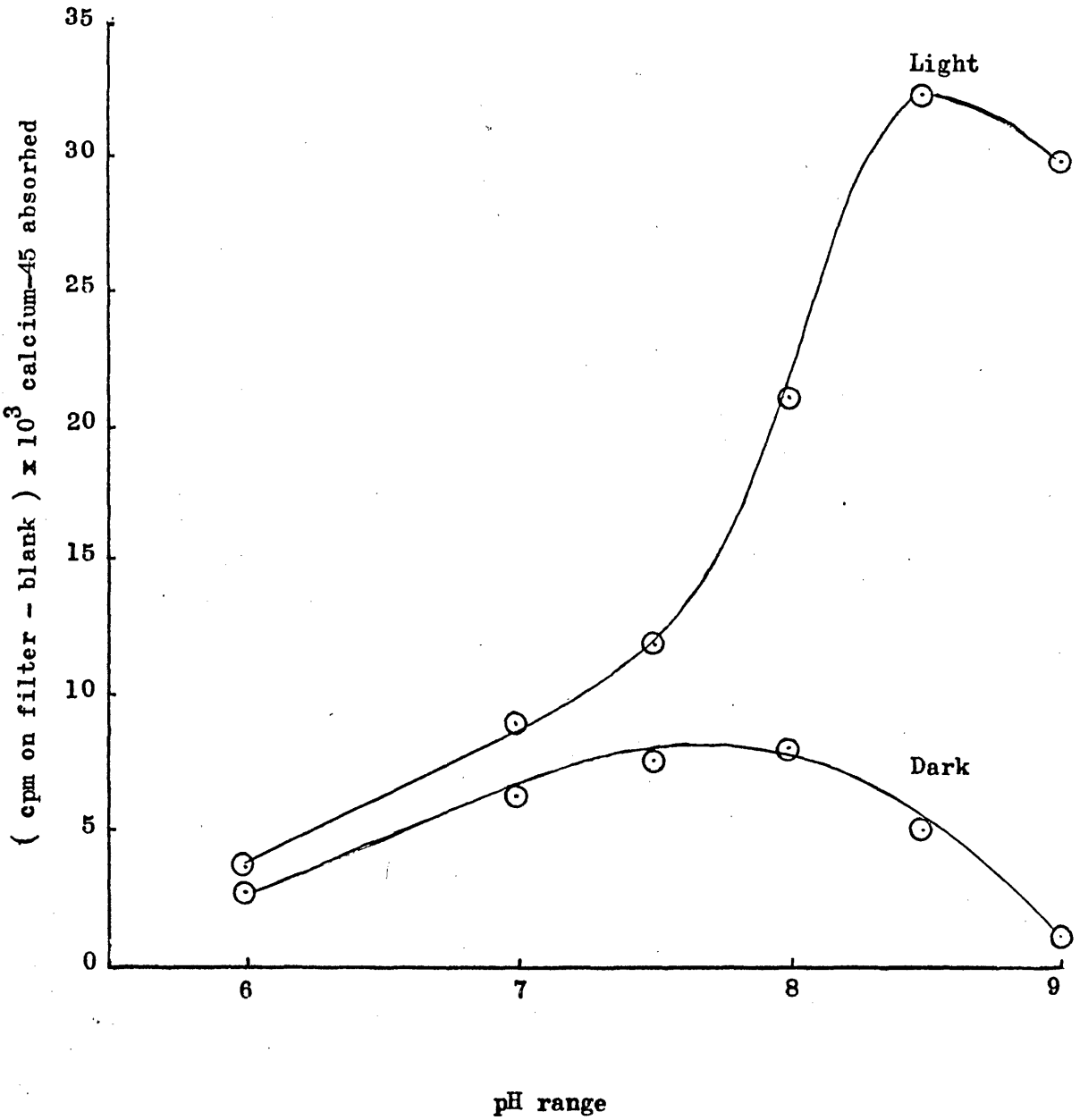


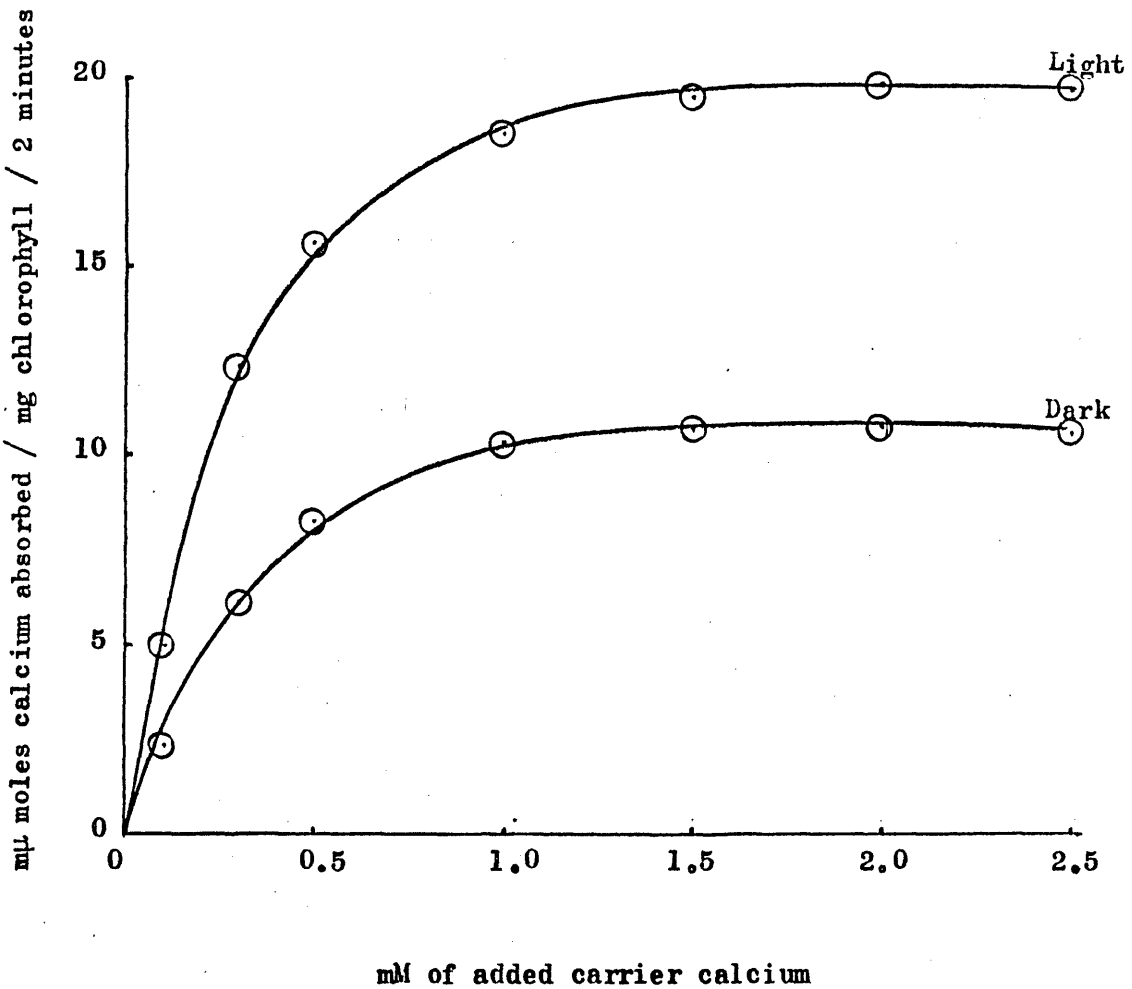
Figure 16 Calcium uptake by plastids as a function of calcium concentration

Plastids (0.120 mg chlorophyll in 2.5 ml.) incubated with 2.5 μc calcium-45 and the indicated amounts of carrier calcium

Light intensity : 1500 Lux

Temperature : 15°C

Uptake measured by filtering 2.5 ml. samples, washing and counting the filters for calcium-45 activity



uptakes observed some 4% of the calcium-45 supplied was removed from the incubation medium, but in most experiments the uptake was about 1% for a 2 minutes incubation in the light.

The effects of white light on calcium uptake were initially investigated using the apparatus, described by Delieu and Walker (179) capable of providing 8 different intensities. However, when it became apparent that uptake was saturated at low light intensity, the problem of stray light was solved by using black nylon netting as a neutral filter. Light intensity was then varied by wrapping differing numbers of layers of the netting around the centrifuge tubes used to contain the experimental solutions

Figure 17 shows the relation between calcium uptake rate and light intensity. Uptake was light saturated between 200 and 400 Lux.

The effects of selected wavelengths of visible light upon calcium uptake was examined using narrow waveband interference filters. The results shown in table 16 indicate that 650 mμ light was more effective in promoting calcium uptake then 725 mμ light. Enhancement was not observed.

Table 16 Effects of wavelength upon calcium uptake

	cpm on filter - blank			
Wavelengthmμ	650	725	(650 + 725)	Dark
Experiment				
1	22.939	17.613	22.710	11.775
2	20.624	15.428	21.125	11.238

Temperature: 20°C. Incubation time: 7 minutes

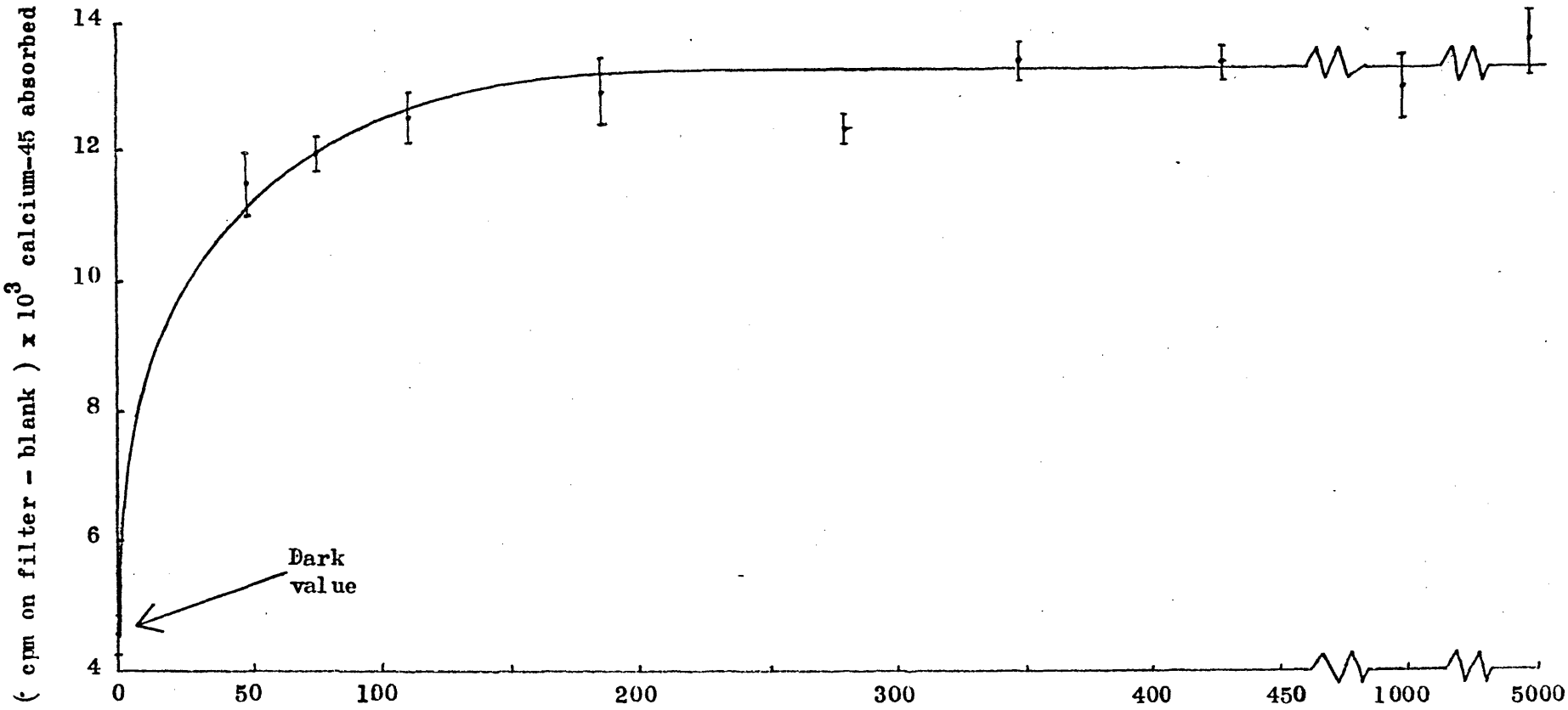
In several of the experiments recorded, it was noted that replicates of a treatment did not always agree to within 5% of each other. This

Figure 17 Effect of light intensity on calcium uptake by plastids

Plastids (0.120 mg chlorophyll in 2.5 ml.) incubated with 2.5 μc calcium-45 and 10^{-4} M carrier calcium

Light intensity varied by the use of neutral filters Temperature : 15°C

Uptake measured by filtering 2.5 ml. samples, washing and counting the filters for calcium-45 activity



effect was particularly noticeable when the replicates were done more than 15 minutes apart. So the decay of calcium uptake capacity on storage was investigated at two temperatures. Figure 18 shows that on storage of the chloroplasts in the dark at 15°C the light-activated uptake disappeared within 1 hour whereas chloroplasts stored in the dark at 1°C still retained over 85% of the light-activated uptake after 1 hour. The dark calcium uptake was also subject to decay. After 1 hour storage at 15°C, uptake was 50% of the original activity, whereas after one hour's storage at 1°C the plastids retained almost 90% of their original activity.

