# ION TRANSPORT AND PHOTOSYNTHESIS
# OF *Elodea densa*

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Doctor of Philosophy

by

WILLIAM HENRY BETTS, B.Sc.(HONS.)

Department of Botany
University of Adelaide

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## DECLARATION

This Thesis contains no material that has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge contains no material previously published or written by any other person, except where due reference is made in the text.

W.H. BETTS

16.3.79

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# SUMMARY

The work described in this Thesis is concerned with establishing mechanisms for ion transport and photosynthesis in *Elodea*. Although *Elodea*, and other aquatic angiosperms, show a remarkable similarity to the giant-celled algae, such as *Chara* and *Nitella*, in patterns of $HCO_3$ use and $OH^-$ efflux, there are essential differences. Models are presented that account for these observed differences in *Elodea*.

The work initially required a lengthy and detailed review of the published literature. As a large proportion of the literature on the appropriate aspects of the physiology of *Elodea* and other aquatics had been published in the early part of this century and mostly in foreign journals, this reviewing involved large amounts of translation into English. Considering the time interval between these early studies and the present work, much of the experimental work described in the Thesis was done to confirm or otherwise this earlier work using more sophisticated and quantitative methods, such as the use of radioactive tracers, pH and $O_2$ electrodes.

At high external pH values, normally found where *Elodea* is growing, photosynthesis, using $HCO_3^-$ as the exogenous carbon source, cannot proceed in the absence of $Ca^{++}$ in the intercellular phase of the leaf tissue. This differs from the established view that a majority of the $Ca^{++}$ is intracellular, and that during $HCO_3^-$ fixation is transported actively through the cells from the lower to the upper surface of the leaf. The model presented differs in that

$Ca^{++}$ moves from the lower to the upper surface in the intercellular phase. $Mg^{++}$, $K^{+}$ and $Ba^{++}$ are not able to replace $Ca^{++}$ in *Elodea*, as previously found in this and other aquatics. At low pH, photosynthesis ($CO_2$ fixation) occurs at very high rates in the presence and absence of $Ca^{++}$.

In an analogous way to the "pH banding" in *Chara*, *Elodea* has a hydroxyl efflux at the upper leaf surface which causes $CaCO_3$ precipitation, during $HCO_3^-$ fixation. In experiments to study this hydroxyl efflux, it was also found that when net photosynthesis was reduced by low exogenous carbon, there was a very pronounced $H^+$ efflux, which caused the pH of the experimental solution to fall to very low values. It is suggested that this proton efflux is partly due to the formation of carboxylate salts ($K^+$) in the lower cell layer. Furthermore it is suggested that this proton efflux also occurs during $HCO_3^-$ fixation although masked by the $OH^-$ efflux; and that it may act as a primary chemiosmotic energy source as suggested by Smith (1970).

Studies on the light-stimulated $Cl^-$ influx suggest that the transport may only occur when both photosystems are functioning normally and producing ATP and NADPH. However it is further suggested that such anion influxes may be influenced by the more important and larger exchanges of $H^+$ and $HCO_3^-$, and to some extent intercellular $Ca^{++}$.

The models proposed for *Elodea* accounting for the more complex behaviour of carbon assimilation probably arise because in the case of this aquatic angiosperm we are dealing with highly organised multicellular tissue, but with the charophyte algae, only single cells.

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To my wife Lee

# CHAPTER ONE

## INTRODUCTION

### 1.1 A Well-known Experiment

A simple experiment demonstrates the ability of *Elodea densa* to change the pH of its surrounding solution. Prepare a dilute solution of phenol red in distilled water and add dilute sodium bicarbonate so the solution pH is just below the end point where it changes from yellow to pink. Take a small sprig of *Elodea* that has been in the dark, rinse it several times in the above solution and then submerge it completely in the solution and allow to stand in a bright light. After only several minutes the solution adjacent to the upper leaf surfaces will change to bright pink, indicating an increase in pH, and after about 20 minutes the entire solution will change to pink. The original yellow colour may be obtained again by placing the sprig in the dark. This is a much slower process and may require up to three hours.

This experiment demonstrates quite clearly the ability of *Elodea* to increase the pH of its bathing solution in the light, and in particular, that the upper leaf surfaces are responsible for this change. Similar experiments have also been used to demonstrate the ability of other aquatic angiosperms (*Vallisneria*, *Potamogeton*, etc.) and giant celled algae (*Chara*, *Nitella*, etc.) to similarly change the solution pH during photosynthesis.

### 1.2 Early Work using Aquatic Angiosperms

Although the work of Arens, Ruttner and others is commonly cited, the details of their experiments have generally been over-

looked, possibly due to the difficulty in obtaining translations from the language in which they were written.

Since there has been surprisingly little experimentation done on this aspect of aquatic angiosperms since the early work of the 1930's, the details of these experiments are quite relevant to the development of this thesis. Furthermore it demonstrates quite clearly how little our understanding of this aspect of aquatic plant physiology has progressed over the past fifty years.

The "Well-known Experiment" (1.1) was first performed by F. Ruttner in 1921 using phenolphthalein rather than phenol red, and Ruttner described the ability of the upper leaf surfaces to change the solution pH as "physiological differentiation", recognising the cellular differences that must exist between the upper and lower layers that result in this differentiation.

However, Arens (1933), after carrying out extensive studies on this process, referred to the phenomenon as "polarised mass action exchange" because there was an apparent net transport of compounds across the tissue. When similar observations were made on *Nitella flexilis* whereby bands of high pH were observed along the length of these giant algal cells, Arens described the process as "physiological multi-polarity" recognising now that even within a single cell, one can find the different processes of transport going on in different locations and in different directions.

Although similar processes appear to occur in both aquatic angiosperms and giant algal cells, it would appear that in the angiosperms the physiological processes must necessarily be more

complex because we are dealing with multicellular tissue. Despite this complexity, most of the early experimental work on carbon sources for photosynthesis and related ion transport requirements was performed on these tissues. This was due to the relative ease by which the upper and lower surfaces of some angiosperms (*Potamogeton* and *Vallisneria*) could be isolated.

### 1.2.1 The Experiments of Arens

In similar experiments to that described above (1.1), Arens was able to show that a large number of other aquatic angiosperms exhibit similar abilities to alkalinize solutions only at their upper leaf surface in the light and also that some plants (*Potamogeton pectinatus* and *Vallisneria spiralis*) were able to increase solution pH on both upper and lower sides (Table 1.1). It is interesting to note how clearly Arens was able to differentiate colour changes at the top (adaxial) and bottom (abaxial) of the leaves and to show that there were differences between species of *Potamogeton*. 0.2% v/v chloroform and 0.4% v/v ether reversibly inhibited the adaxial pH increase in the light with *Elodea canadensis*, *Hydrilla verticillata* and *Potamogeton crispus*. These three species also showed red colour changes in $HCO_3^-$ solutions of Sr, Mg, Ba, Li, K and Na (Table 1.2). Final solution pH, also measured in these experiments, increased over the course of time. However, the ability of these species to survive in these solutions over a period of time differed greatly, and survival was in the following decreasing order Ca > Sr > Mg > K > Na > Li > Ba, (see Table 1.15, below).

In a further experiment, Arens showed that an adaxial pH change could be observed in solutions of $CaCl_2$, $Ca(NO_3)_2$, KCl, and $KNO_3$, in both *Elodea canadensis* and *Potamogeton crispus*, but that the extent of the observed changes depended on the calcium concentration of the pretreatment solution (Table 1.3). Similar observations were made in distilled water, and distilled water without $CO_2$ (Table 1.4). In this experiment it is important to note that the pH changes were greater in distilled water where $CO_2$ had been removed, suggesting that where $CO_2$ may be available for photosynthesis the observed adaxial pH changes are reduced. The differences between the resultant pH changes due to the pretreatment in different calcium concentrations suggested to Arens that leaf tissue was able to hold reserves of various ions that enabled the pH changes to occur in $HCO_3^-$-free solutions.

As a result he showed that leaves soaked in 10mM $CaCl_2$ showed a decreasing ability to produce $O_2$ and to change solution pH with time of soaking (Table 1.5). He attributed this to depletion of $CO_2$ from $HCO_3^-$ for photosynthesis and suggested that *Elodea densa* had greater reserves of $HCO_3^-$ than *Potamogeton crispa*.

When *Elodea*, treated to remove endogenous carbon reserves, was placed in various solutions for a further pretreatment before being placed in experimental solutions, the solution pH only increased when $HCO_3^-$ was supplied in either the pretreatment or experimental solution (Table 1.6). The pH change was greater when the $HCO_3^-$ was in the experimental solution. *Elodea* pretreated in solutions with $HCO_3^-$ was only able to slightly change solution pH in distilled water without any $CO_2$, but when small amounts of $CO_2$

were present, the solution pH did not change at all. Similarly, leaves pretreated in distilled water containing $CO_2$ showed a much reduced pH change in the $HCO_3^-$ solutions than leaves pretreated in $HCO_3^-$ solutions.

There was no significant effect of temperature in the range 10 to 34$^\circ$C on the $O_2$ production and solution pH change with *Elodea densa*.

Having demonstrated a polarisation between the upper and lower surfaces of the leaves in their ability to change the solution pH, Arens demonstrated that there was also polarity in the production of starch between chloroplasts in the upper and lower layers of cells of *Elodea* and *Hydrilla* (Table 1.7). He found that more starch was formed in the chloroplasts of the upper layer of cells.

Arens was not able to demonstrate any polarisation of $O_2$ production between the upper and lower layers of cells in *Elodea*, *Potamogeton* or *Hydrilla*.

### 1.2.2 Isolation Chamber Experiments

Arens (1933) used small isolation chambers and other methods to separate the upper and lower surfaces of leaf tissue and consequently was able to expose the two surfaces to different solutions at the same time.

In Table 1.8, the solution on the upper side was distilled water with phenolphthalein as indicator and it clearly demonstrated in all the species that there was a red change at the upper surface only when the solution on the bottom contained $HCO_3^-$. Phenol-

phthalein was also placed in the solutions at the lower surface, but no changes were observed here. The numbers indicate the percentage of leaves counted where the red change was observed. The leaves had been soaked in distilled water for 24 hours to exhaust the tissue reserves of endogenous carbon. In similar experiments where the lower surface was in contact with distilled water containing phénolphthalein and the upper the same solutions as Table 1.8, none of the leaves produced any significant pH change at either the upper or lower surfaces.

Hence Arens demonstrated quite successfully that $HCO_3^-$ was taken up into the tissue from the lower leaf surfaces in species of *Elodea*, *Hydrilla* and *Potamogeton*, and that it was this uptake which resulted in the pH change at the upper surfaces.

By measuring the presence of calcium by precipitation of calcium oxalate, and potassium by the precipitation of potassium-sodium cobalt nitrite, Arens was able to demonstrate that in the above species, calcium and potassium were transported from the lower to the upper surface in the light but not in the dark (Table 1.9).

From this work Arens modified the hypothesis of Nathansohn (1907) and Gicklhorn (1927) - see Figure 1.1 - where $Ca^{++}$ played no active part in the metabolism and $HCO_3^-$ acted as a supply of $CO_2$ that was constantly removed by the leaves by absorption from the upper surface. Arens' model (Figure 1.2) suggested that $Ca(HCO_3)_2$, $Ca^{++}$, $HCO_3^-$, and $CO_2$ all entered from the lower surface and that $Ca(OH)_2$ was transported across the leaf to the upper surface, while $O_2$ was released at both surfaces.

Arens (1938) then went on to do some valuable work examining the distribution of the $Ca(OH)_2$ extrusion sites on the upper surface (Table 1.10). These sites were identified by manganese hydroxide precipitation, and he showed that the area of precipitation on each leaf increased downwards from apex - with increasing cell age. This was important in that it demonstrated that not all cells in the leaf can equally utilise $Ca(HCO_3)_2$ but that this ability increases with age.

The work of Arens has been taken as a starting point for this dissertation because it points to many interesting questions that investigators have since been attempting to answer.

### 1.2.3 Polar Transport of Ions

Although Arens found that the leaves of some aquatic plants could absorb $Ca(HCO_3)_2$ or $KHCO_3$ from the lower leaf surface while $Ca(OH)_2$ or KOH respectively was released at the upper surface, other workers (Gessner, 1937; Steemann Nielsen, 1947) concluded that $HCO_3^-$ can actually be absorbed from both surfaces. Steemann Nielsen measured the $O_2$ production when exogenous carbon (either $CO_2$ or $HCO_3^-$) was available at either of the two surfaces of *Potamogeton lucens* and compared these with the rates when exogenous carbon was available at both sides simultaneously. His results appear in Table 1.11.

From the details of the experiment, however, it is not clear what the relative concentrations of $HCO_3^-$ and $CO_2$ were, and it is possible that sufficient $CO_2$ was available at all times to diffuse into the cells. Hence these experiments did not conclusively prove that $HCO_3^-$ can enter from both the upper and lower surfaces.

Steemann Nielsen (1947) repeated Arens' experiments and found that at pH 8.0, $OH^-$, $CO_2$ and $HCO_3^-$ were transported from the lower to the upper surface when either natural lake water or 5mM $KHCO_3$ were present in solutions at the lower leaf surfaces (Tables 1.12 and 1.13) confirming the earlier observations of Arens (1938). He was, however, unable to observe the polar transport of $OH^-$ to the upper surface in another species - *Potamogeton natans*, and also *Hydrocharis morsus ranae*. On the other hand he was able to show that photosynthesis in the marine alga *Ulva lactuca* was accompanied by pH increases on both sides of the thallus, no matter what side $HCO_3^-$ was supplied (Table 1.14).

In 1939, Arens observed banding on *Nitella flexilis*, which he attributed to the non-homogeneous transport of $OH^-$ from the cell during photosynthesis. He compared this phenomenon with the situation in *Elodea* and *Potamogeton*, where there were areas where $HCO_3^-$ was taken up and areas where $OH^-$ was released.

### 1.2.4 Growth Experiments

Although Arens (1933, 1938) demonstrated that cations such as Sr, Mg, Ba, Li, K, Na and Ca were able to sustain short-term photosynthesis of aquatic angiosperms in the presence of $HCO_3^-$ at high pH (Tables 1.2 and 1.8), he also showed that the long-term survival of these plants was reduced with some of these ions. Survival was longest in $Ca(HCO_3)_2$ and shortest in $Ba(HCO_3)_2$ following the order Ca, Sr, Mg, K, Na, Li, Ba in all species. Survival was shortest in *Elodea* that had been grown in a pond containing a higher $Ca^{++}$ concentration (Table 1.15).

The effect of pH on the growth of *Elodea densa* was first demonstrated by Olsen (1921). Plants grown at pH 3.5 and 4.5 died within the 60 day study, whereas plants grown at pH 5.5, 6.5, 7.5, and 8.0 showed respective fresh weight increases of 130, 165, 220, and 320 percent (see Table 1.16). Steemann Nielsen (1944) repeated these experiments using *Elodea canadensis* and *Ceratophyllum demersum*. He critidised Olsen's experiments on the grounds that the experimental conditions were not optimised to demonstrate the maximum pH effect on growth. Steemann Nielsen obtained much greater increases in fresh weight over shorter periods of time. The results for these growth studies are shown in Tables 1.17 and 1.18. With both species there was an increase in fresh weight as the pH was increased from 3.5 to 8.5, under conditions of low $CO_2$ (0.03%). In the presence of excess $CO_2$ ( > 2%), there was no difference in the fresh weight between pH 4.5 and 8.0. When there was no stirring of the solutions with 0.03% $CO_2$, the growth of *Ceratophyllum* was reduced even further, due most probably to the reduction in the rate of diffusion of not only $CO_2$ but other solutes (Gessner, 1940; Steemann Nielsen 1942). Note also that *Elodea* showed a decrease in the ratio of fresh weight to dry weight with increasing pH under both limiting and high $CO_2$ levels. Steemann Nielsen suggested that this was probably due to different assimilates in the tissue - that at high pH, sugars were probably formed. The presence of sugar was demonstrated by anthocyanin formation at the higher pH values (Table 1.17). In an attempt to distinguish between the direct effects of pH, free $CO_2$ and $HCO_3^-$, Steemann Nielsen cultured both *Elodea* and *Ceratophyllum* under conditions of increa-

sing pH and $HCO_3^-$ concentration, while a constant $CO_2$ concentration (0.6 mg/l) was maintained. Under these conditions, an increase in $HCO_3^-$ from 0.006 mg/l to 0.03 mg/l (pH increase from 4.5 to 5.5) resulted in a dry weight increase of *Ceratophyllum* from 18 to 86% - the total carbon concentration only increasing by 16%. However, from pH 5.5 to 8.2, where the total carbon concentration increased 40 times, and $HCO_3^-$ increased a 1000 times, the increase in dry weight was only 3 fold. Hence, small increases in the $HCO_3^-$ concentration at levels less than the free $CO_2$ level, caused large changes in the weight increase of both species, and there was no direct effect of pH as such.

### 1.2.5 Sources of Carbon for Photosynthesis - $CO_2$ or $HCO_3^-$

Ruttner (1921) demonstrated successfully that while *Elodea* was able to assimilate carbon from alkaline solutions, the moss *Fontinalis*, was unable to do so. Steemann Nielsen (1947) demonstrated more clearly that *Myriophyllum*, *Potamogeton*, *Cryptocoryne* and *Cladophora* were able to utilise both $CO_2$ and $HCO_3^-$, but *Fontinalis*, along with other plants commonly found in more acid water, were only able to utilise $CO_2$ for photosynthesis. These experiments differed from his earlier growth studies in that he was now measuring actual $O_2$ production by sodium thiosulphate titration. This work neatly complemented Arens' more indirect methods of measuring photosynthesis. It furthermore showed that while weight increase and $OH^-$ extrusion were smaller in the presence of small amounts of $CO_2$, the presence of free $CO_2$ did increase the rate of $O_2$ production quite markedly compared to rates when $HCO_3^-$ was the only source of carbon.

### 1.2.6 Ions and Photosynthesis

While Arens (1933, 1938) was able to demonstrate that cations were able to sustain $OH^-$ release in many species, Steemann Nielsen (1947) was the first to measure the effects directly on photosynthesis as measured by $O_2$ evolution. He demonstrated that anions such as $Cl^-$ and $SO_4^=$ and cations such as $K^+$, $Na^+$ and $Ca^{++}$ had no influence on the rate of photosynthesis at pH 4.1 (Table 1.19), but that at pH 8.3 the presence of cations and anions did exert a differential effect. In particular, the addition of $Cl^-$ and $SO_4^=$ to $K^+$ and $Ca^{++}$ solutions increased $O_2$ production from 79% to 94% of the maximal rate observed in natural lake water (Table 1.20).

## 1.3 Further Experiments with *Potamogeton*

### 1.3.1 Tracer Experiments

Tracer experiments, using $^{45}Ca^{++}$, were first used by Lowenhaupt (1958). He was able to confirm the earlier experiments of Arens and Steemann Nielsen, that in the light $Ca^{++}$ was accumulated into the lower surface of *Potamogeton crispus* leaves and excreted at the upper surface. He further showed that the $^{45}Ca^{++}$ content of leaves increased in the light compared with the dark. It is unfortunate however, that he made no further attempt to quantify these results. In another experiment, he demonstrated that the uptake of $^{45}Ca^{++}$ was greater at pH 7 than at pH 3.8. The pH in this case was increased from 3.8 to 7 by the addition of 8 mM $K_2CO_3$, and then the solution was bubbled with $N_2$ (or air) causing

the pH to fall again. However, in the first hour when the pH would have been sufficiently high, he found a twofold increase in the $^{45}Ca^{++}$ content of the leaves.

Further experiments using tracers (Helder and Boerma, 1972), demonstrated that in *Potamogeton lucens* $Rb^+$ may be transported across the leaf from the lower surface against a $Rb^+$ concentration gradient. Although Steemann Nielsen (1960) had not demonstrated a potential difference across *Potamogeton*, Helder and Boerma concluded that the $Rb^+$ transport was active and that the rate of transport was independent of the concentration gradient, or at least coupled to an active transport of anions, most probably the influx of $HCO_3^-$ during photosynthesis. For there to be a net polar transport of anions, as observed, they suggested that the lower cell layer was more permeable to $HCO_3^-$ and the upper cell layer more permeable to $OH^-$. Such net polar transport of anions may then be accompanied by polar transport of cations (Helder and Boerma, 1972, 1973).

### 1.3.2 Potential Measurements across Leaves

More recently Helder (1975a) measured a potential difference (PD) across *Potamogeton* leaves of about 25 mV in the presence of 1.0 mM $KHCO_3$. This PD was abolished in the dark or by 10 μM DCMU. The net transport of labelled $Rb^+$ under the influence of this PD was much greater than that predicted by the Nernst equation, suggesting an active transport of $Rb^+$. However on comparing the values for the $Rb^+$ flux in the upward and downward directions with the theoretical ones derived from the simplified Ussing-Theorell equa-

tion, Helder found it necessary to propose the simultaneous presence of an active unidirectional $Rb^+$ flux, possibly occurring in the symplasm of the leaf, and a possible flux, bidirectional, restricted to the apoplast.

### 1.3.3 External pH Changes

Following Arens' (1933, 1938) and Steemann Nielsen's (1947) experiments, Helder and Zanstra (1977) measured continuously the pH of solutions at both the upper and lower leaf surfaces using a glass pH electrode and found that when $HCO_3^-$ was supplied to both surfaces the pH of the upper solution increased to 11.8 after about 30 hours, confirming all previous work done under similar conditions. However, they also showed that the pH of the lower solution increased to a steady value of 9.8 when the upper solution only contained $HCO_3^-$.. This agreed with Steemann Nielsen's observations (1947) that $O_2$ evolution of *Potamogeton* will proceed when $HCO_3^-$ is supplied to the upper surface only (1.2.3.).

As pointed out by the authors, there was a curious inflexion in the curve of the pH change at about four minutes after the light was switched on. The authors suggested that this phenomenon may be related to a switching from $CO_2$ to $HCO_3^-$ utilisation by the leaf.

Furthermore, they also observed a fall in the pH at the lower surface from 8.4 to 6.8 when $HCO_3^-$ was supplied to both surfaces. They suggested that this was not primarily due to $HCO_3^-$ assimilation but to the influx of either cations or other alkaline substances, or the release of some acid substance - these exchanges being dependent on $HCO_3^-$ absorption and assimilation. Although they did

describe a fall at both surfaces but ascribed this change solely to the production of $CO_2$ by respiration.

## 1.4 Photosynthesis

### 1.4.1 Bicarbonate Assimilation

As already shown, some aquatic angiosperms appear to utilise $HCO_3^-$ for photosynthesis rather than $CO_2$ under conditions of low or zero $CO_2$. However, there are possibilities of misinterpretation of the experimental data, as pointed out by Raven (1970). For example, it is possible that $HCO_3^-$ may be acting as a reservoir for exogenous $CO_2$. To overcome these problems, it may be necessary to perform estimates of photosynthesis - either $O_2$ production or carbon fixation - at low pH where 90% of the total inorganic carbon exists as $CO_2$, and at a higher pH where 95% of the total inorganic carbon exists as $HCO_3^-$. If the photosynthetic rate is greater at the higher pH than can be accounted for by the remaining 5% of $CO_2$, then $HCO_3^-$ must be acting as a primary exogenous source for photosynthesis. This has been shown for a large number of species (Raven, 1970; Smith, 1968; Jones and Osmond, 1973).

When the $HCO_3^-$ has entered the cell, carbon is supplied to the chloroplasts by disturbing the equilibrium reaction, $HCO_3^- \rightarrow OH^- + CO_2$, as $CO_2$ in the chloroplasts is continually utilised by ribulose-1,5 bisphosphate (RuBP) carboxylase in the case of $C_3$ photosynthesis or phosphoenolpyruvate (PEP) carboxylase in $C_4$ photosynthesis (Hatch and Slack, 1970). This process as it applies

to *Elodea* will be discussed in more detail in 1.4.3. Provided that fixation results in the formation of sugars rather than organic acids such as malic or oxalic acid, which may be stored in the vacuoles, for each mole of $HCO_3^-$ fixed, one mole of $OH^-$ is produced in the cytoplasm. This $OH^-$ production must result in an $OH^-$ efflux or an $H^+$ influx if cytoplasmic pH is to be maintained at a constant level (Smith and Walker, 1975).

Ruttner (1947) used an alternative method of demonstrating $HCO_3^-$ utilisation in *Elodea*. He demonstrated that the leaves could increase the pH of an $HCO_3^-$ solution at a much greater rate than could be done by bubbling with $CO_2$-free air, whereby $HCO_3^-$ was converted to $CO_2$ by the shift of the equilibrium reaction. In the current work (chapter 4), these results were confirmed using the former method.

Although recognising that *Elodea (Egeria) densa* and *Lagarosiphon major* were able to produce high solution pH levels in field experiments in the light, Brown, Dromgoole, Towsey and Browse (1974) were unable to demonstrate that these two species (and *Elodea canadensis*) were able to utilise exogenous $HCO_3^-$ in laboratory experiments. As soon as the free $CO_2$ concentration was reduced to zero, they found that photosynthetic gas exchange and $^{14}C$ incorporation ceased. In addition, they found that in their laboratory experiments, the pH drifts indicated "acid" leaching ($H^+$ efflux) rather than the "base" leaching ($OH^-$ efflux), observed by others (Ruttner 1947), and expected if $HCO_3^-$ was being utilised. They suggested a possible explanation for this difference in behavior of field and laboratory plants. Whole plants (field) have

intact air spaces joining the leaf tissue to the roots, from which $CO_2$ may move (Wium Anderson, 1971) and under these conditions increases in the lake water pH may come about through base excretion ($OH^-$) following photosynthesis of this "non-local" substrate.

A further method for determining whether $HCO_3^-$ is used for photosynthesis is to determine the $CO_2$ compensation point. This is the calculated $CO_2$ concentration when pH changes in the light are maximal. This pH is referred to as the "compensation pH" and the $CO_2$ compensation point is also the $CO_2$ concentration at which $CO_2$ uptake for photosynthesis is balanced by $CO_2$ produced by respiration, and this value may be compared with the value obtained at a low pH where $CO_2$ is the only inorganic carbon source. If the above "compensation pH" is higher than the calculated "compensation pH" - estimated from the $CO_2$ compensation at low pH - then $HCO_3^-$ assimilation must account for this increase. It must be noted that one limitation of this analysis is that it is assumed that there are no other cellular processes that may change solution pH - such as ammonium utilisation in nitrogen assimilation or other cation and anion fluxes (Helder and Zanstra, 1977). Photorespiration is another process that may affect the $CO_2$ compensation point (Servaites and Ogren, 1977). Using calculated values of the equilibrium constants of the $CO_2/HCO_3^-/H_2CO_3$ equilibrium system, Lucas (1975) demonstrated that $CO_2$ could not be supplied at a sufficient rate by the dehydration of $H_2CO_3$ near the cell surface to account for the rate of carbon assimilation of *Chara corallina* at pH 9.0. These calculations assumed that there was no carbonic anhydrase activity near the cell surface and points to a further considera-

tion. Is it possible that carbonic anhydrase produced in the cell wall can catalyse the production of $CO_2$ from exogenous $HCO_3^-$ to levels greater than those predicted by normal equilibrium (Buch, 1960)? Evidence for extracellular carbonic anhydrase is slight (Litchfield and Hood, 1964; Bradfield, 1947). Findenegg (1976) was unable to find active carbonic anhydrase in the cell wall fraction of *Scenedesmus obliquus*, although he was able to find it in the cytoplasmic fraction and he demonstrated that this fraction was important in regulating the use of $HCO_3^-$ and $CO_2$ for photosynthesis (Graham and Reed, 1971; Rybova and Slavikova, 1974). Raven (1970) suggested that this would have been a more efficient action of carbonic anhydrase as extracellular enzyme would still only be able to supply a low equilibrium concentration of $CO_2$ at the cell wall when the pH of the surrounding medium was high.

### 1.4.2. Products of Assimilation

The pathways of carbon fixation following assimilation of $HCO_3^-$ or $CO_2$ have not concerned most investigators studying photosynthesis in conjunction with light-stimulated ion fluxes in aquatic plant cells. Such research is concerned more directly with ion transport, membrane potentials and other cellular and membrane processes, while the analysis of carbon incorporation involves special analytical techniques such as thin layer chromatography (Brown, et al, 1974; Degroote and Kennedy, 1977), and gas-liquid chromatography (Phillips and Jennings, 1976). Consequently, information on the possible alternative pathways of carbon fixation in Characean cells and aquatic angiosperms is scarce. In

*Chara*, Lucas (1975) assumed that the first carboxylating step was typical of $C_3$ photosynthesis because the $K_m$ of $CO_2$ fixation (0.7 mM) was similar to the $K_m$ of RuBP-carboxylase (0.45 - 0.54 mM) *in vitro* (Cooper, Filmer, Wishnick and Lane, 1969; Walker, 1973). However, the value of 0.7 mM found by Lucas was probably much too high because $CO_2$ had to diffuse through unstirred layers (Smith and Walker, personal communication). *Elodea* also shows variation in the rate of photosynthesis depending on the diffusion pathway of $CO_2$ (Hill and Whittingham, 1958). This suggests that any attempt to compare the $K_m$ values of fixation rates with the $K_m$ values of carboxylating enzymes may be extremely misleading.

Assimilation of $CO_2$ during $C_3$ photosynthesis need involve no charge imbalance or large scale change in intracellular pH. Where the cells absorb $HCO_3^-$, as in some aquatic plants, dissociation into $CO_2$ + $OH^-$ can be followed by $OH^-$ loss to the bathing solution: there need be no net accumulation of inorganic cations if the products are uncharged (sugars, starches). In CAM plants, the initial carboxylation is into organic acids which are stored in the vacuole, the pH of which is lowered. Again there is no net transport of inorganic cations from the external phase. In $C_4$ plants, the organic acids formed in the mesophyll cells are transported to the bundle sheath cells where decarboxylation and normal $C_3$ photosynthesis occurs. $H^+$ must also move from the mesophyll to the bundle sheath cells (or $OH^-$ vice versa) to maintain pH at normal levels, and also maintain electroneutrality (Smith and Raven, 1979). Again net accumulation of inorganic cations does not occur.

In contrast when carboxylate salts are stored in vacuoles as found in root cells, pH regulation and electroneutrality are maintained by net $H^+$ efflux or $HCO_3^-$ influx (or both) and net influx of inorganic cations such as $K^+$. The above processes have been discussed in the context of pH regulation by Smith and Raven (1979).

Considering again aquatic plants, it becomes obvious that a knowledge of the type of carbon fixation may be very important in understanding the mechanisms of exchange of cations (including $H^+$), $HCO_3^-$, $OH^-$ and other anions. In short, "photosynthesis" and "ion transport" should not be divorced from each other, as is commonly the case. Furthermore, there may be interactions between the accumulation of photosynthetic assimilates (e.g. carboxylates) and inorganic ions (e.g. $K^+$, $Cl^-$).

### 1.4.3. Photosynthesis by *Elodea*

There is some evidence that the first products of *Elodea* photosynthesis are $C_4$ acids. Brown et al (1974) showed that after 4 seconds, 30% of total, recovered, fixed $^{14}CO_2$ was in $C_4$ acids in *Elodea (Egeria) densa* at pH 4.5 and that after 1000 seconds this amount had dropped to 27%. At 4 seconds sugars and PGA showed 15% and 11% incorporation respectively, but at 1000 seconds sugars had risen to 63%. This contrasted to the situation at pH 9.2, where after 3 seconds, 85% appeared as PGA and only 5% as $C_4$ acids; and after 1000 seconds 7% as PGA and 47% as $C_4$ acids.

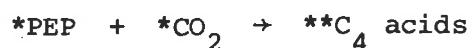
The authors conclude that $^{14}CO_2$ incorporation into $C_4$ acids may have occurred through two mechanisms.

1. $C_4$ photosynthesis

PEP + $*CO_2$ → $*C_4$ acids → *PGA

2. CAM type fixation

RuBP + $*CO_2$ → *PGA → *PEP



*PEP + $*CO_2$ → $**C_4$ acids

The normal CAM fixation did not appear to occur, as light was required for significant $CO_2$ incorporation and in normal CAM fixation, $C_4$ acids are incorporated in the dark. As *Elodea densa* leaves have air spaces in the central vascular bundle, the authors suggested that typical $C_4$ chloroplasts may exist in cells around these air spaces. Hocking, Anderson and Pallaghy (1975) found similar incorporation of $^{14}CO_2$ into organic acids (malate and aspartate) in *Elodea*, that persisted in time, and suggested that such incorporation could be accounted for by the superficial similarity with the Kranz-type anatomy typical of $C_4$ plants. They found that the chloroplasts in the large cells surrounding the central vascular strand were larger in diameter, and contained more extensive starch reserves than the chloroplasts of the small leaf blade cells.

Similar findings were obtained in *Elodea canadensis* (Degroote and Kennedy, 1977) at pH 6.5 and the authors associated this with significant levels of PEP-carboxylase of 14.2 µmoles/mg Chl/hr.. However, they found even higher levels of RuBP-carboxylase of 27.9 µmoles/mg Chl/hr., and they suggested that under various conditions, one, the other, or both of these enzymes may be fixing carbon. The $C_4$ metabolism may be important in maintaining an acid cytoplasm that would result in rapid protonation of $HCO_3^-$ to $H_2CO_3$. However, in contrast to Hocking et al (1974) they found that the

chloroplasts were more typical of $C_3$ plant chloroplasts than Kranz-type chloroplasts.

More recently Browse, Dromgoole and Brown (1977) found that at high levels of exogenous $CO_2$, 90% of the incorporated $^{14}CO_2$ was found in PGA, sugar phosphates and sugars that only 5% was found in malate. This suggested that the Calvin cycle was the primary carboxylation mechanism rather than $C_4$ or CAM type assimilation. They suggested the differences between these results and those previously mentioned were due to the high $K_m$ of the Calvin-cycle enzymes compared to the lower $K_m$ for PEP-carboxylase and other $C_4$ and CAM enzymes. However, these differences may also be due to differences in stirring, causing limitations in $CO_2$ diffusion. This again points to the need for careful experiments on the effect of total exogenous carbon - both $CO_2$ and $HCO_3^-$ and the effect of pH and solution mixing.

## 1.5 Proton Pumps

### 1.5.1 The Proton Influx During $HCO_3^-$ Assimilation

The existence of an apparent $H^+$ influx or $OH^-$ efflux during photosynthesis has been well established for *Chara corallina* (Lucas, 1975a, 1976; Lucas and Smith, 1973; Lucas, Ferrier and Dainty, 1977), for aquatic angiosperms such as *Potamogeton* and *Elodea* (Arens, 1933; Steemann Nielsen, 1947; Hope, Lüttge and Ball, 1972; Helder and Zanstra, 1977), for *Chlamydomonas reinhardii* (Schuldiner and Ohad, 1969; Atkins and Graham, 1971), *Dunaliella parva* (Ben Amotz and Ginzburg, 1969), *Ulva lactuca* (Cummins, Strand and Vaughan, 1969),

and other plants (Brinkman and Lüttge, 1972). Such a proton influx is normally reversed in subsequent dark periods.

Several mechanisms have been proposed for this apparent light-induced proton influx.

1. The changes may be caused by the light-induced $H^+$ uptake by chloroplasts, as observed in higher plants (Packer, Murakami and Mehard, 1970; Ben-Amotz and Ginzburg, 1969).
2. There may be $CO_2$ uptake followed by $HCO_3^-/CO_2$ re-equilibration in the external solution:

$$HCO_3^- \rightleftharpoons CO_2 + OH^-$$

3. The changes may be due to $HCO_3^-$ assimilation, that for each mole of carbon fixed, one mole of $H^+$ enters the cell. Such an $H^+$ influx helps maintain constant intracellular pH (Smith and Raven, 1976).

Atkins and Graham (1971) found that the ratio of added carbon (either $CO_2$ or $HCO_3^-$) : $H^+$ influx : evolved oxygen was 1:1:1 in *Chlamydomonas*, and Hope, Lüttge and Ball (1974) found a 1:1 ratio for $H^+$ influx/efflux : $O_2$ evolved/consumed for *Elodea canadensis* in the light/dark. In contrast, the relationship of $H^+$ exchange to $CO_2$ fixation was not 1:1, but quite variable (Hope et al, 1974).

However, it is generally agreed that the third alternative above is the one operating in cells that are utilising $HCO_3^-$, and that for each mole of $HCO_3^-$ fixed, either one mole of $H^+$ enters the cell or one mole of $OH^-$ leaves. "$OH^-$ efflux" is usually the

preferred term as it represents the loss of $OH^-$ formed from the reaction $HCO_3^- \rightleftharpoons CO_2 + OH^-$, occurring intracellularly. In this dissertation, the term "$OH^-$ efflux" is used to describe this pH change.

## 1.5.2 The Proton Efflux

In addition to the $OH^-$ efflux described above, Raven and Smith (1977) proposed that an active $H^+$ efflux mechanism is widespread in prokaryote and eukaryote cells, and that it serves several functions - the primary evolutionary one being to regulate cytoplasmic pH. The need for such a cytoplasmic "pH stat" during nitrogen metabolism, excess ion influx and hormone responses is well discussed by Smith and Raven (1976) and Raven and Smith (1976, 1977). In this appraisal of proton efflux pumps, I shall examine the evidence for their existence in relation to $CO_2$ and $HCO_3^-$ assimilation.

In *Chara*, Lucas and Smith (1973) demonstrated the existence of distinct acid regions between the alkaline bands. In these regions, the pH was as much as half a unit below the bathing solution pH, whereas the alkaline bands were as much as four units more alkaline. The acidic bands were abolished in the dark. This evidence was used to support the proposed electrogenic $H^+$ pump of Kitasato (1968). Its influence on the membrane potential is discussed in 1.6.

Steemann Nielsen (1947) observed in his isolation chamber experiments that the solution adjacent to the lower leaf surfaces of *Potamogeton* decreased from 8.2 to 8.12. Although this small

change could be accounted for by ion uptake during the experiment, Helder and Zanstra (1977) also found pH decrease from 9.8 to 8.9 in 12 hours at the lower surface of *Potamogeton*. Similar observations have also been made by Lowenhaupt (1954). In the experiments of Helder and Zanstra (1977), the pH decrease became more marked in the dark and they attributed this extra fall to the production of $CO_2$ by respiration. However, they also attributed the pH fall in the light to the intake of cations (principally $K^+$) at the lower surface.

Jeschke (1970) found a rapid fall in the solution pH in the light with *Elodea* leaves in solutions bubbled with air or $CO_2$-free $N_2$, which he attributed to $H^+$ excretion. Similar observations were found by Brown et al (1974) with *Elodea densa*, *Elodea canadensis* and *Lagarosiphon major* when the free $CO_2$ concentration fell to zero. Under these conditions a net $O_2$ uptake was also observed, suggesting that the pH change may be due to respiratory $CO_2$ release. Photorespiration also occurred to a large extent in these species. In these experiments it must be emphasised that although the free $CO_2$ levels approached zero, the $HCO_3^-$ levels remained significant. This was interpreted as these species' inability to fix $HCO_3^-$ as already discussed in 1.4.3..

As suggested by Smith and Lucas (1973), this proton extrusion may occur while $OH^-$ extrusion is occurring at another site, but such extrusion may be masked by the greater $OH^-$ efflux especially during $HCO_3^-$ fixation. Spanswick (1973) proposed that an electrogenic $H^+$ efflux comprises a large component of the large membrane (vacuolar) potential of -296mV for *Elodea canadensis* (see section

1.6). The existence of an $H^+$ efflux under conditions of low exogenous $CO_2$ is examined in chapter 5.

## 1.6 The Tissue Potential Differences

### 1.6.1 The Membrane Potential

The membrane potential of green cells is hyperpolarised in the light. Changes have been observed in leaves of higher plants (Lüttge and Pallaghy, 1969; Pallaghy and Lüttge, 1970), Characean cells (Saito and Senda, 1973, 1974; Vredenberg and Tonk, 1973; Spanswick, 1974; Lucas and Smith, 1973), aquatic angiosperm leaves (Jeschke, 1970; Bentrup, Gratz and Unbehauen, 1973; Spanswick, 1973; Prins, 1974). For reviews see Bentrup (1971) and Higinbotham (1973 ). The observed PD changes may be either transient, lasting only a few seconds, or stable, lasting several hours.

The membrane PD in some cases may be explained by the passive distribution of ions across the membrane as described by the Goldman equation, but in other cases, particularly the light hyperpolarised potentials, the values exceed the Goldman passive diffusion potential. This suggests a need for additional sources for the PD such as an electrogenic proton pump (Jeschke, 1970; Kitasato, 1968; Spanswick, 1973) or an electrogenic chloride pump (Prins, 1974).

### 1.6.2 The Membrane Potential of *Elodea*

Jeschke (1970) obtained estimates of the vacuolar potential ($E_{vo}$) of *Elodea densa* leaf cells using a fine (1-2 micron) glass

microelectrode. His estimates of between -120 and -160mV were much smaller than those obtained by Spanswick (1973) for *Elodea canadensis* (-296mV). Jeschke demonstrated that light caused a biphasic change in $E_{vo}$, composed of a rapid transient hyperpolarisation followed by a slower hyperpolarisation that reached a new equilibrium value in less than 5 minutes. Darkness resulted in a fast depolarisation. The light-dependent hyperpolarisation occurred in 0.5 mM $CaSO_4$ solutions in the presence and absence of KCl, and occurred in the pH range 4 to 10. However, at pH 4.0, the fast component was not a hyperpolarisation but a pronounced depolarisation. If the medium was changed from 5 mM KCl and 0.5 mM $CaSO_4$ to one containing only 10 mM $CaSO_4$, the rise of the slow phase was inhibited and equilibrium was reached only after 15 minutes. Spanswick (1973) only observed a single phase hyperpolarisation with about 20mV overshoot in the light with *Elodea canadensis*. Both DCMU (0.5 μM) and CCCP (2.5 μM) caused a rapid depolarisation of the membrane potential in the light in *Elodea densa* and inhibited the hyperpolarisation/depolarisation changes in subsequent light/dark changes. (Azide and $CN^-$ produced similar effects in *Elodea canadensis*).

Using the Goldman equation, Jeschke demonstrated that in the light, the membrane PD could not be accounted for by cytoplasmic exchanges of $K^+$, $Na^+$, $Cl^-$ and $H^+$, but depended possibly on membrane permeability changes (rapid phase) or electrogenic ion pumps (slow phase) - the latter being consistent with the rapid $E_{vo}$ changes at the end of the light phase and after the addition of inhibitors of photosynthesis. This explanation has also been used by many

other authors to account for the deficiency of the Goldman equation to describe the membrane PD of Characean cells (Kitasato, 1968; Spanswick, 1972; Richards and Hope, 1974).

### 1.6.3. The Effect of pH on the Membrane Potential

The effect of solution pH on the membrane potential may be evidence to support the view that the $H^+$ pump is electrogenic. The membrane potential of *Chara corallina* was temporarily hyperpolarised by 100 - 120mV when the solution was changed from pH 5.75 to values greater than 9 (Smith and Lucas, 1973). Smaller changes have been observed in *Nitella* (Saito and Senda, 1973 ; Spanswick, 1973). However such an effect was not observable with *Elodea densa* (Jeschke, 1970), where the pH effect on the dark membrane potential was consistent with the Goldman equation and the relatively high $K^+$ concentration to $H^+$ concentration ratio. A lack of a pH effect may also be due to an $H^+$ pump being independent of external $H^+$ concentration (Smith and Raven, 1979).

### 1.6.4 Potential Differences Between Acid and Alkaline Sites

As already described (1.3.2), Helder (1975) was able to measure a potential difference between the upper and lower surfaces of *Potamogeton* of up to 25mV (upper side negative) in the light. This PD was generated in the presence of $HCO_3^-$, presumably during $HCO_3^-$ fixation, although the pH of the solutions was not given. The PD was abolished by inhibitors of photosynthesis such as DCMU. The PD was a result of the net transfer of anions from the lower surface to the upper surface during photosynthesis.

Helder used the Nernst potential for $K^+$ in an attempt to determine whether the observed potential could account for the redistribution of $K^+$ ions. He found that the $K^+$ concentration was much greater than that predicted and suggested that $K^+$ was actively transported or coupled to electrogenic anion transport. Such an analysis however is inadequate. Under these conditions the Goldman equation is the correct equation, and should include terms for the $H^+$, $K^+$ and $HCO_3^-$ ions. The application of this equation, however, awaits determinations of the permeabilities of $K^+$, $H^+$ and $HCO_3^-$ for *Potamogeton* and the knowledge of the pathways through the leaf.

Similarly Walker and Smith (1977) found potential differences of up to 7mV between the acid and alkaline bands of *Chara corallina*. As these potential differences were abolished in the dark, they proposed that the current (0.5 - 1.0 μA per zone) between these regions was carried by $HCO_3^-$ and $H^+$ or $OH^-$ ions.

## 1.7 The Calcium Ion

### 1.7.1 "Membrane Integrity"

"Membrane integrity" has been used to refer to the role of calcium in maintaining the normal function of any cell membrane (Lucas and Dainty, 1977; Lucas, Spanswick and Dainty, 1978). In actual fact we have very little understanding of the actual role of $Ca^{++}$ in maintaining this integrity. It has been recognised for a considerable time that $Ca^{++}$ may reverse the toxicity of monovalent alkali salts to plants (Kearney and Cameron, 1902). Hanson

(1960) demonstrated that removal of calcium from roots of *Glycine max* and *Zea mays* by treatment with EDTA resulted in these roots losing their ability to absorb and retain solutes. Similarly, the absorption of $Rb^{+}$ by barley roots (Epstein, 1961) was impaired in solutions without $CaCl_2$, and Maas and Legget (1968) found that membranes became "leaky" to $K^{+}$ in the absence of $Ca^{++}$, so that $K^{+}$ followed its own diffusion gradient (Laüchli and Epstein, 1970). Similarly, $Ca^{++}$ was able to reverse the effects of low pH on $K^{+}$ absorption and retention in barley roots (Jacobson, Moore and Hannapel, 1960). Similar results have been reported with *Ricinus communis* (Minchin and Baker, 1973). As a result, $Ca^{++}$ has a very important role in cell organisation because the membrane is the primary component that results in compartmentation of cell organelles. Marinos (1962) showed that cells of barley shoots contained fragmented membranes, vesicular and amorphous inclusions, when grown in $Ca^{++}$-deficient media.

Chloroplasts contain large amounts of $Ca^{++}$ (Stocking and Ongun, 1962; Grouzis, 1978) and active $Ca^{++}$ transport across chloroplast membranes has been described (Nobel, 1969; DeFilippis and Pallaghy, 1973).

### 1.7.2 $Ca^{++}$ and the $HCO_3^-$ Pump

In addition to observing the role of $Ca^{++}$ in maintaining membrane integrity of *Chara corallina*, $Ca^{++}$ also plays a role in $HCO_3^-$ uptake (Lucas, 1976a; Lucas et al, 1977, 1978) and cytoplasmic streaming (Lucas, 1976a). Whether $Ca^{++}$ deficiency exerts its effects on $HCO_3^-$ influx at the transport site, the fixation pro-

cesses, or general membrane function in the chloroplast is unclear, but Lucas and Dainty (1977) suggested that although gradual elution of $Ca^{++}$ had no effect on the $K_m$ of $HCO_3^-$ influx (fixation), then there must be a specific divalent cation requirement for the $HCO_3^-$ transporter complex or the cation may act as a transport (enzyme) activator. It should be noted however that in these experiments the value obtained for the $K_m$ of the $HCO_3^-$ influx was probably limited by diffusion in unmixed solutions in which case their argument is not valid. A similar proposal has been made for a $Ca^{++}$-stimulated $H^+$ (or $OH^-$) transport system in the skeletal muscle membrane (Connett, 1978). Hasselbach (1977) has demonstrated the existence of a $Ca^{++}$-transporting protein and a $Ca^{++}$-binding protein in sarcoplasmic reticulum.

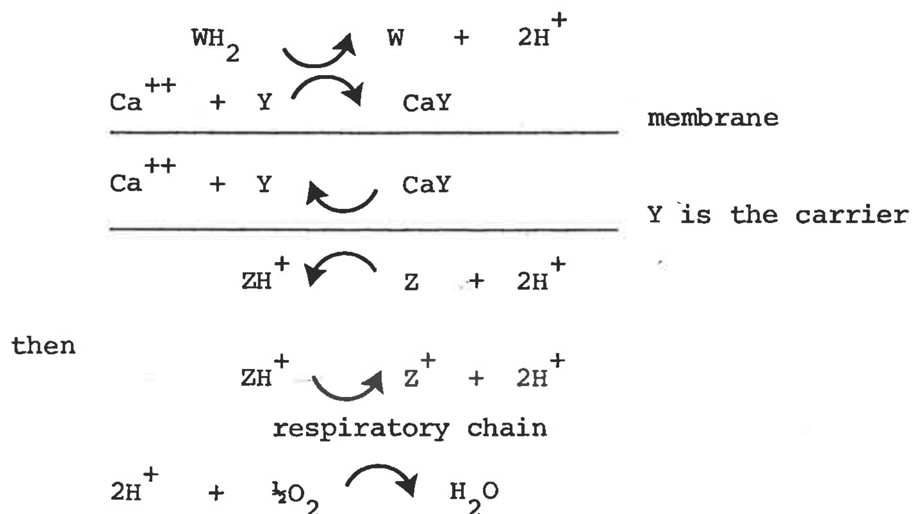
### 1.7.3 The Active $Ca^{++}$ Pump

The uptake of $Ca^{++}$ to both plant roots and leaves has been extensively studied (Lowenhaupt, 1956, 1958; Mazia, 1938, 1938a - see 1.7.4; Dunlop, 1973; Ludlow and Durham, 1977; Wagner and Bellini, 1976 - see 1.7.5.; Rubenstein, Johnson and Rayle, 1977). A general conclusion is that $Ca^{++}$ uptake is a passive process (Penot, Floc'h and Penot, 1976). However Lowenhaupt (1956) proposed a role for not only one but two active $Ca^{++}$ pumps in leaves of aquatic angiosperms that "pump" $Ca^{++}$ and/or other cations from the lower surfaces to the upper surfaces. These pumps are located at the plasma membranes of the cells at these surfaces. In the light these pumps operate initially by the attachment of the cation to a binding group, which is followed by the reorientation of this

group towards the other surface of the active membrane, resulting in the release of the cation. This transport mechanism is part of the respiratory chain and as such cation attachment occurs when the carrier is in the reduced form. Cations are released when an oxidising agent, formed as the result of photosynthesis, liberates them from the carrier. Furthermore, Lowenhaupt proposed that the above transport reactions synthesize a $HCO_3^-$ accepting compound at the plasma membrane that enables $HCO_3^-$ to penetrate the cytoplasm. To support this hypothesis, Lowenhaupt (1956) observed that in *Potamogeton*

1. $Ca^{++}$ release depends on the presence of oxygen,
2. during transport the $Ca^{++}$ content of leaves reaches a steady state level,
3. aeration of one side of the leaf in the dark causes a shift of $Ca^{++}$ towards this side independently of the upper/lower orientation of the leaf,
4. $H^+$ reversibly releases $Ca^{++}$ from the leaves.

The mechanism of the carrier and its associated redox reactions is depicted below:



This mechanism would result in a solution pH reduction from the uptake site and an increase at the release site - both observed by Lowenhaupt (1956) in the absence of $HCO_3^-$. Since the vacuoles in these plants contain no $Ca^{++}$ (Mazia, 1938), Lowenhaupt suggested that the cytoplasm was the medium for the redistribution of $Ca^{++}$ between the lower and upper surfaces and that cytoplasmic streaming facilitated this redistribution.

### 1.7.4 Calcium Binding in *Elodea*

It has been clearly established that cations may be transported across leaf tissue from the lower to the upper surface although the pathways across and the location within the leaf tissue have not been established.

Mazia (1938) demonstrated that certain treatments such as plasmolysis in 500 mM sucrose or dextrose, or direct electric currents (Mazia and Clark, 1936) caused precipitation of calcium oxalate in the vacuoles of *Elodea*. Calcium oxalate was not found in untreated cells indicating that $Ca^{++}$ was not normally present and that the plasmolysis caused the tonoplast to become leaky to $Ca^{++}$ and similarly other cations. In leaves that had been treated with 50 mM potassium citrate, no calcium oxalate precipitation occurred after plasmolysis, indicating that the citrate treatment removed $Ca^{++}$ from its normal location in the cell tissue. Follow-up experiments further indicated that $Ca^{++}$ was not free but bound either in the cytoplasmic layer, the cell membranes themselves or the cell walls. This binding was assumed to be fairly weak as the $Ca^{++}$ was rapidly removed by immersion in potassium citrate or

sodium chloride. However, $Ca^{++}$ would not diffuse out significantly if the tissue was immersed in distilled water, as after 14 days of such treatment he was still able to obtain normal calcium oxalate precipitation in the vacuoles. The fact that $Ca^{++}$ remained bound in acidic media (acetic acid, $CO_2$ or HCl) as low as pH 4.5 suggested that $Ca^{++}$ was either bound in a highly buffered environment or else bound otherwise than to free carboxyl groups.

Similarly he found strontium was bound in the tissue in a similar fashion as $Ca^{++}$ and that barium was bound in the ratio of 2:1 compared to $Ca^{++}$. Similarly the binding ratios with $Mg^{++}$, $K^{+}$ and $Na^{+}$ were determined to be 1:20, 1:100 and 1:100 respectively.

Mazia found that the amount of $Ca^{++}$ bound in *Elodea* immersed in a solution of 0.26 mM $Ca^{++}$ was as high as 10 mg/gram dry weight or 0.80 mg/GFW. If one estimates that one gram of fresh tissue contains 100% water (Mazia suggested 92%) then this was equivalent to a concentration greater than 10 mM. After citration treatment this value still remained as high as 2 mM and as the vacuoles occupy up to 90% of the tissue and $Ca^{++}$ is not present to any great degree in the vacuoles this means an even greater concentration elsewhere in the tissue. This compares favourably with the value of 1.6 mM for $Ca^{++}$ in *Elodea densa* obtained by Jeschke (1970a) in a solution of 1.3 mM $Ca^{++}$.

By a method of snap freezing, DeFilippis and Pallaghy (1973) measured the $Ca^{++}$ content of *Elodea densa* chloroplasts to be 150 mM in the dark and 110 mM in the light with an efflux in the light of 8 pmoles/$cm^2$/sec.

The accumulation of $Ca^{++}$ in the citrated tissue of *Elodea* is independent of the external $Ca^{++}$ concentration in the range

0.5 mM to 100 mM (Mazia, 1938) and Mazia concluded this to be evidence that $Ca^{++}$ does not move into the tissue by simple diffusion but by exchanging $Na^{+}$ or $K^{+}$ which is bound to organic anions in the tissue. These organic anions must have a low mobility and the $Na^{+}$ and $K^{+}$ salts very different dissociation constants from $Ca^{++}$ salts.

### 1.7.5 Calcium and Protoplasmic Streaming

Protoplasmic streaming is very common in leaf cells of *Elodea* and evidence has accumulated (Ridgway and Durham, 1976; Wagner and Bellini, 1976; Braatz and Komnick, 1973; Chi and Francis, 1971; Ludlow and Durham, 1977; Hatano, 1970) that in other species, $Ca^{++}$ is important in cytoplasmic streaming.

In the myxomycete *Physarium polycephalum*, $Ca^{++}$ has definitely been associated with cytoplasmic streaming which occurs as a result of pressure differences which are due to fluctuating $Ca^{++}$ concentrations. These fluctuations exert their influence on a $Ca^{++}$ sensitive actomyosin system (Ridgway and Durham, 1976). This suggests a similar role for $Ca^{++}$ in the plant cell as in animal muscle cells, where $Ca^{++}$, released from the muscle cell membrane acts on the actin and myosin fibrils causing contraction in the presence of ATP.

In the green alga *Mougeotia sp.*, Wagner and Bellini (1976), found the $Ca^{++}$ influx across the plasmalemma was greatly increased in the light and the $Ca^{++}$ was stored in a water-insoluble form in the cell. This process could be quickly reversed to enable $Ca^{++}$-dependent reactions to proceed in the cell independently of the

external $Ca^{++}$ concentration - chloroplast movement being one such reaction and dependent on the internal $Ca^{++}$ concentration. Other probable $Ca^{++}$-dependent processes include cytoplasmic streaming in *Chara* (Pickard, 1972; Lucas, 1976a; Williamson, 1975) and the cleavage of sea urchin egg cells (Schroeder, 1972).

## 1.8 Cation and Anion Transport

Although the polar transport of divalent cations especially $Ca^{++}$ and to a lesser extent monovalent cations such as $K^+$ and $Na^+$ had been well established by 1965, the actual mechanisms of light-stimulated transport, either active or passive, of both types of ions, had not been established. Around this time, ions that were active and light-stimulated were intensely studied to determine the various sources of energy supply.

MacRobbie (1965, 1966) and Raven (1967, 1968) established that in *Nitella translucens* and *Hydrodictyon africanum* respectively, the light-stimulated influx of $Cl^-$ was dependent on electron transport in photosystem II (Boardman, 1970), which produces a supply of NADPH, and that the light-stimulated $K^+$ influx and $Na^+$ efflux were dependent on energy from ATP formed by photophosphorylation in photosystem I (Boardman, 1970). These deductions came from experiments with uncouplers of phosphorylation such as imidazole, CCCP, inhibitors of electron flow such as DCMU, spectral changes and by the exclusion of dissolved gases by nitrogen aeration of the bathing medium. Raven (1969) working with inhibitors such as HOQNO, salicyladoxime, NaCN, DNP, desaspidin and antimycin A was able to further conclude that coupled $K^+/Na^+$ exchange was

powered by oxidative phosphorylation in the dark by photophosphorylation in the light; that the $Cl^-$ influx was powered by mitochondrial electron transport (NADH) in the dark, and the entire photosynthetic electron transport chain (NADPH) in the light.

However, Jeschke (1967, 1972a) demonstrated that in *Elodea densa*, both light-stimulated $Cl^-$ influx and $K^+$ influx were linked and dependent on photophosphorylation, and similarly Barber (1968) found that both light-induced uptake of $K^+$ and $Cl^-$ were driven by ATP from cyclic and non-cyclic photophosphorylation in *Chlorella pyrenoidosa*.

Jeschke's conclusions were drawn from experiments where the $Cl^-$ influx into *Elodea* was not affected by concentrations of DCMU that inhibited $O_2$ evolution by 95% and in experiments where the $Cl^-$ influx was inhibited by 100% at concentrations of atebrin ($10^{-4}$M) that had no effect on the $O_2$ evolution. However, Betts (1970) criticised these experiments because in the DCMU experiments, different $CO_2$ levels were used for the $Cl^-$ uptake measurements compared to the $O_2$ evolution experiments. This is particularly relevant as Jeschke showed in the same paper that small concentrations of $CO_2$ (0.5%) did increase the inhibition of the $Cl^-$ influx by DCMU. Furthermore, Betts (1970) pointed out that since light stimulated the $Cl^-$ influx only twofold over the dark value (Jeschke, 1967), then in the DCMU experiments an inhibition of 50% of the total $Cl^-$ influx in the light would represent a 100% inhibition of the light-stimulated portion. Making corrections for this, the progressive inhibition of the $Cl^-$ influx by DCMU would have been

the same as the $O_2$ evolution inhibition (Jeschke, 1967), as Betts (1970) found in his experiments on *Elodea densa*.

Betts (1970) also criticised Jeschke's experiment in which the $Cl^-$ influx, presumably driven by ATP from either cyclic or non-cyclic photophosphorylation was completely inhibited by concentrations of atebrin that had no effect on $O_2$ evolution. Betts pointed out that with $CO_2$ fixation acting as the only terminal electron acceptor via NADPH *in vivo*, if phosphorylation was inhibited, no $CO_2$ fixation could occur and hence $O_2$ evolution would also be inhibited; and suggested that atebrin exerted its effect on the $Cl^-$ influx via another mechanism, possibly at the plasmalemma. In 1972, Jeschke reported the effects of CCCP on the $Cl^-$ influx and $O_2$ evolution and here both of these processes showed equal inhibition with increasing CCCP concentration, in agreement with Betts (1970).

Although Jeschke concluded that this was further evidence for ATP as the energy source for the $Cl^-$ influx, these experiments were unable to distinguish between ATP or NADPH, as the primary energy sources. Rather they suggested that both photophosphorylation and electron flow mechanisms need to be functional.

Poole (1978), in a review paper, pointed out that in the experiments of MacRobbie (1965), Raven (1968, 1969) and Jeschke (1967, 1972), simultaneous measurements on the actual ATP levels were not made. Miller and Spanswick (1977) recently showed that under aerobic conditions , the ATP levels in *Nitella* were unchanged in the light. Similar observations have also been made in barley leaves (Lüttge et al, 1976), bean leaves (Steinitz and Jacoby,

1974), *Griffithsia* (Lilley and Hope, 1971), *Zea mays* (Brinckmann and Lüttge, 1972), and *Euglena gracilis* (Holm Hansen, 1970).

However, in *Elodea densa* the levels of ATP in the cytoplasm and chloroplasts were increased 6-fold and 2-fold respectively (Santarius, Heber, Ullrich and Urbach, 1964; Heber and Santarius, 1970). These authors further concluded that there was a rapid exchange of ATP between the chloroplasts and the cytoplasm of *Elodea* (see 1.9).

Lüttge, Schöch and Ball (1974) demonstrated that externally applied ATP could stimulate $K^+$ ($Rb^+$) uptake into *Zea mays*, *Panicum milliaceum* and *Sorghum*, in both light and dark with a $K_m$ of approximately 1 mM ATP: however, in conclusion they emphasised that this stimulation may not be due to direct effects of ATP but rather indirect effects such as the reversal of $Ca^{++}$-inhibited $K^+$ influx, by chelation of $Ca^{++}$ by ATP.

Poole (1978) concluded that in studies where ATP levels were measured along with $Cl^-$ influx measurements, correlation between both rates had been good and that in all probability the current evidence was such to suggest that ATP was the ultimate energy source for all anion transport.

## 1.9 Models of Photosynthesis and Ion Transport

Although the various energy sources for ion transport have been elucidated, the mechanisms by which these sources actually provide energy at the transport site are still to be unravelled. It has been necessary to propose "shuttle" mechanisms to transport the products of photosynthesis and respiration to the trans-

port sites as these products - ATP and NADPH - are generally impermeable to the chloroplast and mitochondrial membranes (Heber and Santarius, 1965, 1970; Stokes and Walker, 1971). Such models include the glycolate- glyoxylate shuttle of Kisaki and Tolbert (1969), whereby NADPH reduces glyoxylate to glycolate, which migrates out of the chloroplast to reduce cytoplasmic NAD to NADH. Similarly, the malate-oxaloacetic acid (OAA) shuttle of Heber and Krause (1971, 1972) increases cytoplasmic NADH by the oxidation of malate from the chloroplasts to OAA, which then re-enters the chloroplasts to be reduced to malate again. The PGA-DHAP (dihydroxyacetone phosphate) shuttle (Heber, 1973) associated with the malate-OAA shuttle enables cytoplasmic metabolite control of the chloroplast/cytoplasm exchange. In this model, ATP and NADH are formed in the cytoplasm under the control of the reaction glyceraldehyde-3-phosphate to 3-phosphoglycerate.

The generation of ATP and NADH in the cytoplasm is therefore indirectly linked to the formation of ATP and NADPH in the chloroplasts and provides the supply of energy for the various transport mechanisms. Similar shuttle mechanisms also occur between mitochondria and the cytoplasm and hence it is possible to obtain good interactions between chloroplasts and mitochondria, that result in good feedback control of cytoplasmic levels of ATP and NADH (Jeschke, 1976) and diagrammatically represented in Figure 1.3, In this model, the actual delivery of either ATP or NADH to the plasmalemma depends on the ability to switch between photosynthetic and respiratory energy production, feedback from ion transport, light induced changes in respiration and the transfer of carbohydrates between the two organelles.

The role of $HCO_3^-$ transport and the control of intracellular pH have not been introduced into this model. $HCO_3^-$ transport and fixation could well be the major feedback mechanism whereby ion transport itself controls the levels of cytoplasmic ATP and NADH, pH also plays a similar role (Smith and Raven, 1976). Smith (1970, 1972, 1973) and Raven and Smith (1973) suggested a model for accumulation of inorganic salts and organic salts based on Mitchell's chemiosmotic hypothesis of energy coupling in chloroplasts and mitochondria (Mitchell, 1961). In this model (Figure 1.4) metabolic energy drives an $H^+$ pump that is coupled to a cation influx and an $OH^-$ efflux is coupled to an anion influx. During assimilation of $HCO_3^-$ when there is a net production of $OH^-$, the $OH^-$ efflux may possibly be passive and coupled to an active anion influx, or active and coupled to a passive anion influx. This "pH stat" could therefore be incorporated into Jeschke's model (Figure 1.3) to provide feedback control to the chloroplast and mitochondrial activities and to the sites of ion transport.

## 1.10 The Current Work

Evidence has now accumulated to suggest that both single cells of giant algae and the multicellular tissues of aquatic angiosperms can produce alkaline regions during $HCO_3^-$ fixation. Characean cells such as *Chara corallina* have been studied in detail, and acidic regions where $HCO_3^-$ is absorbed and alkaline regions where $OH^-$ is excreted from the cells, have been demonstrated (Lucas and others). Exchanges of cations such as $K^+$ and $Ca^{++}$ have been shown not to play a major role in these processes, although in

aquatic angiosperms, they have been shown to play an important and necessary role (Arens, 1938; Lowenhaupt, 1956).

The current work, therefore, examines the role of $Ca^{++}$ in relation to $CO_2$ and $HCO_3^-$ fixation, and associated pH changes.

Recent work has emphasised the view that energy for light-stimulated anion and cation transport can only be supplied to the plasmalemma when both ATP and NADPH are being produced in the chloroplasts (1.9). Formally the polarised views that only ATP or NADPH, and not both, could supply energy, produced much debate. This work attempts to elucidate this debate with the study of light stimulated $Cl^-$ uptake studies.

In addition there are experiments to quantify the ability of *Elodea* to utilize $HCO_3^-$, and to study the formation of acid and alkaline regions that form at the lower and upper surfaces respectively.

*Elodea* is a multicellular system that shows remarkably similar properties to the giant-celled algae during $HCO_3^-$ fixation and one of the aims of this thesis is to produce generalised models to describe these phenomena.

TABLE 1.1 SPECIES OF PLANTS THAT SHOW "PHYSIOLOGICAL POLARITY"

% of experimental sprigs with red colouring on the leaves

| Species | Tap Water | | $Ca(HCO_3)_2$ Solution | | Dark Control (tap water) | |
|---|---|---|---|---|---|---|
| | U | L | U | L | U | L |
| *Elodea canadensis f. callitrichoides* | 100 | 0 | 100 | 0 | 0 | 0 |
| *Elodea densa* | 100 | 0 | 100 | 0 | 0 | 0 |
| *Elodea crispa* | 100 | 0 | 100 | 0 | 0 | 0 |
| *Najas minor* | 100 | 0 | 100 | 0 | 0 | 0 |
| *Hydrilla verticillata* | 100 | 0 | 100 | 0 | 0 | 0 |
| *Potamogeton densus* | 100 | 0 | 100 | 0 | 0 | 0 |
| " *crispus* | 100 | 0 | 100 | 0 | 0 | 0 |
| " *pusillus* | 100 | 0 | 100 | 0 | 0 | 0 |
| " *alpinus* | 100 | 0 | 100 | 0 | 0 | 0 |
| " *lucens* | 100 | 0 | 100 | 0 | 0 | 0 |
| " *zizii* | 100 | 0 | 100 | 0 | 0 | 0 |
| " *pectinatus* | 100 | 100 | 100 | 100 | 0 | 0 |
| *Vallisneria spiralis* | 100 | 100 | 100 | 100 | | |

Table shows the percentage of leaves where a pH rise (red colour using phenolphthalein as indicator) at the upper surface (U) and lower surface (L), in tap water, $Ca(HCO_3)_2$ and in the dark.

(From Arens, 1933)

TABLE 1.2 ABILITY OF THREE SPECIES TO INCREASE THE pH AT THE UPPER SURFACE WITH DIFFERENT CATIONS IN THE SOLUTION

| Solutions | *Elodea Canadensis* | | *Hydrilla verticillata* | | *Potamogeton crispus* | |
|---|---|---|---|---|---|---|
| | % | pH | % | pH | % | pH |
| $Sr(HCO_3)_2$ | 100 | 11.4 | 100 | 10.8 | 100 | 10.9 |
| $Mg(HCO_3)_2$ | 100 | 10.1 | 100 | 9.9 | 100 | 10.3 |
| $Ba(HCO_3)_2$ | 100 | 11.8 | 100 | 10.7 | 100 | 11.5 |
| $LiHCO_3$ | 100 | 11.0 | 100 | 10.4 | 100 | 10.1 |
| $KHCO_3$ | 100 | 11.5 | 100 | 11.3 | 100 | 10.9 |
| $NaHCO_3$ | 100 | 11.0 | 100 | 11.1 | 100 | 10.8 |

Table shows the percentage of leaves where a red colour (pH rise) was observed at the upper surface using phenolphthalein as indicator. The pH value represents the maximum solution pH obtained during the course of the experiment. (From Arens, 1933)

TABLE 1.3 DEGREE OF pH CHANGE AT THE UPPER LEAF SURFACES WITH DIFFERENT EXTERNAL SOLUTIONS

| Solutions | *Elodea* I | *Elodea* II | *Potamogeton* I | *Potamogeton* II |
|---|---|---|---|---|
| 1.0 mM $CaCl_2$ | + | ++ | + | ++ |
| 10 mM $CaCl_2$ | ++ | +++ | ++ | +++ |
| 1.0 mM KCl | + | ++ | + | + |
| 10 mM KCl | + | ++ | + | ++ |
| 10 mM $Ca(NO_3)_2$ | ++ | +++ | ++ | +++ |
| 10 mM $KNO_3$ | + | ++ | + | ++ |

Table shows the ability of *Elodea* and *Potamogeton* grown in different environments to increase pH at the upper leaf surfaces. Weak (+), medium (++) and strong (+++) colour changes (red) using phenolphthalein indicator are shown.
*Elodea* I, cultured in water with 8.5 mg/l $Ca^{++}$;
*Elodea* II, 57 mg/l $Ca^{++}$; *Potamogeton* I, 11.7 mg/l $Ca^{++}$;
*Potamogeton* II, 57 mg/l $Ca^{++}$. (From Arens, 1933)

TABLE 1.4 ABILITY TO RAISE SOLUTION pH IN DISTILLED WATER

| Solutions | | *Elodea* I | *Elodea* II | *Potamogeton* I | *Potamogeton* II |
|---|---|---|---|---|---|
| distilled $H_2O$ | Red Colour | + | ++ | + | ++ |
| | pH | 7.9 | 8.8 | 7.7 | 9.3 |
| distilled $H_2O - CO_2$ | Red Colour | ++ | +++ | ++ | +++ |
| | pH | 8.6 | 10.4 | 9.2 | 10.2 |

Table shows the ability to increase pH at the upper leaf surfaces and the effect this has on the final solution pH. Each species was cultured in two different $Ca^{++}$ solutions as in Table 1.3. Symbols (+) to (+++) also as in Table 1.3. (From Arens, 1933)

TABLE 1.5 ABILITY TO RAISE pH AT UPPER LEAF SURFACES AND TO PRODUCE OXYGEN WITH INCREASED SOAKING TIMES IN SOLUTIONS TO REMOVE ENDOGENOUS CARBON

| Time in Soaking Solution | | *Elodea densa* I | *Elodea densa* II | *Potamogeton crispa* I | *Potamogeton crispa* II | *Potamogeton densa* I | *Potamogeton densa* II |
|---|---|---|---|---|---|---|---|
| 30 mins | Red | ++ | +++ | ++ | +++ | - | + |
| | $0_2$ bubbles | 10 | 22 | 6 | 11 | 0 | 2 |
| 60 mins | Red | +++ | ++++ | + | ++ | 0 | - |
| | $0_2$ bubbles | 6 | 21 | 5 | 9 | 0 | 0 |
| 90 mins | Red | + | +++ | + | ++ | - | - |
| | $0_2$ bubbles | 2 | 8 | 3 | 7 | 0 | 0 |
| 120 mins | Red | - | + | - | 4 | - | - |
| | $0_2$ bubbles | 0 | 5 | 0 | 0 | 0 | 0 |
| 180 mins | Red | - | - | - | - | - | - |
| | $0_2$ bubbles | 0 | 0 | 0 | 0 | 0 | 0 |

Table shows the ability of three species (all cultured in different solutions - Table 1.3) to produce a pH increase (indicated by phenolphthalein) at the upper leaf surfaces and to produce $0_2$ bubbles from the intact sprig stem (number represents the number of $0_2$ bubbles in 5 minutes). The actual experimental solution contained 10 mM $CaCl_2$. Symbols (+) to (++++) as in Table 1.3, (-) indicates no pH change. (From Arens, 1933)

TABLE 1.6 UPPER LEAF SURFACE pH CHANGE IN DIFFERENT SOLUTIONS USING *Elodea* WITH DIFFERENT 24 HOUR PRETREATMENT

| Experimental | *Elodea* without Reserves | *Elodea* 24 hrs. in $Mg(HCO_3)_2$ | *Elodea* 24 hrs. in $Ca(HCO_3)_2$ | *Elodea* 24 hrs. in $CaCl_2$(10 mM) | *Elodea* 24 hrs. in $CO_2$ solution |
|---|---|---|---|---|---|
| 10 mM $CaCl_2$ | - | ++ | ++ | - | - |
| $Ca(HCO_3)_2$ | +++ | +++ | +++ | ++ | ++ |
| $Mg(HCO_3)_2$ | +++ | +++ | +++ | ++ | + |
| Dist. $H_2O - CO_2$ | - | + | + | - | - |
| Dist. $H_2O + CO_2$ | - | - | - | - | - |

Table shows the effect of pretreating leaves without endogenous carbon reserves for 24 hours in various solutions on their ability to increase upper surface pH in various experimental solutions. Symbols as in Table 1.3, and 1.5. (From Arens, 1933)

**TABLE 1.7** STARCH FORMATION IN THE UPPER AND LOWER LAYERS OF CELLS

| | | *Elodea densa* | *Elodea canadensis* | *Hydrilla verticillata* |
|---|---|---|---|---|
| After 10 mins | U | + | + | + |
| | L | - | - | - |
| After 20 mins | U | ++ | ++ | ++ |
| | L | - | + | + |
| After 30 mins | U | +++ | +++ | +++ |
| | L | ++ | ++ | ++ |

Table shows the effect of time on the formation of starch (iodide staining) in the chloroplasts in the upper (U) and lower (L) layers of the cells. Symbols represent no (-), weak (+), medium (++) and strong (+++) starch formation. (From Arens, 1933)

TABLE 1.8 pH CHANGES AT THE UPPER AND LOWER LEAF SURFACES WITH DIFFERENT SOLUTIONS AT THE LOWER SURFACES

| Solutions On the underside | *Elodea densa* | | *Hydrilla verticillata* | | *Potamogeton alpinus* " *zizii* " *crispus* | |
|---|---|---|---|---|---|---|
| | U | L | U | L | U | L |
| | % | % | % | % | % | % |
| 10 mM KCl | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 mM $CaCl_2$ | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 mM $KNO_3$ | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 mM $KHCO_3$ | 100 | 0 | 90 | 0 | 100 | 0 |
| 10 mM $NaHCO_3$ | 100 | 0 | 100 | 0 | 100 | 0 |
| $Ca(HCO_3)_2$ | 100 | 0 | 100 | 0 | 100 | 0 |
| $Mg(HCO_3)_2$ | 100 | 0 | 87 | 0 | 100 | 0 |
| $Ba(HCO_3)_2$ | 100 | 0 | 100 | 0 | 100 | 0 |
| distilled $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 |

Table demonstrates the ability of five species to increase pH at the upper and lower leaf surfaces (phenolphthalein indicator) with different solutions at the lower surfaces. Numbers indicate the % of leaves showing a red change at each surface. (From Arens, 1933)

TABLE 1.9 ABILITY OF LEAVES TO TRANSPORT EITHER $K^+$ OR $Ca^{++}$ FROM THE LOWER LEAF SURFACE TO THE UPPER SURFACE

| On the underside | *Elodea densa* | | | *Hydrilla verticillata* | | | *Potamogeton crispus* | | |
|---|---|---|---|---|---|---|---|---|---|
| | Red % | Ca % | K % | Red % | Ca % | K % | Red % | Ca % | K % |
| $Ca(HCO_3)_2$ light | 100 | 100 | 0 | 100 | 100 | 0 | 100 | 100 | 0 |
| $Ca(HCO_3)_2$ dark | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| distilled $H_2O$ light | 7 | 7 | 0 | 0 | 0 | 0 | 4 | 4 | 0 |
| distilled $H_2O$ dark | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $KHCO_3$ light | 96 | 0 | 96 | 100 | 0 | 100 | 100 | 8 | 100 |
| $KHCO_3$ dark | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table shows the ability of three species to transport $K^+$ (indicated by precipitation of potassium- sodium cobalt nitrite) and $Ca^{++}$ (indicated by precipitation of calcium oxalate) from the lower surfaces to the upper surfaces, and to show pH increases (red) at the upper surfaces. Numbers represents percentage of leaves showing precipitation or colour change. (From Arens, 1933)

TABLE 1.10 EFFECT OF DIFFERENT SOLUTIONS AT UPPER AND LOWER SURFACES OF *Elodea* AND *Potamogeton* ON PHOTOSYNTHESIS (MANGANESE PRECIPITATION)

| Lower Surface Solution | Upper Surface Solution | Mn-Precipitation |
|---|---|---|
| $Ca(HCO_3)_2$ | $MnCl_2$ | + |
| $KHCO_3$ | $MnCl_2$ | + |
| $Ca(HCO_3)_2$ + $MnCl_2$ | $H_2O$ | +++ |
| $KHCO_3$ + $MnCl_2$ | $H_2O$ | +++ |
| $MnCl_2$ | $H_2O$ | + |
| AIR | $Ca(HCO_3)_2$ + $MnCl_2$ | - |

Table shows the incidence of manganese precipitation (which indicates $OH^-$ efflux and $O_2$ production - photo synthesis) on the leaf upper surfaces. Symbols indicate no (-) through to strong (+++) precipitation as in Table 1.6. (From Arens, 1938)

TABLE 1.11 ASSIMILATION OF $HCO_3^-$ FROM BOTH UPPER AND LOWER SURFACES OF *Potamogeton lucens*

| Upper Surface Solution | Lower Surface Solution | Net $O_2$ Production |
|---|---|---|
| 2.7 mM $NaHCO_3$ | 2.7 mM $NaHCO_3$ | 100% |
| $H_2O$ ($CO_2$ FREE) | 2.7 mM $NaHCO_3$ | 74% |
| 2.7 mM $NaHCO_3$ | $H_2O$ ($CO_2$ FREE) | 58% |
| $H_2O$ ($CO_2$ FREE) | $H_2O$ ($CO_2$ FREE) | -5% (Resp.) |

(From Steemann Nielsen, 1947.)

**TABLE 1.12** POLAR TRANSPORT ON ANIONS ACROSS THE LEAVES OF *Potamogeton lucens* IN LAKE WATER

| | UPPER SURFACE | | | | LOWER SURFACE |
|---|---|---|---|---|---|
| | pH | $C_{OH^-}$ | $C_{CO_3^{=}}$ | $C_{HCO_3^-}$ | pH |
| | | (nmoles/litre) | | | |
| Before | 7.9 | 0.5 | 0 | 30 | 8.2 |
| After | 10.6 | 250 | 370 | 100 | 8.12 |

Natural Lake Water (2.7 mM $HCO_3^-$) at the lower surface, distilled water at the upper suface. 24,000 Lux, 20°C, experimental time, three hours. (From Steemann Nielsen, 1947)

TABLE 1.13 POLAR TRANSPORT OF ANIONS ACROSS THE LEAVES OF *Potamogeton lucens* IN 5.0 mM $KHCO_3$

| | UPPER SURFACE | | | | LOWER SURFACE |
|---|---|---|---|---|---|
| | pH | $C_{OH^-}$ (nmoles/litre) | $C_{CO_3^=}$ | $C_{HCO_3^-}$ | pH |
| Before | 7.5 | 0.2 | 0 | 30 | 8.4 |
| After | 10.0 | 63 | 84 | 81 | 8.4 |

A solution of 5.0 mM $KHCO_3$ at the lower surface. Distilled water at the upper surface. 24,000 Lux, 20°C, experimental time, 150 minutes. (From Steemann Nielsen, 1947)

**TABLE 1.14** pH CHANGES AT THE SURFACES OF *Ulva lactuca*

| Expt. | Side | | pH Before | pH After |
|---|---|---|---|---|
| 1 | A | SEA WATER | 8.40 | 8.90 |
| | B | ARTIFICIAL SEAWATER | 6.60 | 9.45 |
| 2 | B | SEAWATER | 8.6 | 8.97 |
| | A | ARTIFICIAL SEAWATER (NO C SOURCE) | 6.6 | 9.08 |

Table shows that the solution pH is raised on both sides of the thallus of *Ulva lactuca*. (From Steemann Nielsen, 1947)

TABLE 1.15 SURVIVAL RATE OF *Elodea canadensis*, *Potamogeton crispus*, AND *Potamogeton alpinus* IN VARIOUS 5 mM BICARBONATE SOLUTIONS

| | Survival Time in Days | | | |
|---|---|---|---|---|
| Solutions | *Elodea canadensis* I | *Elodea canadensis* II | *Potamogeton crispus* | *Potamogeton alpinus* |
| $Ca(HCO_3)_2$ | 38 | 25 | 30 | 16 |
| $Ba(HCO_3)_2$ | 5 | 3 | 4 | 3 |
| $Sr(HCO_3)_2$ | 24 | 18 | 23 | 17 |
| $Mg(HCO_3)_2$ | 15 | 14 | 16 | 10 |
| $LiHCO_3$ | 8 | 4 | 5 | 3 |
| $KHCO_3$ | 12 | 10 | 8 | 9 |
| $NaHCO_3$ | 10 | 9 | 9 | 6 |

*Elodea canadensis* I came from a pond containing 8.5 mg/litre $Ca^{++}$ and *Elodea* II 57/mg/litre $Ca^{++}$. (From Arens, 1933)

TABLE 1.16 GROWTH EXPERIMENTS WITH *Elodea densa*

| pH | Growth |
|---|---|
| 3.5 | Dead |
| 4.5 | Dead |
| 5.5 | 130% |
| 6.5 | 165% |
| 7.5 | 220% |
| 8.0 | 320% |

Growth expressed as the percentage increase in fresh weight over a period of 60 days. (From C. Olsen, 1921)

TABLE 1.17 GROWTH EXPERIMENTS WITH *Elodea canadensis*

| pH | Supply of $CO_2$ | Fresh weight at end of experiment in percentages of value at the start | Dry weight at end of experiment in percentages of value at the start | Fresh weight: dry weight | Average length of shoots in cm | Percentage of plants with roots | Length of longest root in cm | Pecentage of plants with distinct development of anthocyanin |
|---|---|---|---|---|---|---|---|---|
| 3.5 | 2 p.c. $CO_2$ blown through | 80 | 100 | 8.0 | 2.0 | 0 | | 0 |
| 4.5 | " | 256 | 274 | 7.5 | 5.5 | 100 | 40 | 0 |
| 5.5 | " | 298 | 340 | 7.8 | 5.4 | 50 | 18 | 33 |
| 6.5 | " | 284 | 369 | 7.0 | 4.9 | 17 | 5 | 100 |
| 7.5 | " | 282 | 361 | 7.0 | 4.9 | 17 | 7 | 83 |
| 8.0 | " | 250 | 336 | 6.7 | 4.6 | 50 | 7 | 67 |
| 3.5 | Atm. air(0.03 p.c. $CO_2$) blown through | | | | | | | |
| 5.0 | " | 132 | 111 | 10.7 | 3.2 | 0 | | 0 |
| 6.0 | " | 156 | 132 | 10.6 | 3.4 | 0 | | 0 |
| 7.0 | " | 173 | 184 | 8.4 | 3.5 | 0 | | 0 |
| 8.2* | " | 202 | 264 | 7.0* | 3.9 | 0 | | 50 |
| 8.2* | " | 201 | 255 | 7.1* | 4.1 | 0 | | 67 |

Six tips of shoots in each glass. Fresh weight at the start about 0.80gm. Experiment started on the 15 June 1943 at 5 p.m., concluded on the 24 June 1943 at 3 p.m. Light: constant about 15,000 lux from 1,000 watt lamp. Temp. 23°C

* At the end of the experiment layer of lime especially on the upper side of the leaves. (From Steemann Nielsen,1944)

TABLE 1.18 GROWTH EXPERIMENTS WITH *Ceratophyllum demersum*

| pH | Supply of $CO_2$ | Fresh weight at end of experiment in percentages of value at the start | Dry weight at end of experiment in percentages of value at the start | Fresh weight: Dry weight | Average length of shoots at end of exp in cm | Notes |
|---|---|---|---|---|---|---|
| 3.5 | 5 p.c. $CO_2$ blown through | | | | | Plants nearly dissolved at end of exp. |
| 4.5 | " | 462 | 532 | 12.8 | 17 | |
| 5.5 | " | 424 | 485 | 13.1 | 15 | |
| 6.5 | " | | | | | Scrapped (deficiency in iron?) |
| 7.0 | " | 614 | 500 | 18.4 | 22 | |
| 7.4 | " | 608 | 546 | 16.6 | 25 | |
| 3.5 | Atm. air(0,03% $CO_2$) blown through | | | | | Plants nearly dissolved at end of exp. |
| 4.5 | " | 129 | 118 | 16.9 | 5 | |
| 5.5 | " | 181 | 186 | 14.4 | 6 | |
| 6.5 | " | 300 | 280 | 16.4 | 10 | |
| 7.5 | " | 370 | 320 | 17.0 | 9 | |
| 8.2 | " | 476 | 400 | 17.9 | 14 | |
| 3.5 | No movement (0,03% $CO_2$) | | | | | Plants nearly dissolved at end of exp |
| 4.5 | " | 100 | 85 | 15.9 | 2 | |
| 5.5 | " | 150 | 142 | 15.6 | 5 | |
| 6.5 | " | 180 | 167 | 16.0 | 5 | |
| 7.5 | " | 237 | 217 | 16.6 | 7 | |
| 8.2 | " | 246 | 232 | 15.8 | 9 | |

Two tips of shoots in each glass. Fresh weight at the start about 0.45gm. Fresh weight:dry weight at the start 15. Experiment started on the 12 May at 11 a.m. concluded on the 27 May at 2 p.m. Light: indirect daylight. Temperature about 20 - 23$^{0}$C. (From Steemann Nielsen, 1944)

**TABLE 1.19** EFFECT OF CATIONS AND ANIONS ON PHOTOSYNTHESIS ($CO_2$ ASSIMILATION) OF *Myriophyllum spicatum*

| $CO_2$ concentration (mM) | Cations except $H^+$ | Anions except $OH^-$ | % rate of photosynthesis |
|---|---|---|---|
| 0.2 | | $Cl^-$ | 102 |
| 0.2 | $K^+$ | $Cl^-, SO_4^=$ | 101 |
| 0.2 | $K^+, Na^+, Ca^{++}$ | $Cl^-, SO_4^=$ | 100 |
| 0.4 | | $Cl^-$ | 104 |
| 0.4 | $K^+, Na^+, Ca^{++}$ | $Cl^-, SO_4^=$ | 100 |

Table shows that cations and anions have no effect on $CO_2$ assimilation. Experimental conditions: pH 4.1, light intensity 37,000 lux at 20°C. $CO_2$ concentrations as indicated. (From Steemann Nielsen, 1947)

TABLE 1.20 EFFECT OF CATIONS AND ANIONS ON PHOTOSYNTHESIS ($HCO_3^-$ ASSIMILATION) OF *Myriophyllum spicatum*

| Cations except $H^+$ | Anions except $HCO_3^-$, $CO_3^=$, and $OH^-$ | % rate of photosynthesis |
|---|---|---|
| $K^+$ | | 42 |
| $Na^+$ | | 50 |
| $Ca^{++}$ | | 71 |
| $K^+$, $Ca^{++}$ | | 79 |
| $K^+$ | $Cl^-$ | 91 |
| $K^+$, $Na^+$, $Ca^{++}$ | $Cl^-$, $SO_4^=$ | 94 |

Table shows that anions and cations do affect $HCO_3^-$ assimilation. All solutions contain 1.0mM $NaHCO_3$ in natural lake water, 37,000 lux at 20°C and pH 8.3. (From Steemann Nielsen, 1947)

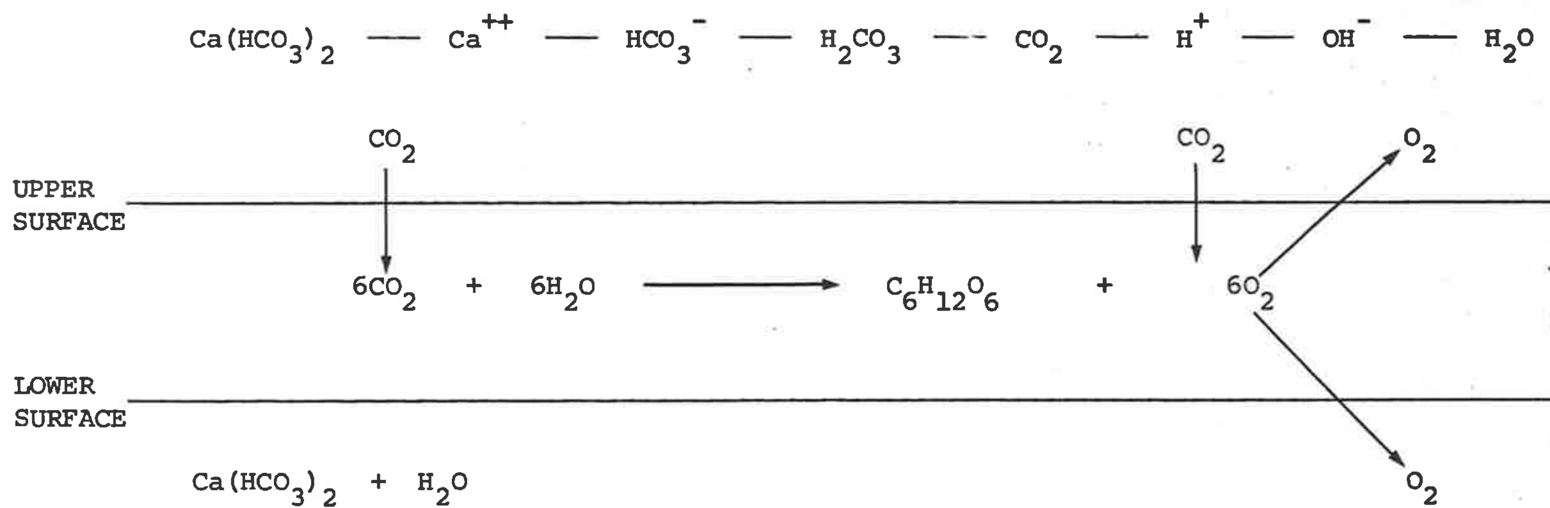



FIGURE 1.1 Scheme of photosynthesis of *Elodea* leaves, taken from Gicklhorn (1927) on the basis of the hypothesis of Nathansohn (1907).