Since the calcium uptake in both light and dark was so labile, this strongly suggested that an enzymatic system was responsible for calcium movement. Accordingly the effect of temperature upon calcium uptake was studied and the temperature coefficients (Q_{10}) were calculated. These are shown in table 17.

Table 17 Q_{10} ranges for calcium uptake

Temperature range °C	Light	Dark	(Light-Dark)
5 - 15	1.6 - 2.1	1.6 - 1.9	1.5 - 2.0
15 - 25	1.4 - 2.3	1.1 - 1.7	1.7 - 2.8

Incubation time: 45 seconds.

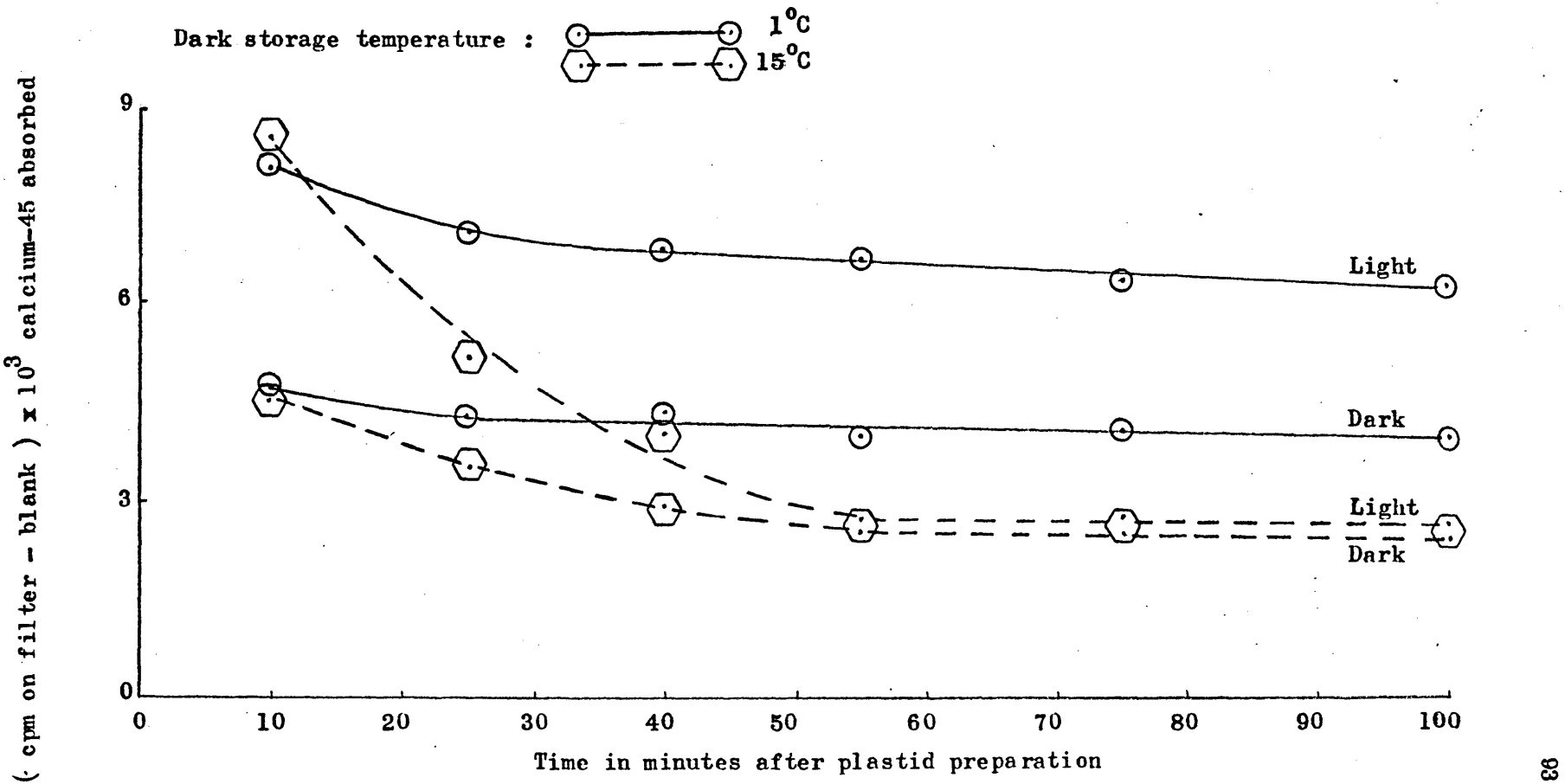
In the temperature range 5 - 15°C the temperature coefficients for the uptake categories listed do not appear to be significantly different. In the higher range however the temperature coefficients for the light and (light minus dark) uptakes were higher than the dark uptake.

In order to characterise the calcium uptake system further, the specificity of the mechanism was examined. Incubations were performed

Figure 18 Decay of calcium uptake capacity by plastids on storage

Plastids (0.120 mg chlorophyll in 2.5 ml.) stored in the dark as indicated. 2.5 ml. aliquots removed and quickly brought to 15°C and incubated with 2.5 μc calcium-45 and 10^{-4} M carrier calcium

Light intensity : 1500 Lux Temperature : 15°C



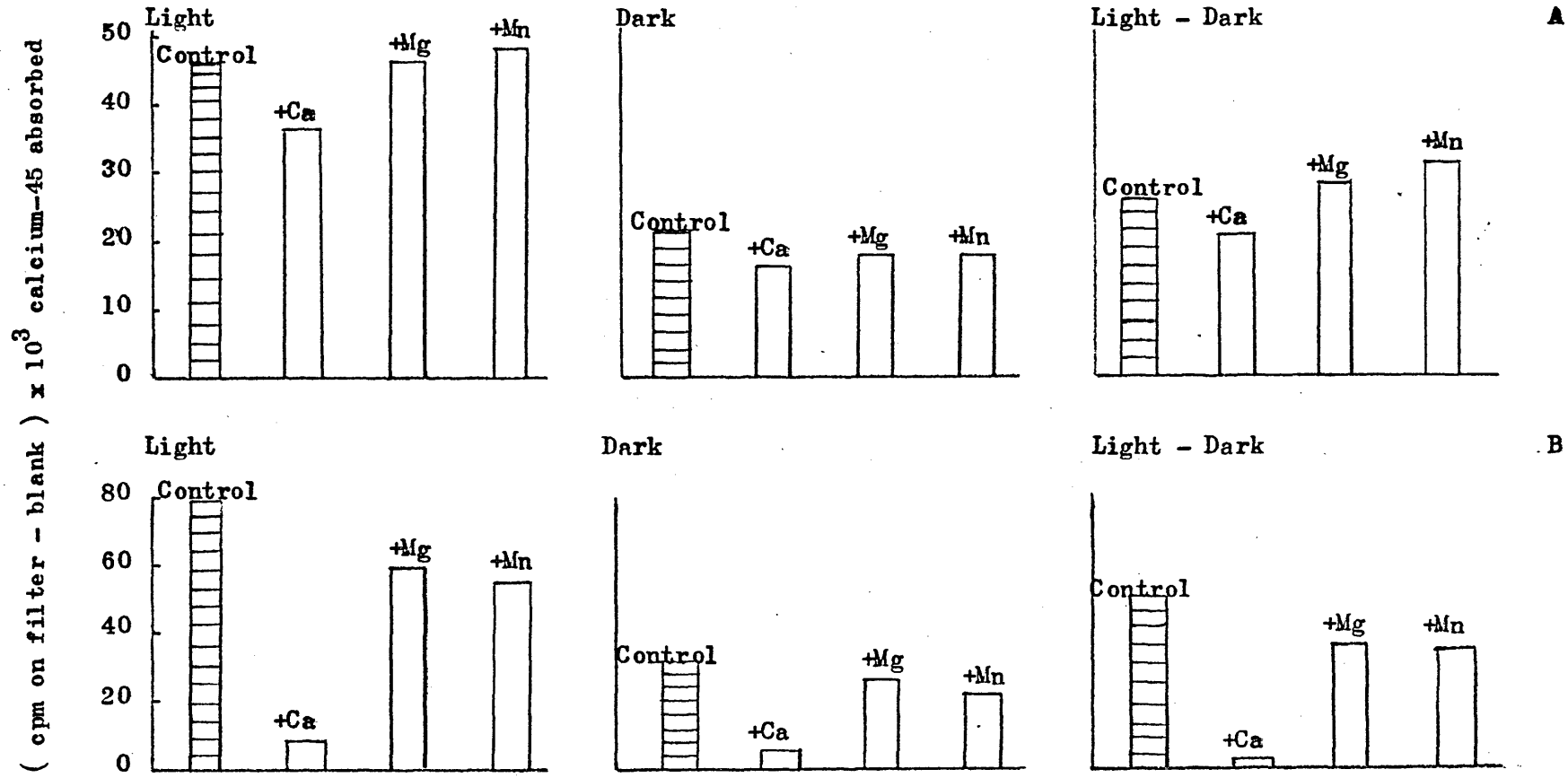
in which calcium-45 was diluted with cold calcium chloride, or with other cations (as the chlorides) at equivalent concentrations. The results of two experiments at two concentrations are illustrated in figure 19. At 5 x 10^{-5}M concentrations, neither magnesium or manganese showed any competitive effects with respect to calcium uptake (as measured by calcium-45) in the light or dark, a slight stimulation being observed for the light uptake. At 1mM concentrations however, magnesium and manganese are depressants to the light and dark uptakes of calcium-45 though not to the same extent as calcium itself.

(2) Metabolic aspects of calcium uptake

The results described in the previous section showed that light accelerated calcium uptake. In an attempt to define this effect more clearly, the effects of certain photosynthetic systems, inhibitors and treatments were investigated. The results of these experiments are presented in this section. Conditions were as described in section (1) except 10^{-4}M calcium chloride was added as carrier to the calcium-45. Carrier was added to the calcium-45 as other additions were given to the experimental solutions and these may have contained trace amounts of calcium thereby diluting the calcium-45 and thus masking the true effect of the addition.