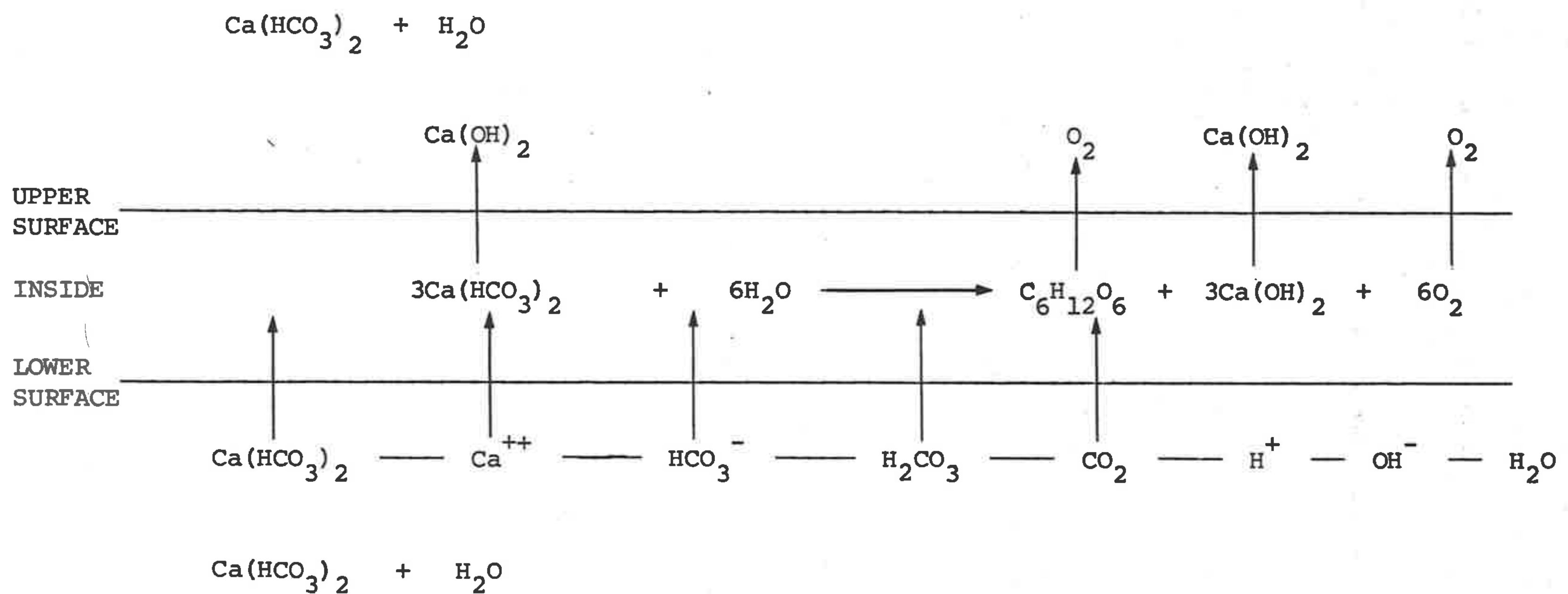



FIGURE 1.2 Scheme of photosynthesis for *Elodea* leaves as a result of the "physiological polarisation of the distribution of molecules". (From Arens, 1938)

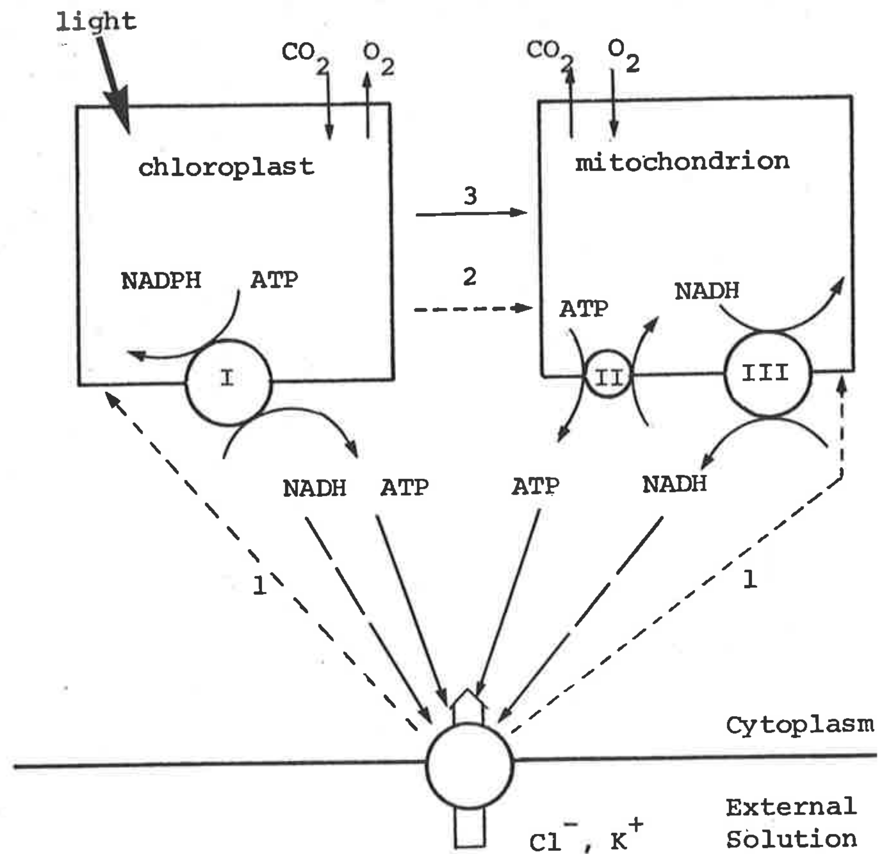



FIGURE 1.3 Possibilities of energy supply to ion transport across the plasmalemma of a leaf cell. Energy delivery by ATP or NADH may depend on the ability to switch from photosynthetic to respiratory energy production and on feedback from ion transport (1); photoinhibition of respiration (2); transfer of carbohydrates (3). I shuttle mediating the ATP and/or NAD(P)H transport by chloroplasts; II mitochondrial adenylate carrier; III mitochondrial malate shuttle. Similar energy balances and feedbacks would pertain to transport across the tonoplast. (From Jeschke, 1976)

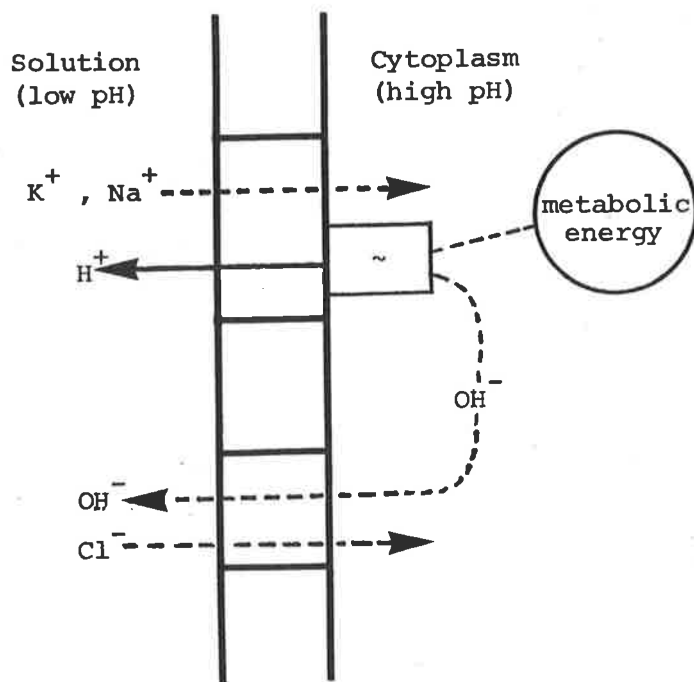



FIGURE 1.4 Hypothetical scheme for salt uptake across the plasmalemma involving two exchange sites. The system is dependent on a supply of metabolic energy from chloroplasts or mitochondria. This is used primarily to separate $H^+$ and $OH^-$ at a site indicated by " ~ ". Active (thermodynamically uphill) ion fluxes shown by solid lines, and passive (downhill) fluxes shown by broken lines. (From Smith, 1970).

# CHAPTER TWO

## METHODOLOGY

### 2.1 Introduction

In this section the various culture techniques, apparatus and procedures will be described and in addition the various alternative procedures used during the course of the study will be outlined and discussed. In the other sections of the work, wherever the methods vary from those described below or involve other special techniques, they will be presented in those sections.

### 2.2.1 Material Sources

Shoots of *Elodea densa* were obtained from either the Adelaide Zoological Gardens or the Botany Department Pond and placed in indoor tanks in the Botany Department. Shoots to be used within two weeks were allowed to float in the tanks until used. However, most shoots were planted in garden soil in the bottom of the tanks and cultured until used. Initially many problems were encountered in culturing suitable material for study - the tendency being to produce long, very narrow, thin leaves with low turgor pressure rather than shorter, broader leaves as discussed in 2.2.3. Towards the end of the study, successful culture methods were established, and a continuous supply of appropriate material cultured under similar conditions was available. It must be emphasised, that provided healthy young leaves were chosen, without large deposits of calcium carbonate on the upper surfaces, without excessive epiphyte growth, and with sufficiently rapid protoplasmic streaming, then

there was very little difference in the comparative responses of the leaves to the various experimental treatments. The absolute values of parameters such as $O_2$ evolution, $Cl^-$ influx and $^{14}C$-fixation did show differences.

### 2.2.2 Culturing Methods

*Elodea* is an ideal plant material for student study and experimentation, and many educational and research institutions have attempted culturing with mixed success. Palmcrantz (1953) developed an extremely detailed culturing medium (Table 2.1) which was used by Jeschke and Simonis (1965). The plants were placed in sand under artificial lights at intensities of 1600 to 3000 lux for 14 hours with 10 dark hours at 24°C. In contrast to this, Olsen (1954) studied the natural habitat of *Elodea* in New Zealand and found that greatest growth was obtained in culture with conditions that closely mimicked those occurring naturally - in particular in thick black mud containing sufficient quantities of ferrous sulphide. In his growth studies, Steemann Nielsen (1944) found that the greatest shoot growth was obtained when 2% $CO_2$ was bubbled through the culture medium.

The most successful culture method in the current study was a combination of the above, with further refinements made from experience gained by trial and error. Garden soil was added to a plastic tank (100 litre) and enriched with ferrous sulphide chips and powder. Deionized water was added to fill the tank and allowed to stand for several days until the water cleared before planting small shoots of *Elodea*. The water was then bubbled with 1 or 5%

$CO_2$ in air. Lighting was by several 40 Watt fluorescent tubes giving a light intensity ranging from 10 to 30 $W/m^2$, in a 12 hour light/12 hour dark cycle.

Sufficient material was generally available for experimentation after a period of 8 to 10 weeks. In any one experiment, shoots were always removed from the same tank, as there was considerable variation between the leaves grown in the different tanks with respect to the parameters measured - mainly due to the differing chlorophyll content of the leaves. High light intensities (greater than 10 $W/m^2$) resulted in rapid growth of leaves with low chlorophyll content, whereas low intensities produced slower growth and generally more suitable study material with higher chlorophyll content.

### 2.2.3 Morphology

The morphology of *Elodea densa* grown under culture conditions in the laboratory showed marked variability. In some cases the stems showed marked elongation and a decrease in the number of leaves per whorl, whereas other plants under similar growth conditions showed very dense leaf growth with little stem elongation. In fact, at times the morphology approached that of *Elodea canadensis* (Rich). In general, however, under conditions of high light intensity, growth was rapid, stem elongation excessive, leaves were long and thin (20 - 30 mm by 2 - 3 mm) had low turgor and were low in chlorophyll. They were generally not suitable for the study. These plants were short-lived as chlorosis occurred soon after maturity. At low light intensities growth was much

slower, leaves were short and broad (10 - 15 mm x 5 - 8 mm) had high turgor pressure and high chlorophyll content and noticeable anthocyanin development in the region of the leaf mid vein. Leaves were suitable for study and generally survived for greater periods after maturity before becoming chlorotic. Because of their prolonged survival, these leaves developed the familiar calcium carbonate deposits on their upper surfaces. In general this material resembled closely that found in the natural habitat, although displaying the advantages of reduced epiphyte infestation and animal damage.

## 2.3 Apparatus and Procedures

### 2.3.1 Oxygen Exchanges

Net oxygen uptake and release was measured using a thermostated Rank Oxygen Electrode connected to either a Heathkit Chart recorder (IR-18M) or a Rikadenki Chart recorder (B-281HS). A full scale deflection was obtained by the addition of distilled water saturated with air, giving an $O_2$ concentration of 250 μmoles/litre, and zero $O_2$ concentration was obtained by the addition to the above of several crystals of sodium dithionate. The experimental media were thermostated at 25°C, and stirred with a small magnetic flea. The leaves were separated from the flea by a coarse polyethylene screen that allowed adequate mixing through the leaves but held the leaves in place.

A mirror was placed behind the electrode at 60 degrees to the incident beam to ensure more uniform illumination of the leaves

in the electrode. The light from a quartz iodide lamp (240 V, 150 Watt) was passed through 2 cm of water (in addition to the water in the thermostat jacket - 1 cm) to filter out infra-red radiation.

In some experiments where the effects of various treatments were monitored over long periods (greater than 6 hours) successively in the light and dark, the recorder was fitted with a set of micro-switches which turned on the light when the partial pressure of oxygen in the solution reached a low value (30 - 40 μmoles/litre) and switched off the light at values just below saturation (210 - 230 μmoles/litre).

### 2.3.2 pH Changes

Net pH changes of the solution surrounding the *Elodea* leaves as a result of photosynthesis, respiration and the various treatments was monitored by a small Beckman frit junction combination electrode of tip diameter 3 mm connected to a Beckman pH meter (1019) and Rikadenki 2 channel Chart recorder. These changes were measured in the Rank Oxygen Electrode chamber under much the same conditions of light and temperature as the oxygen exchange experiments themselves. The combination electrode was passed down the chamber so that the tip was just below the polyethylene screen and just above the magnetic flea. Under constant stirring conditions, the pH reading was quite stable and the effects on the junction potential caused by the stirring were minimised by calibrating the electrode with standard buffers under the same stirring conditions.

With the pH electrode in place, it was not possible to seal the chamber in the usual way, so the system was sealed using liquid

paraffin, which had no demonstrable effect on any of the measurements, as was similarly shown by Lucas (1975). Inhibitors and uncouplers etc., were added to the chamber by plunging a microcap pipette through the paraffin layer.

In experiments where the $O_2$ exchanges were also measured simultaneously, no electrical interference between the two systems was observed.

### 2.3.3 $Cl^-$ Influx Experiments

Leaves were cut from the stems of several plants and placed in 0.5 mM $CaCO_3$ overnight under low intensity light at room temperature. In experiments where the influx was expressed in pmoles/$cm^2$/sec the leaves were cut on the morning of the experiment into 1 cm lengths (longer or shorter depending on the actual size of the leaves) using a sharp scalpel with the leaves placed in a small plastic petri dish over millimeter-square graph paper. The average width of the leaves was obtained from the mean of 10 - 12 leaves. The errors in using this method for obtaining leaves of the same surface area were less than 5%.

In order to correlate the values obtained by this method with the values of Jeschke (1967), in some experiments the leaves were also weighed (fresh weight) before being dried on the planchettes. This enabled the $Cl^-$ influxes to be expressed in pmoles/sec/GFW. This method was tedious, involved a large amount of handling of radioactive material and generally resulted in errors greater than 10%, and so was not often used.

After cutting, leaves were placed in the required solutions

under the required experimental conditions for pretreatment periods of generally 60 minutes before being transferred to solutions containing $^{36}Cl^-$ tracer. $^{36}Cl^-$ uptake was measured over the following 40 - 60 minutes and then the leaves were rinsed in 100 ml of unlabelled experimental solution for 60 minutes before being placed on planchettes. 1 drop of 2% sucrose was added to adhere the leaves to the planchettes, which were then dried and counted. Specific activities of the experimental solutions were obtained by aliquoting 0.2 ml onto planchettes, adding sucrose and counting.

2.3.4 Carbon Fixation Experiments

The preparation and pretreatment of the tissue for $HCO_3^-$ and $CO_2$ fixation experiments was similar to that for the $Cl^-$ influx experiments. During the pretreatment and experimental procedure all vials containing the leaves and solutions were sealed with clear polythene ("Glad-Wrap") to minimise interference from atmospheric carbon dioxide.

Where low concentrations of exogenous $CO_2$ and $HCO_3^-$ were to be used, the solutions were bubbled for at least 10 minutes with $N_2$, scrubbed by bubbling through 100 mM NaOH to ensure complete removal of small traces of $CO_2$. After this, freshly prepared $NaHCO_3$ was added to give the correct concentration, and then finally 10 mM NaOH (freshly prepared) or 1.0 mM HCl was added to give the required pH.

Fixation was initiated by transferring the leaves to vials containing the experimental solutions labelled with $^{14}C$. Fixation was rapidly stopped by emptying the contents of the vial on to a

polythene strainer followed by several rapid rinses (10 seconds) of distilled water. The leaves were then placed on planchettes to which 1 drop of 2% sucrose had been added. Two drops (0.2 mls) of 2% propionic acid were then added to the planchettes, which were then dried slowly over a hot plate. The propionic acid ensured the removal of unfixed $CO_2$ (and $HCO_3^-$) during the drying process.

The specific activity was obtained by a pipetting 0.2 ml of experimental solution to a planchette containing 0.2 ml of 100 mM NaOH (freshly prepared). One drop (0.1 ml) of 2% sucrose was then added and the planchettes rapidly dried and counted immediately. The planchettes used for the above specific activity determination had been previously coated (3 sprays) with "estapol" clear lacquer to ensure that the 100 mM NaOH was not able to react with the aluminium. Although similar methods of determining specific activity have not been used successfully (Hope et al, 1972), provided the above procedure was carried out swiftly and counting commenced immediately after drying, the method gave highly reproducible specific activity determinations.

### 2.3.5 $Ca^{++}$ Influx Experiments

The pretreatment conditions and methods for these experiments were similar to those used for the $Cl^-$ influx experiments. $CaCl_2$ solutions were prepared using a stock solution of high purity 100 mM $CaCl_2$ prepared by Selby's (Adelaide). Radioactive solutions were prepared by the addition of the required amounts of labelled $CaCl_2$ to give an activity of approximately $5 \times 10^4$ µC/mole.

Because of the large deposits of calcium carbonate on the upper surfaces of mature leaves, leaves were treated with 5 mM MES at pH 5.0 for several hours or overnight to remove these desposits before being placed in the pretreatment solutions.

After the uptake period the leaves were then soaked in distilled water or 10 mM $CaSO_4$ for 1 hour before being placed on planchettes with 0.2 ml of 2% sucrose. Specific activities were determined as for $Cl^-$ influx experiments.

Influxes were determined with reference to surface area and no attempt was made to determine them on a fresh or dry weight basis.

### 2.3.6 $Ca^{++}$ Efflux Determinations

Leaves were cut and loaded with $^{45}Ca^{++}$, by soaking in 5.0 mM $^{45}CaSO_4$ overnight. Leaves with visible coatings of calcium carbonate were not selected, and in any case to prevent interference from this phase, leaves were given pretreatment in 5.0 mM MES at pH 5.0 for several hours or overnight before loading.

Aliquots (1.0 ml) of the washing solution were prepared in perspex agglutination trays. Washout was commenced by removing three leaf sections from the loading solution into the first aliquot of washing solution. The three sections were gently agitated for 30 seconds before being transferred to the next washing solution. This process was then repeated every 30 seconds for the complete washout period. For counting, 0.5 ml of each washout aliquot was pipetted onto a planchette and dried. The final sample contained only the three leaf sections. The $Ca^{++}$ content of the leaves remaining after each washout time interval could then be calculated.

### 2.3.7 Analysis of the $Ca^{++}$ Efflux Curves

The efflux kinetics were analysed using the non-linear programme package of SPSS (Statistical Package for the Social Sciences) of the Vogelback Computing Centre, North Western University, U.S.A. Analysis for first, second and even third order kinetic reactions was attempted. Single order kinetic reaction parameters were also computed using an HP-25 calculator (Hewlett Packard). These kinetic determinations were attempted in order to isolate $Ca^{++}$ effluxes from the various phases in the *Elodea* leaf tissue, and to obtain evidence for the polar transport of $Ca^{++}$ ions.

### 2.3.8. Radioactive Counting Equipment

Beta radiation from the decay of $^{36}Cl$, $^{14}C$, and $^{45}Ca$ isotopes was measured using either an end window Geiger tube (G.E.C. E.H.M. 25) connected to an Ekco automatic scaler (N 530f) which was coupled to a Berthold automatic sampler changer, (LB 271), or in later experiments, a Nuclear Chicago gas-flow counter (1043) using 0.5% butane in helium as carrier gas. This second apparatus, had an efficiency approximately 10 times the former, enabling lower specific activity solutions to be used.

Counting times were chosen so that counting error was less than 2%.

### 2.3.9 Calculation of Fluxes and C-fixation

The calculation of these values was improved by the use of an HP-25 pocket calculator (Hewlett Packard) using a special programme devised for this purpose. This programme appears in

Appendix C. Standard deviations of the values were also computed on the same calculator using the built-in programme modified to convert the standard deviation to the standard error of the mean.

### 2.3.10 Solutions and Reagents

Glass distilled water was used to prepare solutions using Analar grade reagents.

The normal bathing media used contained 1.0 mM NaCl, 0.1 mM KCl, 0.5 mM $CaSO_4$, 1.0 mM $NaHCO_3$ using the required buffers depending on the pH of the final medium. Variations from this ionic composition are noted in the text, and legends to the Figures and Tables.

$HCO_3^-$-free and $CO_2$-free solutions were prepared by bubbling the above solutions (with no $HCO_3^-$) with $N_2$ gas scrubbed by passing through 100 mM NaOH and subsequently freshly distilled $H_2O$ for at least 30 minutes. Final pH was adjusted by titration with freshly prepared 10 mM NaOH or 1.0 mM HCl. In all cases solutions containing $HCO_3^-$ were prepared by the addition of freshly prepared 100 mM $NaHCO_3$.

In the $Ca^{++}$ influx and efflux experiments, the solutions were prepared from a stock solution of high purity 100 mM $CaCl_2$. The use of this $CaCl_2$ to replace the normal $CaSO_4$ is noted in each case in legends to the Figures and Tables.

Solutions were all prepared at 20°C using B-grade pipettes and volumetric flasks.

Stock solutions of 1.0 mM CCCP and 10 mM CMU were prepared by dissolving in absolute ethanol. Required amounts were then pipetted into solutions using microcap syringes. The small volumes of ethanol had no effect on the plant material or the $O_2$ and pH electrodes (Betts, 1970).

TABLE 2.1 CULTURE SOLUTION FOR *Elodea densa*

| | | | | | |
|---|---|---|---|---|---|
| $NaHCO_3$ | 1.06 | mM | $Na_2WO_4$ | 6 | nM |
| $Na_2SO_4$ | 0.08 | " | $K_2Cr_2O_7$ | 50 | " |
| $Na_2CO_3$ | 0.012 | " | $MgSO_4$ | 10 | µM |
| $K_2SO_4$ | 0.106 | " | $SnCl_2$ | 1.4 | " |
| $K_2HPO_4$ | 0.038 | " | $Al_2(SO_4)_3$ | 0.25 | " |
| $CaCl_2$ | 0.52 | " | $TiO_2$ | 2 | " |
| $Ca(NO_3)_2$ | 0.1 | " | $MnSO_4$ | 0.25 | " |
| $CaSO_4$ | 0.03 | " | $ZnSO_4$ | 0.1 | " |
| $FeSO_4$ + $Na_2EDTA$ | 0.025 | " | $CoCl_2$ | 60 | nM |
| $H_3BO_3$ | 5.0 | µM | $NiCl_2$ | 60 | " |
| $Na_2MoO_4$ | 0.3 | " | $CuCl_2$ | 80 | " |
| | | | $VOSO_4$ | 18 | " |

Culture medium developed by Palmcrantz (1953) for *Elodea densa*

# CHAPTER THREE

# THE $Cl^-$ INFLUX OF *Elodea densa*

## 3.1 The Light-stimulated $Cl^-$ Influx

The light-stimulation of the $Cl^-$ influx as determined from continuous $^{36}Cl^-$ uptake experiments is shown in Figure 3.1. In this experiment, the external $Cl^-$ concentration was 2.0 mM, and the leaves were from plants taken directly from the Zoological Gardens. The mean of the light-stimulated influx was 1.78 ± 0.2 pmoles/cm$^2$/sec., compared with 0.15 ± 0.02 pmoles/cm$^2$/sec. for the dark influx. Thus, light does stimulate the $Cl^-$ influx by approximately 10-fold over the dark value (see Table 3.1). This is in agreement with Betts (1970), although the stimulation is much greater than the 2-fold stimulation observed by Jeschke (1967) from 0.1 mM $Cl^-$ solutions.

The values obtained for the $Cl^-$ influx, however, were quite variable and depended on the source, culture and storage conditions, as may be seen from Table 3.1. Leaves from the Botany Department Pond, which grew in strong sunlight and had large $CaCO_3$ deposits, generally showed very high $Cl^-$ influx values compared to leaves collected from the Zoological Gardens, which were grown under much lower light conditions. However, leaves from the Botany Department Pond showed a rapid fall in the $Cl^-$ influx during storage in indoor tanks, and fell to between 10 and 20% of the control value in 10 days. Leaves from the Zoological Gardens did not show such rapid deterioration. Of the leaves grown in the indoor tanks,

there was a large variation from tank to tank and this emphasised the need for proper controls for each experiment.

## 3.2 The Effect of External $Cl^-$ Concentration

The effects of increasing the external $Cl^-$ concentration at pH 4.5 and 5.5 are shown in Figures 3.2 and 3.3 respectively. Saturation was achieved at both pH values with the external $Cl^-$ less than 1.0 mM in the light and dark. However, further increases in the external $Cl^-$ concentration (4 to 6 mM) resulted in an inhibition of the $Cl^-$ influx. As the uptake time for this experiment was 80 minutes it was possible that at 6 mM, the leaves may have become saturated with $^{36}Cl^-$ within this period, resulting in a lower estimate for the $Cl^-$ influx. In Figure 3.2, the KCl and $CaCl_2$ concentrations were both 0.1 mM, except when the external $Cl^-$ was 0.1 mM, and here the KCl and $CaCl_2$ concentrations were 0.01 mM. However, in Figure 3.3 the KCl and $CaCl_2$ concentrations were kept constant at 0.01 mM throughout.

## 3.3 The Effect of Solution pH

The effect of solution pH on the $Cl^-$ influx is shown in Figure 3.4. It shows data for three separate experiments, and indicates that there was a pH value between 5.5 and 6.5 where the light-stimulated $Cl^-$ influx was maximal. There was no significant pH effect on the dark $Cl^-$ influx. These effects are similar to those obtained by Smith and Walker (1976) for *Chara*, although they obtained maximum $Cl^-$ influx values between pH 6.5 and 7.5. The buffers used for each experimental point are shown

(Figure 3.4). Smith (1970) found that these buffers did not affect the $Cl^-$ influx in *Chara*, although buffers such as TRIS and imidazole did increase the $Cl^-$ influx values noticeably.

Smith (1970) suggested that an inhibition of the $Cl^-$ influx by increasing solution pH, would provide evidence for this proposed model for ion transport (1.9 and Figure 1.5), whereby the $Cl^-$ influx was coupled to an $OH^-$ efflux. The results here, may also provide evidence for this model in *Elodea densa* (see also Chapter 8). However, the inhibition at low pH values may reflect inhibition by $CO_2$ (Jeschke, 1967).

## 3.4 The Effects of $HCO_3^-$ and $CO_2$

Tables 3.2, 3.3, 3.4 and Figure 3.5 show that at both low and high pH, the effect of adding $HCO_3^-$ to the bathing solutions was to minimally increase the $Cl^-$ influx. At pH 6.0, the addition of $HCO_3^-$ would have reflected an effect of $CO_2$ since the concentration of $CO_2$ at this pH would have been approximately 0.6 mM, whereas at pH 9, the effect would have primarily been due to $HCO_3^-$. The effects of treatment with potassium citrate and $CaCO_3$ replacement are discussed in 3.5 and in detail in Chapter 6. The effects of $HCO_3^-$ in the absence of $Ca^{++}$ due to citrate treatment, made it possible to differentiate between the direct effects on the $Cl^-$ influx of $HCO_3^-$, and the effects due to $HCO_3^-$ influx and fixation. The results therefore show that there was very little effect of $CO_2$ and $HCO_3^-$ on the $Cl^-$ influx. This is in agreement with the results obtained with *Elodea* (Jeschke and Simonis, 1969) and *Vallisneria* (Prins, 1974). However, if $HCO_3^-$ did compete with $Cl^-$

for transport sites as observed in *Vallisneria* (van Lookeren Campagne, 1957), then this may account for the lack of stimulation of the $Cl^-$ influx in both treated and untreated leaves (Table 3.2).

The results at low pH, while only showing a small stimulation of the $Cl^-$ influx by $CO_2$ did not agree with Jeschke (1967) who found an inhibitory effect of $CO_2$. Betts (1970) also found a stimulatory effect of $CO_2$ on the $Cl^-$ influx compared to values in $N_2$-bubbled solutions and this is interpreted as evidence that the $Cl^-$ influx requires the operation of noncyclic electron transport in the production of either or both ATP and NADPH.

## 3.5 The Effect of $CaSO_4$

The effect of $CaSO_4$ on the $Cl^-$ influx at high pH in normal and citrate-treated leaves is shown in Tables 3.2 and 3.5. The normal $Cl^-$ influx of the leaves in Table 3.2 was less than in Table 3.5 due to their different sources (3.1), but both demonstrate that $Ca^{++}$ replacement did reverse the inhibition caused by removal of $Ca^{++}$ by citrate. In the solutions that also contained 1 mM $NaHCO_3$, one cannot distinguish between a primary effect of $Ca^{++}$ as such or an indirect effect via photosynthesis. This will be discussed more fully in Chapters 6 and 9.

## 3.6 The Effect of CCCP

The effects of CCCP on processes such as photosynthesis and respiration (see 4.1) show noticeable time-dependent changes. This was well illustrated in *Vallisneria* by Prins (1974), where 1 μM CCCP

stimulated photosynthesis in the second and third hour after addition, but in the fifth and sixth hour resulted in a 40% inhibition. This is undoubtedly due to CCCP's action as an uncoupler of photosynthesis, which causes an increase in electron transport until there is a depletion of reactants. In contrast, respiration was stimulated by over 200% in *Vallisneria* and this took three to four hours to reach a maximum and remained at this value into the sixth hour.

Similarly, studies in this work not only showed that CCCP produced time dependent changes on $O_2$ exchange - photosynthesis and respiration - but also differences depending on the presence of light during the initial phase of interaction as described in Chapter 4.

In addition, CCCP showed a differential effect depending on the pH of the bathing solution. Table 3.6 shows this effect on the $Cl^-$ influx. Here 2 μM CCCP was added to the solutions containing the leaves at four pH levels for one hour before being transferred to solutions at pH 6.5 (containing no CCCP) for the estimation of the $Cl^-$ influx. As the effects of CCCP are partially reversible (Prins, 1974), these values do not represent definitive inhibition values of CCCP. It does demonstrate that CCCP administered to leaves at pH 6.5 and 8.0, reduced the $Cl^-$ influx in a subsequent solution at pH 6.5 by 42% and 9% respectively. As the $pK_a$ of CCCP is 6.8, it was obviously a differential effect between the ionised and unionised forms of the compound. Thus, inhibition studies in this thesis using CCCP, were all carried out by adding CCCP to solutions with pH values less than 6.5, and then if necessary transferring the leaves to other solutions.

As CCCP showed unpredictable effects on $O_2$ evolution (Chapter 4), it was decided to study its effects on the $Cl^-$ influx while monitoring the $O_2$ evolution at the same time. Hence for each experimental run about 10 - 14 leaves, cut and pretreated overnight in the usual way, were placed into the oxygen electrode in $N_2$-bubbled solutions. The required amount of CCCP and $NaHCO_3$ was added and allowed 30 to 60 minutes in this pretreatment solution before being replaced by labelled $Cl^-$ solutions without CCCP. Otherwise the methods were the same as described in 2.3.3. The importance of these experiments is that the $Cl^-$ influx was measured under exactly the same conditions of stirring and light saturation as photosynthetic $O_2$ production measurements. Tables 3.7 and 3.8 show the effect of CCCP treatment under these conditions. It was surprising to see that with concentrations of CCCP less than 1 µM, the $Cl^-$ influx was stimulated, but at concentrations above 2 µM it was inhibited in both $HCO_3^-/CO_2$ solutions and $HCO_3^-/CO_2$-free solutions. In Table 3.9, two experiments are shown where the $Cl^-$ influx was measured by the method described in 2.3.3. The results are very much the same as in Tables 3.7 and 3.8, and a summary of all these experiments is shown in Figure 3.6.

Further discussion and comparison with the effect of CCCP on $O_2$ evolution will appear in the general discussion (Chapter 9) after the effects of CCCP on photosynthesis have been presented (Chapter 4).

## 3.7 The Effect of CMU on the $Cl^-$ Influx and Photosynthesis

The presence of CMU at pH 6.5 does have a marked inhibitory effect on the $Cl^-$ influx (Table 3.10). As most of the exogenous

carbon at this pH was $CO_2$, this confirmed the results of Jeschke (1967). The results of several further experiments with and without 1.0 mM $NaHCO_3$ are shown graphically in Figure 3.7. Again the $Cl^-$ influx in $N_2$ was less sensitive to the presence of CMU - the $Cl^-$ influx at 10 μM CMU was still 90% of the control values - whereas the $Cl^-$ influx and the $O_2$ evolution in the presence of exogenous carbon were reduced to 30% and 10% of controls respectively.

The $O_2$ evolution results are shown here because CMU had a more reproducible effect on photosynthetic $O_2$ production in comparison to CCCP, and its effects did not require careful consideration. These results support the interpretation of Jeschke (1967, 1972) that the $Cl^-$ influx, when occurring concurrently with photosynthesis shows similar sensitivity to photosynthetic $O_2$ production. However, when there is no net $O_2$ production - photosynthesis and respiration balancing each other - the $Cl^-$ influx is less sensitive to CMU. Jeschke interpreted this as cyclic photophosphorylation (ATP) powering the $Cl^-$ influx in $N_2$ conditions, whereas under conditions of $CO_2$, noncyclic electron flow occurred and the $Cl^-$ influx had to compete with C-fixation for energy. It should be pointed out again that in these experiments, the $Cl^-$ influx with no CMU was greater in the presence of $CO_2$ than in $N_2$ (Table 3.10), suggesting that the $Cl^-$ influx depends on the production of both ATP and NADPH in the light.

## 3.8 Summary

1. Light stimulates the $Cl^-$ uptake by as much as 10 fold, although the actual values obtained

vary greatly depending on the growth and storage conditions.

2. Maximum values for the $Cl^-$ influx are obtained when the external $Cl^-$ concentration exceeds 0.2 mM.
3. The light-stimulated $Cl^-$ influx has a pH maximum at pH 6.
4. $CO_2$ and $HCO_3^-$ have little effect on the $Cl^-$ influx except when photosynthesis is occurring maximally, then $CO_2$ and $HCO_3^-$ stimulate the $Cl^-$ influx.
5. $Ca^{++}$ and an operating photosystem are necessary for maximal $Cl^-$ influx.
6. Low concentrations of CCCP stimulate the $Cl^-$ influx and inhibit it at higher concentrations.
7. The $Cl^-$ influx is less sensitive to CMU in $N_2$ bubbled solutions than when $CO_2$ or $HCO_3^-$ fixation is occurring; and both these rates are less sensitive to CMU than is $O_2$ evolution.

TABLE 3.1 EFFECTS OF ORIGIN, CULTURE AND STORAGE ON THE $^{36}Cl^-$ INFLUX OF *Elodea densa*

| Source of Material | Culture Conditions | Length of Storage | Solution pH | External $Cl^-$ Concentration | $^{36}Cl^-$ influx (pmoles $cm^2$/sec.) light | dark |
|---|---|---|---|---|---|---|
| Zoological Gardens 1 | Indoor Tanks | 24 hours | 4.5 | 2 mM | 1.78 ± .2 | 0.15 ± .02 |
| " " | " | 10 days | 4.5 | 2 " | 2.46 ± .2 | 0.37 ± .04 |
| " " | " | 40 " | 4.1 | 2 " | 1.54 ± .1 | - |
| " 2 | " | 8 " | 5.0 | 1 " | 8.5 ± .2 | 0.56 ± .11 |
| " " | " | 12 " | 5.0 | 1 " | 3.4 ± .3 | - |
| Botany Dept. Pond 1 | " | 2 " | 5.0 | 2 " | 9.0 ± 1.3 | - |
| " " | " | 6 " | 5.0 | 1 " | 3.0 ± .1 | 0.86 ± .03 |
| " " | " | 6 " | 5.0 | 1 " | 4.9 ± .4 | - |
| " " | " | 12 " | 5.0 | 1 " | 0.59 ± .07 | 0.12 ± .01 |
| " 2 | " | 4 " | 5.0 | 2 " | 4.7 ± .3 | 0.33 ± .04 |
| " " | " | 7 | 5.0 | 2 " | 1.4 ± .07 | - |
| " " | " | 20 " | 5.0 | 2 " | 1.7 ± .09 | - |
| Grown in the indoor tanks | Tank 1 | - | 6.5 | 1 " | 2.58 ± .3 | |
| " " | Tank 2 | - | 6.0 | 1 " | 1.21 ± .11 | |
| " " | Tank 3 | - | 6.0 | 1 " | 6.6 ± .3 | |

All experimental solutions contain 1.0 mM $NaHCO_3$ 0.1 mM KCl, 0.1 mM $CaSO_4$ at the pH indicated.

TABLE 3.2 EFFECT OF $Ca^{++}$ AND $HCO_3^-$ ON THE $Cl^-$ INFLUX AT pH 6.0 AND 9.0 IN NORMAL AND CITRATE TREATED LEAVES

| TREATMENT AND EXPERIMENTAL SOLUTIONS | $Cl^-$ INFLUX (pmoles/$cm^2$/sec) | % OF CONTROLS |
|---|---|---|
| No citrate treatment | | |
| pH 6.0 no $Ca^{++}$ $N_2$ | 0.52 ± .05 | 42 |
| " " no $Ca^{++}$ + 1.0mM $NaHCO_3$ | 0.70 ± .08 | 58 |
| " " 0.1mM $CaSO_4$ + 1.0mM $NaHCO_3$ | 1.21 ± .11 | 100 |
| pH 9.0 no $Ca^{++}$ $N_2$ | 0.17 ± .008 | 100 |
| " " no $Ca^{++}$ + 1.0mM $NaHCO_3$ | 0.26 ± .01 | 152 |
| " " 0.1mM $CaSO_4$ + 1.0mM $NaHCO_3$ | 0.17 ± .02 | 100 |
| Citrate treated leaves | | |
| pH 6.0 no $Ca^{++}$ $N_2$ | 0.11 ± .02 | 9 |
| " " no $Ca^{++}$ + 1.0mM $NaHCO_3$ | 0.28 ± .02 | 23 |
| " " 0.1mM $CaSO_4$ $N_2$ | 0.63 ± .05 | 52 |
| " " 0.1mM $CaSO_4$ + 1.0mM $NaHCO_3$ | 0.81 ± .09 | 67 |
| pH 9.0 no $Ca^{++}$ $N_2$ | 0.074 ± .007 | 44 |
| " " no $Ca^{++}$ + 1.0mM $NaHCO_3$ | 0.053 ± .004 | 31 |
| " " 0.1mM $CaSO_4$ $N_2$ | 0.14 ± .03 | 82 |
| " " 0.1mM $CaSO_4$ + 1.0mM $NaHCO_3$ | 0.18 ± .03 | 106 |

The $Cl^-$ influxes of the untreated leaves with 0.1mM $CaSO_4$ and 1.0mM $NaHCO_3$ were taken as controls. Experimental conditions: 5.0mM HEPES at pH 6.0, 5.0mM TAPS at pH 10, 1.0mM NaCl, 0.1mM KCl 0.1mM $CaSO_4$. Leaves from tank 2. Citrate treatments as described in 6.3

TABLE 3.3 EFFECT OF $HCO_3^-$ LEVELS ON THE $Cl^-$ INFLUX WITH CITRATE TREATED LEAVES AT pH 9.0

| Experimental Conditions | | $^{36}Cl^-$ Influx (pmoles/$cm^2$/sec) | % Of Controls |
|---|---|---|---|
| No citrate | $N_2$ | 5.7 ± .2 | 100 |
| " " | 1.0mM $NaHCO_3$ | 6.6 ± .3 | 114 |
| citrate | $N_2$ | 2.9 ± .2 | 52 |
| " | 0.05mM $NaHCO_3$ | 3.2 ± .3 | 54 |
| " | 0.01 " " | 3.4 ± .4 | 59 |
| " | 0.2 " " | 4.0 ± .3 | 70 |
| " | 0.5 " " | 3.5 ± .2 | 61 |
| " | 1.0 " " | 3.8 ± .3 | 66 |

Experimental conditions as described in Table 3.2. Leaves from tank 3.