The effects of an added cyclic photophosphorylative system upon calcium uptake were studied. Table 18 shows that such an added system completely abolished the light-activated uptake and further decreased by over 50% the dark calcium uptake. (A similar result was found when a non-cyclic photophosphorylative system was added). The effects of

Figure 19 Calcium uptake in the presence of other divalent cations



Plastids (0.120 mg chlorophyll in 2.5 ml.) incubated for 5 minutes with 2.5 μc calcium-45 alone (control) or with isotope and 5×10^{-5}M (A) or 1mM (B) of the indicated divalent cations
Light intensity : 1500 Lux Temperature : 15°C
Uptake measured by filtering 2.5 ml. samples, washing and counting the filters for calcium-45 activity

the added systems were thus over and above any effect due to photosynthetic activity.

Table 18 Effect of a cyclic photophosphorylative system upon calcium uptake

	cpm on filter - blank	
	Cyclic system	Control
LIGHT 1	3238	15,862
2	3412	15,419
DARK 1	3454	7,350
2	3079	7,622
(LIGHT-DARK)	58	8,158

Incubation time: 4 minutes

With the same preparation of chloroplasts as used in the last experiment, the uncoupling action of calcium upon photophosphorylation was investigated. It was found that calcium concentrations up to 3×10^{-4}M had no effect upon the initial rates (up to 8 minutes) of cyclic photophosphorylation, although a marked inhibition was seen after this period. Concentrations of 5×10^{-4}M and 10^{-3}M calcium chloride inhibited the initial rates of photophosphorylation by 46% and 80% respectively.

The effect of ADP upon calcium uptake was investigated. Table 19 shows that ADP inhibited completely the light-activated uptake and depressed it below the dark incubation with ADP which itself was inhibited by almost 20%.

Table 19 Effect of 1mM ADP upon calcium uptake

	cpm on filter - blank	
	+ ADP	Control
LIGHT 1	1720	7450
2	1691	7511
DARK 1	3031	3742
2	2938	3701

Incubation time: 2 minutes

As the literature contains several reports of a calcium uptake mediated by a light-triggered ATP-ase (155)(159), the ATP-ase activity of these chloroplasts was assayed. Negligible activity was detected, the highest rate of ATP-hydrolysis being 0.2 μ mole Pi liberated /mg chlorophyll /hour.

Incubation of the chloroplasts with an added ATP-ase system did not enhance calcium uptake in the light or dark. As seen in table 20, the added ATP-ase system was 62% inhibitory to the light uptake. Further the uptake in the light with the ATP-ase system present was decreased virtually to the dark uptake with the system present.

Table 20 Effect of an added ATP-ase system on calcium uptake

	cpm on filter - blank	
	+ ATP-ase	Control
LIGHT	2783	11,004
DARK	2495	6,523
(LIGHT-DARK)	288	3,481

Incubation time: 4 minutes.

The effect of ATP concentration upon calcium uptake was studied. Table 21 shows that in some instances low ATP concentrations were capable of slightly stimulating uptake. Higher concentrations however were inhibitory. The results also show that ATP like ADP was more inhibitory to the uptake in the light than in the dark.

Table 21 Effect of ATP upon calcium uptake

ATP concentration (μM)	0	50	100	200	1000
LIGHT 1	100%	110%	93%	-	40%
2	100%	98%	-	71%	41%
DARK	100%	108%	-	82%	46%

Chloroplasts isolated and resuspended in Tricine-sucrose pH 8. Incubation time: 5 minutes. 0.120 mg chlorophyll /2.5 ml. with 2.5 μc calcium-45 and 10^{-4}M calcium chloride.

In an attempt to demonstrate the persistence of the effect created by light upon calcium uptake i.e. the uptake mechanism was light-triggered and not light dependent, a series of incubations were carried out in which transitions from light to dark and vice versa were made. One such experiment is illustrated in figure 20 and shows that the effect of light did not apparently continue when the reaction mixture was transferred from light to dark. Further in the period 10-13 minutes transfer from light to dark resulted in a decrease of the calcium activity of the chloroplasts. Using a different order of illumination figure 21 shows the effect of pre-illumination, in the absence of calcium, upon the uptake of calcium in a succeeding dark period with calcium present. The reaction mixture

Figure 20 **Effect of interrupted illumination on calcium uptake by chloroplasts**

Plastids (0.120 mg chlorophyll in 2.5 ml.) incubated with 2.5 µc calcium-45 and 10^{-4} M carrier calcium

Light intensity : 1500 Lux

Temperature : 15°C

Uptake measured by filtering 2.5 ml. samples, washing and counting the filters for calcium-45 activity

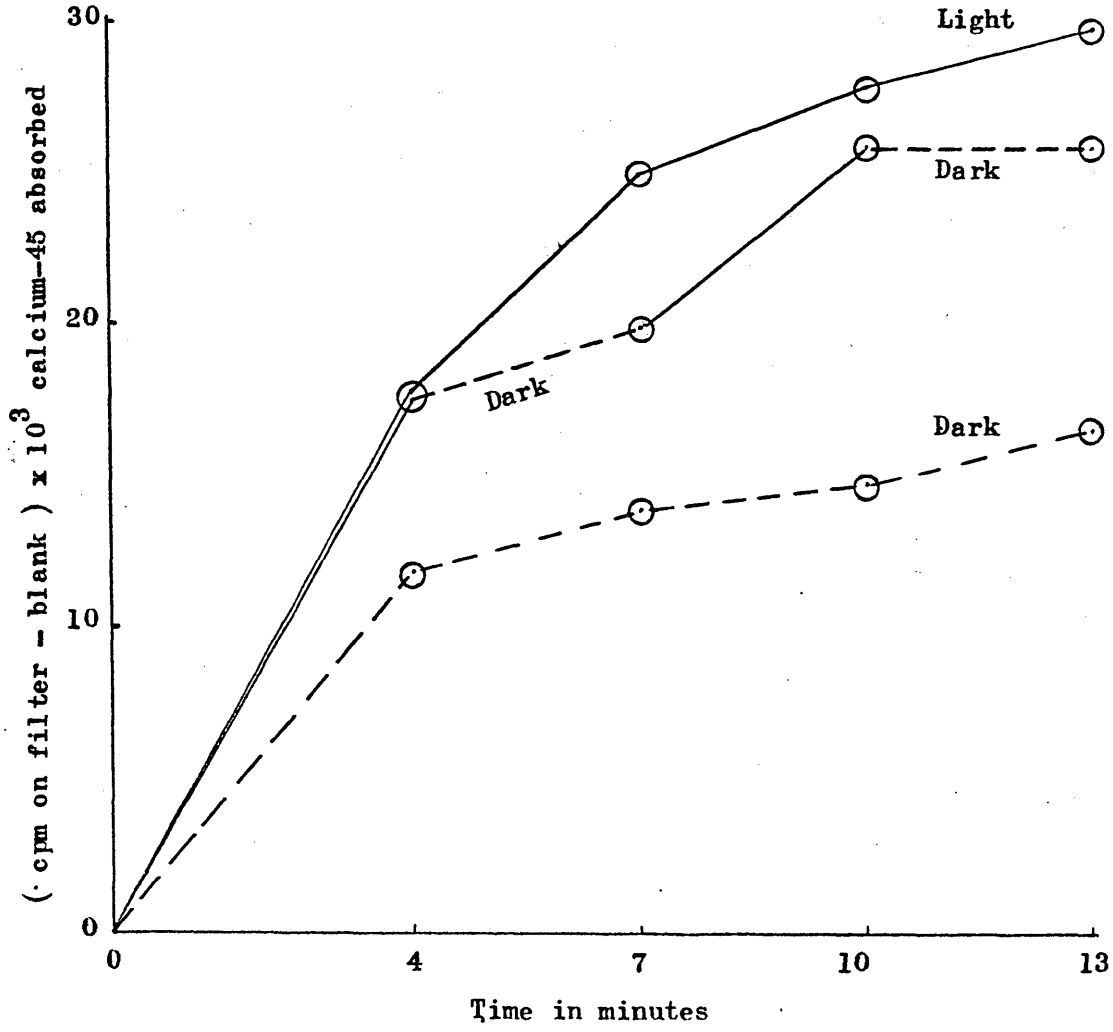
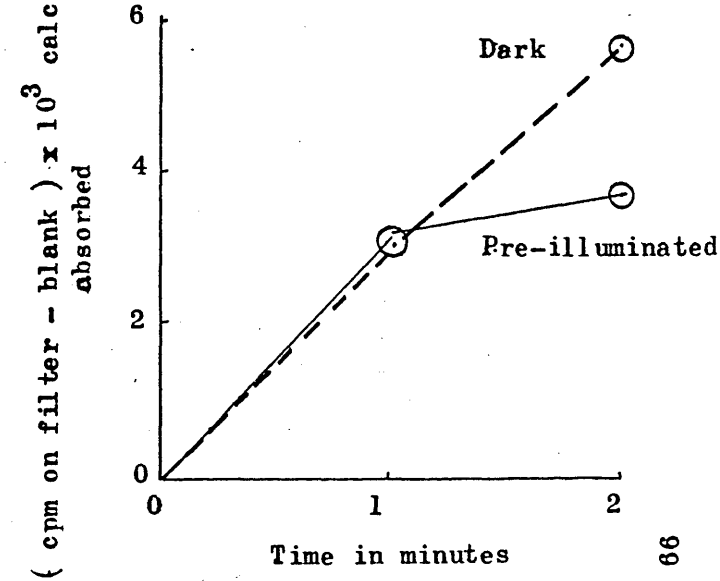


Figure 21 **Effect of pre-illumination on calcium uptake in the dark**

Conditions as for figure 20 except the plastids were pre-incubated in the light or dark in the absence of calcium



that was pre-illuminated shows a significant decrease in calcium uptake in the dark after one minute.

CMU is a potent inhibitor of oxygen evolution by isolated chloroplasts (181), and its effect upon calcium uptake was tested, together with other additions designed to relieve the inhibitory action of CMU. The results of such experiments are listed in table 22 as percentages of the appropriate controls without any additions.

Table 22 Effect of CMU and other additions upon calcium uptake

		% uptake		
Addition	Concentration	Light	Dark	(Light-Dark)
NONE	-	100	100	100
CMU	10^{-5}M	41	75	22
PMS	3×10^{-7}M	77	92	36
CMU and PMS		85	61	109
Ascorbate /DPIP	10^{-3}M/10^{-4}M	50	50	50
CMU and ascorbate /DPIP		75	53	88

Incubation time: 4 minutes

Thus the inhibition in the light of calcium uptake by CMU could be partially removed by the addition of either PMS or the ascorbate DPIP couple. The situation regarding the dark and (light minus dark) uptakes is complicated by the fact that both PMS and ascorbate /DPIP were somewhat inhibitory and the addition of these substances to a CMU-poisoned system in the dark did not result in an additive inhibitory effect.

Other inhibitors of photosynthesis were tested upon the calcium uptake, and the results of these experiments are shown in table 23 as percentages of the control uptake.

Table 23 Inhibitors of calcium uptake

Addition		Concentration	% uptake Light	Dark
NONE		-	100	100
Ammonium chloride	1.	10^{-3}M	71	60
	2.	10^{-4}M	110	102
Antimycin A		4 x 10^{-5}M	70	-
CCCP	1.	2 x 10^{-5}M	29	76
	2.	2 x 10^{-6}M	44	75
Desaspidin	1.	10^{-5}M	50	100
	2.	10^{-7}M	100	100
EDTA		10^{-2}M	12	24
PMA		10^{-4}M	18	39

Incubation: 4 minutes

The inhibitions due to CCCP could be partially relieved by cysteine. Table 24 shows that 5x 10^{-5}M cysteine reduced the inhibition of the (light-minus dark) uptake from 100% to 87% with 2 x 10^{-5}M CCCP and almost halves the inhibition due to 2x 10^{-6}M CCCP in the(light minus dark) uptake. By itself cysteine was stimulatory to uptake in the light but slightly inhibitory to the dark uptake.

Table 24 Effect of cysteine on the inhibition of calcium uptake by CCCP

Addition		Concentration	% uptake Light	Dark
NONE		-	100	100
CCCP	1.	2×10^{-5}M	29	76
	2.	2×10^{-6}M	44	75
CCCP + Cysteine	1.		33	83
	2.		65	83
Cysteine		5×10^{-5}M	110	93

Incubation time: 4 minutes

Ammonium chloride at a concentration of 10^{-4}M inhibited cyclic photophosphorylation by over 50%, but gave a small stimulation to the light uptake. At a concentration of 10^{-3}M cyclic photophosphorylation was completely inhibited whereas calcium uptake was only cut by approximately one-third. Antimycin A - a weak uncoupler and inhibitor of ferredoxin catalysed cyclic photophosphorylation (66) (182) - inhibited calcium uptake in the light by 30%. CCCP is a potent uncoupler of photophosphorylation (183) (184) and is particularly severe at low light intensities (185). At a concentration of 2×10^{-5}M the light stimulated uptake was inhibited by 71% and the dark uptake by 24%, a concentration of 2×10^{-6}M gave a 54% inhibition to the uptake in the light and a 25% inhibition to the dark uptake. Desaspidin uncouples photophosphorylation (87) (186) and at concentrations of 10^{-5}M completely abolishes any light-activated uptake whilst leaving the dark uptake unaffected. Concentration of 10^{-7}M were without effect upon calcium uptake. EDTA is a powerful

chelator of divalent cations (187) and in these experiments it was added in excess to the reaction mixture in an attempt to chelate all the calcium present. EDTA completely abolished the light-stimulated uptake and reduced the dark uptake by 76% such that light and dark uptakes were the same. PMA inhibits electron flow and stimulates ATP-ase (119) and severely inhibited the light uptake by 82% and the dark by 61%.

Photosystem II of photosynthesis has been shown to be irreversibly destroyed by heating chloroplasts to 50°C for a few minutes (188). Such treatment of the plastids followed by incubation at 15°C resulted in the complete loss of the light-activated calcium uptake and further decreased the dark uptake by some 40%. This is shown in table 25.

Table 25 Effect of heating chloroplasts upon calcium uptake

(cpm on filter - blank)

Experiment		Heated	Control
Light	1	4850	13.224
	2	4947	13.102
Dark	1	4856	8053
	2	5052	7825

Electron transport is uncoupled from photophosphorylation when chloroplasts are isolated with EDTA (189) and electron flow is further stimulated. Chloroplasts were isolated and resuspended in Tricine-sucrose media at pH8. The suspension was divided into two aliquots, one of which was incubated in the dark for 10 minutes at 1°C with 1mM EDTA present. The other aliquot was kept in the dark at 1°C for 10 minutes. Both aliquots

were then re-centrifuged and the pellets resuspended in Tricine-sucrose medium at pH 8. Calcium uptake by the treated and untreated chloroplasts was then compared in the light and dark. Table 25 illustrates that the EDTA treatment did not affect calcium uptake by these chloroplasts.

Table 26 Effect of EDTA treatment upon calcium uptake

Experiment		EDTA-treated	Control
Light	1.	7769	7290
	2.	8755	8723
Dark	1.	3883	3807
	2.	5344	4903

Simultaneous measurements of calcium uptake and basal ferricyanide reduction were made in an attempt to demonstrate some stoichiometry between the two processes. Table 27 shows that calcium uptake was initially coupled closely to electron flow with a Ca : 2e of 0.58. However this ratio was decreased after five minutes to 0.20. Calcium itself had no effect upon ferricyanide reduction.

Table 27 Stoichiometry of calcium uptake and FeCy reduction.

Time in minutes	FeCy reduction (Light-Dark)	Calcium uptake (Light-Dark)	Ca : 2e
1.	0.0207 μ mole	0.006 μ mole	0.58
5.	0.1296 μ mole	0.013 μ mole	0.20

Light intensity: 3000 Lux.

Much interest has been focussed upon the possible existence of exchange-diffusion systems in isolated chloroplasts, following the publication of the chemi-osmotic theory (150). Further interest in

this theory has been prompted by the demonstrations of a light-induced proton uptake by chloroplasts (132) (133) and of ATP - formation in the dark by chloroplasts subjected first to acid then to basic conditions (190). Further evidence suggests that ATP - formation and the proton uptake in chloroplasts are intimately linked. In an attempt to demonstrate a connection between these processes and calcium uptake, the following experiments were conducted.

Chloroplasts were isolated in Tricine sucrose media at pH 7 and resuspended in 0.35M sucrose. Samples of the suspension were pre-incubated for 5 minutes in the dark with 25mM Tricine-sucrose at acidic (pH 5.5) or basic (pH 8.0) conditions. The suspensions were then injected into calcium-45 and 10^{-4}M calcium chloride solutions in the dark containing the necessary amounts of NaOH, Tricine or sucrose, to make the incubation phase alkaline, acid or the same as the pre-incubation phase. Table 28 shows the results of such a series of experiments.

Table 28 Effect of pH transitions upon calcium uptake

		cpm on filter - blank
Pre-incubation pH	Incubation pH	Dark
5.5	8.0	3558
8.0	8.0	4007
8.0	5.5	2251

Incubation time with calcium: 2 minutes.

Transfer from acid to basic pH results in an 11% inhibition of calcium uptake, transfer from basic to acid conditions results in a

56% inhibition to uptake, when both are compared to the suspension kept at pH 8 throughout.

A similar experiment was done in which 1 mM malic acid was present throughout every incubation. These results are presented in table 29.

Table 29 Effect of pH transition (with malate present) upon calcium uptake

Pre-incubation pH	Incubation pH	% uptake Dark
5.5 (malate absent)	8.0 (malate absent)	93
5.5	8.0	86
8.0 (malate absent)	8.0 (malate absent)	100
8.0	8.0	62

Incubation time with calcium: 2 minutes.

Transfer from acid to basic conditions with malate present increases the inhibition due to transference from acid to basic conditions itself. Transfer from acid to basic conditions with malate present however resulted in a greater uptake than suspensions maintained at pH 8 with malate present. The effects observed in these experiments are thus not comparable to the results obtained with phosphorylation in the experiments of Jagendorf and Uribe. The results shown here would appear to be related to the presence or absence of malate rather than to the pH transition, and so the uptake of C^{14} - labelled malic acid was investigated at three pH values in the light and dark. Table 30 shows that at acid pH the amount of malate present in the plastids in the light incubation was fractionally greater than in the dark. As the pH was

raised so the light/dark ratio increased but the absolute amount of malate accumulated decreased.

Table 30 Accumulation of malate

	cpm on filter - blank		
Incubation pH	Light	Dark	Light/Dark
6.2	5730	5426	1.06
7.1	2278	1483	1.53
8.0	1977	1242	1.59

Chloroplasts isolated in Tricine-sucrose pH 8 and resuspended in 0.35M sucrose. 0.10 mg chlorophyll per 2.5 ml. sample with 1 μc C^{14}-malate. Incubation time: 4 minutes. Temperature: 10°C. Light intensity: 1500 Lux. Filters pre-washed with 0.1M malate and after chloroplast filtration washed with 0.35M Nacl.

The effect of transferring suspensions from basic to acid conditions was investigated further. Chloroplasts were isolated and resuspended in 5mM Tricine-sucrose media at pH 8, and were initially incubated at pH 8 with calcium - 45 and carrier present. Uptake was measured in the light and dark for a period of 4 minutes at which time sufficient Tricine was injected into the suspension to lower the pH to 6.3. Uptake measurements were then continued for a further period. Figure 22 shows the results of this experiment. It can be seen that a part of the previously accumulated calcium is lost on exposing the chloroplasts to acid conditions.

This experiment concluded the work done to investigate the metabolic nature of the observed calcium uptake. The final section on calcium uptake was concerned with the localisation of the calcium within the chloroplast.

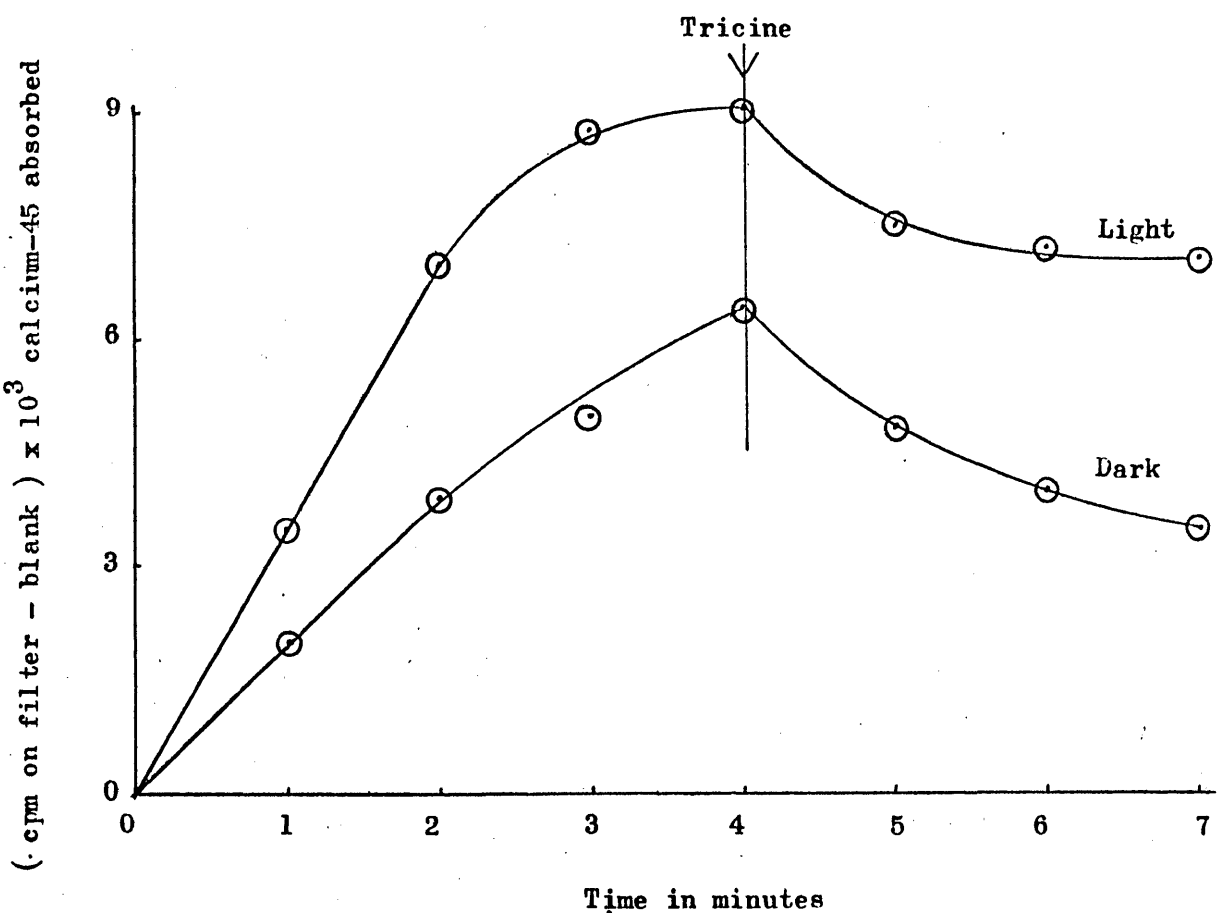
Figure 22 Effect of acid conditions upon previously accumulated calcium by chloroplasts

Plastids (0.120 mg chlorophyll in 2.5 ml.) incubated at pH 8 with 2.5 μc calcium-45 and 10^{-4} M carrier calcium. Tricine was injected into the incubation mixture to alter the pH to 6.3

Light intensity : 1500 Lux

Temperature : 15°C

Uptake measured by filtering 2.5 ml. samples, washing and counting the filters for calcium-45 activity



(3) Localisation of the accumulated calcium

The experiments of Nobel et al (156) indicate that the calcium accumulated by spinach chloroplasts is deposited upon the granal system of the plastids (probably as insoluble phosphate salts). Further Nobel (155) found that inorganic phosphate itself markedly stimulated calcium uptake and that phosphate uptake required the presence of calcium. In the experiments performed in this work, phosphate was found to produce almost a 20% stimulation to calcium uptake in the light when the concentrations of calcium and phosphate were less than 50 μM. At higher concentrations (not high enough to exceed the solubility of calcium phosphate at pH 8 in the medium) the stimulation to the light uptake was less than 10%. The dark calcium uptake was slightly stimulated (less than 5%) at all phosphate concentration used. Table 31 shows the effect of phosphate concentration upon the rate of calcium uptake (at equivalent concentrations) in the light.