TABLE 3.4 EFFECT OF EXOGENOUS CARBON ON THE $Cl^-$ INFLUX AT DIFFERENT pH

| Experimental Conditions | | | $^{36}Cl^-$ Influx (pmoles/$cm^2$/sec) |
|---|---|---|---|
| pH 4.4 | light | $N_2$ | 2.9 ± .3 |
| " " | " | 1.0mM $NaHCO_3$ | 4.5 ± .1 |
| pH 6.5 | light | $N_2$ | 5.1 ± .6 |
| " " | " | 1.0mM $NaHCO_3$ | 4.9 ± .5 |
| " " | dark | " " " | 0.56 ± .1 |
| pH 7.3 | light | $N_2$ | 3.6 ± .2 |
| " " | " | 1.0mM $NaHCO_3$ | 3.8 ± .4 |

Experimental conditions as described in Table 3.2. Leaves from tank 1.

TABLE 3.5 EFFECT OF CITRATE TREATMENT AND $Ca^{++}$ ON THE $Cl^-$ INFLUX AT pH 9.0

| Experimental Conditions | | $^{36}Cl^-$ Influx (pmoles/$cm^2$/sec) |
|---|---|---|
| No citrate | No $Ca^{++}$ | 1.28 ± .2 |
| " " | 1.0mM $CaSO_4$ | 1.24 ± .1 |
| Citrate | No $Ca^{++}$ | 0.17 ± .01 |
| " | 0.5mM $CaSO_4$ | 0.22 ± .01 |
| " | 0.1 " " | 0.23 ± .01 |
| " | 0.5 " " | 0.52 ± .05 |
| " | 1.0 " " | 0.42 ± .04 |
| " | 5.0mM $CaSO_4$ | 0.31 ± .04 |

Experimental solutions and treatments as in Table 3.2 except all contained 1.0mM $NaHCO_3$. Leaves from tank 2.

TABLE 3.6 EFFECT OF pH ON THE CCCP INHIBITION OF THE $Cl^-$ INFLUX

| | $Cl^-$ Influx (pmoles/$cm^2$/sec) | | | |
|---|---|---|---|---|
| Pretreatment pH | 6.5 | 7.0 | 7.5 | 8.0 |
| Controls | 2.6 ± .3 | 3.2 ± .3 | 2.7 ± .3 | 2.2 ± .3 |
| + 2μM CCCP | 1.5 ± .2 | 2.4 ± .3 | 2.5 ± .3 | 2.0 ± .2 |

Treatment with 2μM CCCP at appropriate pH for 1 hour then leaves washed and transferred to solutions (-CCCP) at pH 6.5 for flux measurement (40 mins). Solutions as in 2.3.10.

TABLE 3.7 EFFECT OF CCCP ON THE $Cl^-$ INFLUX

| | Experimental Conditions | $^{36}Cl^-$ Influx (pmoles/cm$^2$/sec) | % Of Controls |
|---|---|---|---|
| A | light | 1.8 ± .2 | 100 |
| | dark | 0.3 ± .02 | 16 |
| | light + 0.25μM CCCP | 3.2 ± .4 | 183 |
| | " + 1.25 " " | 1.9 ± .2 | 108 |
| B | light | 0.77 ± .1 | 100 |
| | " + 1.0μM CCCP | 0.66 ± .1 | 86 |
| | dark + " " " | 0.05 ± .002 | 6 |
| | light + 0.25" " | 1.85 ± .2 | 242 |
| | " + 0.5 " " | 1.76 ± .2 | 230 |
| C | light | 8.9 ± 1 | 100 |
| | " + 0.25μM CCCP | 16.8 ± 2 | 186 |

Three different experiments with different material: A,B - tank 2, C from Zoological Gardens. Experimental solutions as in 2.3.10, at pH 6.5. CCCP pretreatments at pH 6.5, as described in Table 3.6. All experiments performed in the Rank $O_2$ electrode.

TABLE 3.8 EFFECT OF $CO_2$ ON CCCP INHIBITION OF THE $Cl^-$ INFLUX

| Experimental Conditions | $^{36}Cl^-$ Influx (pmoles/cm$^2$/sec) | % Of Controls |
|---|---|---|
| HEPES APW + 1.0mM $NaHCO_3$ | 5.2 ± 0.24 | 100 |
| + 1.5 μM CCCP | 6.0 ± 0.19 | 115 |
| + 2.0 μM " | 2.5 ± 0.4 | 48 |
| | | |
| HEPES APW + $N_2$ | 5.6 ± 0.2 | 100 |
| + 1.5μM CCCP | 5.7 ± 0.2 | 102 |
| + 1.7μM " | 4.7 ± 0.3 | 84 |
| + 2.0μM " | 4.3 ± 0.3 | 77 |

Experimental solutions as in 2.3.10 at pH 6.5 CCCP pretreatments as described in Table 3.7. Experiments performed in Rank $O_2$ electrode.

TABLE 3.9 EFFECT OF CCCP ON THE $Cl^-$ INFLUX

| | CCCP (µM) | $Cl^-$ Influx ($pmoles/cm^2/sec$) | % Of Control |
|---|---|---|---|
| Exp I | 0 | 1.2 ± 0.08 | 100 |
| | 0.5 | 1.7 ± 0.16 | 143 |
| | 0.75 | 2.1 ± 0.2 | 170 |
| | 1.0 | 2.0 ± 0.2 | 163 |
| | 1.25 | 1.8 ± 0.2 | 146 |
| | 1.5 | 1.4 ± 0.2 | 114 |
| | 1.75 | 1.3 ± 0.2 | 106 |
| Exp II | 0 | 1.3 ± 0.09 | 100 |
| | 1.0 | 1.1 ± 0.04 | 91 |
| | 2.0 | 0.05 ± 0.01 | 43 |
| | 5.0 | 0.06 ± 0.004 | 5 |

Experiments I and II were using the same material, but run at different times. Experimental solutions and pretreatments as in Table 3.7.

TABLE 3.10 THE EFFECT OF EXOGENOUS $CO_2$ ON THE CMU INHIBITION OF THE $Cl^-$ INFLUX

| Experimental Conditions | $Cl^-$ Influx (pmoles/cm$^2$/sec) | % Of Controls |
|---|---|---|
| $N_2$ | 2.62 ± 0.3 | 100 |
| " + 1 µM CMU | 3.2 ± 0.2 | 121 |
| " + 2 µM " | 2.5 ± 0.2 | 96 |
| " + 5 µM " | 3.1 ± 0.3 | 118 |
| " + 7 µM " | 3.0 ± 0.3 | 114 |
| " + 10 µM " | 2.4 ± 0.2 | 90 |
| " + 50 µM " | 1.3 ± 0.2 | 39 |
| 1 mM $NaHCO_3$ | 3.6 ± 0.2 | 100 |
| " " + 1 µM CMU | 2.3 ± 0.1 | 63 |
| " " + 2 µM " | 1.8 ± 0.1 | 49 |
| " " + 5 µM " | 2.1 ± 0.1 | 57 |
| " " + 7 µM " | 2.6 ± 0.2 | 70 |
| " " + 10 µM " | 1.0 ± 0.1 | 27 |
| " " + 20 µM " | 1.1 ± 0.07 | 29 |
| " " + 50 µM " | 0.94 ± 0.07 | 26 |

Experimental conditions as in 2.3.10, at pH 6.5. Pretreatment in CMU for 1 hour before uptake measurement commenced.

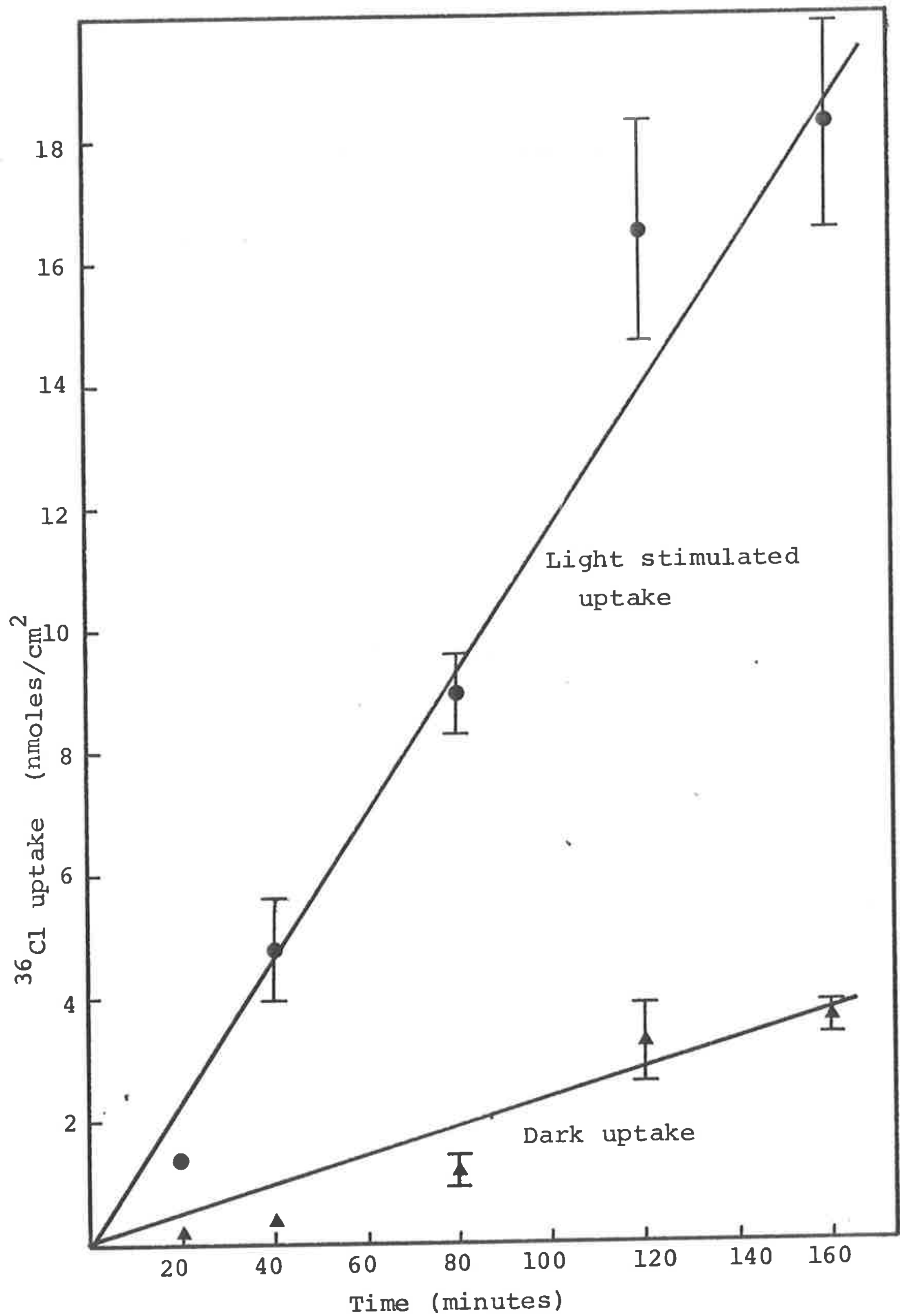



FIGURE 3.1. The continuous uptake of $^{36}$Cl in the light (●) and dark (▲). Distance between error bars represents 2 x S.D.

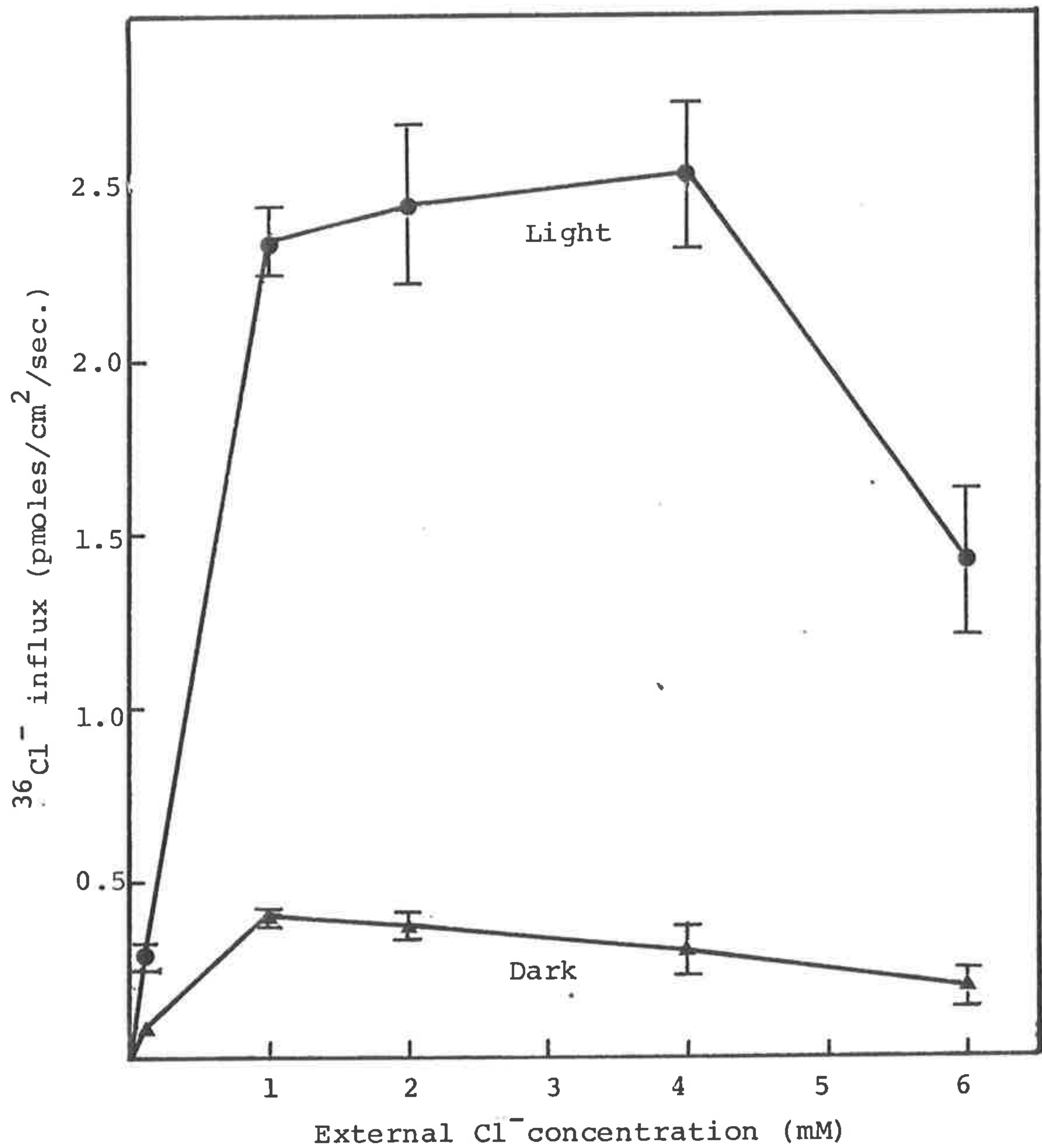



FIGURE 3.2 Effect of external $Cl^-$ concentration on $Cl^-$ influx at pH 4.5. Material cultured in indoor tanks.

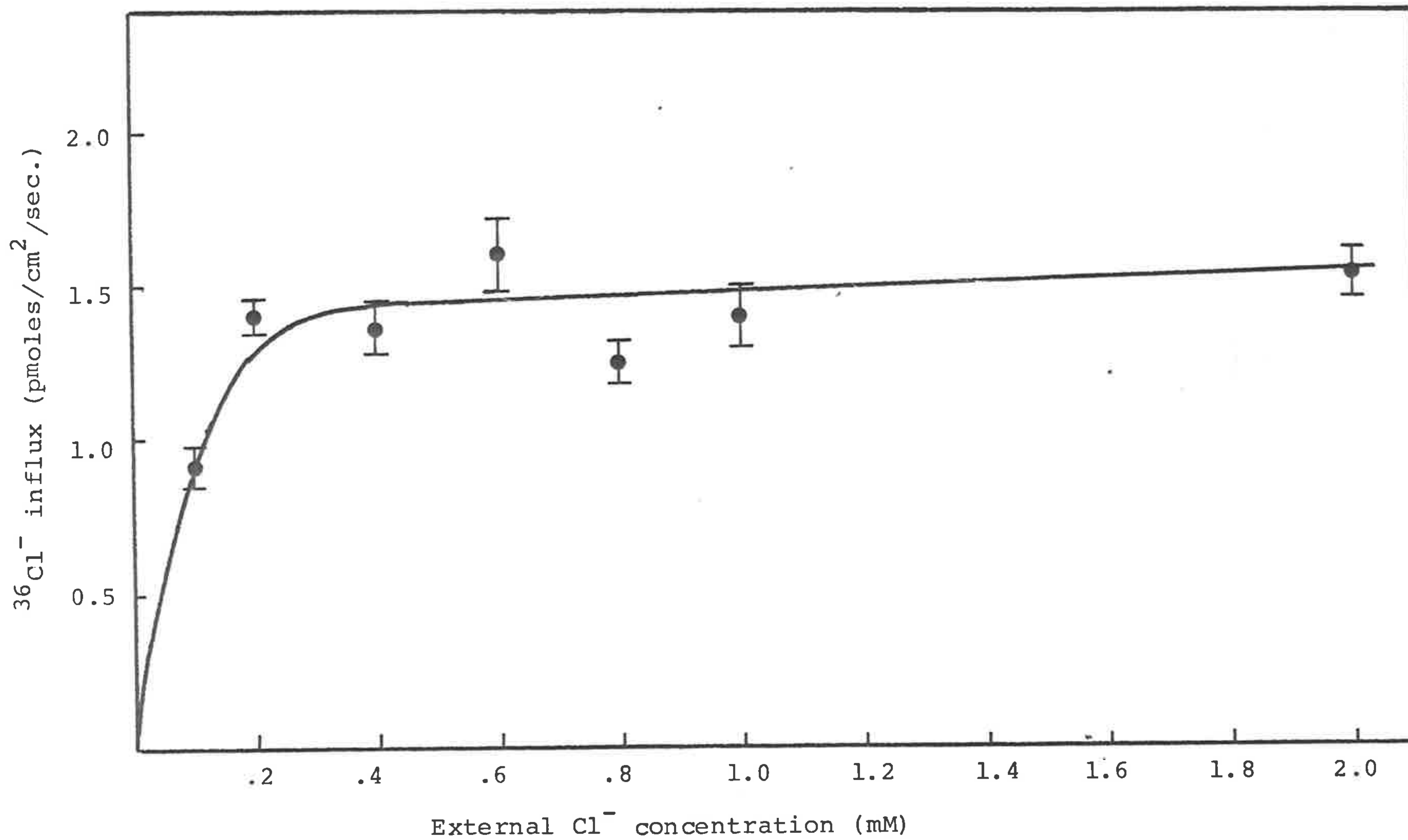



FIGURE 3.3. Effect of external $Cl^-$ concentration on the $Cl^-$ influx at pH 5.5. Material collected from the Zoological Gardens.

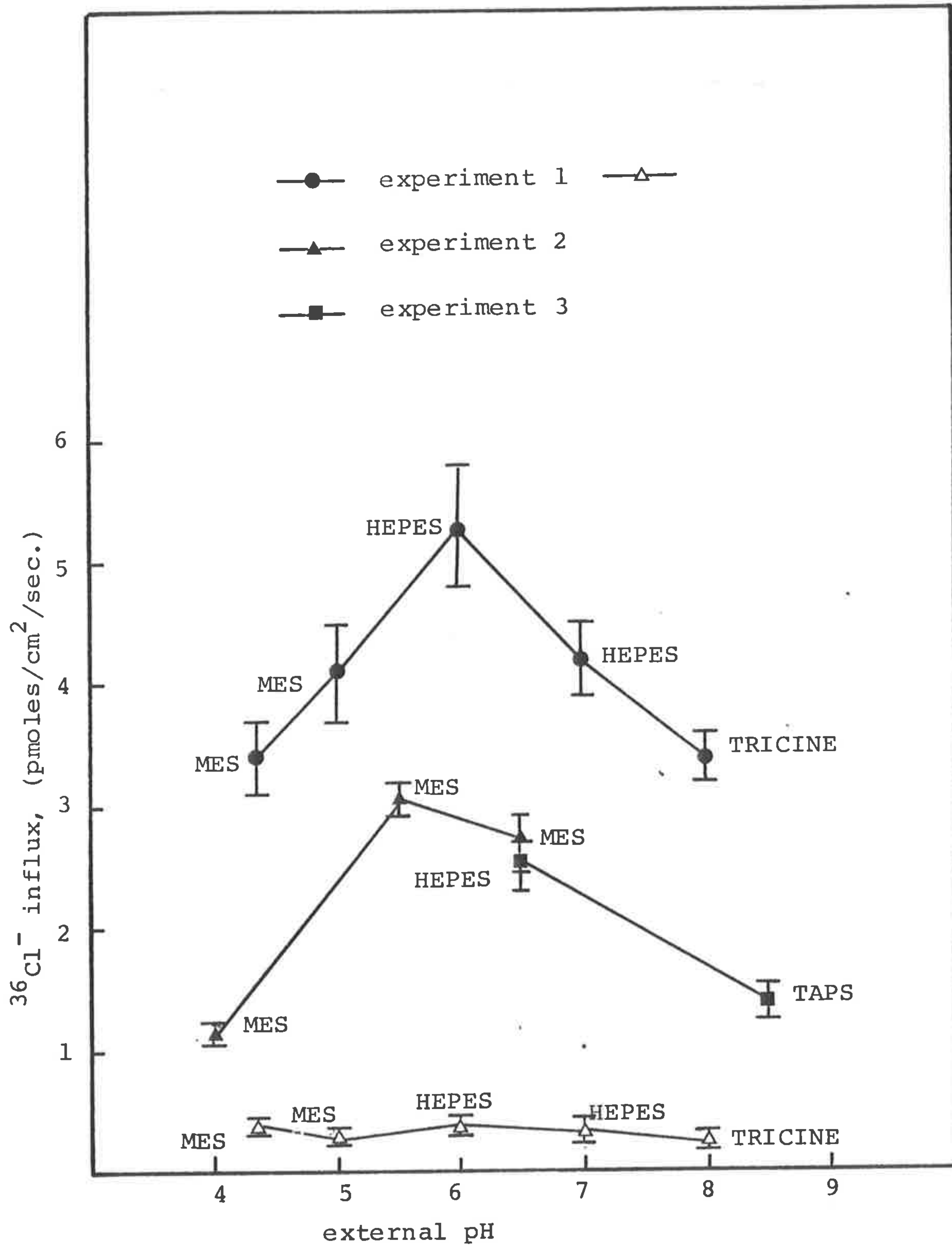



FIGURE 3.4. The effect of external pH on the $^{36}Cl^-$ influx in the light (●, ▲, ■) and dark (△). External Cl concentration 1.3 mM. The buffers used at each pH are as indicated.

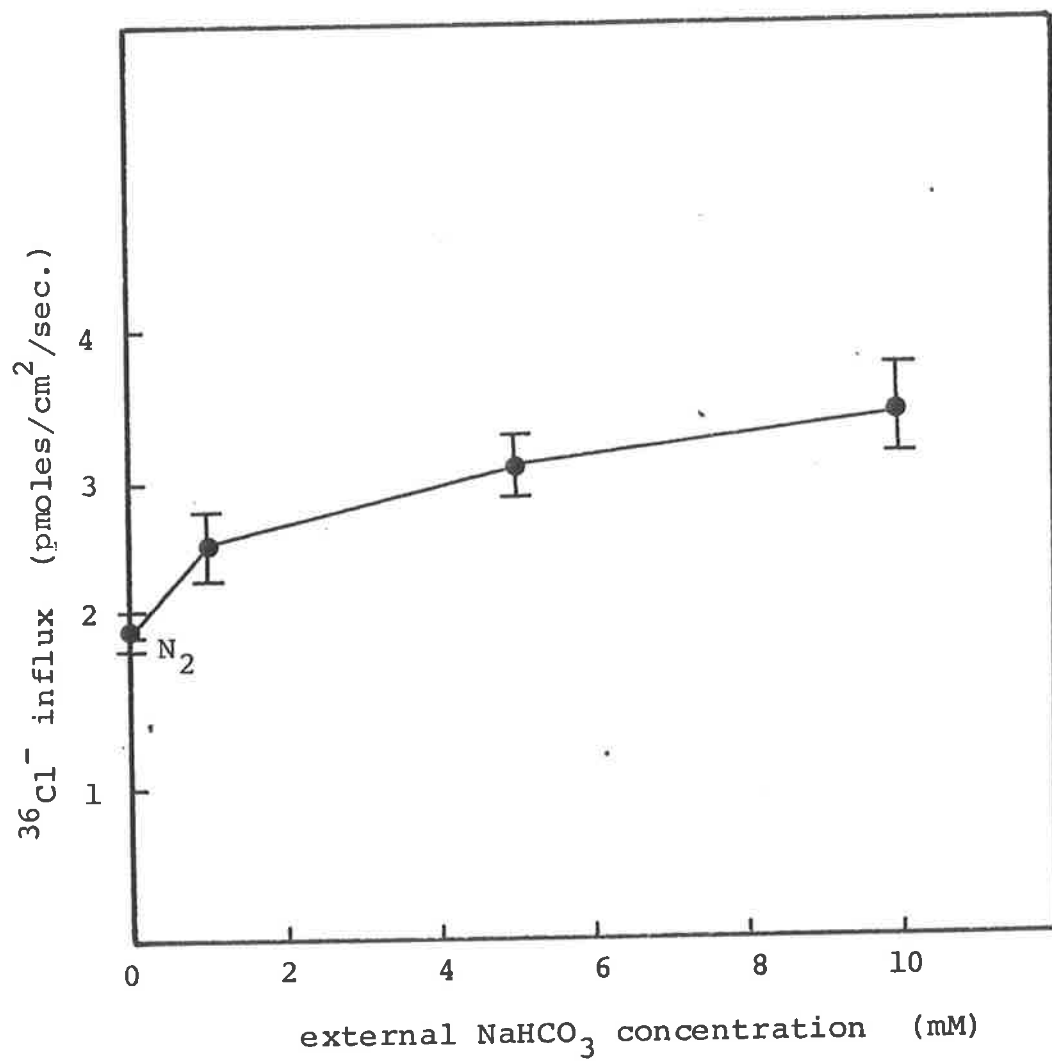



FIGURE 3.5. The effect of external $NaHCO_3$ on the light stimulated $Cl^-$ influx at pH 6.5. The solution at zero $NaHCO_3$ was bubbled with $N_2$. 5.0 mM HEPES buffer.

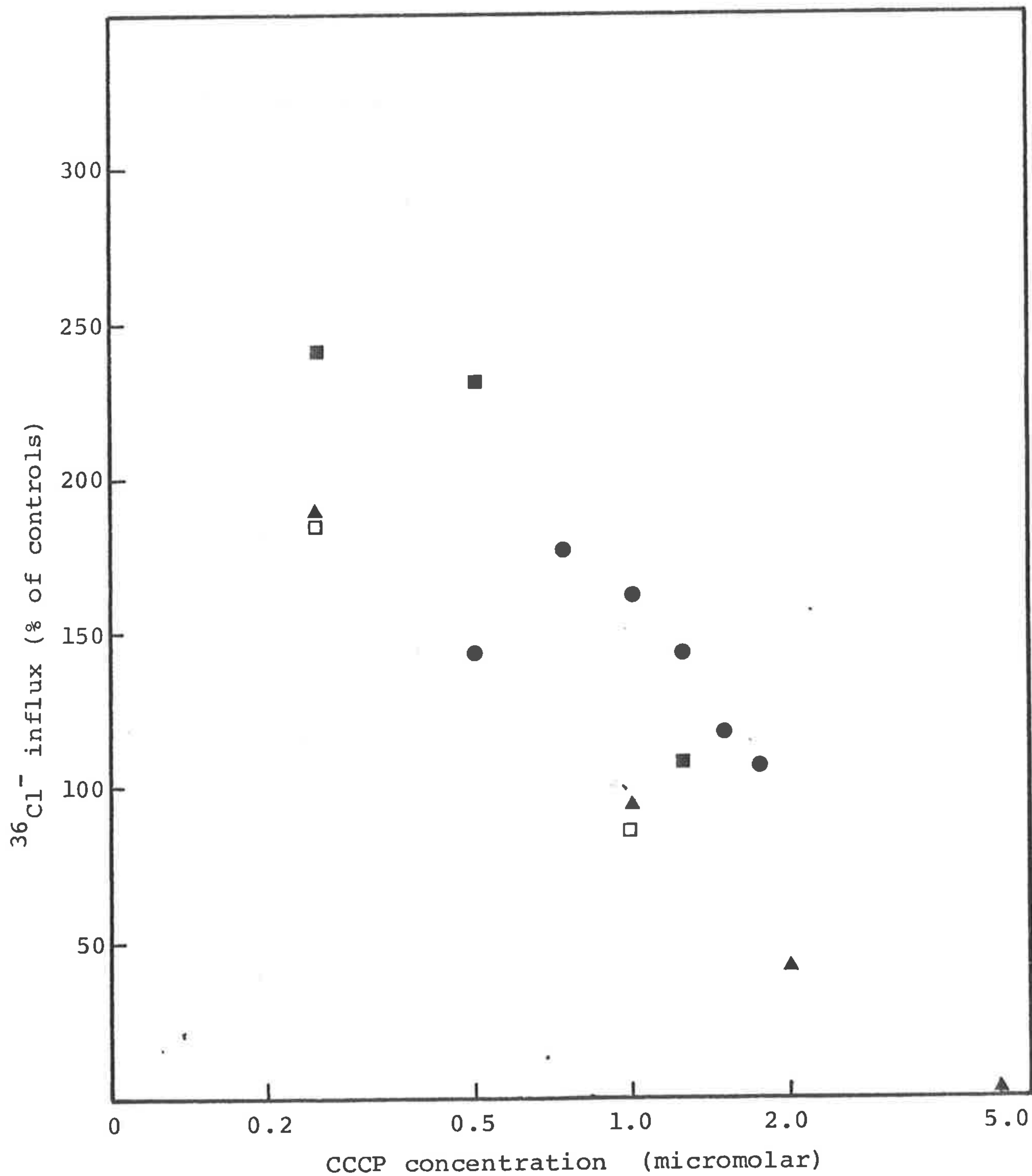



FIGURE 3.6 The effect of CCCP on the $Cl^-$ influx. The different symbols represent different experiments from Tables 3.7 to 3.9. Experimental conditions as described in those tables. Control values represented by 100%.

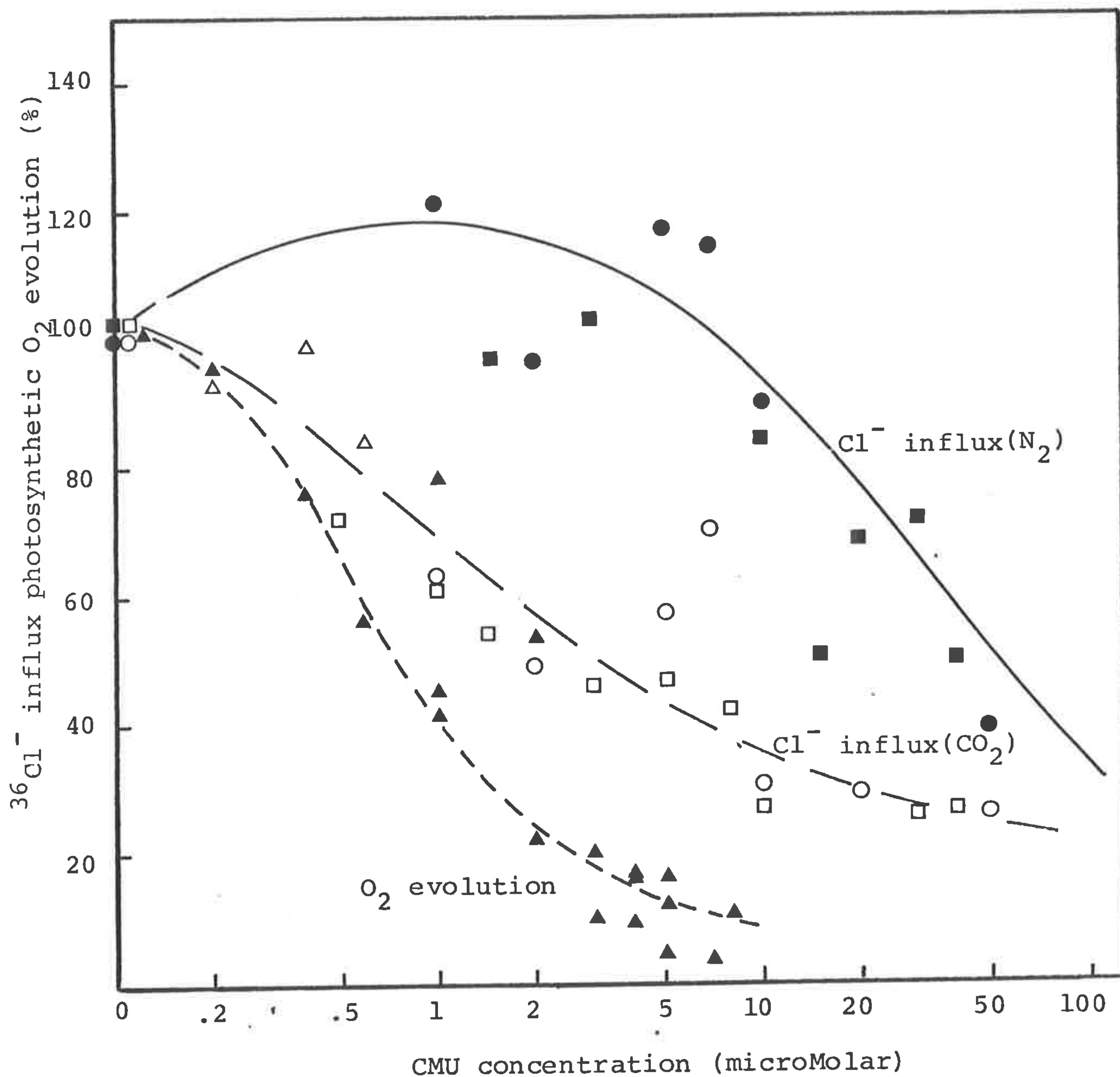



FIGURE 3.7. The effect of CMU on the $Cl^-$ influx and $O_2$ evolution. The different symbols in each group represent different experiments. $Cl^-$ influx in $N_2$ (●,■), $Cl^-$ influx in 1.0 mM $NaHCO_3$ (○,△,□) and $O_2$ evolution (1.0 mM $NaHCO_3$) (▲). All experiments at pH 6.5.

# CHAPTER FOUR

## PHOTOSYNTHESIS AND RESPIRATION OF *Elodea densa*

### 4.1 Introduction

Although the early literature (Arens, 1938; Steemann Nielsen, 1947) demonstrated that *Elodea densa* was able to assimilate $HCO_3^-$, Brown et al (1974) found that in the presence of $HCO_3^-$ ions, photosynthesis - as measured by $^{14}C$-fixation - ceased when the calculated $CO_2$ level fell to zero. This suggested that *Elodea* could not utilise $HCO_3^-$ for carbon assimilation. These conflicting reports warrant further investigation.

The chloroplasts of *Elodea* not only showed rapid cytoplasmic streaming in low intensity light (Seitz, 1964), but at higher light intensities showed a second phototactic response whereby the chloroplasts 'clump' into a spherical formation in the middle of the cells (De Filippis and Pallaghy, 1973). This clumping was reversible in the dark. Similar observations by Betts (1970) were used to explain the fall in net $O_2$ evolution and $Cl^-$ influx after 60 minutes of illumination ($10^4$ ergs/sec/cm$^2$). In addition, photorespiration has been shown to occur in *Elodea* (Brown et al, 1974; Hope, Lüttge and Ball, 1972). Considering the observations above, it was considered important to study exhaustively oxygen evolution and C-fixation under a large variety of conditions.

Inhibitors and uncouplers have been used to study photosynthesis and respiration in many plant species (Raven, 1969; Jeschke, 1967, 1972). Such studies have been extended to establishing

sources of energy for such processes as active ion accumulation and nitrogen metabolism, (see 1.8).

CCCP is a strong uncoupler of photosynthetic and oxidative phosphorylation (Gould and Bassham, 1965; Avron and Neumann, 1968; Gromet-Elhanan, 1968) and at higher concentrations inhibits electron transport in the region of photosystem II (Duysens, 1971; Siggel, Rengel, and Rumberg, 1971). Because of its uncoupling action, CCCP stimulates the rate of respiratory oxygen consumption and photosynthetic oxygen production with *in vitro* experiments of isolated chloroplasts. However, *in vivo*, the uncoupling appears more complex and in many cases, has an inhibitory effect on photosynthetic $O_2$ production, presumably by a build up of NADPH, which cannot be oxidised by the Calvin cycle in the absence of ATP (Prins, 1974; Betts, 1970). The effects of CCCP may be even more complex than this. Prins (1974) showed that both respiratory and photosynthetic $O_2$ exchange in *Vallisneria* leaves changed continuously under the influence of $1 \times 10^{-6}$M CCCP, as discussed in 3.6.

## 4.2 Continuous Measurement of $O_2$ Exchanges in Light/Dark Periods

Continuous measurement of $O_2$ exchanges in the Rank oxygen electrode are shown in Figure 4.1. Here the light/dark periods were controlled by a micro-switching system that ensured a light change at a particular oxygen concentration (see 2.3.1).

The rates of photosynthetic oxygen production and respiratory uptake under these conditions were very stable, except under conditions of $CO_2/HCO_3^-$ depletion when the compensation point between

$O_2$ production and uptake was approached (Figure 4.2). Such depletion was rapidly reversed by the addition of $NaHCO_3$, which acts as a supply of $CO_2$ at low pH and $HCO_3^-$ at higher pH values.

These light/dark studies were undertaken to determine whether there were any diurnal changes in the rates of $O_2$ exchange. Figure 4.2 shows a typical experiment, where over a period of 14 hours, there was no significant change in the rate of respiratory $O_2$ uptake and in the rate of photosynthetic $O_2$ evolution, except when the exogenous carbon became depleted at about 10 hours.

Leaves given this type of treatment before any experimental runs, showed no significant differences in $O_2$ evolution rates when compared to leaves freshly removed from tanks and placed in the $O_2$ electrode.

Figure 4.1 also shows that the ratio of photosynthetic $O_2$ evolution to respiratory consumption was approximately 8 to 1. In other experiments the ratio was in the range 5 - 10 to 1. Most experiments were performed using light/dark periods so that the $O_2$ concentration in solution could return to a level where photosynthetic $O_2$ production could be measured again. Another alternative to this method was to change the solution with one having a low $O_2$ concentration (due to $N_2$-bubbling). However, this method was not used for long periods because chloroplast phototaxis and photorespiration (4.3) may have become problematical, particularly when the rates of photosynthesis were very slow (Betts, 1970; Servaites and Ogren, 1977).

## 4.3 Photorespiration

Figure 4.1 demonstrates that the rate of respiratory $O_2$ consumption changed during the dark period. When the light was turned off the rate was faster than near the end of the dark period. This also had a small effect on the total $O_2$ evolution in the light - the rate being slightly slower at the end of the light period than at the beginning. Similar observations were made by Hope, Lüttge and Ball (1972) with *Elodea canadensis*. In their experiments, as in these, the photosynthetic $O_2$ evolution was calculated by adding the maximum net $O_2$ production obtained immediately after turning on the light, and the respiration rate obtained immediately before turning on the light.

This stimulation of respiratory $O_2$ uptake in the light may also have been due to the increased $O_2$ concentration at the end of a light period. However, Figure 4.3 shows that the rate of respiration, when interrupted by short periods of light, declined even though the $O_2$ concentration did not change significantly. Hence this phenomenon is attributed to photorespiration.

Brown, Dromgoole, Towsey and Browse (1974) demonstrated photorespiration in *Elodea densa* and *Lagarosiphon major* under similar conditions. However, they were unable to find an effect of glycolate, and αHPMS (2-pyridylhydroxymethane - sulphonic acid) and INH (isonicotinyl hydrazide) - inhibitors of glycolate pathway, suggesting that in these two species photorespiration originated from $C_4$ acids which were the major early products of $^{14}CO_2$ fixation with low exogenous $CO_2$ and low pH.

## 4.4 Photosynthetic $O_2$ Evolution Saturation Curves

The rates of photosynthetic $O_2$ evolution are dependent on exogenous $CO_2$ or $HCO_3^-$ in the bathing solutions. When leaves were first placed into the $O_2$ electrode, there was always a small amount of net $O_2$ evolution in the light, due to endogenous carbon reserves (Arens, 1933). This was not observed by Hope, Lüttge and Ball (1972) with *Elodea canadensis*. However, for these experiments it was essential to ensure that these residual carbon reserves were exhausted before exogenous carbon was added. This normally only required several minutes in the light or several light-dark periods in the case of leaves that had been encrusted with carbonate deposits before the pretreatment period (see 2.3.5).

Figures 4.4 and 4.5 show that photosynthetic $O_2$ evolution was saturated at concentrations of approximately 0.4 mM $NaHCO_3$ at pH 6.5. Such concentrations are much lower than those obtained by Blackman and Smith (1911) and Brown, Phillips and Rattigan (1974) using *Elodea canadensis*.

As pointed out by Hill and Whittingham (1958) in their discussion of Blackman and Smith's (1911) results, this was possibly due to the long diffusion pathways particularly in unstirred solutions. In the experiments described here the solutions were vigourously stirred.

## 4.5 Effects of CCCP on Photosynthesis and Respiration

As already mentioned in 3.6, CCCP has a highly variable influence on oxygen exchange in *Elodea densa*. One of the most

significant observations has not been reported before and this was its differential effect in the light and dark. If CCCP was added to solutions containing *Elodea* in the light the effect on $O_2$ evolution was much reduced compared to its effects when added in the dark prior to a light period. In this light period, the inhibition was apparently much greater for a comparative CCCP concentration. However, when CCCP was added in the light, in a subsequent light period - following a period in the dark - the inhibition became maximal again. As a result of this phenomenon, and the differential time effect of CCCP (Prins, 1974), all CCCP additions were made in the dark and rates of photosynthetic and respiratory $O_2$ exchange measured in the following light-dark periods, respectively. In all cases, it was necessary to compare the influence of a particular CCCP concentration with a control, using new material for each concentration. For a complete set of data, this involved measurements over several days, using different plants, and hence there was a considerable scatter in the results.

From Figure 4.6, it may be seen that at CCCP concentrations below 0.5 µM, photosynthetic $O_2$ evolution was stimulated and that evolution was only completely inhibited at 2 µM. Similar effects are shown on the $^{14}CO_2$ fixation (pH 6.5), although there was no stimulation at the lower concentrations, indicating that CCCP was acting as a true phosphorylation uncoupler. CCCP stimulated respiratory $O_2$ uptake at all concentrations used - Figure 4.7. These results are in agreement with Betts (1970) in that $CO_2$ fixation was not markedly inhibited by CCCP concentrations less than 1 µM. However, $O_2$ production was less sensitive to CCCP than observed by

Jeschke (1972), where 1 μM CCCP maximally inhibited the $O_2$ production. In concurrent experiments (3.6), the $^{36}Cl^-$ influx was also stimulated by low concentrations of CCCP in the light suggesting that the $Cl^-$ influx may be coupled closely to the rate of photosynthetic electron flow.

The effect of pH on the CCCP inhibition of $^{14}CO_2$ fixation is shown in Table 4.1. As with the inhibition of the $Cl^-$ influx, CCCP added to solutions at high pH had little effect on the $^{14}CO_2$ fixation. In fact, at pH 9.5, 5 μM CCCP only inhibited the $^{14}CO_2$ fixation by 39% of the control value. Similar observations were also made with $O_2$ evolution at high pH (see Figure 4.6). Since Jeschke's experiments (1972) were performed at pH 5.2, pH effects on CCCP inhibition cannot explain the differences between his results and those presented here.

The interpretation of these CCCP experiments and the CMU experiments (3.7) in terms of the energy sources of the light stimulated $Cl^-$ influx will be discussed in Chapter 9.