Table 31 Effect of phosphate upon calcium uptake in the light

	Rate of uptake: mμ moles Ca/min	
Concentration μM	$CaCl_2$	$CaCl_2 + K_2HPO_4$
25	0.40	0.47
50	0.61	0.73
100	1.00	1.05
200	1.31	1.40

Incubation time: 1 minute

The comparative abilities of intact and broken chloroplasts to absorb calcium in the light and dark were investigated. Table 32 shows

that broken chloroplasts in both the light and dark were able to absorb more calcium than intact plastids.

Table 32 Effect of membrane integrity upon calcium uptake

mμ moles calcium

	Light		Dark	
Time	Whole	Broken	Whole	Broken
1 minute	0.38	0.50	0.30	0.35
4 minutes	0.95	1.33	0.40	0.51

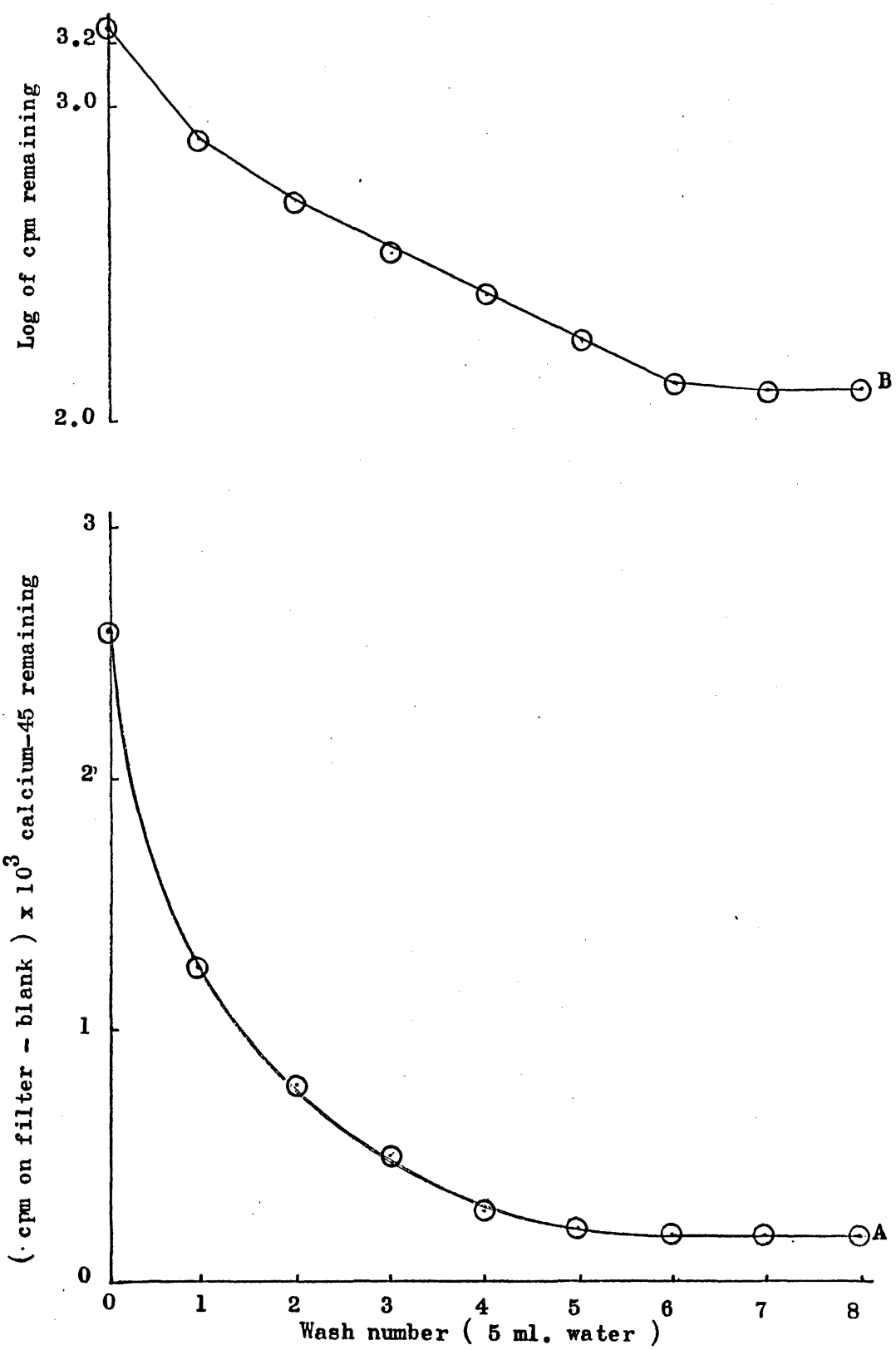
6 ml of chloroplasts contained in a 50 mL conical flask with 6μc calcium-45 and 10^{-4}M calcium chloride. 2.5 ml. samples (0.120 mg chlorophyll) filtered.

In an attempt to define further the location of the absorbed calcium, chloroplasts were incubated with carrier-free calcium-45 and collected by membrane filtration. The filters were dried and then held in place upon a planchette with a metal washer, such that only the membrane area with chloroplasts was exposed. Blanks were also treated in this way. After counting the filters were replaced on the filtration apparatus and washed, using various solvents, dried and re-counted. For some experiments this procedure was repeated several times. The wash solvent was allowed to flow slowly (20-30 seconds) through the filters.

Figure 23 illustrates an experiment in which a series of washes with water were given and the filters counted after each wash. Curve A is the plot of cpm remaining on the filter against the wash number. Curve B is the logarithmic plot of cpm remaining on the filter against wash number. From curve B it can be seen that there is not a linear

Light intensity : 1500 Lux Temperature : 15°C

Plastids filtered, filter washed (NaCl) counted then rewashed with 5 ml. water and recounted (after drying) for calcium-45 activity. Blank similarly treated.



relationship between counts remaining and wash number. This effect is emphasised in figure 24 where the logarithmic plot of cpm remaining against wash number is shown for chloroplasts labelled in the light and dark. Both figures also show that some 10% of the accumulated calcium-45 is relatively difficult to wash out with water from both light and dark treatments.

The fact that the logarithmic plots in figures 23 and 24 are not linear suggests that the calcium may be bound or present on more than one component of the chloroplast. This was investigated further by labelling chloroplasts and then, after counting, washing the filters with various solvents and recounting the washed filters. The results of these experiments are shown in table 33.

Table 33 Effects of washing with various solvents upon the calcium content of chloroplasts

	% activity lost on washing	
Solvent (10 ml)	Light	Dark
Water	61.8	62.3
0.01M EDTA pH 8	91.4	97.6
0.1N HCl	97.0	94.7
0.1N NaOH	92.1	93.3
0.01M $CaCl_2$	90.4	91.6
0.01M Fe Cl_3	91.6	91.5
Chloroform/methanol (2:1 v/v)	5.1	4.3

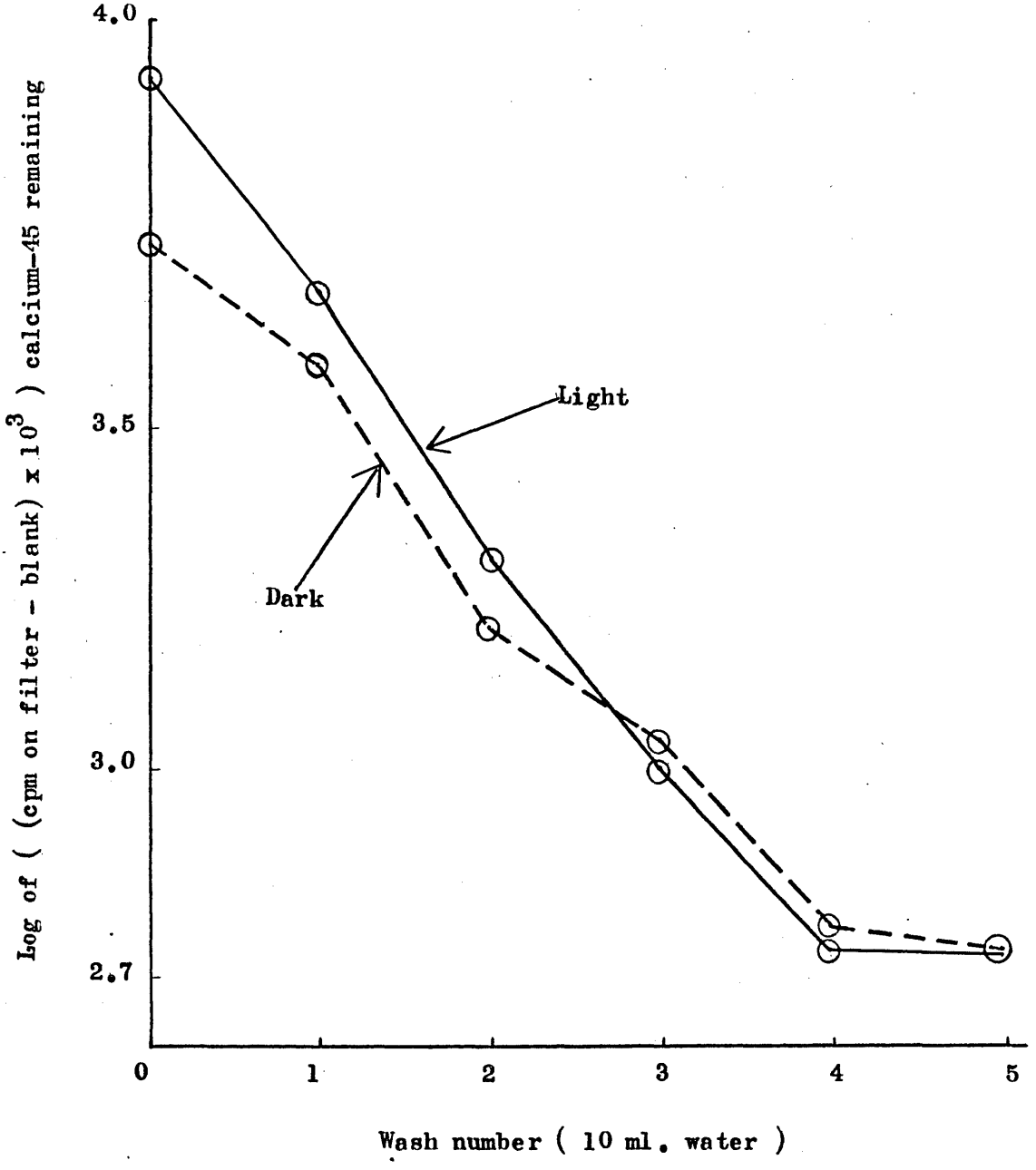
Chloroplasts isolated and resuspended in Tricine-sucrose media pH 8. Incubation time 2 minutes at 1500 Lux with 2.5 μc calcium-45. Filters

Figure 24 Extraction of calcium-45 from labelled plastids

Plastids (0.120 mg chlorophyll in 2.5 ml.) incubated with 2.5 μc calcium-45

Light intensity : 1500 Lux Temperature : 15°C

Plastids filtered, filter washed (NaCl) counted and then rewashed with 10 ml. water and recounted (after drying) for calcium-45 activity. Blank similarly treated.



washed with NaCl(0.35M)prior to counting.

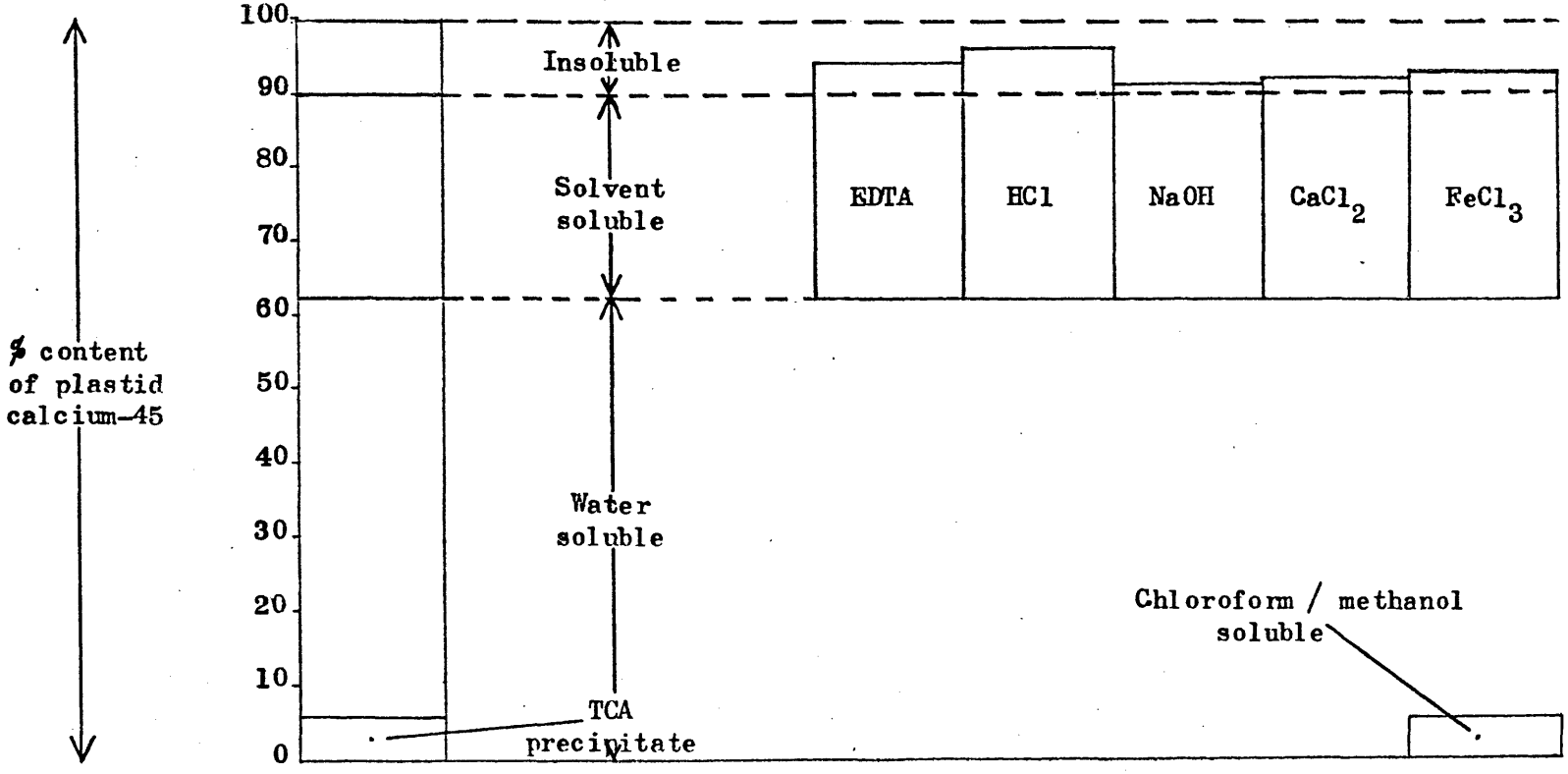
It is concluded that all solvents extract approximately the same percentage of calcium from both the light and the dark. Calcium is present as a water soluble-component representing approximately 60% of the total calcium accumulated. It is assumed that washes with all the other aqueous solvents also remove this aqueous percentage. Using this assumption it can be seen from figure 25 that all the aqueous solvents used extracted a further 30% (approximately). A 10% fraction of the calcium remained unextracted. The chloroform/methanol solvent removed approximately 6% of the accumulated calcium.

The water extract obtained in the previous experiment was further treated with TCA (to a final concentration of 5%) to precipitate any water-soluble proteins present. The precipitate was counted for calcium-45, after a further water wash, and approximately 10% of the total water - soluble calcium fraction was present, equivalent to 6% of the total calcium accumulated. This data, together with the data of table 33 are represented in figure 25.

The samll fraction of calcium associated with the TCA protein precipitate was investigated further. Chloroplasts were labelled in the light with carrier-free calcium-45 and then centifruged. The chloroplast pellet was then resuspended with 2ml. Tricine-NaOH buffer pH 8, to break the chloroplasts. The buffered suspension was kept overnight at 1$0^{\circ}$C and then centrifuged for 10 minutes at 5,000 x g to removed particulate matter. The supernatant was removed and 1mL placed on a Sephadex-G25 column previously equilibrated with Tricine-NaOH buffer pH 8.

Figure 25 Extracted fractions of calcium-45 from labelled plastids

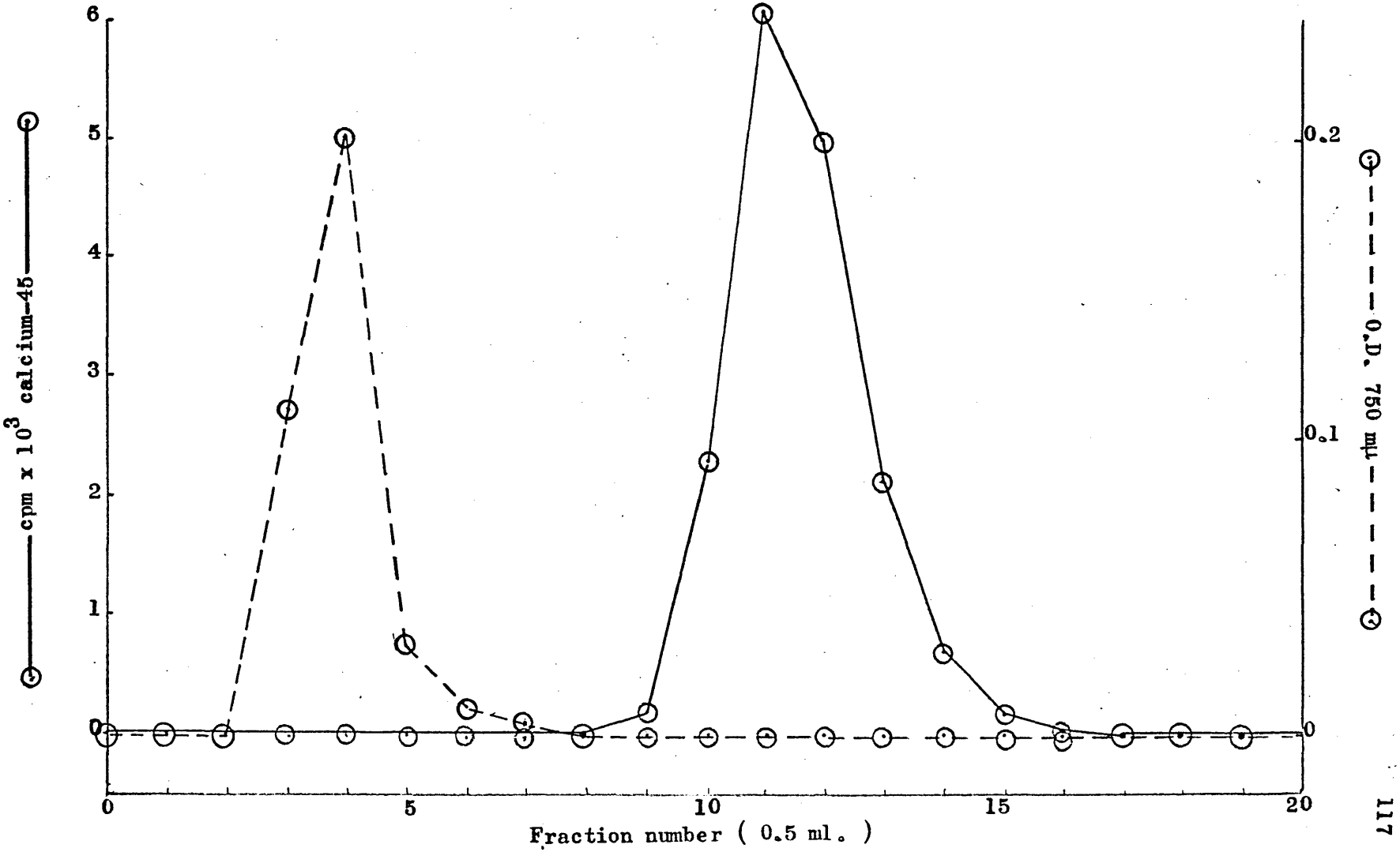
Figure compiled from the data of figures 23 and 24 and table 33



Fractions (0.5ml) were collected and analysed for protein and calcium-45 content. Figure 26 shows the results of this separation, the protein and calcium-45 fractions are quite separate so any calcium bound to a protein must be either very small (beyond the column resolution) or non-existent. These experiments concluded the work done with calcium-45 uptake.

Figure 26 Separation of soluble protein and calcium-45 fractions from labelled plastids

Plastids (0.120 mg chlorophyll in 2.5 ml.) incubated with 2.5 μc calcium-45. Plastids extracted with buffer and the soluble extract fractionated using a Sephadex G-25 column. Collected fractions assayed for protein and calcium-45



DISCUSSION

The results reported here with respect to chloride uptake by Elodea confirm that uptake is stimulated by light. The fact that the values for Km differ for uptake in the light and dark suggests that two separate processes are involved. The observation of Michaelis-Menten type kinetics however, cannot be taken as proof of a chloride-carrier complex, and the application of this type of analysis to an intact tissue or (chloroplasts) is debateable. Further evidence for the existence of two accumulatory processes is provided by the results obtained using anaerobic conditions. Uptake in the dark was inhibited under anaerobic and CO_2-free conditions indicating that chloride absorption is dependent upon respiration and/or a dark CO_2-fixing mechanism. The dark anaerobic-resistant uptake observed is probably accounted for by the free space uptake. Chloride uptake under CO_2-free nitrogen was supported by light to the extent of 84% of the aerobic uptake. This indicates that respiratory processes contribute only a part (16%) to the light-stimulated uptake. The existence of two accumulatory mechanisms in the light and dark for green tissues has also been noted by Kylin (see Introduction). In CO_2-free nitrogen, in the absence of a terminal acceptor for non-cyclic electron flow, only cyclic photophosphorylation should occur (assuming there is no large endogenous acceptor pool). The possibility however of maintaining a green tissue completely anaerobic in the light is remote; a certain amount of oxygen produced by photosynthesis could be maintaining a pseudocyclic flow of electrons as well as a small amount of respiration.

Chloride uptake in the light was stimulated in CO_2-free air, showing that the effect of light is not mediated through carbon fixation but by the partial reactions of photosynthesis. This view is substantiated by the finding that bicarbonate was inhibitory to chloride uptake in the light. Taken together these results suggest that chloride absorption and carbon fixation are in competition for some product of the light reactions of photosynthesis. Further, the very similar $C^{14}O_2/Cl^{36}$ ratios seen under 650 and 707 mμ illumination also suggests that the carbon-fixing and absorption mechanisms are in competition.

The inhibition to the dark uptake in CO_2-free conditions may reflect a dependency of dark uptake upon a dark CO_2-fixing mechanism, which is well developed in Elodea and explains the high $C^{14}O_2/Cl^{36}$ ratios seen in the dark.