## 4.6 Sources of Carbon for Photosynthesis

The effects of increasing pH on the photosynthetic $O_2$ evolution are shown in Figure 4.8. Here, the $O_2$ evolution in leaves from various sources and ages - as indicated in the legend to the Figure - all showed similar falls with increasing pH. Buffers used were MES for pH up to 6, HEPES 6 to 8, TAPS 8 to 9.5, CAPS from 9.5. A similar fall in $^{14}C$-fixation with increasing pH is shown in Figure 4.9. Here the $O_2$ evolution, measured simultaneously, is shown for comparison. Here, 100% $^{14}C$-fixation was equivalent to

85 - 100 pmoles/$cm^2$/sec. Hence, although at high pH values, $^{14}C$-fixation was markedly reduced, it did proceed at rates slightly less than 10 - 15 pmoles/$cm^2$/sec. In these experiments, photosynthesis at pH 10, proceeded at a rate of 10% of the maximal rate (observed at pH 6), whereas the exogenous $CO_2$ concentration had fallen to zero, suggesting that *Elodea densa* can in fact utilise $HCO_3^-$ as a carbon source, Raven (1970), (see 1.4.1). This confirms the indirect observations of Arens (1938) and Steemann Nielsen (1947), although it is in contrast to the results of Brown, et al (1974).

## 4.7 Temperature and C-fixation in *Elodea*

Arens (1938) was not able to demonstrate an influence of temperature ($5^o$ to $35^oC$) on $O_2$ evolution and external pH change. This may possibly be due to the lack of sensitivity (quantitative) of his methods. Table 4.2 shows that there was a significant influence of temperature between $25^oC$ and $35^oC$ on the $HCO_3^-$ and $CO_2$ fixation rates. While the range was limited, $CO_2$ fixation showed a 90% increase from $25^o$ to $35^oC$. This increase cannot be explained by the higher temperature increasing the rate of diffusion of $CO_2$ into the tissue, and must be explained by a stimulation of the Calvin Cycle enzymes in this range. On the other hand, the influence of temperature on the $HCO_3^-$ fixation, which showed a maximum at $30^oC$ may reflect a temperature maximum of some enzyme involved in transporting $HCO_3^-$ across the plasma membrane.

## 4.8 Adaptation to Either $CO_2$ or $HCO_3^-$ Utilisation

In section 4.6, it was demonstrated that *Elodea* was able to utilise $HCO_3^-$ at high pH and $CO_2$ at low pH. In these experiments the *Elodea* was grown or stored in tanks at approximately pH 9.0, where $HCO_3^-$ utilisation would have been predominant. It was of interest to determine whether the ability to utilise either carbon source, could be affected by long culture periods in solutions containing either $CO_2$ or $HCO_3^-$. Findenegg (1976) found that *Scenedesmus obliquus* did show an increasing ability to fix $HCO_3^-$ on adaptation to conditions when $HCO_3^-$ utilisation was necessary and he associated this with the induction of carbonic anhydrase activity. Table 4.3 shows that *Elodea* tissue grown at low pH for 7 days and 25 days could produce fixation rates at high pH that were equivalent to tissue cultured at high pH. This occurred even when the pretreatment times were as short as 60 seconds (Table 4.3B). Similarly, tissue cultured at high pH, could produce $CO_2$ fixation rates at low pH equivalent to tissue continuously cultured at low pH. This suggests that enzyme induction for $HCO_3^-$ transport and fixation is not required in *Elodea* and that such an explanation would not be sufficient to account for the different results of Brown et al (1974), who observed that *Elodea densa* were not able to utilise $HCO_3^-$ for photosynthesis.

## 4.9 Summary

1. Continuous light/dark experiments showed that there is no diurnal change in photosynthetic $O_2$ evolution and respiratory uptake.

2. Photosynthetic $O_2$ production proceeds at a rate in the range 5 to 10 times faster than respiratory uptake.
3. Photorespiration is observed in *Elodea*.
4. At pH 6.5, photosynthesis is saturated at exogenous carbon concentrations of 0.4 mM.
5. CCCP exerts different effects when added to leaves in the light and in the dark. 2 μM CCCP (added in the dark) completely inhibits photosynthetic $O_2$ production at pH 6.5.
6. *Elodea densa* is able to fix both $CO_2$ at low pH and $HCO_3^-$ at higher pH values.
7. Temperature changes both $CO_2$ and $HCO_3^-$ fixation rates differentially.
8. *Elodea* leaves do not change their ability to fix either $CO_2$ or $HCO_3^-$ with very long preconditioning periods.

TABLE 4.1 THE EFFECT OF pH ON THE CCCP INHIBITION OF $^{14}C$-FIXATION

| Pretreatment pH | $^{14}C$-Fixation (nmoles/sec/GFW) | % of Controls |
|---|---|---|
| 6.55(no CCCP) | 19.2 ± 2.3 | 100 |
| 6.55 | 0.11 ± .04 | 0.6 |
| 7.5 | 8.3 ± .3 | 43 |
| 8.5 | 10.9 ± .3 | 57 |
| 9.5 | 11.7 ± .7 | 61 |

Pretreatment with 2 μM CCCP at appropriate pH for 1 hour, then leaves washed and transferred to solutions (-CCCP) at pH 7.0 for $^{14}C$-fixation measurement (5 mins). Solutions as in 2.3.10.

TABLE 4.2 THE EFFECT OF TEMPERATURE ON $^{14}CO_2$ and $H^{14}CO_3^-$ FIXATION.

| pH | Temperature ($^oC$) | $^{14}C$-Fixation (pmoles/cm$^2$/sec) | % of controls |
|---|---|---|---|
| 6.5 | 25 | 90 ± 5 | 100 |
| 6.5 | 30 | 130 ± 6 | 144 |
| 6.5 | 35 | 170 ± 6 | 190 |
| 9.5 | 25 | 12 ± 1 | 100 |
| 9.5 | 30 | 21 ± 1 | 175 |
| 9.5 | 35 | 18 ± 1 | 150 |

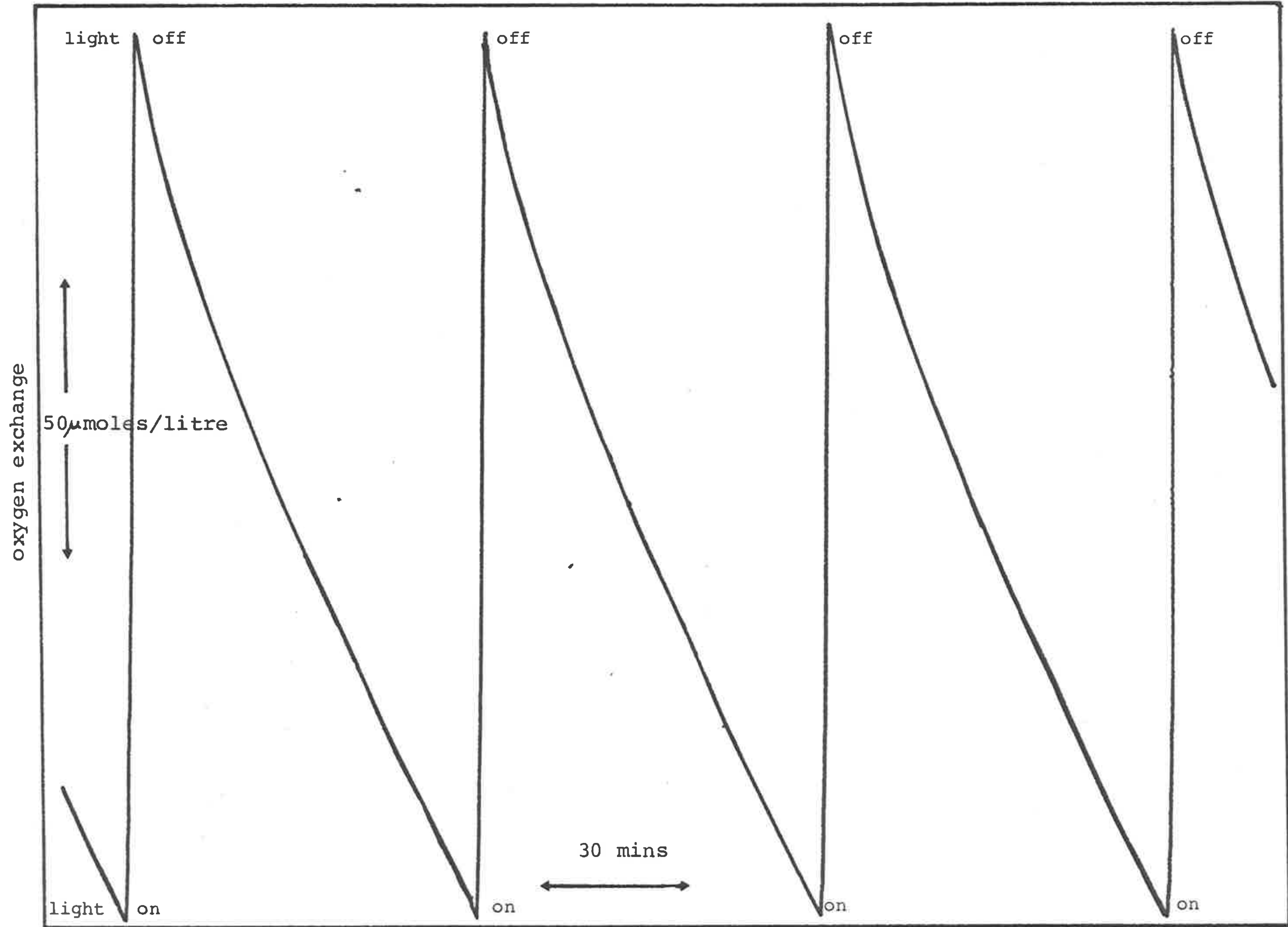
Experiments performed in the Rank $O_2$ electrode. Samples equilibrated for 20 minutes in the solutions at each temperature.

TABLE 4.3 THE EFFECT OF LONG PRETREATMENT AT HIGH AND LOW pH ON *Elodea's* ABILITY TO UTILISE $CO_2$ and $HCO_3^-$.

| | Long pretreatment time and pH | | Experimental Pretreatment Time (Hours) | Experimental pH | $^{14}C$-Fixation (pmoles/cm$^2$/sec) |
|---|---|---|---|---|---|
| A | 7 days | 6.5 | 1 | 9.7 | 15.6 ± .6 |
| | " " | " | " | 6.5 | 127 ± 3.0 |
| | " " | " | 2 | 9.7 | 17.5 ± .6 |
| | " " | " | " | 6.5 | 110 ± 4.0 |
| | " " | 9.5 | 1 | 9.7 | 13.0 ± .5 |
| | " " | " | " | 6.5 | 130 ± 4.0 |
| | " " | " | 2 | 9.7 | 19.0 ± 1.0 |
| | " " | " | " | 6.5 | 109 ± 3.0 |
| B | 25 days | 10.5 | 0.5 | 10.5 | 10.0 ± .6 |
| | " " | " | " | 6.5 | 115 ± 4.0 |
| | " " | 6.5 | " | 6.5 | 99.0 ± 4.0 |
| | " " | " | " | 10.5 | 6.0 ± .2 |
| | " " | " | 0.02 | 6.5 | 101 ± 10.0 |
| | " " | " | " | 10.5 | 1.7 ± .1 |

Solutions at pH 10.5 for long periods were 5.0 mM CAPS, 1.0 mM $NaHCO_3$, 0.5 mM $CaSO_4$ and 0.1 mM KCl. Similarly at pH 6.5, 5.0 mM HEPES was used.

FIGURE 4.1 Continuous $O_2$ exchange experiment in the light and and dark, as described in 2.3.1.



light
off
off
off
off
oxygen exchange
50μmoles/litre
30 mins
light
on
on
on
on

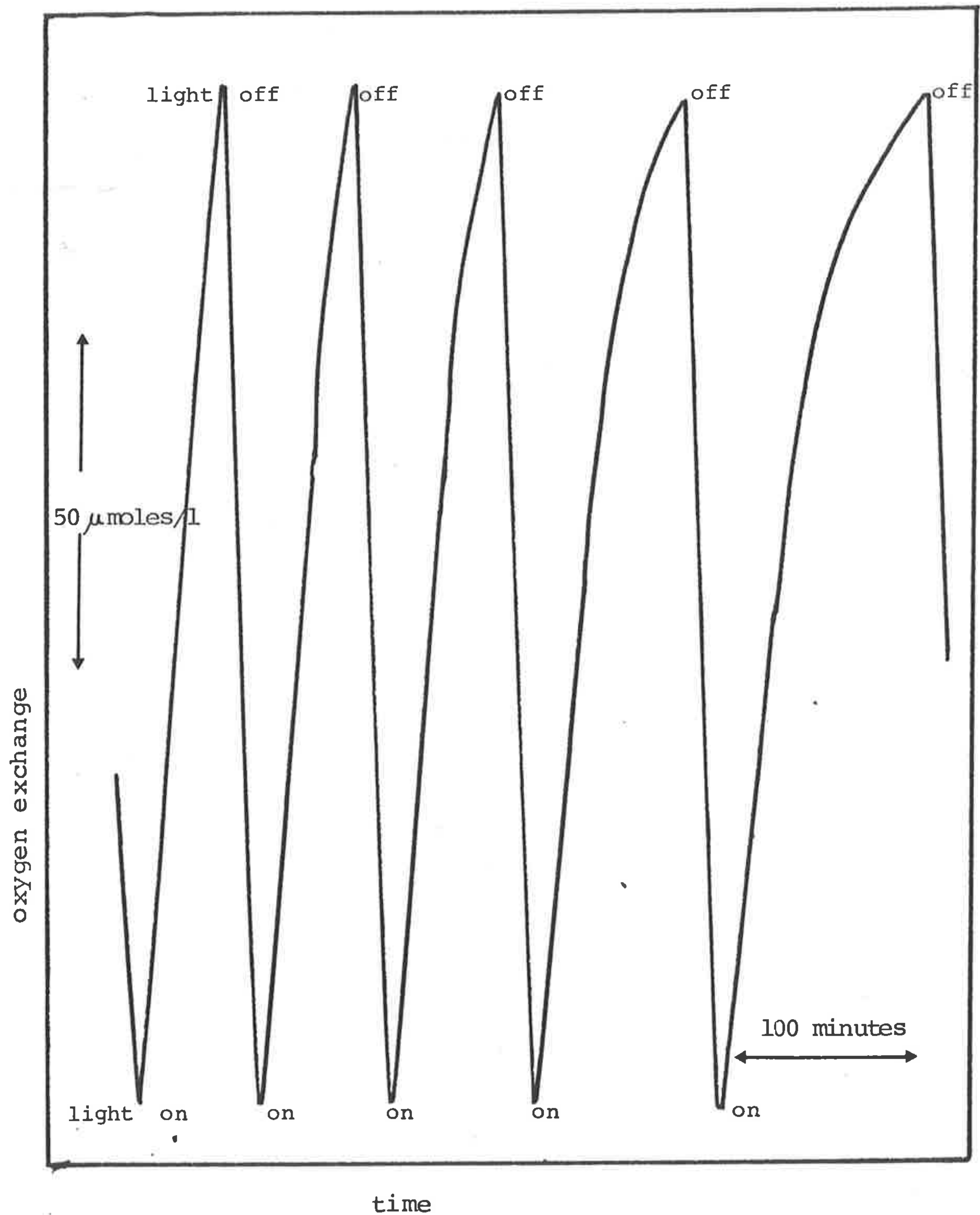



FIGURE 4.2 Continuous $O_2$ exchange experiment in light dark periods (see 2.3.1.) showing the effect of depletion of exogenous $CO_2(HCO_3^-)$ after 12 hours - initial concentration, 1.0 mM.

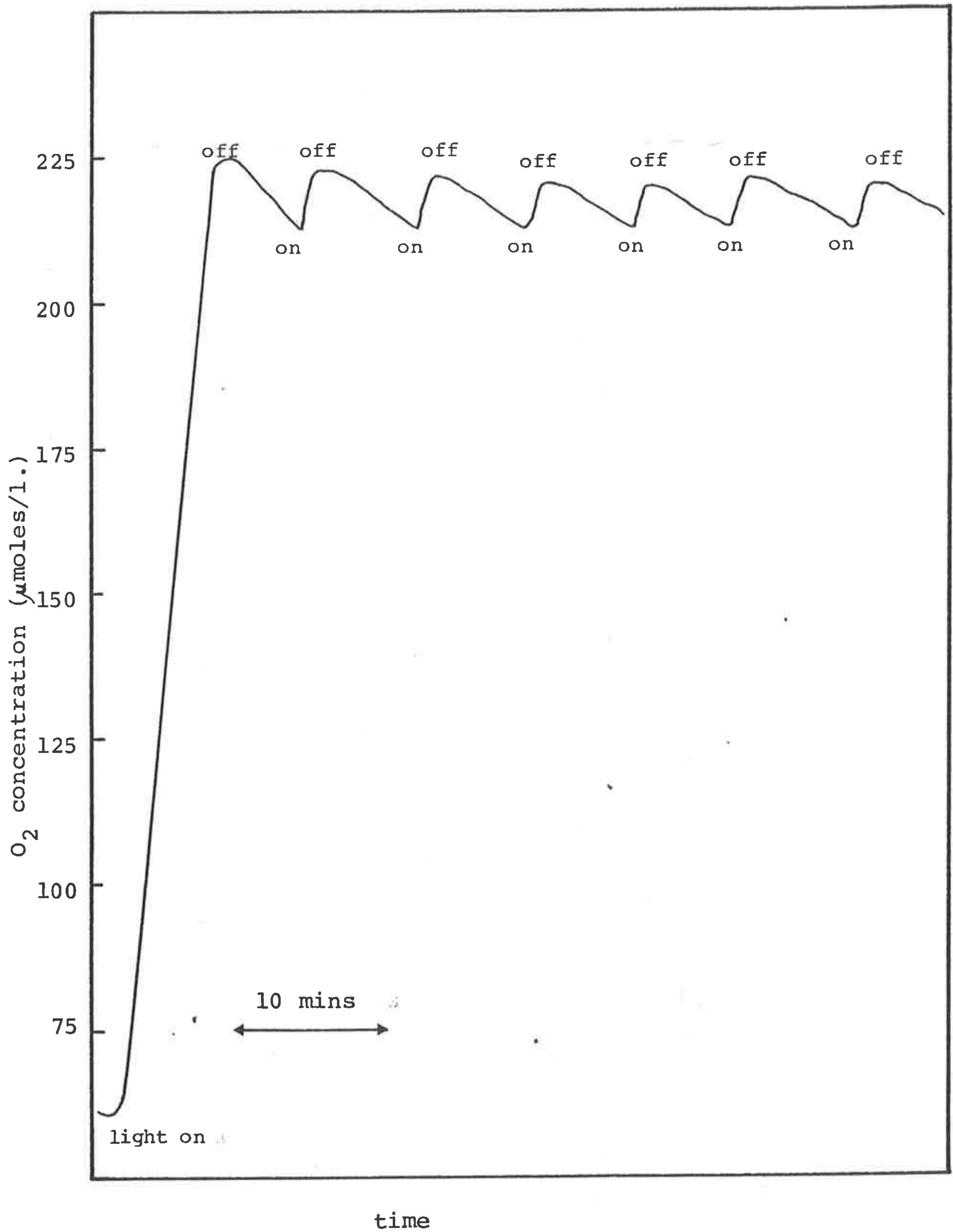



FIGURE 4.3 Continuous $O_2$ exchange in the light and dark at high $O_2$ concentrations - evidence for photorespiration.

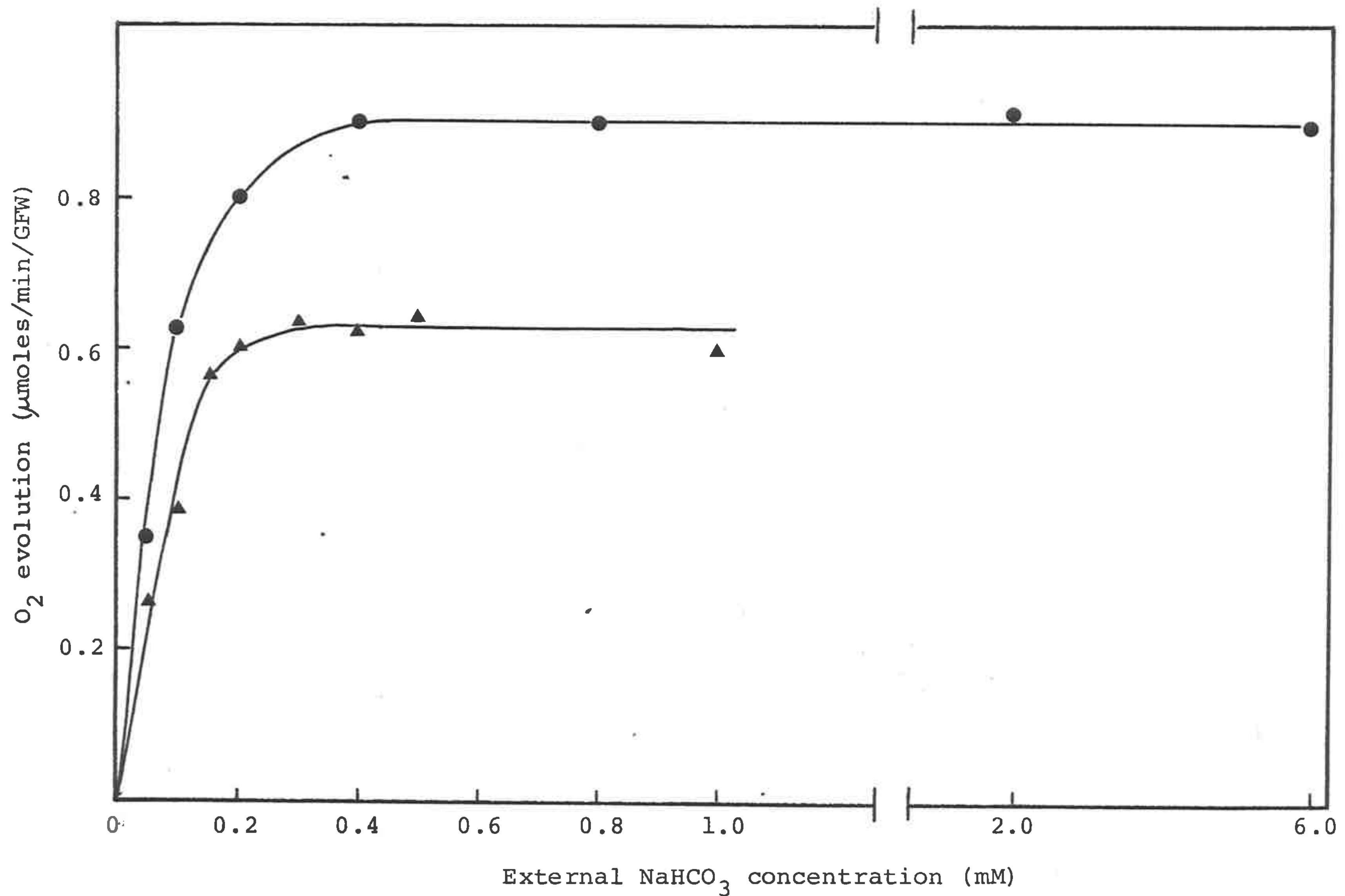



FIGURE 4.4 $O_2$ evolution saturation curves in response to increasing exogenous $CO_2$ concentration at pH 6.5. Symbols represent experiments on *Elodea* from different sources.

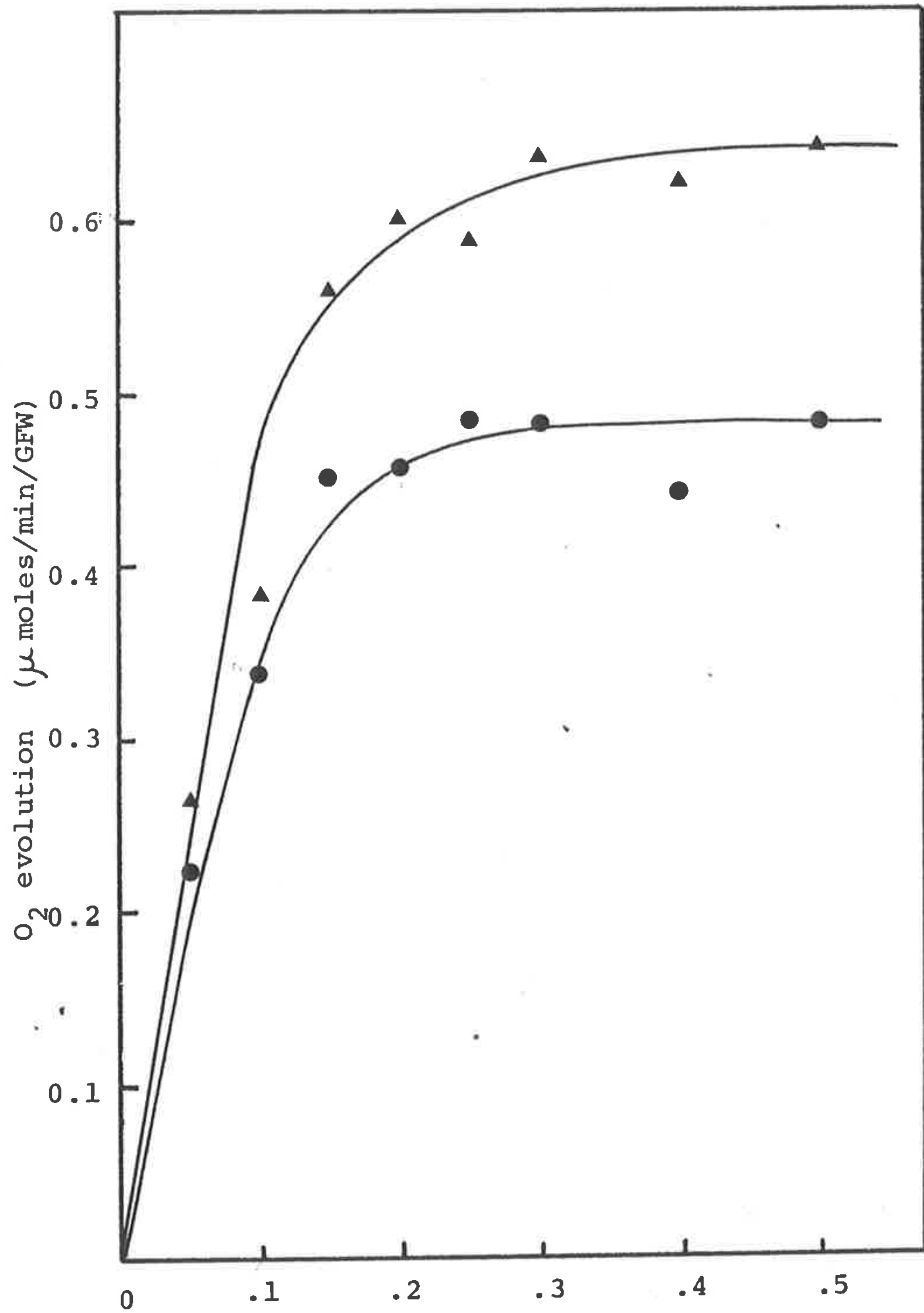



FIGURE 4.5 $O_2$ evolution saturation curves in response to increasing exogenous $CO_2$ concentration at pH 6.5.

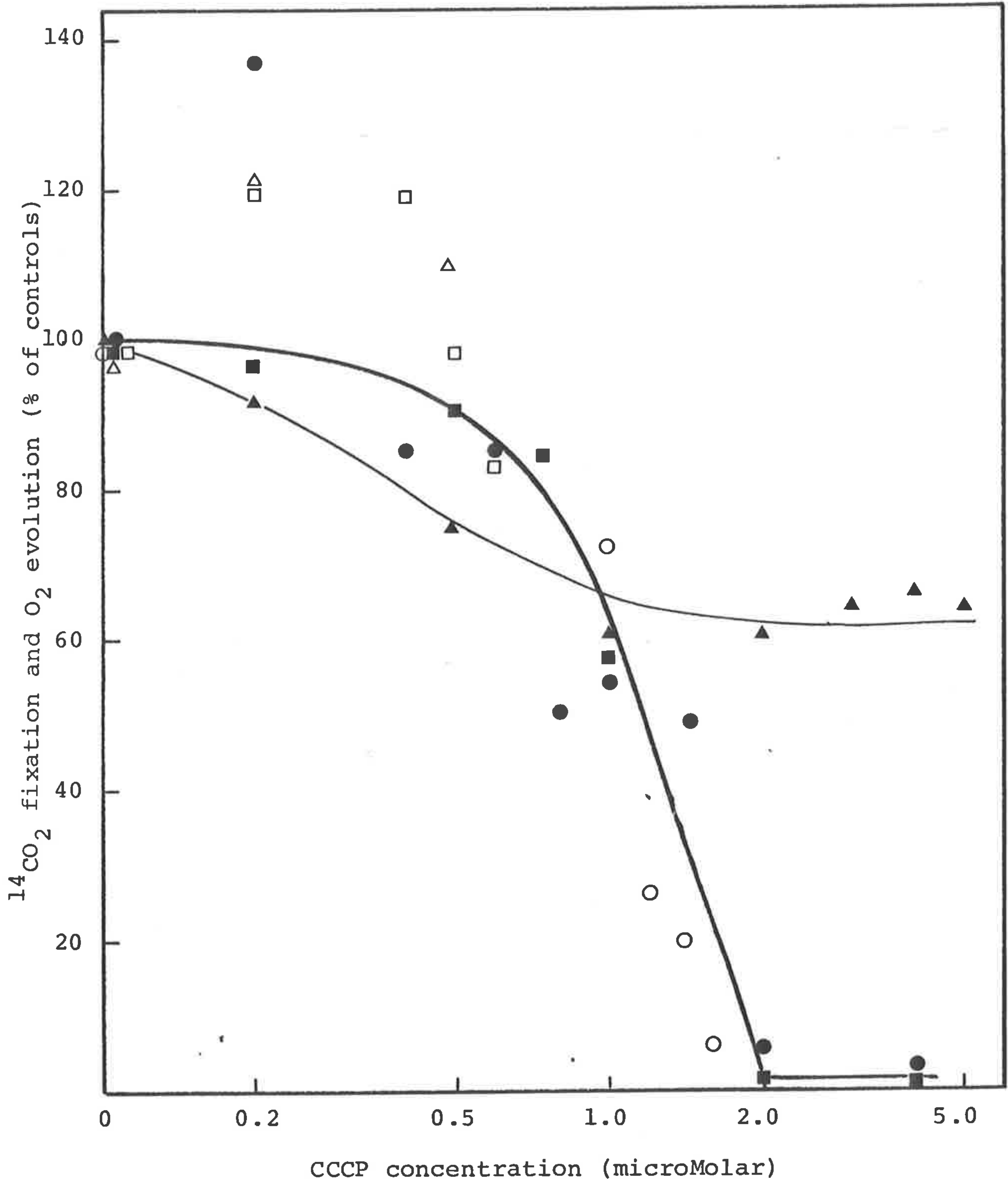



FIGURE 4.6 Effect of CCCP concentrations on net photosynthetic $O_2$ production at pH 9.5 (▲) and 6.5 (●, □, △), and $^{14}CO_2$ fixation (■, ○) at pH 6.5. Symbols represent different experiments with *Elodea* from different sources.

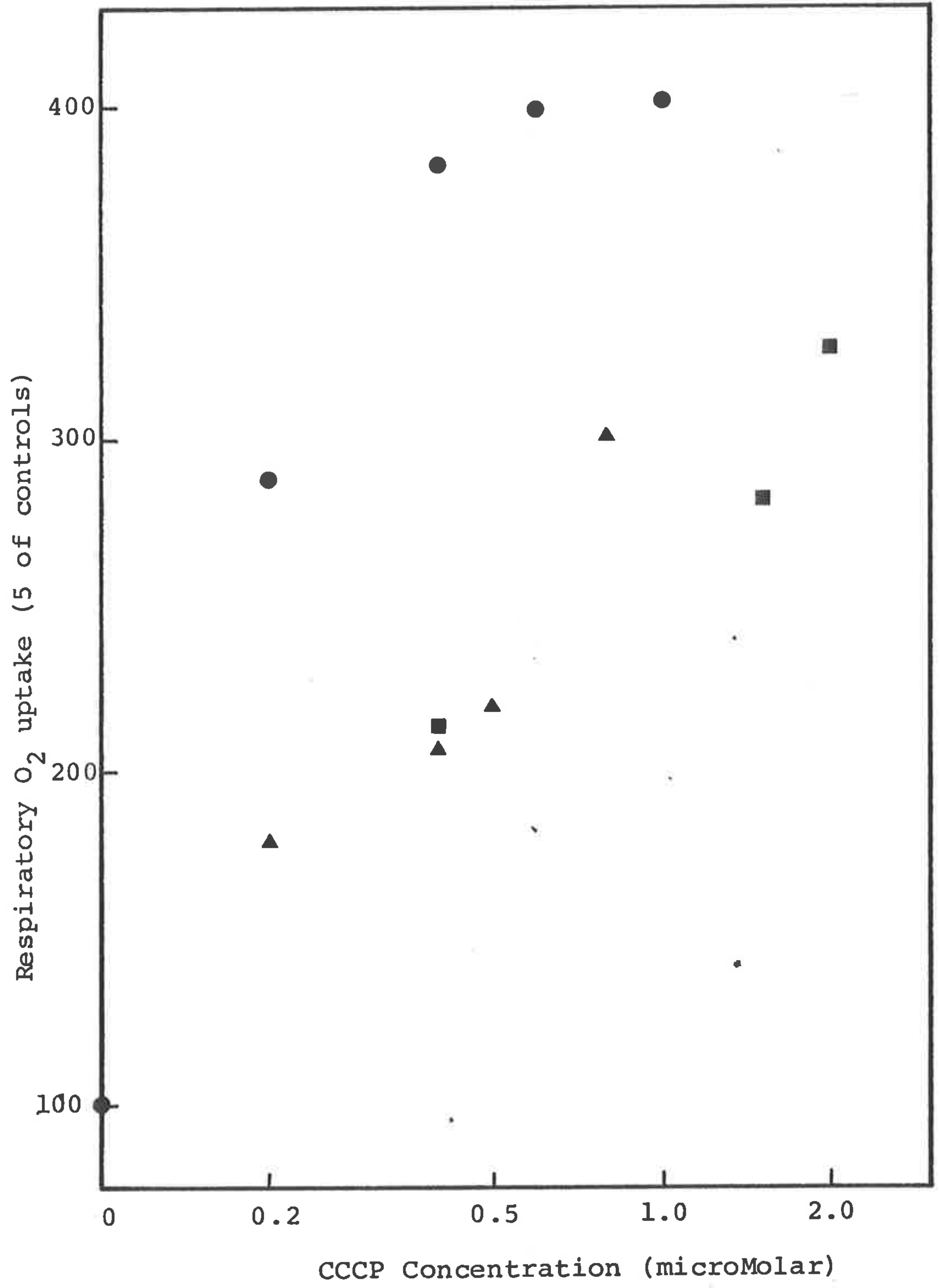



FIGURE 4.7 Effect of CCCP on the respiratory $O_2$ uptake. Symbols represent data from different experiments with *Elodea* from different sources. Experimental solutions at pH 6.5.

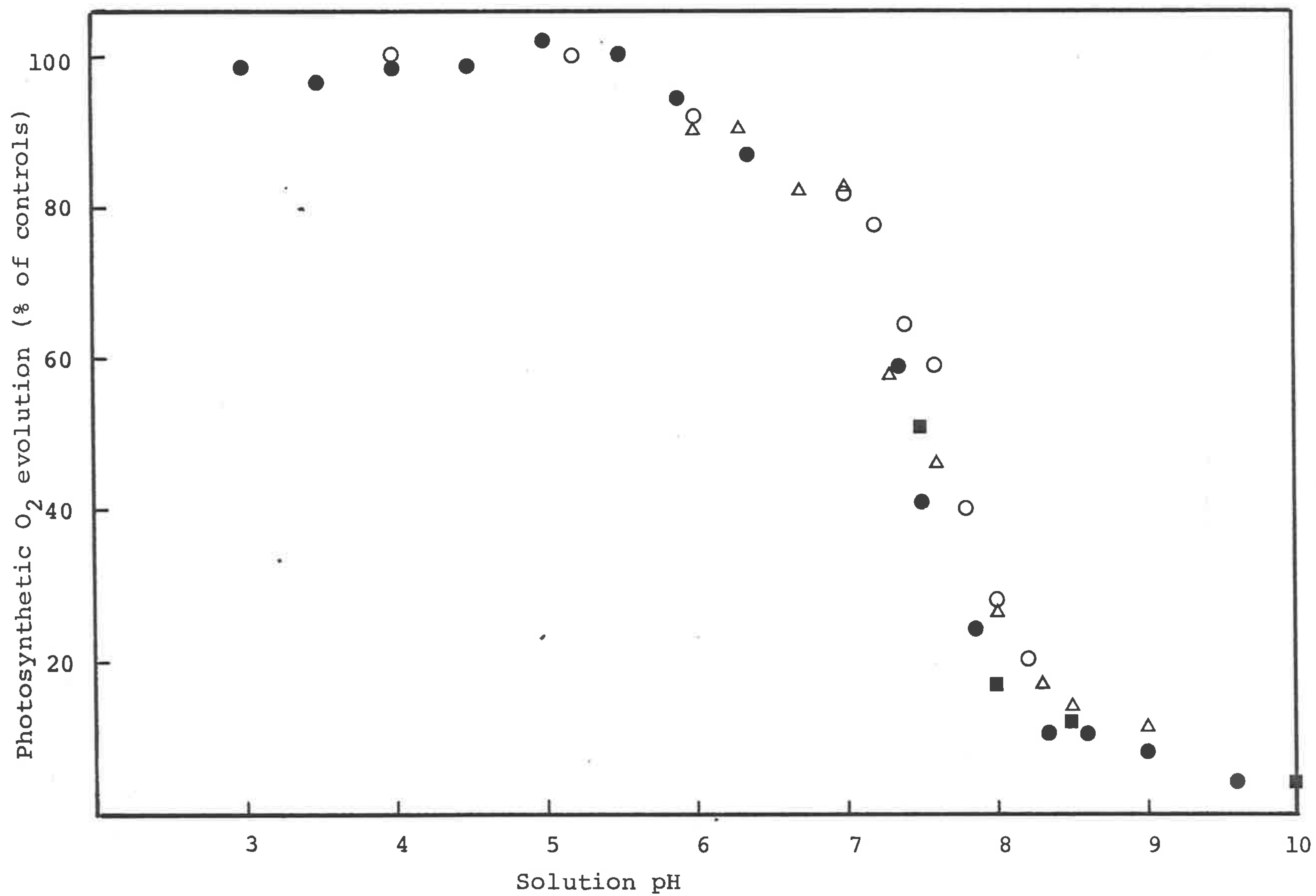



FIGURE 4.8 Effect of pH on the photosynthetic $O_2$ production. Symbols represent *Elodea* collected from the Zoological Gardens (●), and stored for 1 month (■); indoor tanks (○, □).

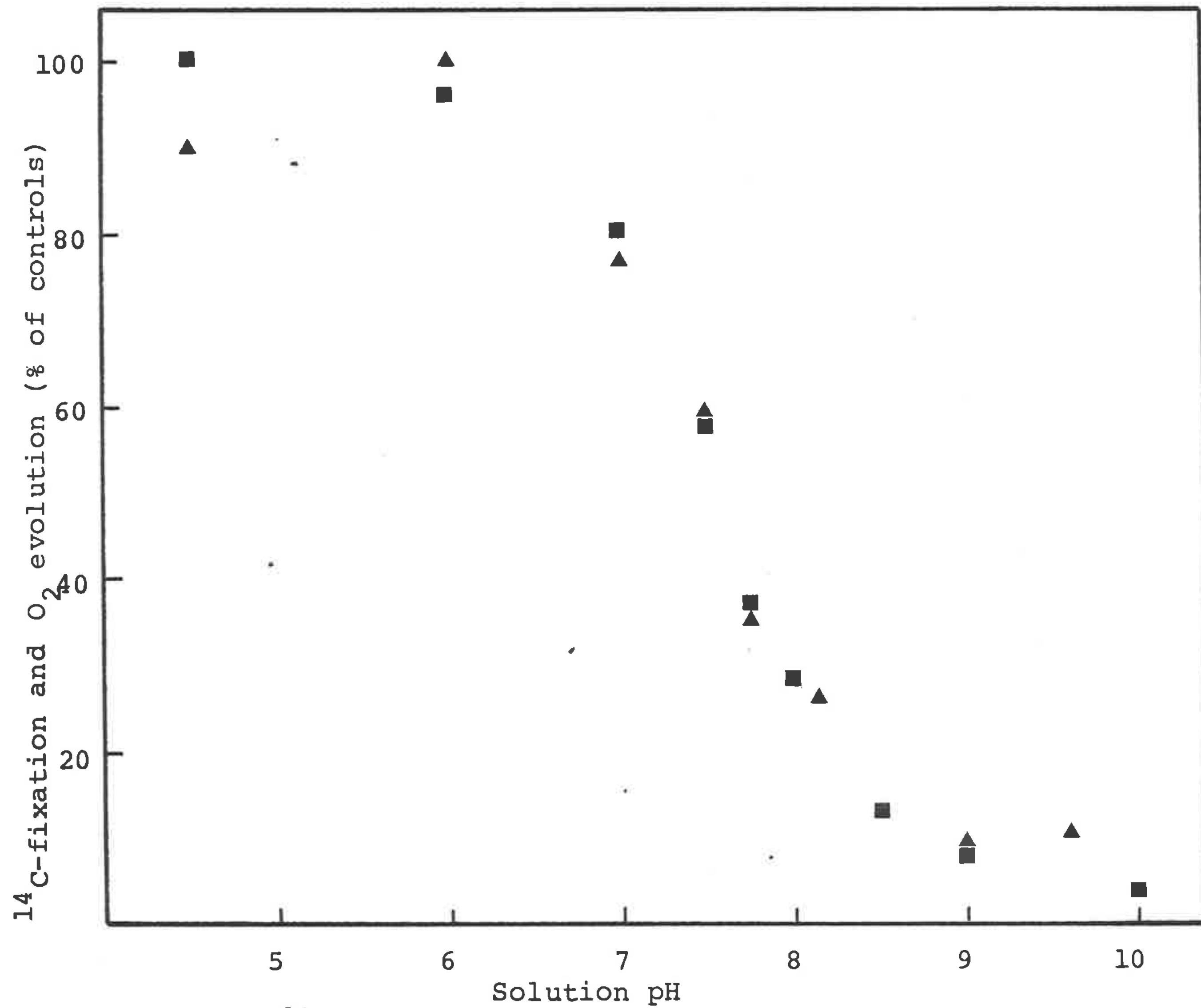



FIGURE 4.9 Effect of pH on $^{14}$C-fixation (▲) and $O_2$ evolution (■). See text for buffers used.

# CHAPTER FIVE

## SOLUTION pH CHANGES IN THE LIGHT AND DARK

### 5.1 Introduction

Preliminary experiments with *Elodea* to measure the effects of light on the bulk solution pH were performed using a Beckman pH electrode inserted into the Rank $O_2$ electrode. The output from the pH meter was plotted manually rather than using a potentiometric recorder. As with all $CO_2$ evolution experiments, the endogenous $CO_2$ and $HCO_3^-$ within the tissue was removed by pretreatment for short periods in the light (see 4.4). During this time it was noted that the pH did not reach equilibrium quickly like the $O_2$ exchange but rose slowly for 5 minutes and then rapidly fell as shown in Figure 5.1A. However, if 1.0 mM $NaHCO_3$ was present in the solution, the pH of the bulk solution rose quite sharply - Figure 5.1B. A review of the literature showed that a pH fall in the light had previously been observed in *Elodea* by Jeschke (1970), who attributed it to a large $H^+$ efflux and by Brown et al (1974), who attributed it to respiratory $CO_2$ release (see 1.5.2). It therefore seemed extremely important to study the conditions under which there were net solution pH changes and relate this to the primary sources of photosynthesis and ion transport.

### 5.2 pH Changes with Low Exogenous Carbon Concentrations

From Figure 5.2, it can be seen that when no exogenous $CO_2$ or $HCO_3^-$ was present in the external unbuffered solution, the pH of the solution fell from a value of approximately 6 to a new equi-

librium value of approximately 4.2, after peaking to pH 6.7. Simultaneous measurement of $O_2$ production showed that initially there was net production before equilibrium was reached. This is the compensation point where the $O_2$ produced by photosynthesis is balanced by the $O_2$ uptake of respiration. Similarly under these conditions there would have been no net $CO_2$ production so that the pH fall cannot be attributed to $CO_2$ release from respiration. In this respect, the results differ from those of Brown et al (1974) as these authors observed a net $O_2$ uptake under similar conditions.