Since carbon-fixation and chloride uptake appear to be in competition for a product of the light reactions, the most likely product of the light reactions is ATP. Light of 650mμ, activating both photosystems, and light of 705 or 725 mμ, activating photosystem I, were capable of supporting a chloride uptake greater than the dark uptake. These results would implicate both cyclic and non-cyclic electron flow and/or photophosphorylation as being able to support chloride absorption in the light. The results of the enhancement experiments showed that simultaneous illumination of Elodea with red and far-red light usually results in less chloride absorption then found using red (650mμ) light alone. This suggests that photosystem I is reducing the efficiency of photosystem II for chloride absorption. This effect may be viewed as an

increased carbon fixation occurring with both wavelengths present thereby reducing the ATP available for chloride uptake.

More chloride absorption was found with 650 mμ light indicating the importance of photosystem II in chloride absorption, a feature that is also emphasised by the inhibiting action of DCMU upon absorption, although a conflicting view is seen in the inhibition of uptake by DCMU with 707 mμ light. Further evidence that the product of the light reactions utilised in uptake is ATP, is provided by the work of Jeschke with Elodea (see Introduction), which shows that both cyclic and non-cyclic photophosphorylation support chloride uptake.

In summary, the competitive effects of bicarbonate and carbon fixation upon chloride absorption, the effect on uptake of far-red light, the stimulation to uptake by CO_2-free air together with the lack of any marked inhibition in anaerobic conditions, all support the view that chloride uptake in the light is mediated through the partial reactions of photosynthesis and in particular by photophosphorylation. The dark uptake of chloride is dependent upon aerobic conditions and CO_2 and thus differs from the uptake in the light.

Thus the mechanism for chloride movement in Elodea differs from that described for Nitella and Hydrodictyon (see Introduction). The greater efficiency of the mechanism under conditions allowing non-cyclic photophosphorylation may be due to low rates of cyclic photophosphorylation in vivo, although it has been shown that cyclic photophosphorylation in vivo has a faster turnover than oxidative phosphorylation (191). Alternatively, the sites of cyclic and non-cyclic photophosphorylations

may be physically separated and only the ATP produced by the non-cyclic mechanism may be easily available for chloride movements.

The results obtained in studying chloride uptake in isolated pea chloroplasts lack precision. This is due in part to the lack of a fast method for separating chloroplasts from solutions and in part to the variability found in the chloroplast preparations.

The chloride content of aqueously prepared chloroplasts was calculated to be 1.9×10^{-2}M. In view of the many contaminants present in these preparations this value is certainly an over-estimate. By contrast plastids isolated non-aqueously from labelled leaves were not contaminated (as judged by the lack of fumarase activity in the suspensions) and had a calculated chloride concentration of approximately 2×10^{-2}M. This result indicates that pea chloroplasts in vivo have the ability to accumulate chloride at least in long term feeding periods - although whether by an active or passive mechanism cannot be determined from this result.

Using the supernatant analysis technique showed that a small light-activated uptake was present and this was confirmed by the Millipore filtration method. However, the kinetics of uptake are completely different when the results of the two methods are compared. A further difference is seen between the two methods when the dark uptakes are compared. Using the supernatant analysis technique no dark uptake was observed, however with the filtration technique a gradual accumulation of chloride in the dark is observed. This difference may reflect a difference in the force applied during the separation of the chloroplasts. It further demonstrates that the uptake in the light results in a

distribution of chloride within the chloroplasts more resistant to centrifugal forces. However, the analysis of chloroplast pellets centrifuged through a layer of buffer failed to detect any increase of chloride-36 activity with time. This may have resulted from a "leaching" effect of centrifugation as noted by Stocking and Ongun (148).

The results found on comparing the abilities of whole and broken plastids to absorb chloride show that the membrane in vitro is acting as a barrier to the entrance of chloride. Uptake by broken chloroplasts is greater than that seen with intact plastids in the dark although the difference is less in the light.

Of all the additives tested to increase the size of the uptake, only ATP was effective and more so in the dark than in the light. The dark uptake decreased after 10 minutes incubation and this effect was not due to breakage of the plastids since microscopic examination of the experimental suspensions at the end of experiments did not indicate any significant increase in the numbers of broken chloroplasts. Chloroplasts shrink in the light and dark on the addition of ATP (as measured by the absorbancy changes at 540 mμ). The dilution of the supernatant by the extruded water was calculated to be not more than 1% of the activity present. This was less than the effect on chloride uptake observed.

The observed effects of ATP upon chloride absorption were not apparently mediated by an added ATP-ase system. This is in agreement with the work of Nobel (see Introduction) who found no chloride absorption in spinach plastids.

The effects of sucrose molarity upon chloride movements show that light exerted an effect upon the water movements due to the sucrose

concentration. At molarities less than 0.4M, the activity (cpm) of the solution increased, more in the light than dark, indicating that water entrance occurred without concomitant chloride influx (unless chloride was effluxed as fast as it was influxed). The existence of a pump solely effluxing chloride from the chloroplast however is not consistent with the finding that in vivo chloroplast accumulate chloride. Water entrance and exit from the plastid is known to be affected by light and certain ions. The existence of a light-activated chloride influx however is not evident in these experiments under conditions of chloroplast volume increase. Further, at 0.8M sucrose where the chloroplasts are shrunken no changes in supernatant activity were detected. This suggests that much less than a 1% dilution of the supernatant by water from the chloroplast internal volume is contributing to a change in activity. Further, the effects induced by ATP (which also shrinks the chloroplasts) must be more specific than an effect of shrinkage.

When the Millipore filtration technique of experimental solutions was tried much more precise data were obtained. Very large and fast movements of chloride occurred in the first minutes of illumination. A general pattern of initial net influx upon illumination followed by net efflux was observed. Examination of the data of Jagendorf and Neuman (134) shows that proton entry into the chloroplast is almost saturated after 30 seconds and fully saturated after 1 minute. The increase in chloride activity in the chloroplasts observed here, although variable, generally increased in the light for 10-60 seconds. This may be interpreted either as an influx of chloride to balance the charge difference created by

proton entry or as an accompanying anion entering with the proton. The subsequent net efflux of chloride would then have to be mediated by an active mechanism. The amount of chloride absorbed in 10 seconds as illustrated in figure 11 was equivalent to 0.75 μ moles/mg chlorophyll. The data of Jagendorf and Neuman show that between 0.65 - 1.0 μ moles of protons/mg chlorophyll were absorbed in approximately 30 seconds. It would thus seem that only part of the chloride influx could be related to proton entry. In several other experiments however all the chloride influx could be accounted for on the magnitude of the proton movement.

The filtration experiments, in contrast to analyses of supernatant activities, showed that in the dark the chloroplasts gradually accumulated chloride. This demonstrates that the plastid membrane is permeable to chloride when in the dark and the greater chloride movement seen in the light may be related to the production of ATP by photochemical processes.

Dark to light transients show that the chloroplasts have a limited net capacity for chloride. This could result from the existence of a definite number of adsorption sites. Figure 12 shows that after a dark incubration with chloride-36, the activity found in the chloroplasts on subsequent illumination does not increase so quickly as when chloroplasts are incubated with chloride in the light. Return to a dark period after illumination causes an efflux of previously accumulated anion. This suggests that light is an essential factor for the maintenance of chloride levels in the chloroplast. The outpumping mechanism however returns the chloride-36 level almost to zero.

The absorption of calcium by pea chloroplasts is stimulated by light, the initial rate of uptake in the light being almost double the dark uptake. Both uptake in the light and dark are very labile and the chloroplasts very quickly lose their absorption capacity on storage in the dark at 15°C. This fact, together with the finding that there are two pH optima, strongly suggests that the absorption process is mediated by an enzymatic mechanism rather than by adsorption phenomena. This view is strengthened by the degree of specificity seen when divalent cations other than calcium are present in the incubations. The Q_{10} values are variable probably because the decay factor will also be a function of temperature.

Uptake in the light is saturated between 200 and 400 Lux, at which intensity negligible photophosphorylation is detectable (at 15°C) although electron-flow is measurable (195). Calcium itself is an uncoupler (probably by activating an ATP-ase (158) and at saturating concentrations for uptake, photophosphorylation was severely reduced. The independence of calcium uptake and photophosphorylation is seen further in the inhibitory effects of added phosphorylative systems, although these systems were also found to inhibit the dark uptake. These effects may be the result of chelation of calcium by the added nucleotide thus decreasing the amount of free calcium present in the medium. Addition of excess EDTA to chelate all the calcium in solutions shows that the chloroplasts, in the light or dark, can absorb little calcium from a chelated form.

An added ATP-ase system was inhibitory to calcium absorption and as

uptake in the light and dark was approximately the same, this again suggests that the added nucleotide is chelating the cation thus reducing uptake. However, low concentrations of ATP alone give almost a 10% stimulation to uptake in both light and dark. It remains possible that some small fraction of the observed uptake is mediated by an ATP-ase system. The ATP-ase system described elsewhere in connection with calcium uptake is activated by light and can function in uptake (after activation) independently of light for several minutes (155); no such effect was found in the experiments reported here. This indicates that the calcium uptake into pea chloroplasts is dependent upon light, although if the decay time (in the dark) of light-activation were of the order of seconds any effect of pre-illumination could have escaped detection in these experiments. An additional complication was that pre-illumination of plastids in the absence of calcium followed by a dark incubation with calcium present resulted in a cessation of the dark uptake after 1 minute. The mechanism of this effect was not investigated further.

Removal of the coupling factor with EDTA and low concentrations of the uncoupler ammonium chloride both allowed calcium uptake to continue unabated. Measurements of the Ca:2e ratio showed that initially approximately 3 calcium are accumulated for every 10 electrons transported and this occurred under saturating light intensity. These results suggest that the effect of light upon calcium accumulation is mediated by a mechanism associated with electron flow. However, the rates of photophosphorylation and Hill reaction did not show the same rate of decay on storage as did calcium uptake. This suggests that calcium

uptake, whilst closely associated with the photosynthetic light reactions may not be mediated by the principal pathway of electron flow but by a related branch or even a labile adsorption site that is influenced by light.

If an adsorption site such as a protein were involved in uptake, the differing pH optima for light and dark uptakes shows that light must be able to alter the charge upon this site. Electrophoretic studies (196) show that spinach chloroplasts are negatively charged and become 15% more negative in the light. This increased light-induced negative charge however was not apparent if the plastids were disrupted, but did show the same low light intensity requirement as does the calcium uptake reported here. Calcium uptake was more efficient with broken chloroplasts in both the light and dark, so it seems that the mechanism of uptake is not wholly concerned with the light-induced change in electronegativity which is associated with the presence of a bounding membrane.

Changes in chloroplast volume, by altering the membrane, might expose or conceal charged groups. The results of Dilley, already discussed, indicate the existence of a Donnan system within the osmotic compartment of the chloroplast. Illumination may result in an increase in the number of negative charges and/or an altered permeability of the membrane to allow cations to enter and neutralise these charges. However, although light-dependent volume changes have been described in ionic media (see Introduction), calcium uptake was just as efficient in sucrose solutions, which inhibit light-activated volume changes (131).

The effects of selected wavelengths of light upon uptake showed that

photosystem II was most efficient in promoting absorption, but appreciable uptake did occur in far-red light showing that cyclic electron flow could also support uptake. Enhancement of uptake was not observed and the effect of simultaneous illumination with 650 and 725 mμ light was approximately the same as that produced by 650 mμ light alone. The effect of far-red light may be inoperative whilst illumination with 650 mμ light is present. Further evidence of the dependence of calcium uptake upon photosystem II is shown by the inhibitory effects of CMU upon absorption. The destruction of photosystem II by heating to 50°C also inhibited calcium absorption.