When the light was switched off there was no change in solution pH although there was a net $O_2$ uptake (Figure 5.2). In subsequent light and dark periods, there was no further change in the solution pH, although there were small changes in the $O_2$ exchange. In all experiments where the effects of exogenous carbon concentration was being determined, this fall in pH was allowed to proceed to ensure depletion of endogenous carbon stores within the *Elodea* leaves. This fall in solution pH was not observed by Hope et al (1972) with *Elodea canadensis*.

The effect of adding small amounts of $NaHCO_3$ to the solutions in the light during the minimum pH equilibrium value (5.2), is shown in Figure 5.3. Here it can be seen that there was a spike in the pH value on the addition of $NaHCO_3$ to give final concentrations of 0.1 mM and 0.2 mM $NaHCO_3$. The new equilibrium pH values after these additions were due to the alteration of the equilibrium $HCO_3^- \rightleftharpoons CO_2 + OH^-$. However, when the concentration of $NaHCO_3$ reached 0.3 mM, there was quite a rapid rise in the pH which reached a new equilibrium value of 9.2 after 90 minutes. Following this

the pH rapidly fell in the dark. In a subsequent light period there was again a rise in pH, but note the inflection after approximately 15 minutes. After 8 hours of light/dark treatments, the pH, after an initial slow rise, peaked and began to rapidly fall again to an equilibrium pH below 5. This is interpreted as being due to a depletion of exogenous $CO_2/HCO_3^-$ as the pH did increase again on the addition of $CO_2$ (as $NaHCO_3$). This type of event was consistently obtained in many repeat experiments using other *Elodea* leaves.

## 5.3 The Rapid pH Fall in the Dark

As shown in Figure 5.3, there was a very rapid fall in solution pH when the light was turned off. This may be interpreted in two ways.

1. $CO_2$ produced by respiration which drives the equilibrium reaction $CO_2 + OH^- \longrightarrow HCO_3^-$ (Helder and Zanstra, 1977).
2. A light stimulated $H^+$ efflux, which is only detected once $HCO_3^-$ fixation ceases, and which continues for some time after the light is switched off.

From the $O_2$ evolution experiments (Chapter 4) it may be seen that $O_2$ uptake in the light proceeds at a much slower rate than photosynthetic $O_2$ production. Provided the ratio $CO_2/O_2$ exchange remains constant for photosynthesis and respiration, then the first alternative above seems inadequate to explain the very rapid pH fall. A similar conclusion was made by Hope et al (1972)

who found that although $H^+/O_2$ equalled 1, the $CO_2/H^+$ was closer to 0.6 - 0.7. They suggested therefore, that there was a component of the $H^+$ flux that was independent of $CO_2$ fixation.

## 5.4 The "All or None" pH Increase/Decrease

The pH changes observed under different total carbon concentrations appeared to be an "all or none" process. Either the pH fell to a minimum value as with 0.1 and 0.2 mM $NaHCO_3$ in Figure 5.3 or it rose to a maximum pH greater than 9 as with 0.3 mM $NaHCO_3$ (Figure 5.3). Under these latter conditions the pH rose again in subsequent light/dark exposures until the exogenous $CO_2$/$HCO_3^-$ level fell to zero and the pH again fell to a minimum. If the $CO_2$ added to the solution at any time was insufficient for a stable pH maximum to be reached the pH would increase and then decrease until a minimum equilibrium value was reached.

This "all or none" phenomena would not be expected if $CO_2$ and $HCO_3^-$ fixation alone were contributing to the pH changes. If this was occurring, equilibrium pH values would be reached that were dependent on the exogenous carbon concentration, or until saturation was reached where the pH could not be increased further. Such a pH was found to be 9.5 as shown in Figure 5.4 using a solution containing 1.0 mM $NaHCO_3$. Here the maximum was reached after approximately 80 minutes.

Hence the apparent "all or none" phenomenon observed, in the presence and absence of $CO_2$ and $HCO_3^-$ in the light, is interpreted as further evidence for an $H^+$ efflux.

## 5.5 The Apparent "Switch On" of $HCO_3^-$ Utilisation

As already noted (5.2) there was an inflection in the rate of pH rise when small concentrations of exogenous carbon were present in the bathing medium (Figure 5.3). These variations in the rate of pH change were always observed between pH 6 and 7 in control tissue. This suggests that at a particular pH, provided the exogenous carbon concentration was sufficient, some mechanism was being 'switched on' and that this mechanism was responsible for an $OH^-$ efflux (or $H^+$ influx). Such a mechanism could well be $HCO_3^-$ uptake and assimilation.

The slower initial rise in pH may be interpreted as the utilisation of $CO_2$ from solution during $CO_2$ fixation disturbing the equilibrium reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ .

As the pH increases, the level of $HCO_3^-$ also increases and then suddenly the $HCO_3^-$ fixation mechanism becomes active and the reaction $HCO_3^- \longrightarrow CO_2 + OH^-$ within the leaf tissue is initiated and the $OH^-$ efflux from the upper leaf surfaces causes the pH of the bulk solution to increase further until a new equilibrium pH is reached.

Similar observations and conclusions were made by Helder and Zanstra (1977) with *Potamogeton* leaves. These authors also noted that with some experiments, at this point, the pH sometimes fell. Whether this "switching on" was determined solely by pH, $CO_2$ to $HCO_3^-$ concentration ratio, or a combination of both is quite difficult to determine. Helder and Zanstra (1977) found that the solution pH at their inflection point was equivalent to the $CO_2$ compensation point.

The inflection in the pH rise was much more obvious when 0.1 mM $(NH_4)_2SO_4$ had been added to the solution (Figure 8.1). In fact the pH rose and started to fall before again rising sharply. This is interpreted as being possibly due to delay in the onset of $HCO_3^-$ fixation in the presence of $NH_4^+$, as discussed in more detail in Chapter 8.

The 'switch on' of $HCO_3^-$ utilisation could be activated in the presence of low exogenous carbon by artificially increasing the pH of the solution by the addition of appropriate quantities of freshly prepared NaOH. Similarly, the utilisation could be 'switched off' by reducing the pH below 5.5 by the addition of HCl. Hence the 'switch on' mechanism appears to be determined by the exogenous $HCO_3^-$ concentration, which in the unbuffered solutions used in these experiments was determined by pH. Further quantification of these observations by the use of strong buffers to control pH and the actual $HCO_3^-$ concentration were not possible using the present experimental set up whereby pH changes in the bulk solution reflect $H^+$ and $OH^-$ fluxes out of and into the leaf tissue. To perform such experiments one needs to be able to separate upper and lower leaf surfaces (Helder and Zanstra, 1977). *Elodea* does not easily lend itself to such experiments because of the size and fragility of the leaves, as discussed in Chapter 9.

## 5.6 The $OH^-$ Efflux

The $OH^-$ efflux that occurs during $HCO_3^-$ utilisation as shown in Figures 5.1 and 5.2 is more extensively studied here. It should be noted at this stage however, that the apparent $OH^-$ efflux mea-

sured is contributed to by the $OH^-$ efflux during $HCO_3^-$ fixation and any $H^+$ efflux that may be occurring, as discussed in earlier sections of this Chapter.

In order to quantify the $OH^-$ efflux, at the end of each experiment known amounts of NaOH were added to the solution containing the leaves in the $O_2$ electrode. Such a calibration curve is shown in Figure 5.5, which although quite linear does not pass through the origin. The pH range for this curve was 5.6 to 5.8. Attempts to calibrate over very large pH ranges were not very successful due to the buffering capacity of the leaves themselves and the $HCO_3^-/CO_2$ in the solution (Hope et al, 1972). However, calibration was reasonably linear in the range 5.6 to 6.5 so estimates of the apparent $OH^-$ exchange in this range were obtainable. In the light, values between 5 and 20 nmoles/sec/GFW were obtained for the $OH^-$ efflux of *Elodea* in the light during $HCO_3^-$ fixation. A typical $OH^-$ efflux curve in the light is shown in Figure 5.6. Duplicate runs after a dark period of 20 minutes are shown, and so is the $H^+$ efflux during a 16 minute dark period. In this experiment the rate of $OH^-$ efflux in the light was 8 times the dark $H^+$ efflux. However, as pointed out in 5.3 the $H^+$ efflux is affected by the length of the immediate light period, which in Figures 5.2 and 5.3 were over 90 minutes. These values for the $OH^-$ efflux are similar to those obtained with *Elodea canadensis*, by Hope et al (1972), who obtained values up to 25 nmoles/sec/GFW.

## 5.7 The Effect of CCCP on the $OH^-$ Flux

The effects of 60 minutes treatment with CCCP at pH between 6.6 and 5.7 (unbuffered solutions) are shown in Figures 5.7 and

and 5.8. CCCP was added in the dark to obtain maximum effect (see 4.2.4). Hence 0.5 CCCP μM inhibited $OH^-$ efflux by approximately 47% of the control values, and 1.4 μM CCCP inhibited the $OH^-$ efflux to a maximum of 20% of the control values (Figure 5.8). However, at both these concentrations of CCCP, the pH changes were not constant and changed during the light period.

This further highlights the problems with experiments using CCCP as shown in 3.6 and 4.5. Figure 5.9 shows that CCCP had no effect on the apparent dark $H^+$ efflux at both 2 and 80 minutes. If the apparent $H^+$ efflux here was due solely to $CO_2$ released during respiration then the pH change should have been much greater as respiration is stimulated by as much as 300% of the control values at this CCCP concentration (4.5). This is taken as further evidence for an active $H^+$ efflux pump in *Elodea*.

## 5.8 Summary

1. At low pH and when the level of exogenous $CO_2$ or $HCO_3^-$ falls to zero there is an observable $H^+$ efflux from *Elodea* leaves.
2. The $H^+$ efflux appears to be light stimulated.
3. There is a very large $OH^-$ efflux during $HCO_3^-$ fixation, (5 - 20 nmoles/sec/GFW).
4. At low levels of exogenous carbon there appears an inflection in the pH rise, which is evidence of a 'switch on' of the $HCO_3^-$ utilisation mechanism.
5. 0.5 μM CCCP inhibits the $OH^-$ efflux by 53% of the control values.

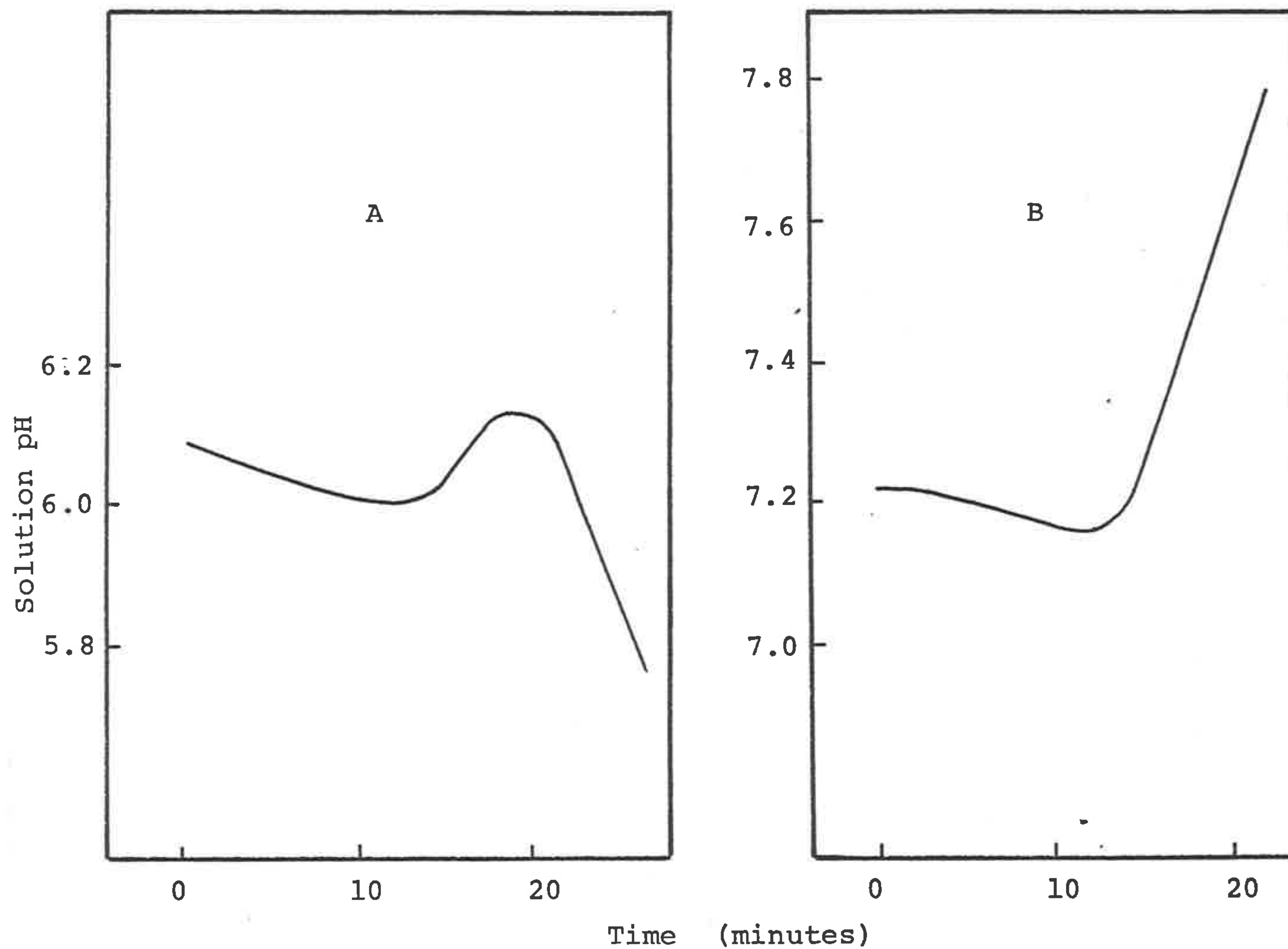



FIGURE 5.1 Solution pH changes in the presence of 1.0 mM $NaHCO_3$ (B) and without exogenous C (A). Solutions contain no buffer but otherwise as in 2.3.10.

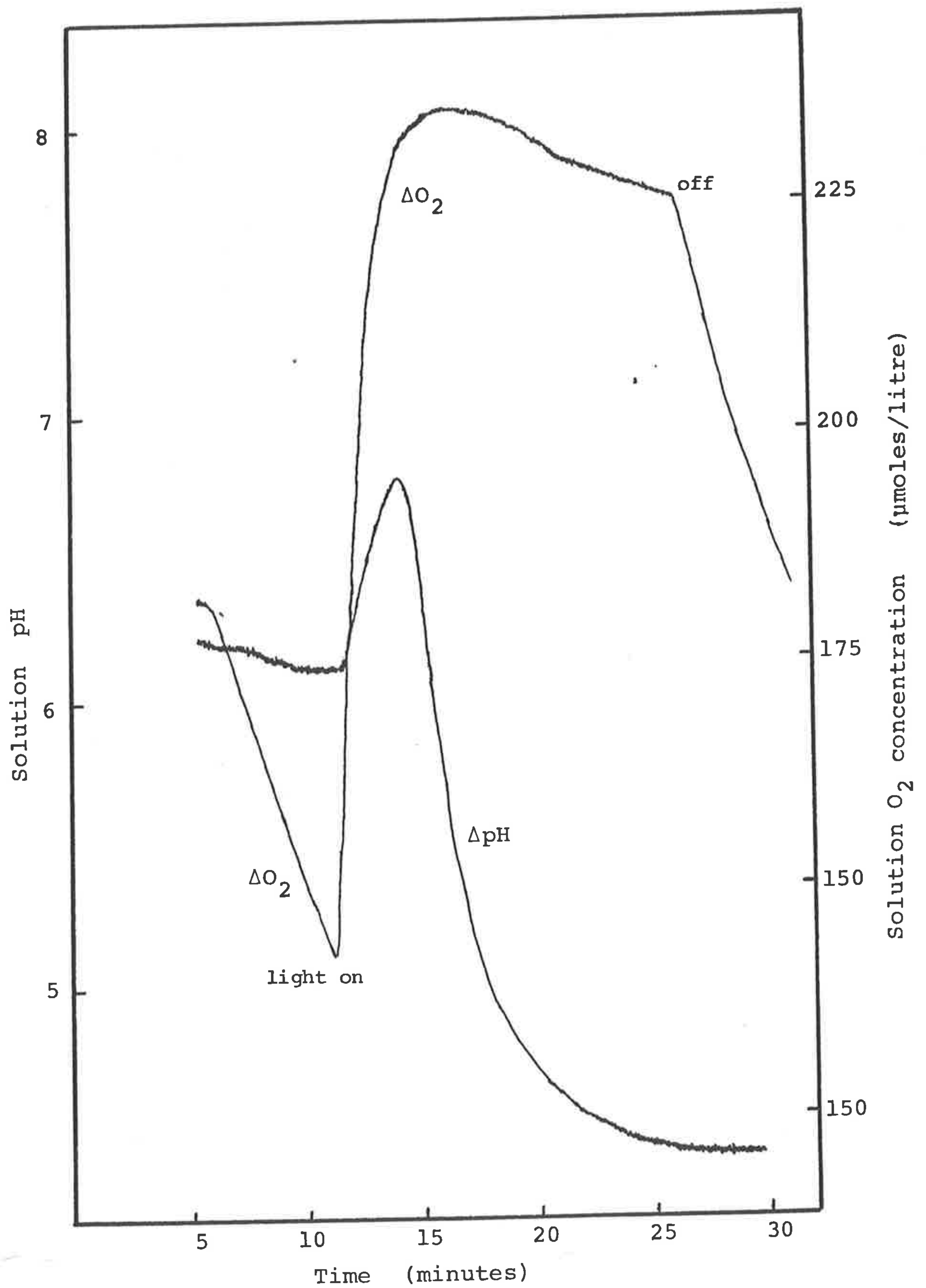



FIGURE 5.2 Solution pH changes when no exogenous $CO_2$ or $HCO_3^-$ is added. Also shown are the changes in solution $O_2$ concentration. For full explanation see text.

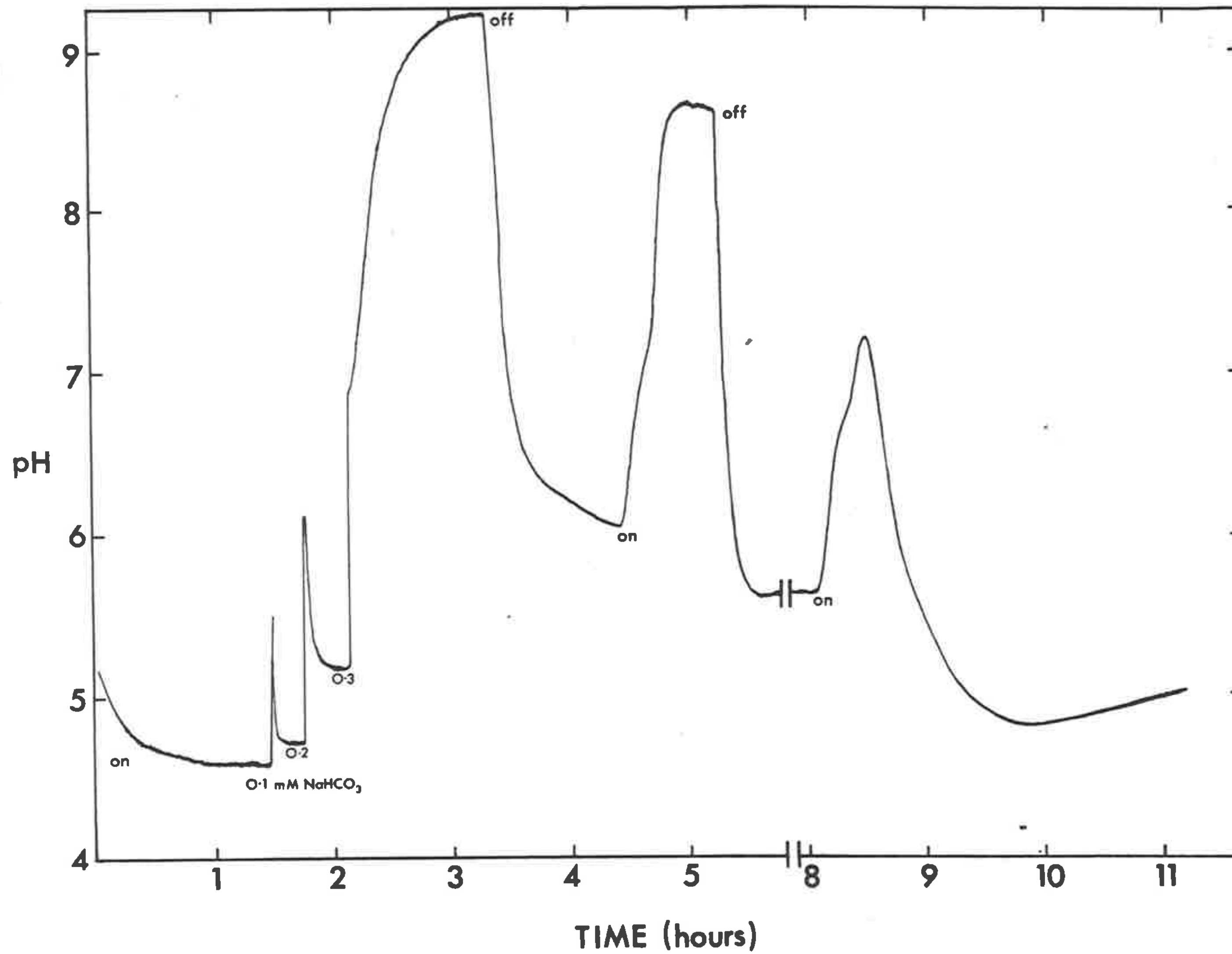



FIGURE 5.3 Effect of adding varying amounts of $NaHCO_3$ at the equilibrium pH value. For explanation see text (5.2).

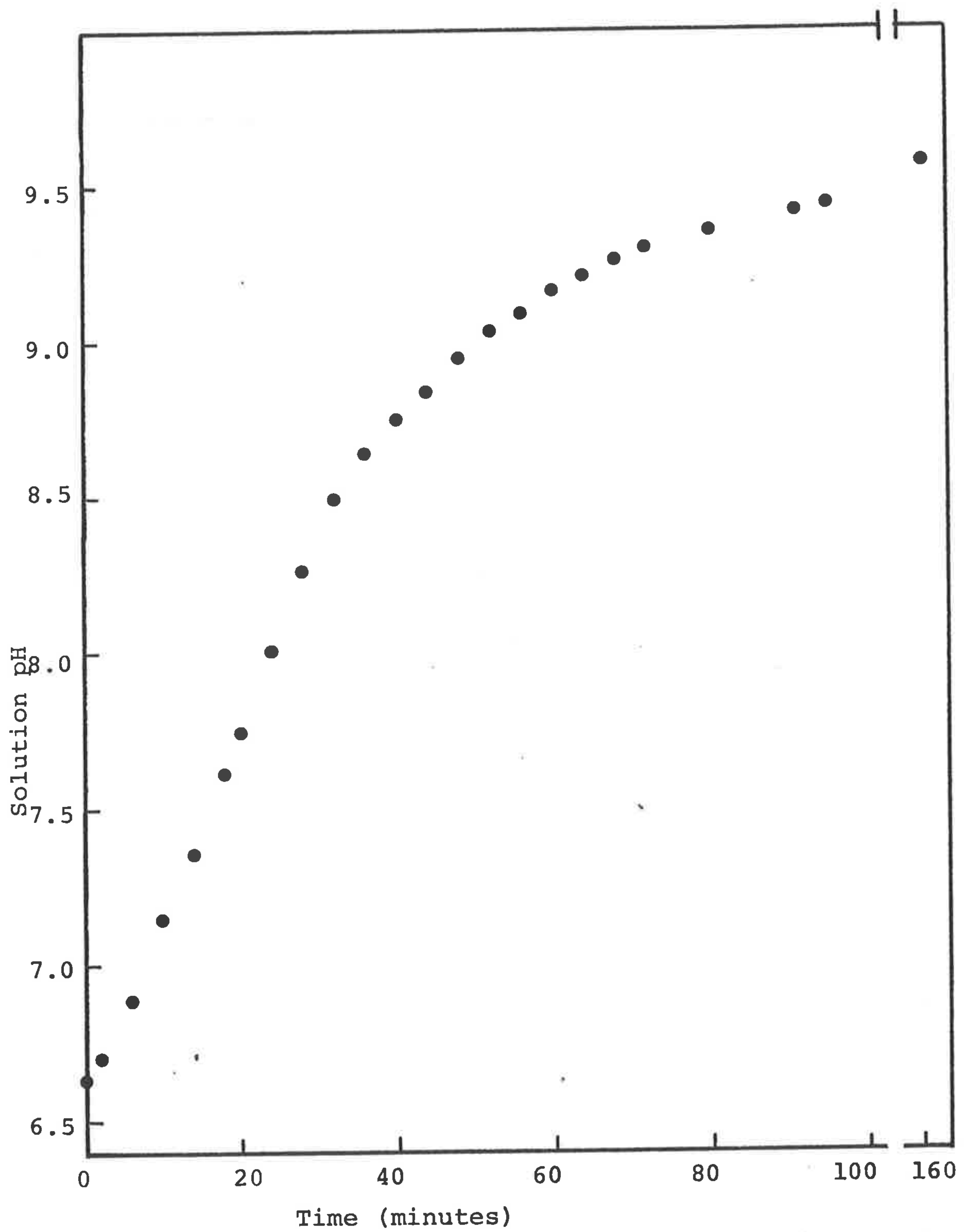



FIGURE 5.4 Solution pH increase in a period of 160 minutes in normal solution containing 1.0 mM $NaHCO_3$ (no buffer).

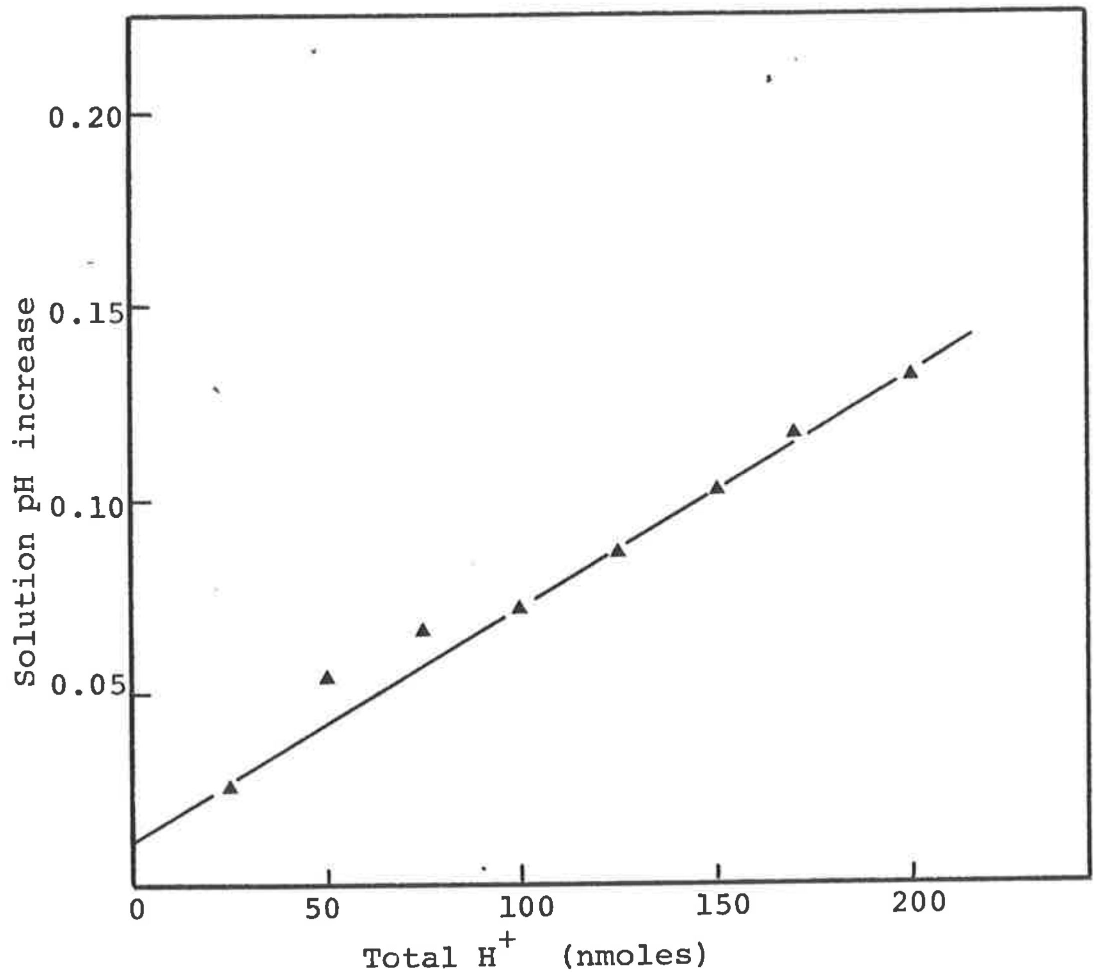



FIGURE 5.5 Calibration of $H^+$ concentration in $O_2$ electrode containing the *Elodea* leaves. Calibration performed by adding known amounts of $H^+$ (as NaOH) to the experimented solution (1 pH unit = 1.681 μmoles $H^+$).

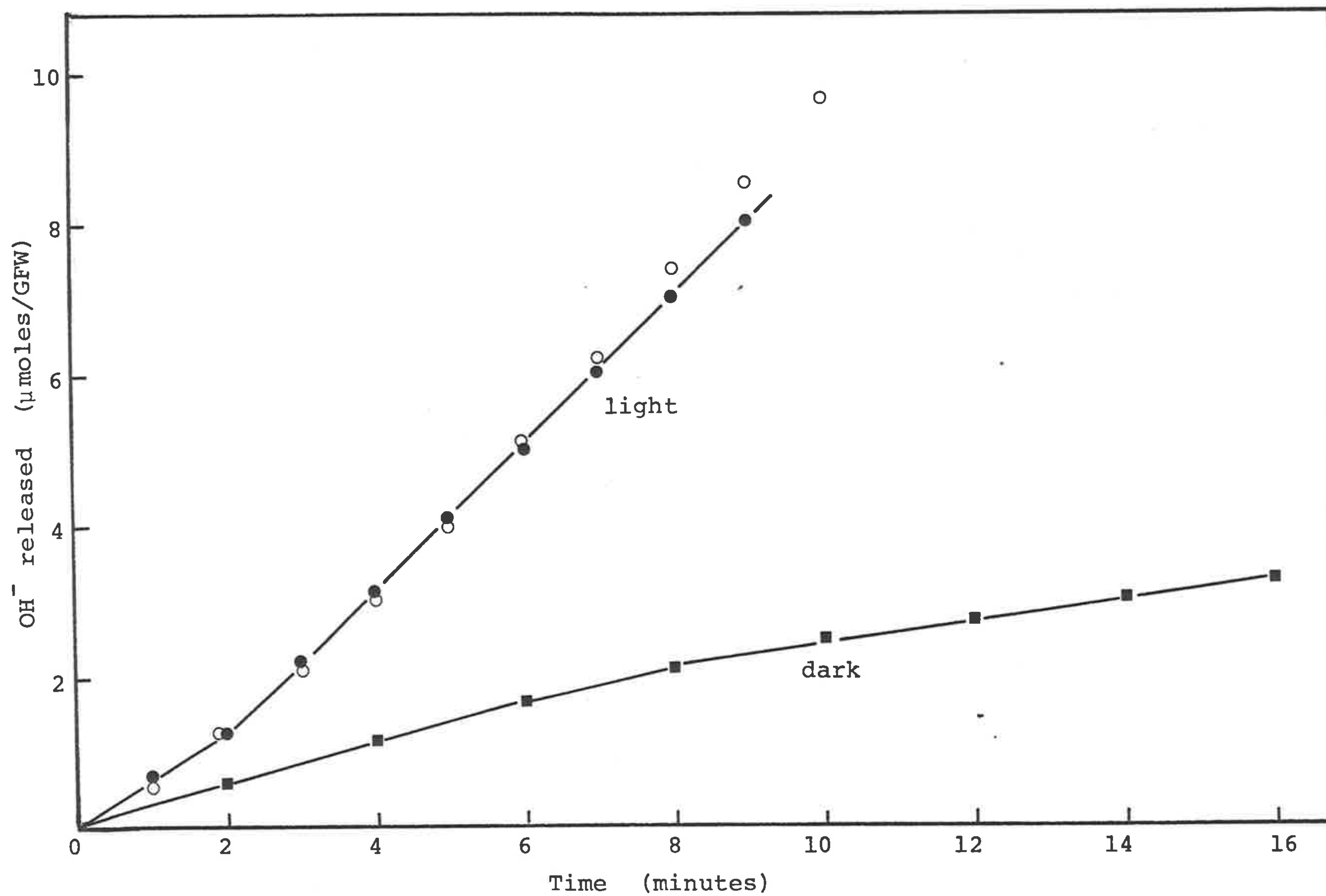



FIGURE 5.6 $OH^-$ release in the light (●) and a repeat after 20 minutes in the dark (○). The dark $H^+$ efflux (■) over a 16 minute interval. Experimental solutions as in Figure 5.4.

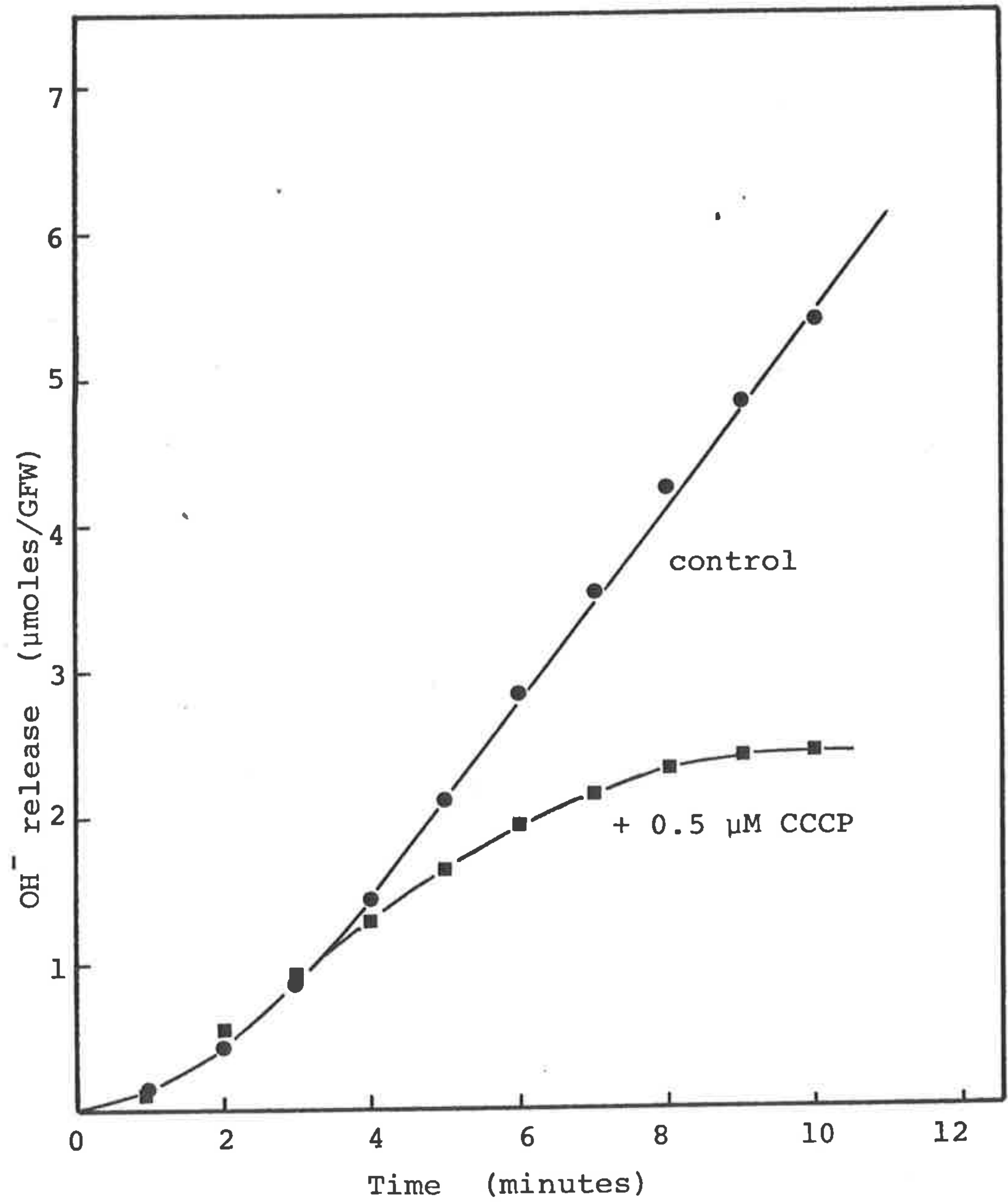



FIGURE 5.7 Effect of 0.5 µM CCCP on the light $OH^-$ efflux. Leaves given 1 hour treatment at pH 6.6 then transferred to experimental solution (same as control) see Figure 5.4.

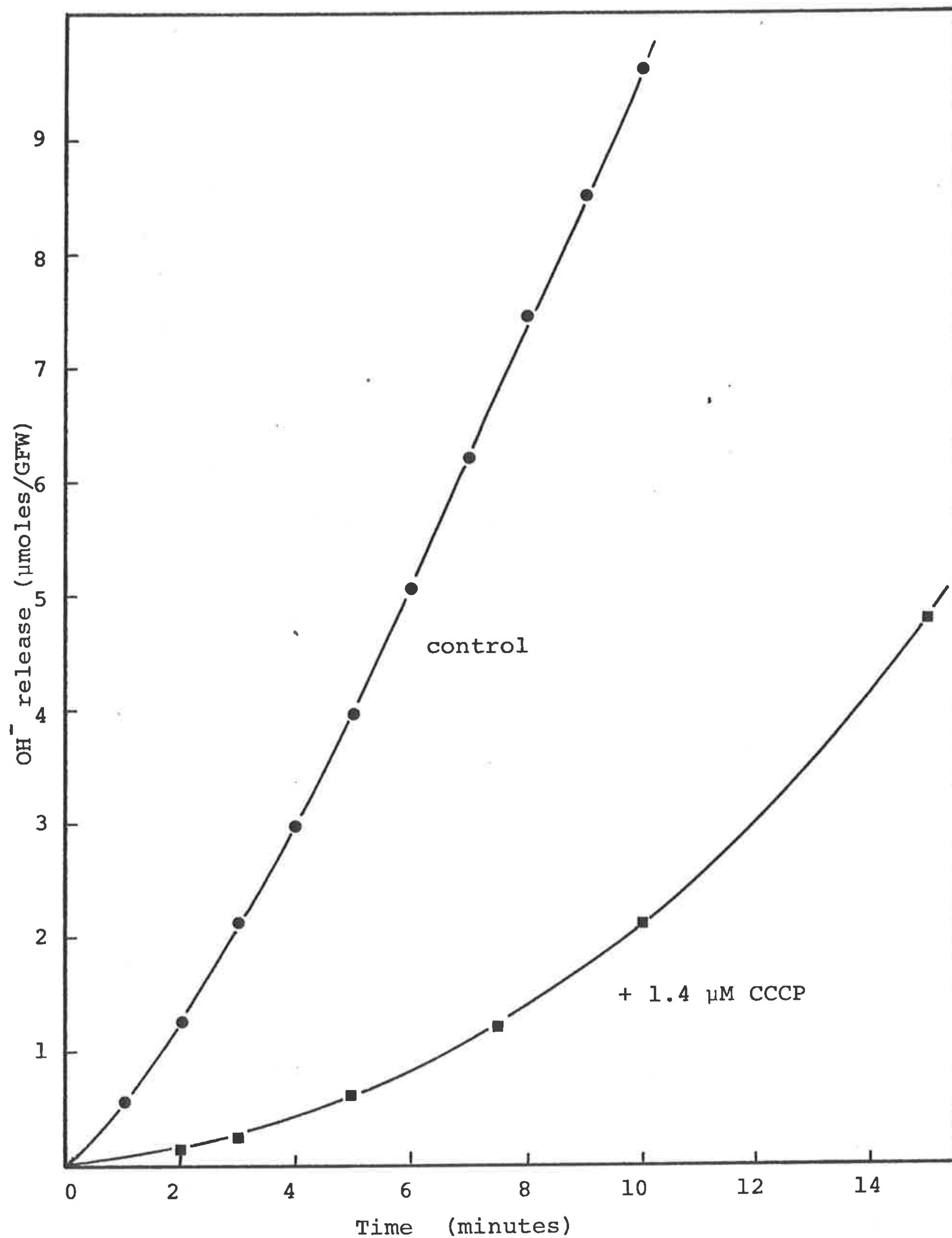



FIGURE 5.8 Effect of 1.4 µM CCCP on the light $OH^-$ efflux. Treatments and solutions as in Figure 5.7.

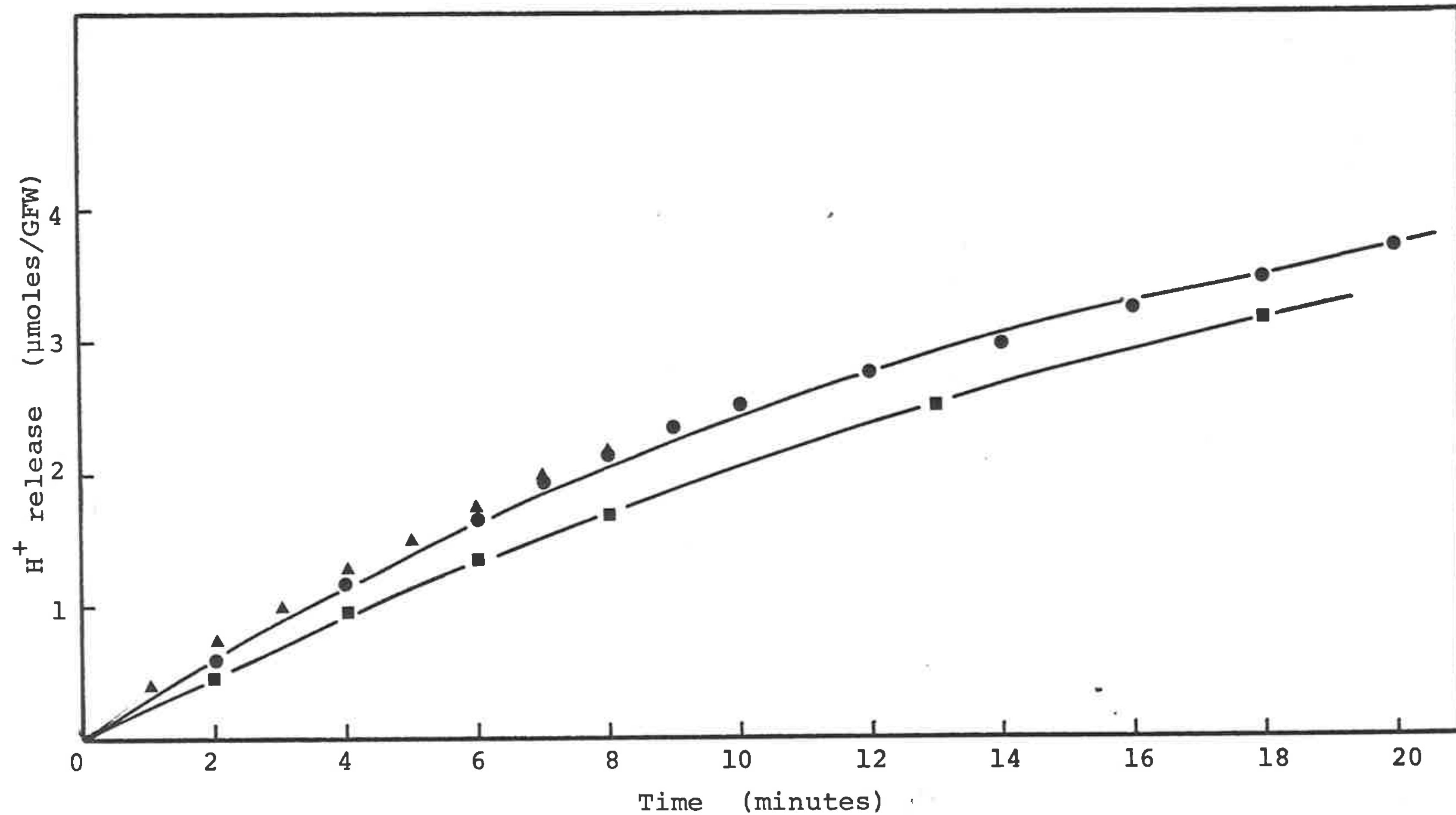



FIGURE 5.9 Effect of CCCP on the dark $H^+$ efflux. Symbols represent the normal $H^+$ efflux (●), $H^+$ efflux 2-12 minutes after the addition of 1.4 μM CCCP (▲), and 60 minutes after 1.4 μM CCCP (■). Experimental solutions contained CCCP at initial pH 6.5, otherwise as in Figure 5.4.

# CHAPTER SIX

## CATION REQUIREMENTS DURING PHOTOSYNTHESIS

### 6.1 Introduction

The large influx of $HCO_3^-$ into the lower surfaces of the leaves of *Elodea* at high pH, and the associated high $OH^-$ efflux from the upper surfaces may provide the potential energy for an electrogenic cation flux across the tissue (Helder, 1975a). Without this cation flux, potentials could develop not only between the surfaces of the tissue but also between the upper and lower layers of cells. This may in fact explain some of the very high potential differences that have been measured between adjacent cells in *Elodea* (Goodwin, 1974). Without this form of charge redistribution the potential difference would build up during photosynthesis, reducing the $OH^-$ efflux and $HCO_3^-$ efflux, and photosynthesis itself would eventually be inhibited.

Steemann Nielsen (1947) and Arens (1933) suggested that in *Elodea, Potamogeton* and *Vallisneria* these cations may be principally $K^+$ or $Ca^{++}$ *in vivo* but under experimental conditions also $Mg^{++}$ (Arens 1933) $Sr^{++}$, $Ba^{++}$ and $Na^+$ (Mazia, 1938; Brilliant, 1947; Baslavskaya and Zhuravlyova, 1948). While Mazia found that these cations could replace $Ca^{++}$ in the tissue he did not attempt any subsequent studies to determine whether they were physiologically active. He did, however, find levels of $Ca^{++}$ in aquarium *Elodea* leaves up to 16 mg/gm of dry weight, or at a tissue concentration of 0.23 mM. Furthermore, he found that 80% of this $Ca^{++}$ was re-

moved by treatment with potassium citrate (or sodium citrate). The citrate ion, having a strong negative charge, binds the $Ca^{++}$ more strongly than the $Ca^{++}$ binds to the tissue, thus effectively removing it (Mazia and Clark, 1936; McLean and Hastings, 1935). This is similar to the removal of $Ca^{++}$ from erythrocytes during anticoagulant treatment with sodium citrate.

This chapter sets out to describe the effects on photosynthesis of chemical removal of $Ca^{++}$ from the tissue by citrate treatment, and the reversibility of this treatment by subsequent replacement of the tissue in solutions containing not only $Ca^{++}$ but also other cations. Also described are the experiments with the $Ca^{++}$ chelating compound EDTA (ethylenediaminetetra acetic acid disodium salt), where the data proved to be more confusing than helpful.

## 6.2 Effects of Changing the External $Ca^{++}$ Concentration

Preliminary experiments, where *Elodea* leaves were washed for long periods in distilled water to elute tissue $Ca^{++}$, did not show any deterioration in either $HCO_3^-$ assimilation or $O_2$ evolution. However, experiments, whereby $Ca^{++}$ was subsequently added to this tissue, showed increases in $HCO_3^-$ assimilation and $O_2$ evolution.

Table 6.1 shows the effect of a 24-hour pretreatment in distilled water on $HCO_3^-$ fixation (pH 9.0). Fixation was stimulated by as much as 50% after a subsequent two hour treatment in 5.0 mM $CaSO_4$ compared to treatment in solutions containing no $CaSO_4$.

Considering the apparent importance of $Ca^{++}$ as a counterion in balancing the potential developed resulting from the high fluxes

of $OH^-$ and $HCO_3^-$ ( > 20 pmoles/$cm^2$/sec) (Arens, 1933), removal of $Ca^{++}$ should have a much greater effect than this. However, if the $Ca^{++}$ in the tissue is still sufficiently high after 24 hours and is sufficiently labile (see Chapter 7), then there may be sufficient remaining for it to act as a counterion. Since citrate treatment does remove an additional 80% of the total $Ca^{++}$ from the tissue, the effect of this treatment was studied.

## 6.3 Effects of Changing the External $Ca^{++}$ Concentration on Photosynthesis of Citrated Leaves

In Tables 6.2 and 6.3 we see that treatment with 50 mM potassium citrate did in fact reduce $HCO_3^-$ fixation by about 80% and that subsequent treatment with 0.5 mM $CaCl_2$ for two hours did reverse this inhibition. However, 0.5 mM KCl had no effect on reversing this inhibition. In these experiments the citration treatment was for 10 minutes, after which the tissue was continuously rinsed in distilled water for another 10 minutes before being placed in the appropriate experimental solutions for 2 hours under low intensity light. In Table 6.3, increasing the concentration of the $Ca^{++}$ in the experimental solutions progressively reversed the inhibition of $HCO_3^-$ fixation due to the citrate treatment.

It is well known that $Ca^{++}$ is required to maintain the integrity of the cell plasma membrane and membrane enzymes (1.7.1) and the results presented above could be interpreted as the re-establishment of the carrier system for $HCO_3^-$ or other membrane processes. The $Ca^{++}$ concentrations normally required to prevent such inhibition are much less than 0.1 mM - a level above which we see further restoration of $HCO_3^-$ fixation (Table 6.3).

## 6.4 Effects of Changing the External Potassium Concentration on Photosynthesis of Citrated Leaves

In Table 6.2 0.5 mM KCl did not reverse the inhibition of photosynthesis by potassium citrate, and as suggested above, this may have been due to the depletion of the $Ca^{++}$ in the tissue. Results of experiments whereby the $Ca^{++}$ level was replaced at sufficient concentration to restore plasma membrane integrity but sufficiently low, not to significantly restimulate $HCO_3^-$ fixation are summarised in Table 6.4. It can be seen that increasing the external $K^+$ concentration did not significantly alter the $HCO_3^-$ fixation rates above that achieved by 0.05 mM $CaCl_2$ treatment alone (32%), although at 0.5 mM KCl, $HCO_3^-$ assimilation was increased by an additional 15%. This demonstrates that $K^+$ cannot replace $Ca^{++}$ efficiently as a counterion during photosynthesis as found by Arens (1933) in *Elodea* and Steemann Nielsen (1947) in *Potamogeton*.

## 6.5 Effect of Citration on Photosynthesis at Low pH

The effect of citration on photosynthesis and other metabolic processes has not appeared in the literature, and it is quite possible that in the above experiments, citration may have had direct effects on the processes of photosynthesis and that such effects could have been reversed by $Ca^{++}$ replacement in the external solution.

However, it can be seen from Table 6.5 that at low pH (5.5), photosynthesis was not inhibited by citration and that subsequent replacement of $Ca^{++}$ did not further stimulate photosynthesis. At this pH, $CO_2$ fixation rather than $HCO_3^-$ fixation, was being measured and the lack of inhibition with citrate supports the concept that

$Ca^{++}$ was only required when $HCO_3^-$ was being transported into the cell for assimilation.

### 6.6 Effect of $Mg^{++}$ and $Ba^{++}$ on $HCO_3^-$ Assimilation in Citrated Leaves

Replacement of the leaves in solutions of $Mg^{++}$ and $Ba^{++}$ after citration treatment may result in a reversal of $HCO_3^-$ fixation (Mazia 1938). However, it can be seen from Table 6.6 that in 1.0 mM $MgSO_4$ or $BaSO_4$, such reversal was only partially achieved and in fact was similar to the reversal achieved by $K^+$ replacement (6.4).

A more detailed study of $Mg^{++}$ replacement is seen in Table 6.7, and in this study, it can be seen that maximum reversibility was achieved at 0.5 mM $MgSO_4$ (75%). At 5.0 mM $MgSO_4$ reversal was less - fixation was only 36% of the controls. It was possible that at high concentrations, $Mg^{++}$ becomes toxic (Heilbrunn, 1937; Ashkenaz, 1938) and that in the presence of $Ca^{++}$, this toxicity was reversed (Procter 1970), as in Table 6.7.

### 6.7 Oxygen Evolution and Potassium Citrate Treatment

Initial experiments using the oxygen electrode indicated that sodium citrate and potassium citrate had little or no effect on the oxygen evolution at low pH where $CO_2$ was the carbon source for photosynthesis. Similarly, at high pH, $O_2$ evolution was not inhibited significantly (Table 6.8). Although the concentrations of sodium citrate were much less in this experiment than in the preceding $H^{14}CO_3^-$ fixation experiments, the $O_2$ evolution was stimulated when

$CaCl_2$ was added to give a final concentration of 1.0 mM. In other experiments when 50 mM potassium citrate was used, $O_2$ evolution was not significantly inhibited nor affected by subsequent $Ca^{++}$ replacement.

Despite the small inhibition of $O_2$ evolution in the above experiments, there was at the same time quite marked inhibition of the pH changes in the external solution (Table 6.9). The ΔpH and $O_2$ evolution experiments were performed concurrently (see 2.3.2).

In experiments where higher concentrations of potassium citrate (50 mM) were used, it was again not possible to demonstrate a significant restimulation of $O_2$ evolution at pH 8.0 by replacing $Ca^{++}$ in the external solution. However, the citrate treatment did inhibit the $O_2$ evolution by 80% of the control value without citrate.

This lack of reversibility of $O_2$ evolution is difficult to reconcile with the reversibility of the $HCO_3^-$ assimilation. Citration appears to have had no effect on $O_2$ evolution at low pH suggesting that the photosynthetic mechanism in the chloroplasts was still intact. This is further discussed in Chapter Nine.

## 6.8 Citration and pH Changes

Treatment with low concentrations of sodium citrate has a dramatic effect on the ability of *Elodea* to raise the pH of the external solution. In Table 6.9, the conditions are the same as those in Table 6.8, and the pH values indicated are the initial pH's of the solutions. 5.0 mM sodium citrate inhibited the ΔpH by 82% and 1.0 mM $CaCl_2$ only slightly reversed this (14%).

The effect of a 10 minute 50 mM potassium citrate treatment is demonstrated in Table 6.10. Here pH changes were measured in unbuffered solutions in 1.0 mM $NaHCO_3$. The effect of citration was to inhibit ΔpH by 100% in the absence of added $Ca^{++}$. However in the presence of 1.0 mM $CaCl_2$ the effect was to reverse the inhibition so that the pH changes were 80% of the control values. Calibration of the system by additions of sodium hydroxide, (Hope et al, 1972) to allow pH changes to be expressed as an efflux of $OH^-$ from the tissue, was difficult (see Chapter 5).

Comparison of Tables 6.8 and 6.9 indicate that citration may have a dramatic effect on ΔpH at concentrations where no effect on the $O_2$ evolution was detected. Similarly $CO_2$ fixation was not affected either (see Table 6.5). As discussed in Chapter 4, $HCO_3^-$ fixation did proceed at significant rates at pH 6.7 and in the above experiment, the exogenous $HCO_3^-$ concentration was 0.5 mM. Hence in this case the ΔpH changes did reflect the effects of citration on external pH changes when $HCO_3^-$ was acting significantly as the carbon source. Hence we see that $HCO_3^-$ fixation and the associated $OH^-$ efflux could not proceed significantly without $Ca^{++}$ in the external solution and in the leaf tissue (see Chapter 7).

## 6.9 Effects of EDTA on Photosynthesis and External pH Changes

EDTA is a well-known agent in medicine for removing $Ca^{++}$ from erythrocytes to prevent fibrin formation which leads to blood clotting. In the presence of unbound calcium or even protein-bound calcium it forms calcium disodium edetate ((ethylene dinitrilo) tetra acetic acid calcium disodium salt) as indicated in Figure 6.1. Hence EDTA should behave in a similar manner to citrate.

In $Ca^{++}$-free solutions EDTA behaved differently in citrated and non-citrated leaves at pH 9.0, as shown in Table 6.11. In non-citrated leaves, $^{14}C$-fixation was inhibited at low concentrations ( < 2 mM) but at higher concentrations (5 mM) was stimulated by as much as 180% of the control values. However, in the case of citrated leaves where the rate of $HCO_3^-$ assimilation was much reduced in the absence of $Ca^{++}$, low concentrations of EDTA did not further inhibit $^{14}C$-fixation. 5.0 mM EDTA had stimulated fixation to values greater than the control (non-citrated) $^{14}C$-fixation levels.

However, the behaviour of EDTA became more complex in the presence of calcium as indicated by Table 6.12. In the case of citrated leaves, the presence together of both EDTA at low concentrations ( < 2 mM) and 0.5 mM $CaCl_2$ did reverse the effect of citration. See also Table 6.13.

The effect on oxygen evolution on non-citrated leaves was much the same. Although EDTA did not inhibit evolution at any concentrations used, as was the case with $HCO_3^-$ assimilation at low concentrations, evolution was stimulated much more in the presence of $Ca^{++}$ than in its absence (see Table 6.8).

EDTA influenced the ΔpH changes in the same manner as citrate treatments - inhibiting it in calcium free solutions by 70%. The addition of 1.0 mM $CaCl_2$ then reversed this inhibition to 92% of the control value (Table 6.9).

These experiments indicate that citrate and EDTA did behave in a similar manner by inhibiting photosynthesis ($H^{14}CO_3$ assimilation). Addition of 0.5 mM and 1.0 mM EDTA did not further inhibit $^{14}C$-fixation of citrated leaves and this inhibition was reversed in

the presence of $Ca^{++}$ as with leaves treated with citrate only. However high concentrations of EDTA did reverse $^{14}C$-fixation inhibition in the absence of $Ca^{++}$ and this did not occur on the addition of excess citrate.

This similarity between citrated leaves and EDTA-treated leaves was not observed with $O_2$ evolution, however, as $O_2$ evolution was not inhibited and was in fact stimulated by all concentrations of EDTA.

This suggests that in addition to acting as a chelating compound, EDTA also acted on *Elodea* by stimulating photosynthesis - non-cyclic electron flow; this is because at higher concentrations it did stimulate $O_2$ evolution and $HCO_3^-$ assimilation and $OH^-$ efflux. These counter effects made EDTA unsuitable for the study of $Ca^{++}$ requirements of tissue undergoing $HCO_3^-$ fixation.

6.10 Summary (see Table 6.14)

1. Addition of $Ca^{++}$ to leaves soaked in distilled water for long periods only partially stimulates $HCO_3^-$ fixation.
2. Addition of $Ca^{++}$ to leaves treated with 50 mM potassium citrate which inhibits $HCO_3^-$ fixation, reverses this inhibition.
3. $K^+$, $Mg^+$, and $Ba^{++}$ cannot restimulate $HCO_3^-$ fixation in citrated leaves.
4. Citration has no effect on (i) $CO_2$ fixation or (ii) $O_2$ evolution (at low and high pH).

5. $Ca^{++}$ only slightly stimulates $O_2$ evolution after citrate treatment, which has no effect on $O_2$ evolution.
6. Citration inhibits the ability of the leaves to increase the solution pH during $HCO_3^-$ fixation and this inhibition is reversed by $Ca^{++}$.
7. EDTA stimulates $O_2$ production and has a variable effect on carbon fixation. EDTA inhibits the ability of the leaves to change solution pH.

TABLE 6.1 EFFECT ON $HCO_3^-$ FIXATION OF 24 HOUR PRETREATMENT IN DISTILLED WATER

| Experimental Solutions | $^{14}C$-fixation ($pmoles/cm^2/sec$) | % of controls |
|---|---|---|
| no $Ca^{++}$ | 25.5 ± 2 | 100 |
| 0.05 mM $CaSO_4$ | 23.0 ± 2 | 92 |
| 0.1 " " | 26.4 ± 2 | 106 |
| 0.5 " " | 32.2 ± 2 | 129 |
| 1.0 " " | 34.0 ± 2 | 136 |
| 5.0 " " | 37.9 ± 2 | 152 |

Pretreatment (2 hours) and $^{14}C$-fixation measured in solutions of increasing $Ca^{++}$, as indicated, and 5.0 mM TAPS, 1.0 mM $NaHCO_3$ at pH 9.0.

TABLE 6.2 EFFECT OF CITRATE TREATMENT AND CATIONS ON $HCO_3^-$ FIXATION

| Pretreatment | Experimental Conditions | | $^{14}C$-fixation | % |
|---|---|---|---|---|
| | $[CaCl_2]$ mM | [KCl] mM | ($pmoles/cm^2/sec$) | of controls |
| no citration | 0.5 | 0.5 | 12.6 ± 1 | 100 |
| citration | 0 | 0 | 2.4 ± .1 | 19 |
| " | 0.5 | 0 | 14.3 ± .5 | 119 |
| " | 0 | 0.5 | 2.6 ± .1 | 22 |
| " | 0.5 | 0.5 | 12.8 ± .7 | 102 |
| citration | 1.0 mM EDTA | | 6.1 ± .2 | 48 |

Pretreatment in 50 mM potassium citrate. Solutions all contain 5.0 mM TAPS, 1.0 mM $NaHCO_3$ at pH 9.0.

**TABLE 6.3** EFFECT OF $Ca^{++}$ ON $HCO_3^-$ FIXATION OF CITRATE TREATED LEAVES

| Experimental Conditions | $^{14}C$-Fixation (pmoles/$cm^2$/sec) | % of controls |
|---|---|---|
| 0.01 mM $CaCl_2$ | 2.3 ± .2 | 24 |
| 0.02 " " | 2.5 ± .1 | 26 |
| 0.05 " " | 3.3 ± .3 | 35 |
| 0.1 " " | 2.5 ± .2 | 26 |
| 0.2 " " | 3.4 ± .3 | 36 |
| 0.5 " " | 6.2 ± .5 | 65 |
| 1.0 " " | 9.3 ± .3 | 98 |
| 2.0 mM $CaCl_2$ | 9.5 ± .2 | 100 |

Citrate pretreatment and solutions as in Table 6.2.

TABLE 6.4 EFFECTS OF $K^+$ IN THE PRESENCE OF LOW $Ca^{++}$ CONCENTRATIONS ON $HCO_3^-$ FIXATION AFTER CITRATION TREATMENT

| Experimental Conditions | | $^{14}C$-fixation ($pmoles/cm^2/sec$) | % of controls |
|---|---|---|---|
| 0.5 mM $CaCl_2$ | | 15.4 ± 1.2 | 100 |
| 0.05 mM $CaCl_2$ | | 4.9 ± .3 | 32 |
| 0.05 mM $CaCl_2$ | + 0.05mM KCl | 5.7 ± .9 | 37 |
| " " " | + 0.1 " " | 4.8 ± .2 | 31 |
| " " " | + 0.2 " " | 6.2 ± .9 | 40 |
| " " " | + 0.5 " " | 7.2 ± .8 | 47 |
| " " " | + 1.0 " " | 5.4 ± .2 | 35 |
| 0.05 mM $CaCl_2$ | + 2.0mM KCl | 5.4 ± .2 | 35 |

Citrate pretreatment and solutions as in Table 6.2.

TABLE 6.5 EFFECT OF $Ca^{++}$ ON $CO_2$ FIXATION OF CITRATE TREATED LEAVES

| Treatment | Experimental Conditions | $^{14}C$-Fixation (pmoles/$cm^2$/sec) |
|---|---|---|
| no citration | 0.5 mM $CaCl_2$ | 96 ± 3 |
| citration | no $Ca^{++}$ | 95 ± 2 |
| " | 0.05 mM $CaCl_2$ | 95 ± 2 |
| " | 0.5 mM $CaCl_2$ | 94 ± 3 |
| " | 1.0 mM $CaCl_2$ | 92 ± 4 |
| citration | 5.0 mM $CaCl_2$ | 95 ± 3 |

Citrate treatment as in Table 6.2. Solutions contain 5.0 mM MES and 1.0 mM $NaHCO_3$ at pH 5.5.

TABLE 6.6 EFFECT OF CATIONS ON $HCO_3^-$ FIXATION OF CITRATE TREATED LEAVES

| Treatment | Experimental Conditions | $^{14}C$-fixation ($pmoles/cm^2/sec$) | % of controls |
|---|---|---|---|
| no citrations | 1.0 mM $CaSO_4$ | 17.4 ± .2 | 100 |
| citration | no $Ca^{++}$ | 3.8 ± .8 | 22 |
| " | 1.0 mM $CaSO_4$ | 19.4 ± .3 | 111 |
| " | 1.0 mM $MgSO_4$ | 5.3 ± .4 | 30 |
| citration | 1.0 mM $BaSO_4$ | 8.0 ± 2 | 46 |

Citrate pretreatment and experimental solutions as in Table 6.2.

TABLE 6.7 EFFECT OF $Mg^{++}$ ON $HCO_3^-$ FIXATION OF CITRATE TREATED LEAVES

| Pretreatment | Experimental Conditions | $^{14}C$-fixation (pmoles/$cm^2$/sec) | % of controls |
|---|---|---|---|
| no citration | | 9.0 ± .7 | 73 |
| no citration | 1.0 mM $MgSO_4$ | 12.4 ± .8 | 100 |
| citration | 0.05 mM " | 5.3 ± .3 | 43 |
| " | 0.1 " " | 4.9 ± .6 | 40 |
| " | 0.2 " " | 5.7 ± .7 | 46 |
| " | 0.5 " " | 9.1 ± .7 | 73 |
| " | 1.0 " " | 5.4 ± .6 | 44 |
| " | 2.0 " " | 7.9 ± 1.0 | 64 |
| " | 5.0 mM $MgSO_4$ | 3.2 ± .3 | 26 |
| " | 0.1 mM $CaCl_2$ | 3.9 ± .4 | 31 |
| " | 1.0 mM $CaCl_2$ | 14.5 ± 1.5 | 117 |
| citration | 1.0 mM $CaCl_2$ | 13.4 ± 1.9 | 108 |

Citrate pretreatment and experimental solutions as in Table 6.2.

TABLE 6.8 EFFECTS OF CITRATE AND EDTA TREATMENTS ON $O_2$ EVOLUTION AT pH 6.5 AND 8.5

| Pretreatment | pH | Experimental Conditions (solutions) | $O_2$-evolution (nmoles/min/GFW) | % of controls |
|---|---|---|---|---|
| no citrate | 6.85 | no $Ca^{++}$ | 197 | 100 |
| | 8.8 | no $Ca^{++}$ | 28 | 14 |
| | 8.8 | 1.0 mM $Ca^{++}$ | 30 | 15 |
| 5.0 mM citrate | 6.5 | no $Ca^{++}$ | 168 | 85 |
| | 8.8 | no $Ca^{++}$ | 24 | 12 |
| | 8.8 | 1.0 mM $CaCl_2$ | 37 | 19 |
| | 6.7 | " " | 158 | 80 |
| 10 mM citrate | 6.6 | no $Ca^{++}$ | 150 | 76 |
| | 8.6 | " " | 30 | 15 |
| | 8.6 | 1.0 mM $CaCl_2$ | 42 | 21 |
| | 6.7 | " " | 139 | 71 |
| 1.0 mM EDTA | 6.6 | no $Ca^{++}$ | 185 | 94 |
| | 8.5 | " " | 54 | 28 |
| | 8.5 | 1.0 mM $CaCl_2$ | 75 | 38 |
| | 6.75 | " " | 199 | 101 |
| 1.0 mM EDTA 5.0 mM citrate | 6.6 | no $Ca^{++}$ | 104 | 53 |
| | 8.5 | " " | 39 | 20 |
| | 8.5 | 1.0 mM $CaCl_2$ | 41 | 21 |
| | 6.7 | " " | 119 | 60 |

All pretreatments were in 5.0 mM HEPES at pH 6.7 with the concentrations of citrate or EDTA indicated. After pretreatment, tissue was washed in distilled water for 10 mins before transferring to oxygen electrode. At the low pH and high pH 0.5 mM MES and 0.5 mM TAPS buffers were used respectively.

TABLE 6.9 EFFECTS OF CITRATE AND EDTA TREATMENTS ON THE EXTERNAL pH INCREASES

| Pretreatment | Initial pH | Experimental Conditions | $\Delta$ pH (pH/min/GFW) | % of controls |
|---|---|---|---|---|
| no citrate | 6.85 | no $Ca^{++}$ | 0.43 | 100 |
| citration | 6.85 | no $Ca^{++}$ | 0.079 | 18 |
| (5.0 mM) | 6.7 | 1.0 mM $CaCl_2$ | 0.37 | 86 |
| citration | 6.6 | no $Ca^{++}$ | 0.092 | 21 |
| (10 mM) | 6.7 | 1.0 mM $CaCl_2$ | 0.281 | 65 |
| 1.0 mM EDTA | 6.6 | no $Ca^{++}$ | 0.133 | 31 |
| | 6.7 | 1.0 mM $CaCl_2$ | 0.396 | 92 |
| 1.0 mM EDTA | 6.6 | no $Ca^{++}$ | 0.067 | 15 |
| + 5.0 mM citrate | 6.7 | 1.0 mM $CaCl_2$ | 0.242 | 56 |

Experimental conditions as in Table 6.8.

TABLE 6.10 EFFECT OF CITRATE TREATMENT ON EXTERNAL pH INCREASES AT INITIAL pH 9.0

| Pretreatment | Experimental Conditions | $\Delta$ pH (pH/min/GFW) | % of controls |
|---|---|---|---|
| no citration | 1.0 mM $CaCl_2$ | 0.27 | 100 |
| citration | no $Ca^{++}$ | 0 | 0 |
| " | 0.1 mM $CaCl_2$ | 0.06 | 22 |
| " | 0.5 mM $CaCl_2$ | 0.17 | 63 |
| citration | 1.0 mM $CaCl_2$ | 0.21 | 78 |

$\Delta$ pH changes at initial pH of 9.0. Citration in 50 mM potassium citrate for 10 minutes followed by 2 hours in 0.5 mM $NaHCO_3$ at pH 9.0 with the different concentrations of $CaCl_2$ as shown, before $\Delta$ pH measurements made.

TABLE 6.11 EFFECTS OF EDTA ON NORMAL AND CITRATE TREATED LEAVES

| Pretreatment | Experimental Conditions | $^{14}C$-fixation (pmoles/$cm^2$/sec) | % of controls |
|---|---|---|---|
| no citrate | no EDTA | 19.1 ± .9 | 100 |
| " | 0.5 mM EDTA | 6.1 ± .5 | 32 |
| " | 1.0 " " | 6.8 ± .7 | 35 |
| " | 2.0 " " | 9.1 ± .3 | 47 |
| no citrate | 5.0 mM EDTA | 34 ± 4 | 177 |
| citrate | no EDTA | 1.6 ± .1 | 8 |
| " | 0.5 mM EDTA | 1.6 ± .2 | 8 |
| " | 1.0 " " | 2.2 ± .2 | 12 |
| " | 2.0 " " | 5.1 ± .6 | 27 |
| citrate | 5.0 mM EDTA | 28.9 ± 2 | 151 |

Citrate pretreatment and experimental solutions as in Table 6.2.

TABLE 6.12 EFFECTS OF $Ca^{++}$ AND EDTA ON $HCO_3^-$ FIXATION OF CITRATE TREATED LEAVES

| Pretreatment | Experimental Conditions | $^{14}C$-fixation (pmoles/$cm^2$/sec) | % of controls |
|---|---|---|---|
| no citration | 0.5 mM $CaCl_2$ | 21 ± .4 | |
| citration | no $CaCl_2$ | 2.8 ± 2.5 | 100 |
| " | 0.5 mM $CaCl_2$ | 18.4 ± .8 | |
| " | 0.5 mM EDTA | 2.0 ± .2 | 71 |
| " | 1.0 " " | 2.8 ± .3 | 100 |
| " | 5.0 mM EDTA | 14.3 ± 2 | 500 |
| citration | 0.5 mM $CaCl_2$ + .2 mM EDTA | 11.4 ± 1.5 | 407 |
| " | 0.5 mM $CaCl_2$ + 1.0 mM EDTA | 15.4 ± 3.2 | 550 |
| " | 0.5 mM $CaCl_2$ + 5.0 mM EDTA | 2.0 ± .2 | 71 |

Citrate pretreatment and experimental solutions as in Table 6.2.

TABLE 6.13 COMPARATIVE EFFECTS OF EDTA AND $Ca^{++}$ ON $HCO_3^-$ FIXATION OF CITRATE TREATED LEAVES

| Experimental Conditions | $^{14}C$-fixation (pmoles/$cm^2$/sec) | % of controls |
|---|---|---|
| no $Ca^{++}$ | 3.1 ± .3 | 100 |
| 0.5 mM $CaCl_2$ | 8.0 ± .6 | 257 |
| 0.5 mM EDTA | 3.2 ± .5 | 101 |
| 1.0 mM EDTA | 5.1 ± .8 | 164 |
| 0.5mM $CaCl_2$ + 0.5mM EDTA | 10.1 ± .9 | 325 |
| " " + 1.0mM EDTA | 22.9 ± 4 | 736 |

Citrate pretreatment and experimental solutions as in Table 6.2.

TABLE 6.14 SUMMARY TABLE (CHAPTER SIX)

| Treatment | $O_2$ evolution | | $^{14}C$-fixation | | $OH^-$ efflux |
|---|---|---|---|---|---|
| | $CO_2$ | $HCO_3^-$ | $CO_2$ | $HCO_3^-$ | |
| citrated leaves | no effect | no effect | no effect | effects | effects |
| citrated leaves + $Ca^{++}$ | slight stimulation | slight stimulation | no effect | reversible | reversible |

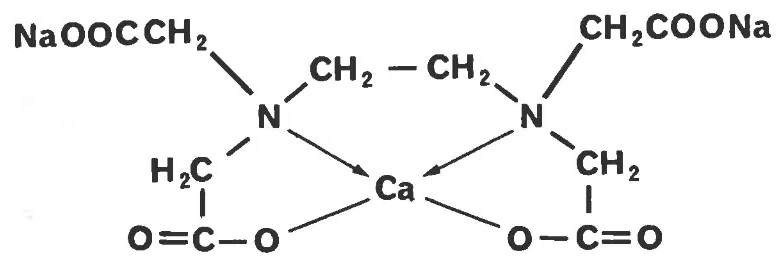



FIGURE 6.1 Calcium disodium edetate

# CHAPTER SEVEN

## THE EFFLUX AND ABSORPTION OF CALCIUM IN *Elodea* LEAVES

### 7.1 Introduction

As shown in Chapter 6, the presence of $Ca^{++}$ in the solution or tissue is a prerequisite for $HCO_3^-$ absorption and fixation but not $CO_2$ fixation. That $Ca^{++}$ and other cations are transported from the lower surface of the leaf to the upper surface has been amply demonstrated (Steemann Nielsen, 1947; Lowenhaupt, 1956; Helder and Boerma, 1973). Lowenhaupt (1956) suggested that this movement is active into the cells at the lower surface and active out of the cells at the upper layer. But in the experiments, Lowenhaupt did not distinguish between $Ca^{++}$ in the cells and in the tissue as a whole, as he measured changes in labelled $Ca^{++}$ in the external solutions. Similarly, Mazia (1938) did not distinguish between intracellular and extracellular tissue $Ca^{++}$ in his uptake experiments. After the uptake of labelled $Ca^{++}$, he washed the tissue in distilled water for 60 minutes. Mazia and Clark (1936) and Mazia (1938) demonstrated that $Ca^{++}$ was not found in the vacuoles of *Elodea* except after the effects of stimulating agents (plasmolysis with 0.5 mM sucrose or dextrose) when large amounts of calcium oxalate crystals were found. They assumed that this $Ca^{++}$ came from the cytoplasm.

If, as demonstrated by Helder and Boerma (1972), the rate of cation transport across the leaf is stimulated in the light during

$HCO_3^-$ assimilation, then one should also find a stimulation of the uptake of labelled cation into the tissue itself and also a light-stimulated efflux of labelled cations from previously labelled cation-loaded tissue. Also, as there is this cation requirement for $HCO_3^-$ fixation as shown in the last chapter such intake and efflux should be affected by parameters that affect $HCO_3^-$ fixation, such as pH and inhibitors. Such effects are examined in this chapter.

## 7.2 Uptake of $Ca^{++}$ into *Elodea* tissue

Uptake of $^{45}Ca^{++}$ into *Elodea* tissue in the light and dark is shown in Figure 7.1 and 7.2. Although the errors are quite large, there appears to be only a small difference between the light and dark uptake. Figure 7.1 shows the uptake into leaves that had been soaked in a $Ca^{++}$ free solution for 24 hours, whereas in Figure 7.2 leaves had been treated for 10 minutes in 50 mM potassium citrate, then washed in the normal pretreatment solution (5 mM TAPS, 1 mM $NaHCO_3$ at pH 9.0) for 3 hours, before being transferred to 0.5 mM $^{45}CaCl_2$ in the same solution. Assuming that there was linearity in the first 10 minutes, then the $Ca^{++}$ influx to untreated tissue is approximately 50 pmoles/$cm^2$/sec, whereas in potassium citrate treated cells, the $Ca^{++}$ influx is 350 pmoles/$cm^2$/sec (assuming linearity in the first 2 minutes - Figure 7.2).

In both of the above experiments the leaves were washed in distilled water for less than 5 minutes. As shown in 7.6, this was not sufficient to wash all the $^{45}Ca^{++}$ from the free space - presumably the cell walls - hence the flux represents uptake into the tissue

as a whole and not only into the cells. Such uptake is referred to as the tissue uptake. Figure 7.3 shows the $Ca^{++}$ uptake into tissue remaining after a 60 minute washout in 1 mM NaCl. Again it appears that 1 mM NaCl did not exchange the $^{45}Ca^{++}$ in the free space. In figure 7.4, when the washout was for 60 minutes in 1.0 mM $CaSO_4$, there is a linear uptake of $^{45}Ca^{++}$ for 40 minutes. This does represent an uptake into the cells of the tissue, and the flux is calculated to be 2.2 pmoles/$cm^2$/sec. This uptake is referred to as the cellular $Ca^{++}$ uptake.

## 7.3 Influence of CCCP on the Cellular $Ca^{++}$ Uptake

To determine whether the cellular $Ca^{++}$ uptake was dependent on energy from photosynthesis or respiration, the effect of 1 µM CCCP was investigated. As CCCP has little effect at high pH, for this experiment the leaves were treated with 1 µM CCCP at pH 6 for 50 minutes before being transferred to the experimental pretreatment solution for 20 hours. Uptake was determined at the end of this period. Figure 7.5 shows the effect of CCCP treatment on the continuous cellular $Ca^{++}$ uptake. The $Ca^{++}$ influx was estimated to be reduced to 20% of the control value by this treatment.

## 7.4 Effect of pH on Cellular $Ca^{++}$ Uptake

As shown in Chapter 6, $Ca^{++}$ was not required for $CO_2$ fixation (low pH) whereas it was at pH 9.0 for $HCO_3^-$ fixation. Hence measurements of the cellular $Ca^{++}$ influx at these two pH values should have given information as to the role of $Ca^{++}$ in maintaining photosynthesis. Figure 7.6 shows that there was very little difference

between the cellular $Ca^{++}$ uptake at the two pH values and in addition, the uptake was the same in the light and dark. The effect of increasing the solution $Ca^{++}$ concentration suggests that the $Ca^{++}$ follows its own diffusion potential and enters the cell passively. This was the conclusion of Mazia (1938) but in his experiments uptake was most probably into the whole tissue not the cells.

7.5 The Effects of CMU and pH on the Tissue $Ca^{++}$ Uptake

The effects on short term uptake of $Ca^{++}$ to the whole tissue are shown in Tables 7.1 and 7.2. In the pH range 5.5 to 10 there was no differential effect on the uptake in $CO_2$ free solutions and in solutions containing $NaHCO_3$. Similarly, CMU in the range 0.1 μM to 50 μM had no differential effect on the $Ca^{++}$ uptake. These experiments were to test the relationship between $HCO_3^-$ fixation, which is both pH dependent and inhibited by CMU, and $Ca^{++}$ uptake into the free-space. They show in conjunction with the experiments of Chapter 6, that while photosynthesis requires the presence of $Ca^{++}$ in the free-space, the rate of photosynthetic $HCO_3^-$ fixation does not influence the rate of exchange of $Ca^{++}$ between the solution and the free-space.

7.6 Calcium Efflux

The efflux of $^{45}Ca^{++}$ into solutions containing increasing $Ca^{++}$ concentrations at pH 9.0 is shown in Figure 7.7. The curves are expressed as a percentage of the initial total $^{45}Ca^{++}$ in the tissue. A is the $Ca^{++}$ efflux into distilled water, and curve G

the efflux into 5.0 mM $CaSO_4$. Analysis of the curves using the computer program non-linear of SSPS (2.3.7) showed that after 90 seconds curves A to D were monophasic and conformed to a one compartment model.

$$[Ca^{++}]_t = [Ca^{++}]_o \, e^{-kt} \qquad (1)$$

where $[Ca^{++}]_t$ is the leaf $^{45}Ca^{++}$ content at any time t after washing commences, $[Ca^{++}]_o$ is the total leaf content at time t = o, and k is the efflux constant. The curves E, F and G show a biphasic efflux of $Ca^{++}$ after 90 seconds, according to the two compartment model

$$[Ca^{++}]_t = [Ca^{++}]_o e^{-kt} + [Ca^{++}]_c \, e^{-k_2 t} \qquad (2)$$

where $[Ca^{++}]_t$ and $[Ca^{++}]_o$ are as in equation (1); $[Ca^{++}]_c$ is the $^{45}Ca^{++}$ remaining in the second compartment which is the slower exchanging compartment, which in these experiments may represent the $Ca^{++}$ efflux from the cytoplasmic or cellular compartment. The faster component represents intercellular $Ca^{++}$ exchange. Two phases were not detected in the washout experiments into low $Ca^{++}$ solutions because most of the $^{45}Ca^{++}$ was still being washed out from the tissue and this was occurring at similar rates to the exchange from the cellular component.

5 μM CCCP had no effect on the fast and slow $^{45}Ca^{++}$ efflux (Figure 7.8), suggesting in the case of the cellular $^{45}Ca^{++}$ that it was not an energy dependent process. Similarly 50 μM CMU had no effect on the cellular $^{45}Ca^{++}$ efflux.

The effect of adding 50 mM potassium citrate to the washout solutions after 5 minutes is shown in Figure 7.9. In the various

washing solutions containing various concentrations of $Ca^{++}$ it can be seen that the apparent exchange from the extracellular phase was extremely rapid. In all washout solutions, the $^{45}Ca^{++}$ exchange after 10 minutes represented an exchange from the cellular components.

## 7.7 Summary

1. $Ca^{++}$ uptake into *Elodea* tissue is into 2 separate compartments. A rapid uptake into the intercellular spaces (cell walls, etc.) (50 pmoles/$cm^2$/sec) and a slower uptake of 2 pmoles/$cm^2$/sec into the cells (cellular uptake).
2. $Ca^{++}$ uptake into potassium citrate treated leaves is extremely rapid (350 pmoles/$cm^2$/sec) but equilibrium is reached in less than 2 minutes.
3. Cellular $Ca^{++}$ uptake was reduced by 20% of the control values by 1 μM CCCP.
4. Cellular $Ca^{++}$ uptake is unaffected by solution pH.
5. $^{45}Ca^{++}$ in the tissue exchanges more rapidly into washout solutions as the $Ca^{++}$ concentration of these solutions increases. Washout into 50 mM potassium citrate solutions is extremely rapid.
6. Washout into 5 mM $CaSO_4$ is biphasic, the slower phase representing a $Ca^{++}$ efflux from the cellular compartment.
7. The cellular $Ca^{++}$ efflux is insensitive to CCCP and CMU.
8. The cellular $Ca^{++}$ concentration is not affected by citrate.

## TABLE 7.1 EFFECT OF pH ON $Ca^{++}$ UPTAKE INTO TISSUE

| pH of External Solution | | $Ca^{++}$ Uptake (pmoles/cm$^2$/sec) |
|---|---|---|
| 5.5 | $-CO_2$ | 92 ± 5 |
| " | + 1.0 mM $NaHCO_3$ | 108 ± 6 |
| 6.5 | $-CO_2$ | 118 ± 4 |
| " | + 1.0 mM $NaHCO_3$ | 116 ± 6 |
| 7.5 | $-CO_2$ | 103 ± 4 |
| " | + 1.0 mM $NaHCO_3$ | 86 ± 7 |
| 8.5 | $-CO_2$ | 98 ± 11 |
| " | + 1.0 mM $NaHCO_3$ | 93 ± 4 |
| 9.0 | $-CO_2$ | 104 ± 4 |
| " | + 1.0 mM $NaHCO_3$ | 116 ± 4 |
| 10.0 | $-CO_2$ | 102 ± 4 |
| " | + 1.0 mM $NaHCO_3$ | 118 ± 9 |

Solutions contained 1.0 mM $CaCl_2$ and 5 mM buffer (MES - pH 5.5, 6.5; HEPES - pH 7.5, 8.5; TAPS - 9.0, 10). All solutions were bubbled with $N_2$ for 20 minutes. 1 mM $NaHCO_3$ then added to required solutions. Pretreatment was then for 2 hours.

TABLE 7.2 THE EFFECT OF CMU ON THE TISSUE UPTAKE OF $Ca^{++}$

| CMU Concentration | $Ca^{++}$ Influx (pmoles/cm$^2$/sec) |
|---|---|
| 0 | 110 ± 5 |
| 0.1 μM CMU | 106 ± 3 |
| 0.2 " " | 100 ± 3 |
| 0.5 " " | 101 ± 2 |
| 1.0 " " | 100 ± 3 |
| 5 " " | 93 ± 3 |
| 10 " " | 106 ± 4 |
| 20 " " | 115 ± 3 |
| 50 " " | 98 ± 3 |

CMU treatment in 1.0 mM $CaCl_2$, 1.0 mM $NaHCO_3$, 5 mM HEPES at pH 7 for 60 minutes. Leaves then transferred to solution 1.0 mM $CaCl_2$, 1.0 mM $NaHCO_3$, 5 mM TAPS at pH 9.0, for 30 minutes before transfer to solutions containing $^{45}Ca^{++}$ for uptake measurements.

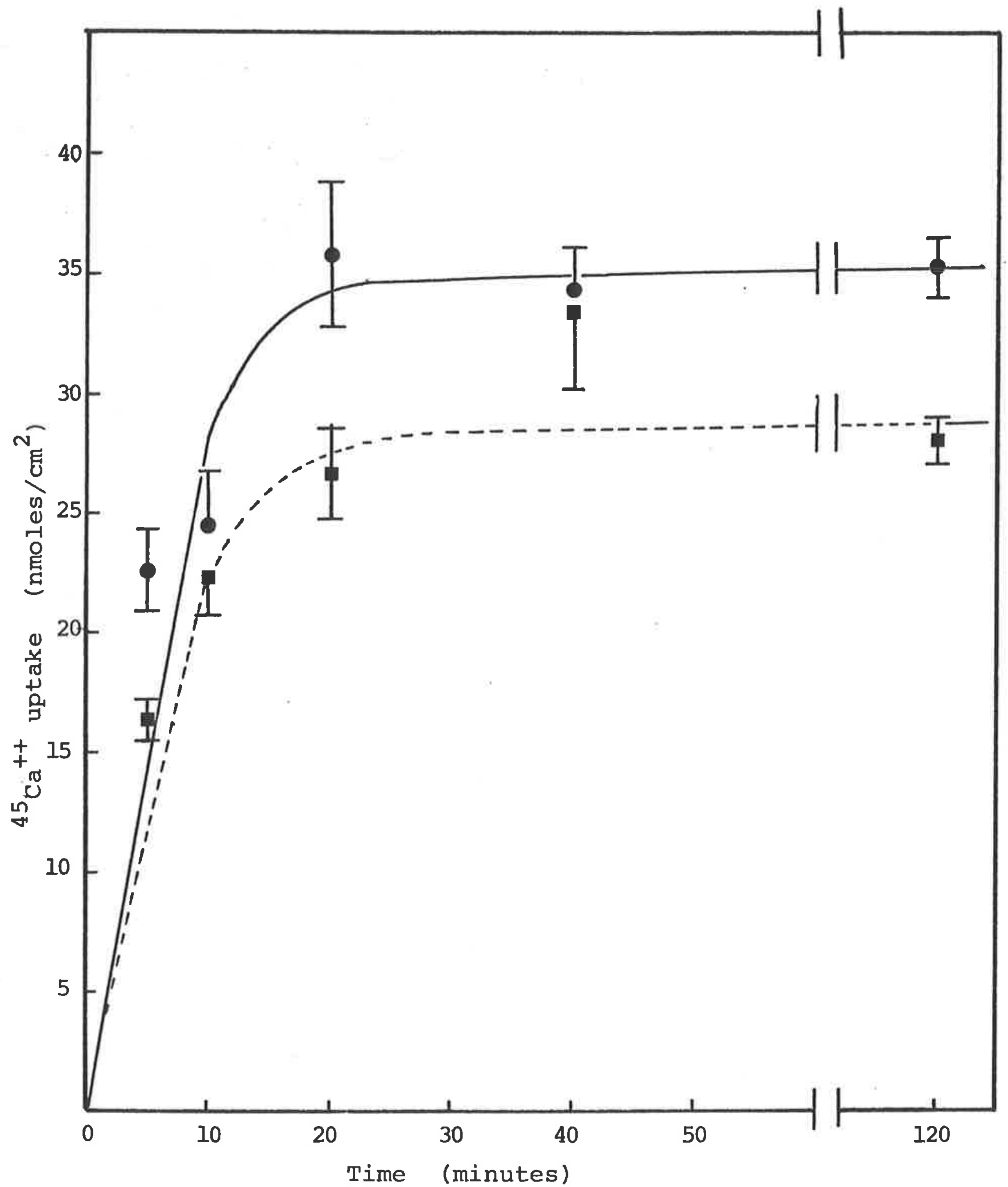



FIGURE 7.1 Continuous tissue uptake of $^{45}Ca^{++}$ in the light (●) and and dark (■) at pH 9.0. Experimental solutions as in 2.3.10 with 1.0 mM $CaCl_2$. Leaves soaked in $Ca^{++}$-free solutions for 24 hours.