The interaction of photosynthesis and calcium was defined further by finding that the inhibition due to CMU was partially relieved by the ascorbate-indophenol couple which supplies electrons to the system beyond the CMU-sensitive site which lies on the reducing side of photosystem II (53). The incomplete inhibition of uptake by CMU is probably due to endogenous cyclic electron flow occurring. That cyclic electron flow can support calcium uptake is shown further by the relief given to a poisoned uptake system by PMS. Some inhibition was seen with PMS itself and this may be due to a cation competitive effect, since PMS is a cation at the pH value used. The inhibition seen with the ascorbate-indophenol couple may result from the over-reduction of a component of the photosystems. Desaspidin at concentrations of 10^{-7}M, which uncouples cyclic photophosphorylation (186), was without effect upon calcium accumulation, at concentrations of 10^{-5}M, which uncouples non-cyclic photophosphorylation, the light-stimulated calcium

uptake was inhibited. Thus it appears that uptake is maintained by a non-cyclic process.

The fact that many of the photosynthetic inhibitors tested affected the dark uptake is surprising, especially as the inhibiting effects due to PMS or CMU were not additive, not were the effects of CMU and the ascorbate-indophenol additive when placed together in the dark incubation mixture. These effects imply that electron flow is occurring in the dark. Although the dark uptake exhibits a similar specificity towards calcium as does uptake in the light, there are sufficient differences between the uptakes to show that they are mediated by separate mechanisms. Further, as the decay curves for uptake capacity at 15°C intersect and then run together after 50 minutes, it appears that the dark uptake continues in the light (and the effect of light is best measured as the difference between light and dark uptakes).

The involvement of sulphydryl (-SH) groups with absorption in the light is seen from the results with PMA and cysteine. PMA drastically inhibited uptake. Cysteine could relieve part of the inhibition due to CCCP and by itself stimulated calcium uptake in the light by 10% whilst inhibiting the dark uptake - a further difference between the two uptakes.

Attempts to demonstrate an exchange of calcium for protons accumulated in plastids showed that calcium uptake was inhibited by protons inside the chloroplasts (derived by incubation in acid media). This inhibition was increased by the presence of a penetrant organic acid (providing protons to the inside of the plastid) as shown using malate.

With malate present transfer from acid to basic conditions however relieved part of the inhibition due to malate in suspensions maintained at pH 8. This effect may be explained either as being due to malate (entering in the plastid at low pH) acting as an anion for calcium or as malate competing with calcium at pH 8 for entry into the chloroplast. At acid pH, malate absorption was independent of light whereas at basic pH, although the amount accumulated was less, a light-stimulation to malate uptake was observed.

Calcium accumulated under basic conditions was partially lost on transfer to acid media. This effect was greater in the dark and suggests that the loss of accumulated calcium was not due to exchange for protons since a greater proton uptake occurs in the light. More probable is that the membranes become "leaky" to calcium in acid conditions or that for the retention of accumulated calcium the accumulatory mechanism must continue to operate and this requires basic conditions.

The loss of previously accumulated calcium in acid media may also be considered from a chemical change. If the calcium were present in the chloroplast either partly or totally as an insoluble phosphate salt at pH 8 the species would be mostly $CaHPO_4$, at pH 6.3 again $CaHPO_4$ would predominate with a very small amount of $Ca(H_2PO_4)_2$. The change in pH may give a change in the form of the calcium salt resulting in a greater amount of calcium ion inside the plastid which then leaves to re-establish the equilibrium.

The effects of inorganic phosphate upon calcium asborption suggest that some deposition of calcium as phosphate salt may occur since the

effect of phosphate is not mediated through added phosphorylative systems. The small stimulus by ATP to calcium accumulation may then result from the liberation of phosphate from the nucleotide by the ATP-ase allowing calcium to be deposited as an insoluble phosphate. This effect, by removing calcium from solution would maintain a sink for calcium entrance.

The results of washing out calcium-labelled chloroplasts with water show that most of the cation is present as a water soluble component (a phosphate deposit may be part or all of this). Some 60% of the accummulated calcium is easily removed by water and the remainder is removed only on prolonged washing. This resistant fraction may indeed be water soluble but could also be the result of fragmentation of the plastids upon the filter by the forces applied during filtration. Of the water soluble fraction a portion equivalent to some 6% of the total calcium absorbed may be present as a protein-cation complex (i.e. that precipitated by TCA). The association must however be a loose one since no combination of protein and calcium was found upon fractionation using Sephadex columns. The amounts extracted by EDTA suggest that only 5% of the calcium is tightly bound to the chloroplast and further the amounts extracted with calcium and ferric chloride solutions suggest that 30% of the total cation accumulated is bound but is readily exchangeable. The increased efficiencies of HCl and NaOH solutions over water as extracting solvents also suggests that the calcium is bound to a chloroplast component, possibly a protein. These results all indicate that some form of binding between chloroplast components and calcium is occurring. Some

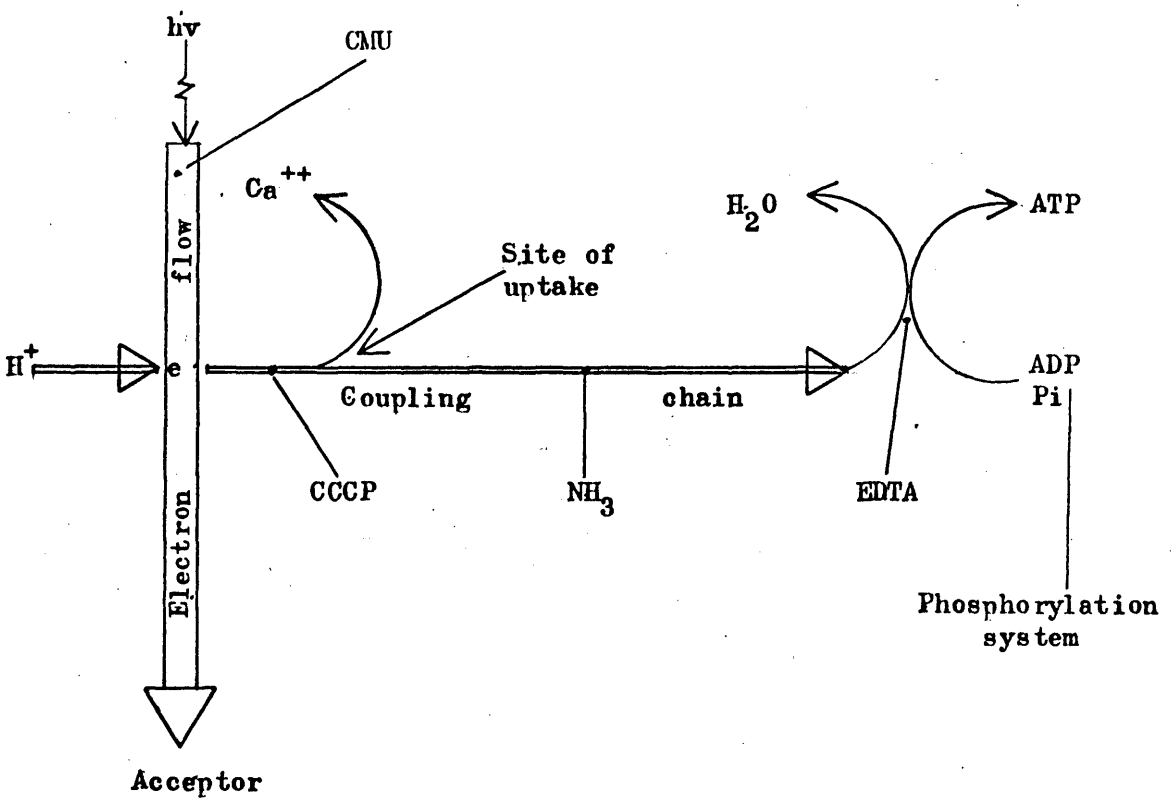
6% of the accumulated calcium is found associated in a chloroform/ methanol soluble fraction and this may represent calcium incorporation into a lipid or chlorophyll fraction.

The amounts of chloride accumulated by pea chloroplasts initially are large enough to act as counterions to calcium, the subsequent efflux (in the light) of chloride is not followed by calcium influx. The counterion to calcium could thus lie in a system of negative charges present in the plastid.

In summary, the uptake of calcium by pea chloroplasts is related to electron flow rather than to phosphorylation or ATP-ase activity. As the decay times on storage of Hill reaction and calcium uptake are different, the connection between the processes could be indirect. The differential effects of ammonium chloride, CCCP, ATP, EDTA uncoupling and phosphorylative systems are consistent with this idea and that the mechanism may lie very close to the site of coupling between electron flow and the phosphorylative enzyme. A possible location is indicated in figure 27.

The proposed sites of uncoupling are in figure 27 indicated by the hierarchy of action of uncouplers upon light-induced conformational changes and the results of Gromet-Elhanan and Avron (197). CCCP by uncoupling at the start of the coupling chain sequence prevents any changes occurring in the proposed related pathway concerned with calcium uptake. Ammonium chloride by operating after the branch point of the coupling chain and calcium pathway allows calcium uptake to continue and may stimulate uptake in the light by diverting more flow through the

Figure 27 Proposed site of calcium uptake in pea chloroplasts



• ——— Possible site of action

side branch. The inhibitory effects of high ammonium concentrations upon calcium uptake may be due to a cation competitive effect. The inhibition of calcium accumulation by added phosphorylative systems on this proposed scheme, would result from the chelating properties of the added nucleotides rather than from a competitive effect. This conclusion is strenthened by the fact that both uptake in the light and dark is reduced to approximately the same level indicating the absence of any effect of photophosphorylation. EDTA also acts after the proposed site of uptake and allows flow through the relevant part of the chain. The stimulatory effect of ATP suggests that a small part of the calcium uptake may be mediated by an ATP-ase system. The absence however, of any light-triggering effects and the inhibitory action of an added ATP-ase system, implies that the effect of ATP upon uptake is not mediated by an ATP-ase system.

The previously reported instances of calcium uptake into chloroplasts (155) (199) differ markedly from the calcium accumulation reported here. Brown, using pea chloroplasts, could find no evidence for a light-activated calcium uptake. This may be due to the rapid decay of uptake capacity in his material. The light-activated calcium absorption reported by Nobel is mediated by an ATP-ase system. It differs from the work reported here in so many aspects as to provide further evidence that the calcium accumulation in pea chloroplasts is not mediated by the same mechanism as in spinach chloroplasts. Some of the differences between calcium uptake in pea and spinach chloroplasts are illustrated in table 34.

Table 34 Comparison of calcium uptake by spinach and pea chloroplasts

Feature		Spinach	Pea
pH optima	(Light	7.9	8.5
	(Dark	7.0	8.0
Saturating Light (Lux)		30,000	400
Saturating calcium concentration (Light)		1mM	1 - 2.5 mM
Optimum ATP concentration		3mM	50μM (approx)
Light/Dark ratio		6	2
Stoichiometry to electron flow (Ca:2e)		0.05	0.58
Omission of P_i		>95% inhibition	20% inhibition
Requirement for			
	Magnesium	Yes	No
	PMS	Yes	No
	Sulphydryl group	Yes	No
Chelating agents		No effect	Inhibition
Stability of accumulated calcium		>90% firmly bound	<10% firmly bound
Light triggered		Yes	No

The function of the ions accumulated by the chloroplast may lie in the cofactor requirements of the photosynthetic reactions. Thus chloride is known to be an essential factor for oxygen evolution. The role of calcium is less certain. A calcium-requiring ATP-ase is known to be present in chloroplasts (158) and insoluble calcium phosphate salts are also present. Calcium may therefore regulate the delivery of phosphate to the photophosphorylation system. As the internal pH of the chloroplast changes on illumination this would result in changes in the form of phosphate to an extent determined by the level of calcium. Also in the dark, the cytoplasm and mitochondria would be unable to sequester phosphate from the insoluble calcium phosphate deposits in the chloroplasts. Calcium is also known to be required for the maintenance of membrane structure and function and may thus play a part in maintaining the large membrane systems of the chloroplasts.

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