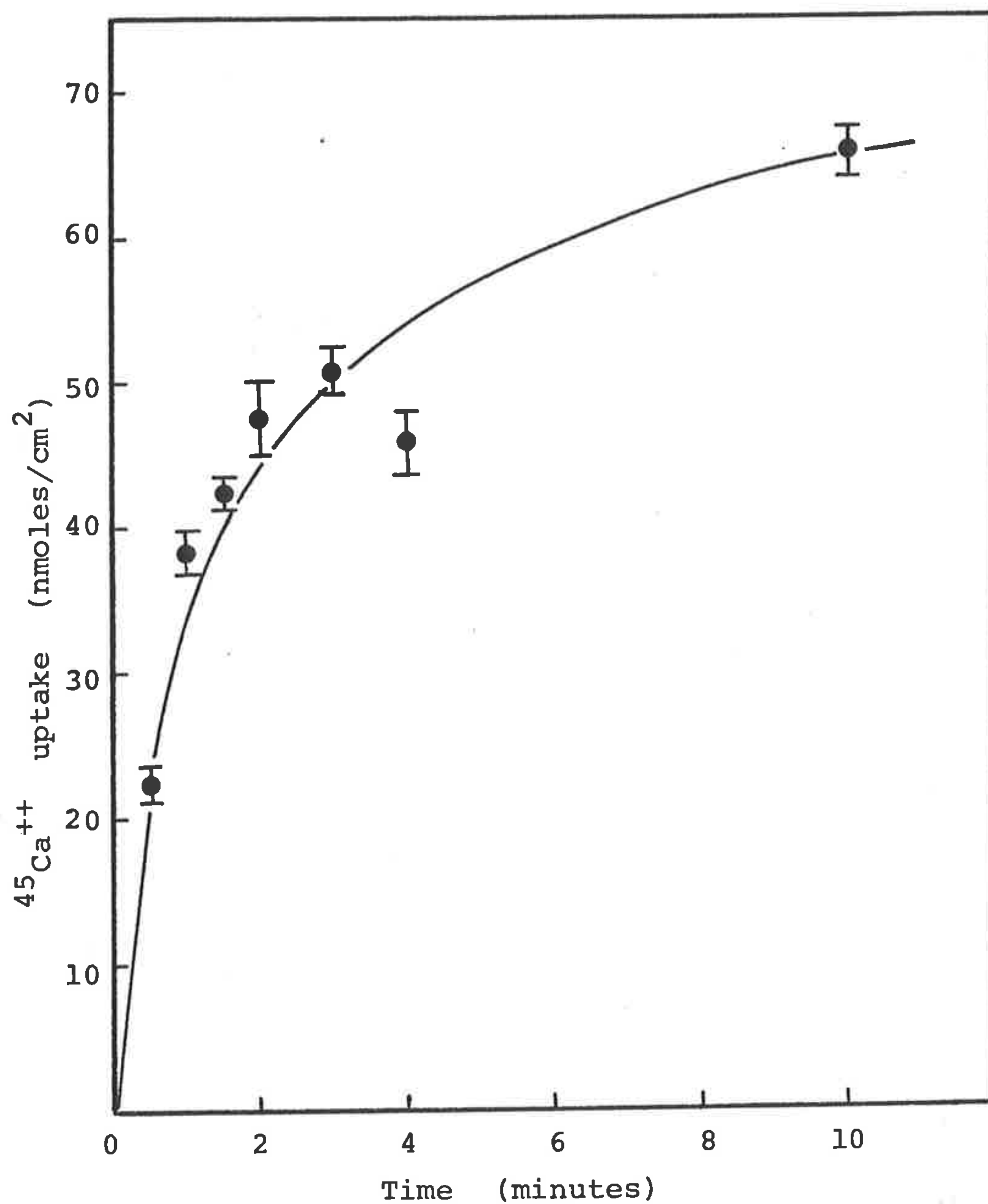



FIGURE 7.2 Short term continuous tissue uptake of $^{45}Ca^{++}$ in the light, after 10 minutes pretreatment with 50 mM potassium citrate. Leaves given 3 hour treatment in experimental solutions.

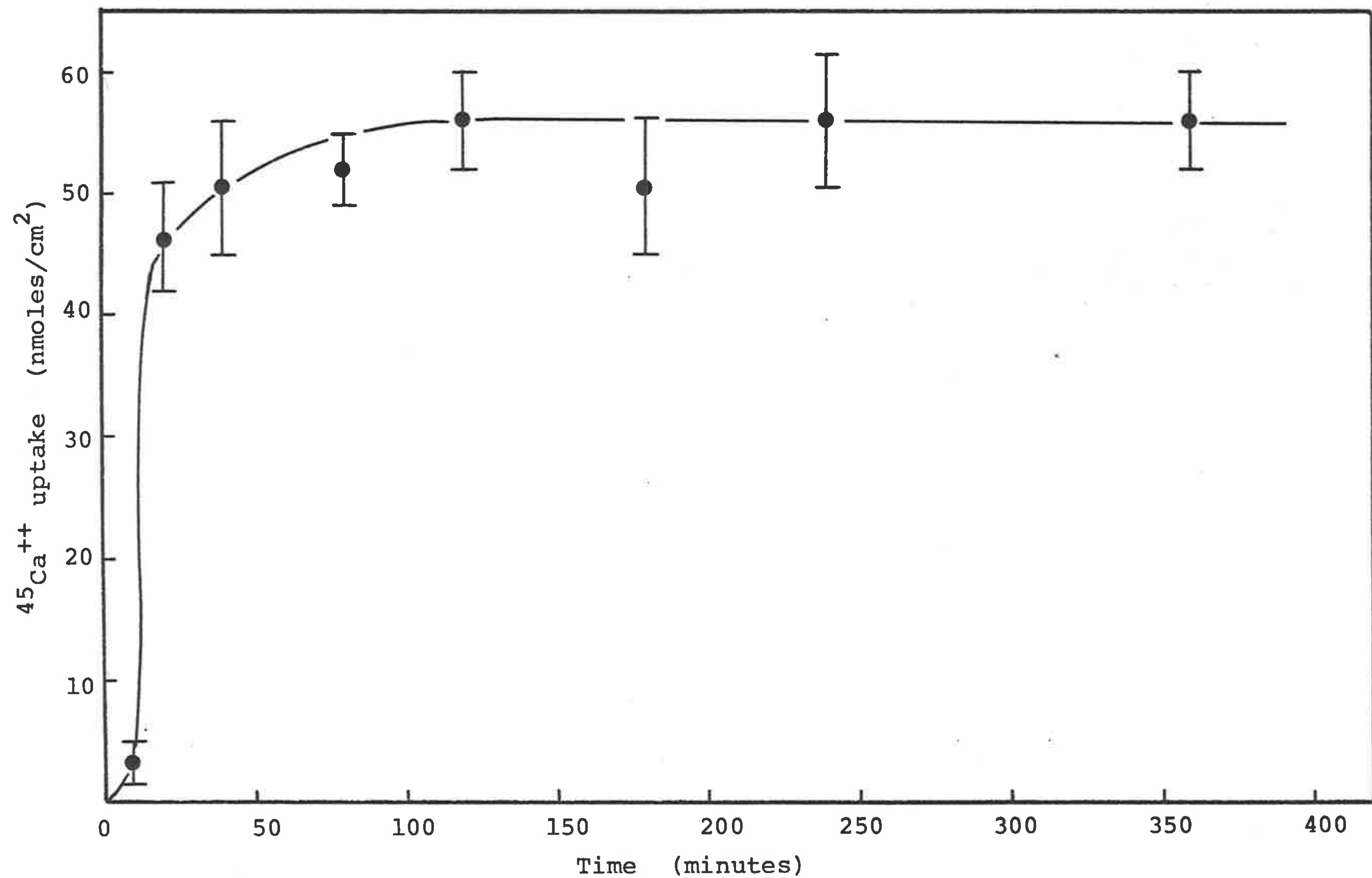



FIGURE 7.3 $^{45}Ca^{++}$ uptake at pH 9.0 followed by 60 minute wash in 1.0 mM NaCl. Solutions as in Figure 7.1.

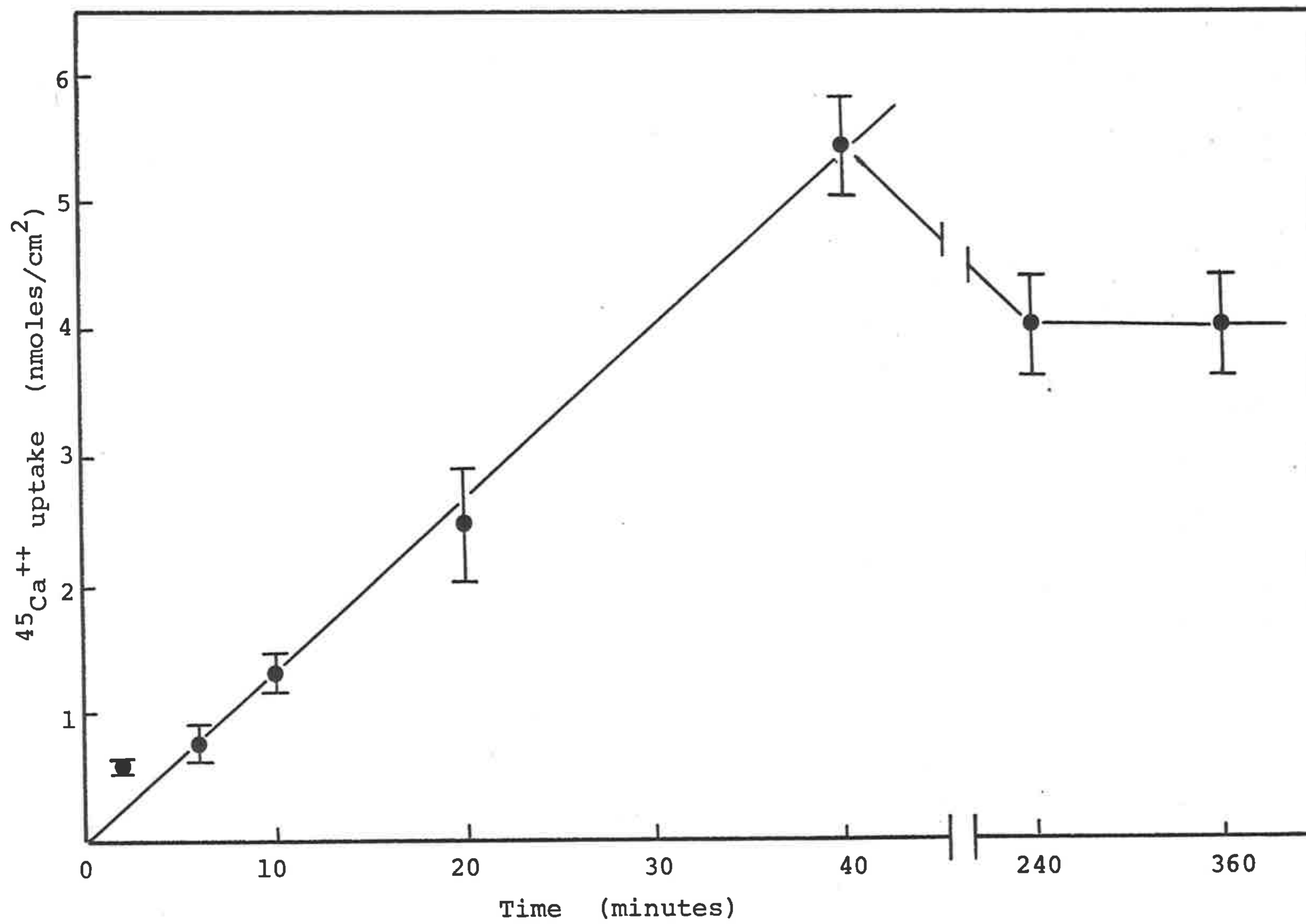



FIGURE 7.4 Cellular $^{45}Ca^{++}$ uptake from 1.0 mM $CaSO_4$ at pH 9.0. Solutions as in 2.3.10.

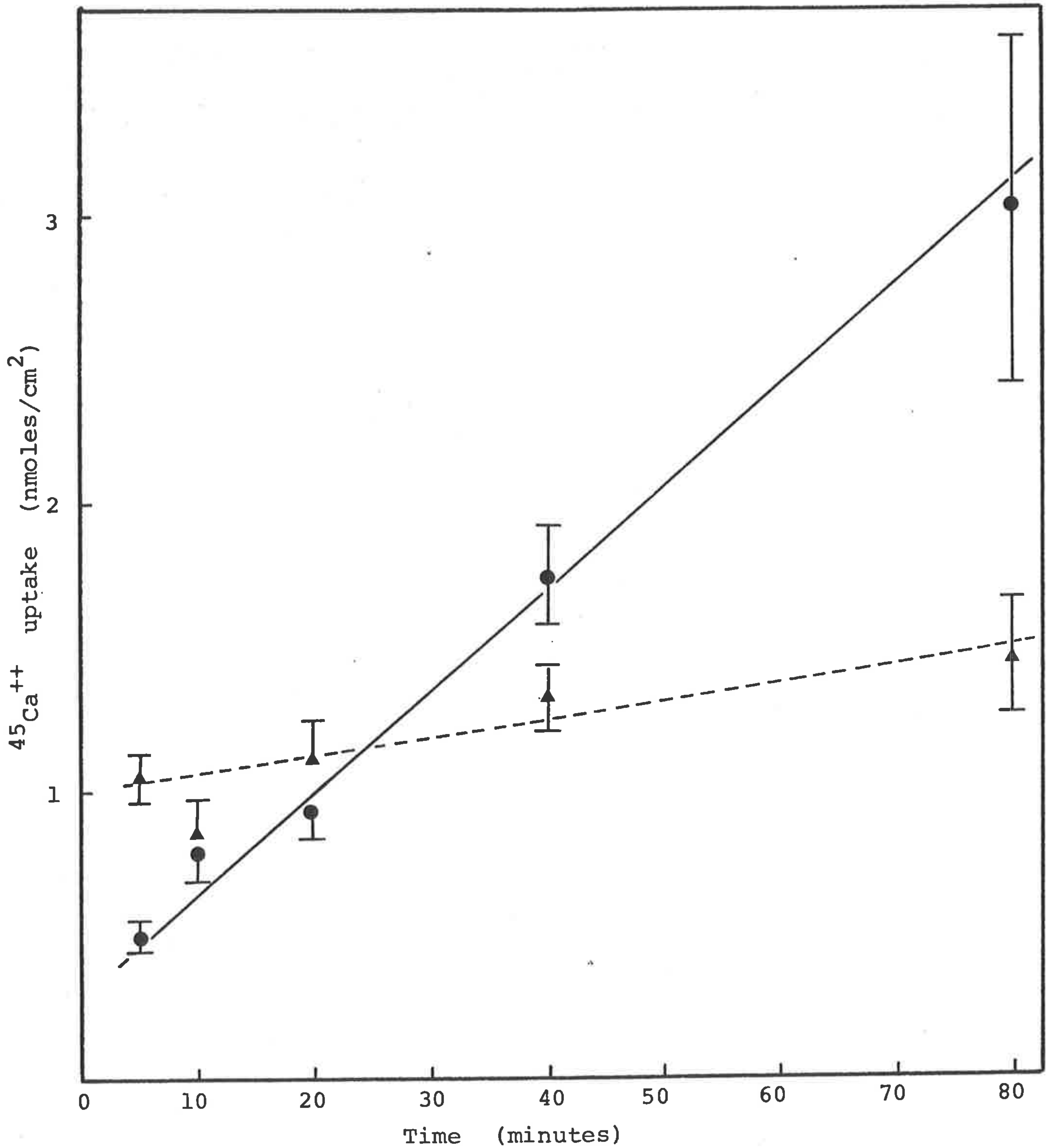



FIGURE 7.5 Effect of 1 μM CCCP (pH 6.0) on cellular $^{45}Ca^{++}$ uptake. Leaves treated with CCCP for 50 minutes then transferred to experimental solutions for 20 hours before uptake measurements. Controls (●), +1 μM CCCP (▲).

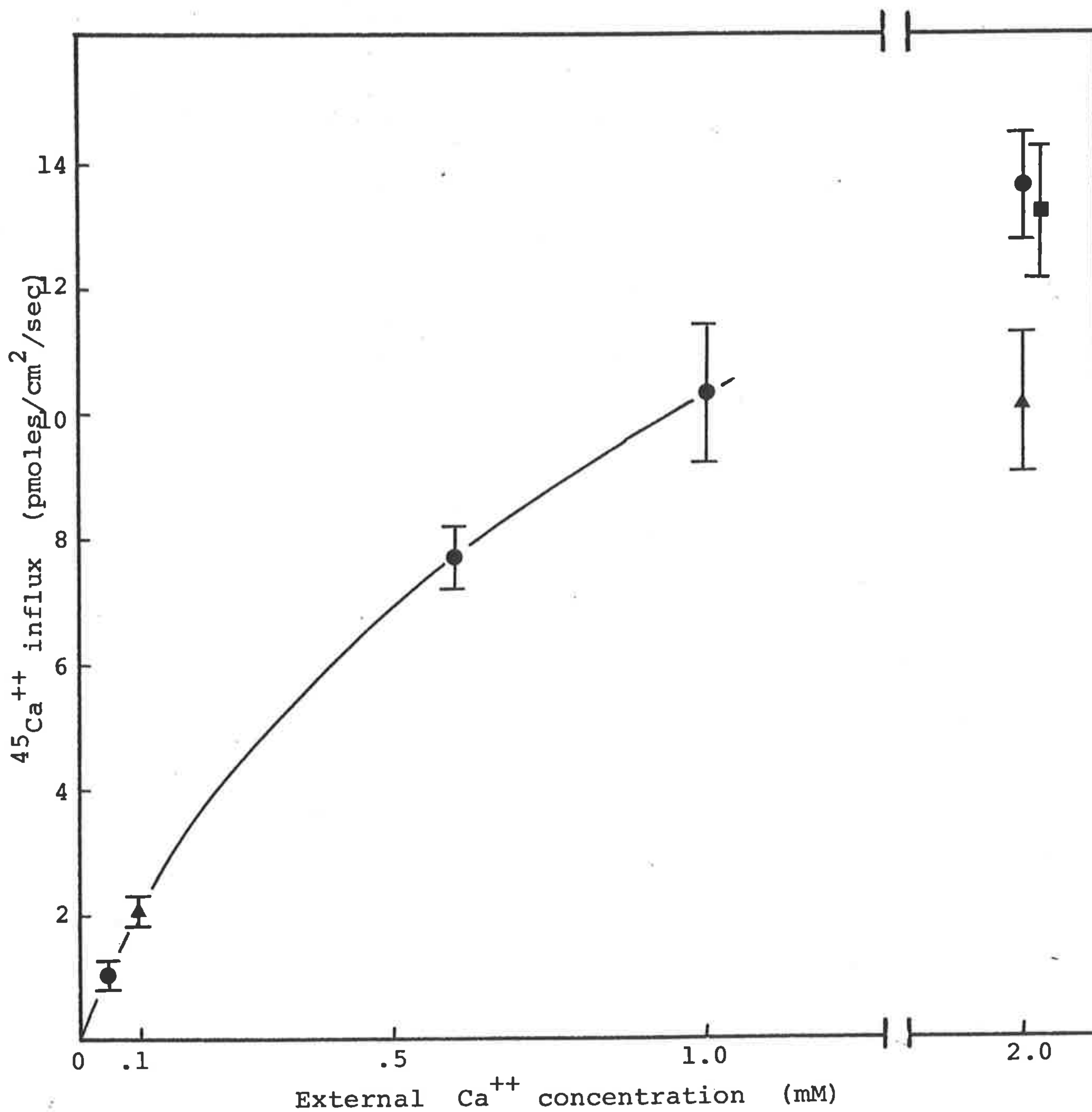



FIGURE 7.6 Effect of pH on cellular $^{45}Ca^{++}$ uptake, pH 9.0 (●); pH 6.0 (■); dark, pH 9.0 (▲). Solutions as described in 2.3.10 with 1.0 mM $CaCl_2$.

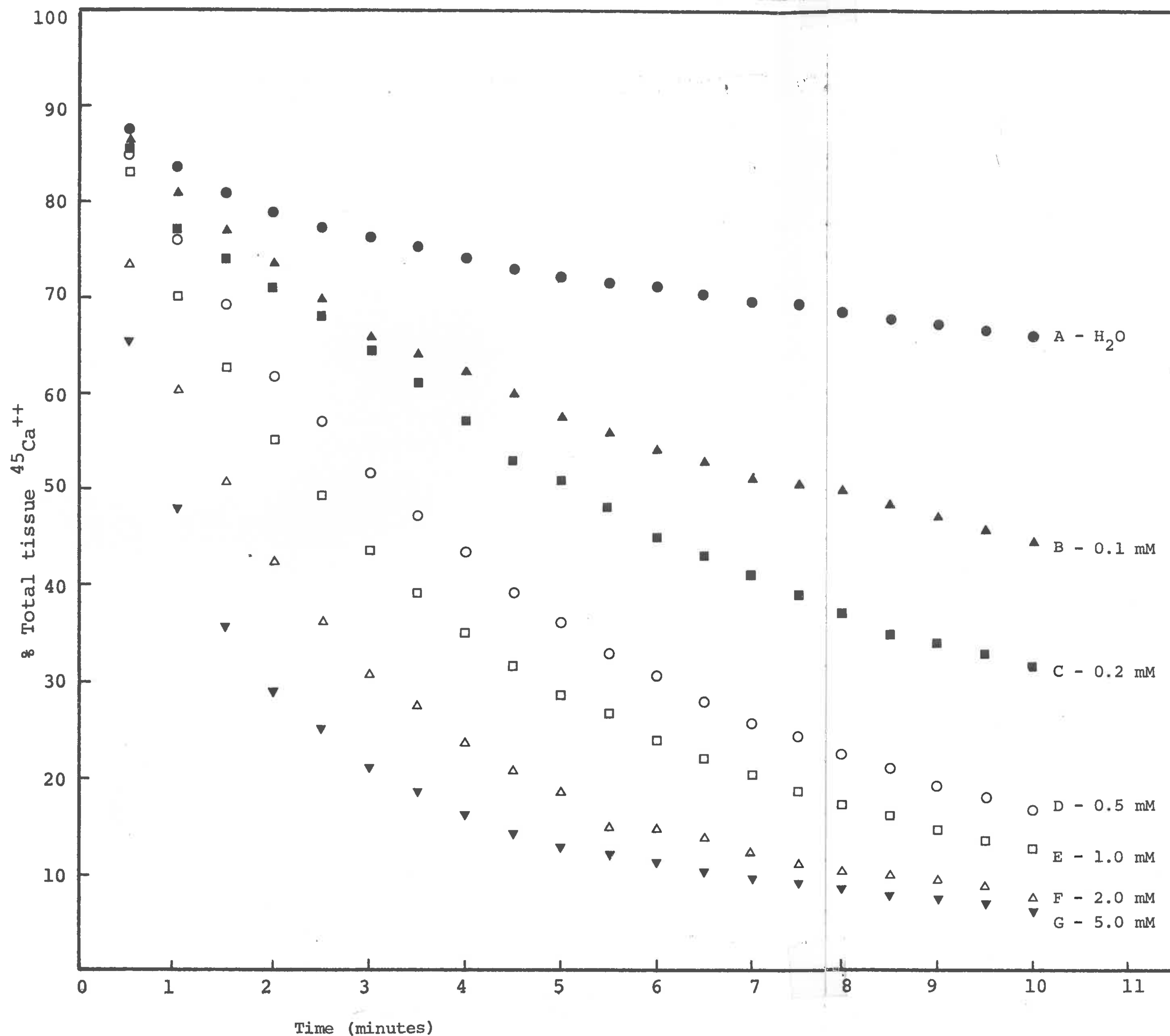



FIGURE 7.7 Efflux of $^{45}Ca^{++}$ into solutions with different $Ca^{++}$ concentrations at pH 9.0 (expressed as a % of the estimated total tissue $Ca^{++}$). Washout solution $Ca^{++}$ concentration as indicated with each curve.

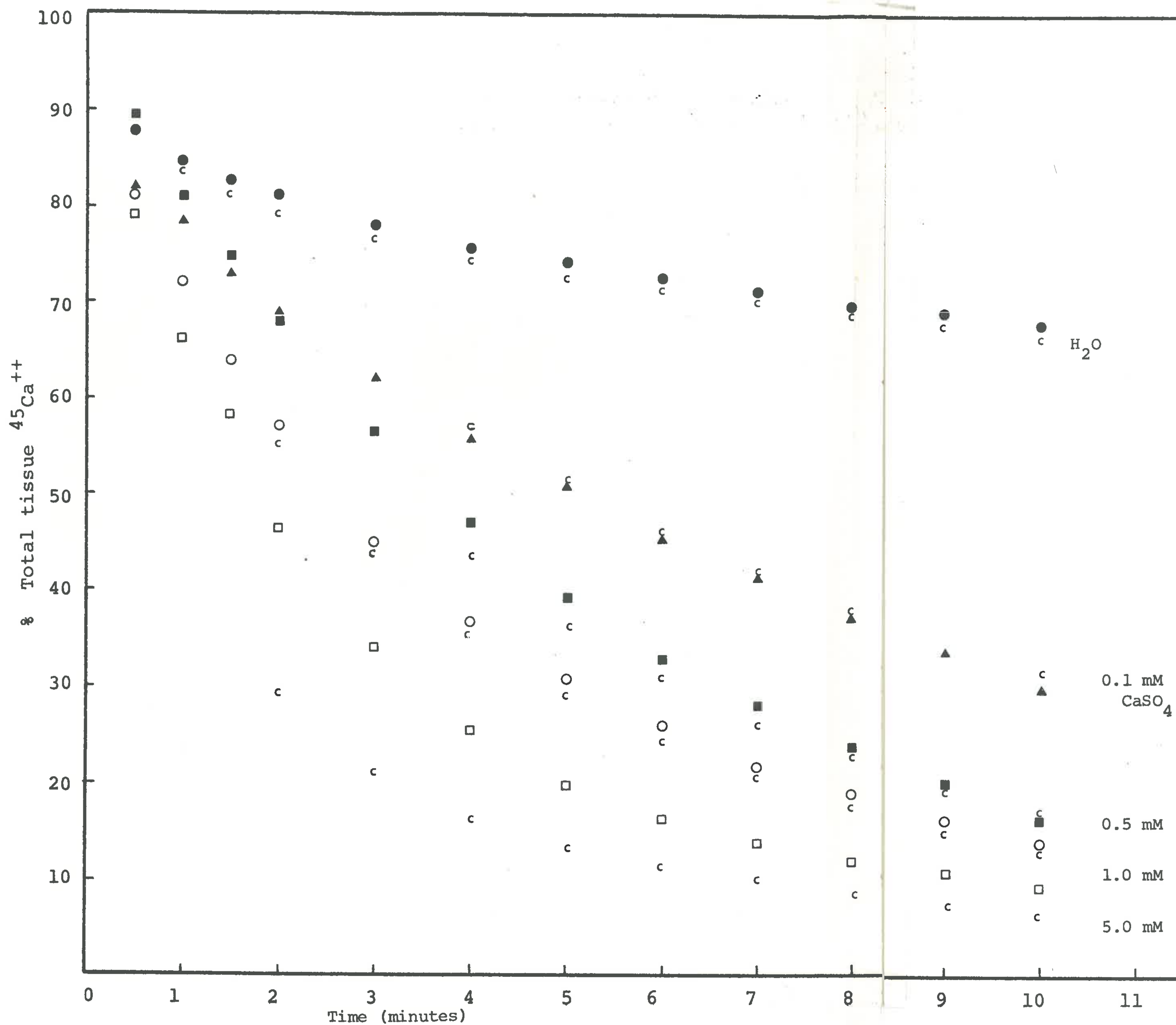



FIGURE 7.8 Effect of 5 μM CCCP on the efflux of $^{45}Ca^{++}$ into various $Ca^{++}$ concentrations at pH 9.0 (expressed as a % of the estimated total tissue $Ca^{++}$). 'c' represent the control value for each curve.

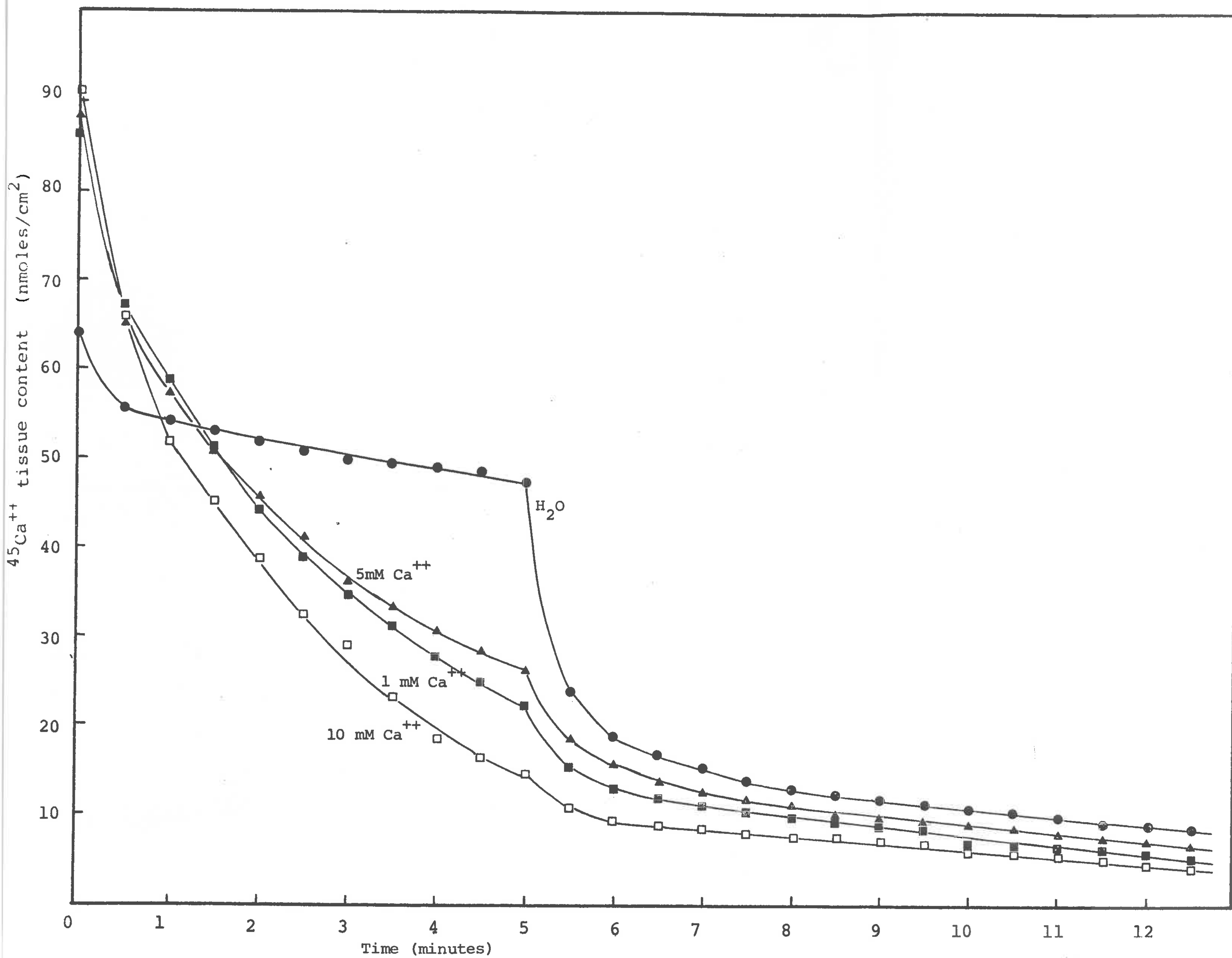



FIGURE 7.9 Effect of adding 50 mM potassium citrate to washout solutions on the $^{45}Ca^{++}$ efflux. Citrate added at the 5 minute mark. Solutions contain $Ca^{++}$ concentrations as indicated.

# CHAPTER EIGHT

## AMMONIUM SULPHATE - ITS EFFECTS ON PHOTOSYNTHESIS AND ION TRANSPORT

### 8.1 Introduction

Raven and Smith (1976) have written a detailed review on nitrogen assimilation and transport in vascular land plants. They point out that nitrogen assimilation involves either $H^+$ production (during $NH_4$ assimilation) or $OH^-$ production (during $NO_3^-$ assimilation). In the aerial parts of land plants, no direct exchange of $H^+$ or $OH^-$ with an external liquid phase is possible, whereas in the roots it is. In aquatic plants, especially algae, $H^+$ may be lost to the bathing solution as may $OH^-$. Some aquatic plants are able to make carboxylates (e.g. *Thalassia testudinum* (Benedict and Scott, 1976) and *Elodea canadensis* (DeGroote and Kennedy, 1977)) as a further alternative to maintaining steady state cytoplasmic pH. Land plants may remove $OH^-$ produced during $NO_3^-$ assimilation in leaves by transport to the roots or by storage in the vacuoles as carboxylates.

As *Elodea* apparently contains large vacuolar concentrations of organic salts such as malate and oxalate - see 1.7 - it seems possible that it (and other similar aquatic angiosperms) may be able to deal with 'pH stat' problems during nitrogen assimilation by both direct exchange with bathing solutions and by storage as carboxylates.

Although the experiments described in this chapter do not deal primarily with nitrogen assimilation but rather with the

direct effects of nitrogenous compounds on photosynthesis and ion transport, the effects described above should be kept in mind.

The effects of $(NH_4)_2SO_4$ on photosynthesis and ion transport in aquatic angiosperms have not been reported in the literature. The hypothesis of MacRobbie (1965, 1966) that photophosphorylation *in vivo* was inhibited by amines (e.g. ammonia and imidazole) was initially challenged by Smith (1967), who showed that $^{14}CO_2$ fixation in *Chara corallina* was insensitive to imidazole and $(NH_4)_2SO_4$ at pH 7.0. Lucas (1977) found that there was a differential effect of $(NH_4)_2SO_4$ on $^{14}C$-fixation with increasing external pH. He found that at high pH (9.0), where $HCO_3^-$ is fixed in *Chara*, 1.25 mM $(NH_4)_2SO_4$ inhibited the $H^{14}CO_3^-$ fixation by 90%; whereas at pH 6.2, there was no inhibition of $^{14}CO_2$ fixation in agreement with Smith (1967). This observation of Lucas may be taken as evidence that $NH_4^+(NH_3)$ does not uncouple photophosphorylation. In *in vitro* experiments with isolated chloroplasts, Hind and Whittingham (1963) had found that such amines uncoupled photophosphorylation. With further experiments using his 'isolation chamber', Lucas (1977) concluded that the $(NH_4)_2SO_4$ exerted its inhibitory effect directly on the $OH^-$ efflux mechanism, thereby exerting an indirect effect on $HCO_3^-$ fixation. Such an indirect effect may be due to alkalinization of the cytoplasm or by charge imbalance. As the inhibition of $HCO_3^-$ fixation was dependent on the external $NH_3$ and not the $NH_4^+$ concentration, Lucas attributed the $OH^-$ efflux inhibition to $NH_3$ not $NH_4^+$, because the inhibition of $HCO_3^-$ fixation was related to external $NH_3$ concentration but not $NH_4^+$. However, it is necessary to postulate that this inhibition

occurred at the plasmalemma, without $NH_3$ necessarily penetrating into the cytoplasm. This is necessary as the $NH_3/NH_4^+$ ratio in the cytoplasm would be different to the external ratio as the cytoplasmic pH is lower than the solution pH (as discussed below).

One point that needs to be considered when discussing the effects of $(NH_4)_2SO_4$, is the fate of $NH_4^+$ or $NH_3$ once it enters the cytoplasm. If, for example $NH_3$ enters the cell (and at high solution pH, $NH_3$ is predominant over the cation $NH_4^+$) then it should be protonated in the cytoplasm, causing cytoplasmic pH to rise. Similarly at lower pH, $NH_4^+$ may predominantly enter and be deprotonated in the cytoplasm, causing the pH to fall. To what extent these processes do occur depends on what other factors are regulating cytoplasmic pH. However, such observations were made by Walker and Smith (1975) with *Chara* at low pH (6.9 to 7.0) in long term (15 hours) experiments in the light. However, in short term experiments, Smith and Walker (1976) found that at an external pH of 6.6, cytoplasmic pH was increased marginally by 0.3 units by 2 mM imidazole. At higher pH (8.0 to 8.1) there was no effect of imidazole. These different effects may also be explained by changes in the permeability of imidazole and other nitrogenous compounds at the different pH values and hence different cytoplasmic concentrations.

Studies of $Cl^-$ transport into cells of *Chara corallina* have shown that amines, $NH_4^+$, imidazole, and methylamine increased the $Cl^-$ influx at low concentrations (Smith and West, 1969; Smith, 1970, 1972; Smith and Walker, 1978, 1979). Recent experiments from Smith (unpublished) have shown that the stimulation of the $Cl^-$ influx is

dependent on the pH of external solution, showing maximal stimulation at 6.5 to 7.5. By measuring the cytoplasmic pH by the distribution of DMO, Smith (unpublished) has also shown that an increase in the $Cl^-$ influx is accompanied by a small increase in cytoplasmic pH (increased by $NH_4^+$ or methylammonium). This supports earlier conclusions that the $Cl^-$ influx in *Chara* involves an $H^+/Cl^-$ symport (as discussed in 1.9).

This chapter reports the effects of $(NH_4)_2SO_4$ on photosynthesis (C-fixation), proton ($OH^-$) transport and the $Cl^-$ influx in *Elodea* and the results are discussed of the available data obtained with the giant-celled algae.

## 8.2 Effects on Photosynthesis

From Tables 8.1 and 8.2, it can be readily seen that at pH 6, concentrations of $(NH_4)_2SO_4$ up to 2.0 mM had a minimal effect on $^{14}CO_2$ fixation. In fact, in the experiment shown in Table 8.2, there was a stimulation of $CO_2$ fixation. In Table 8.1 and 8.2B the pretreatment with $(NH_4)_2SO_4$ was for 2 hours before $^{14}C$-fixation was measured. When $^{14}CO_2$ fixation was measured in the first 20 minutes after the addition of $(NH_4)_2SO_4$, the stimulation of $CO_2$ fixation appeared to be instantaneous (Table 8.2A).

On the other hand, $H^{14}CO_3^-$ fixation at pH 9 and 10 was inhibited by even very low concentrations of $(NH_4)_2SO_4$ (Tables 8.1 and 8.2); and it appeared (Table 8.2A), that the inhibition was a much slower process because in the first 20 minutes after addition, 0.1 mM $(NH_4)_2SO_4$ had inhibited the $H^{14}CO_3^-$ fixation to only 63% of the control value whereas $H^{14}CO_3^-$ fixation had fallen to 4%

after 2 hours. These results are similar to those obtained by Lucas (1977) using *Chara*.

## 8.3 Effects on the External pH Changes

The effects of low concentrations of $(NH_4)_2SO_4$ on the ability of *Elodea* to change the solution pH were qualitatively studied. In Figure 8.1, both pH and $O_2$ changes are shown. Under conditions of low exogenous $CO_2/HCO_3^-$, sufficient to allow $HCO_3^-$ fixation and a measurable $OH^-$ efflux, 0.1 mM $(NH_4)_2SO_4$, added in the dark, caused a gross exaggeration of the inflection during the pH rise (see 5.5). Following this, the rate of pH rise was reduced to approximately 25% of the control value. There was no apparent effect on the $O_2$ evolution rate, even at the higher pH values. However, it should be noted that in these experiments where long exposures to light were used, the solutions were $O_2$ saturated and it was not possible to observe small decreases in $O_2$ evolution.

Figure 8.2 which is taken from the same experiments as Figure 8.1, shows that 0.3 mM $(NH_4)_2SO_4$ caused an even greater inflection in the pH rise. At 1.1 mM $(NH_4)_2SO_4$, the pH rise became a fall and net $O_2$ evolution ceased.

Since it was shown in 8.2 that $NH_4^+/NH_3$ inhibited $HCO_3^-$ fixation, the exaggeration in the inflection of the pH rise with low concentrations of $(NH_4)_2SO_4$ may possibly be due to a delay in the onset of the $HCO_3^-$ fixation mechanism as the pH rises (see 5.5). Because of this delay, the $H^+$ efflux, stimulated by light, causes the pH to fall for a short period. The stimulation of the apparent $H^+$ efflux when the exogenous $CO_2$ was low may be also explained by

an $NH_4^+/H^+$ antiport under these conditions. Such an antiport is only possible if the cells assimilate ammonia or store carboxylates in the vacuoles. Such options will be discussed in more detail in Chapter 9.

## 8.4 The Effect on the $Cl^-$ Influx

As with carbon fixation, $Cl^-$ influx was inhibited at high pH but not at low pH, as shown in two separate experiments (Tables 8.3 and 8.4). In Table 8.3 the $Cl^-$ influx, which was low in this experiment, was measured at pH 6.0 and 9.0, and in Table 8.4 where the $Cl^-$ influx was much higher it was measured at pH 6.0 and 10.0. This inhibition at high pH is consistent with the evidence (Chapter 3) that photosynthesis (both phosphorylation - ATP - and electron transport - NADPH) is the energy source for the $Cl^-$ influx. These results are in contrast to those obtained using *Chara*, where $Cl^-$ influx was maximally stimulated at lower pH and minimally at higher pH values (Smith and Walker, 1978, 1979; Smith, unpublished data).

## 8.5 $(NH_4)_2SO_4$ and the Cellular $Ca^{++}$ Influx

At low and high pH, $(NH_4)_2SO_4$ had little although a variable effect on the cellular uptake of $Ca^{++}$ as shown in Tables 8.5 and 8.6. This was true whether the solutions contained exogenous carbon (Table 8.5) or were bubbled with $N_2$ (Table 8.6). This suggests that $Ca^{++}$ uptake into the leaf cells is not dependent on $CO_2$ or $HCO_3^-$ fixation, even though $HCO_3^-$ fixation does not proceed in leaves with $Ca^{++}$ removed by citrate treatment (Chapter 6).

## 8.6 Summary

1. $(NH_4)_2SO_4$ inhibits $HCO_3^-$ fixation (pH 9.0) but not $CO_2$ fixation (pH 6.0).
2. $(NH_4)_2SO_4$ inhibits the $Cl^-$ influx in a similar manner to C-fixation, e.g. not at low pH but at high pH.. It does not stimulate $Cl^-$ influx as in *Chara*.
3. $(NH_4)_2SO_4$ alters the pH response of leaves possibly by delaying the onset of $HCO_3^-$ fixation.
4. $(NH_4)_2SO_4$ has no significant effect on the cellular $Ca^{++}$ influx at high or low pH.

TABLE 8.1 THE EFFECT OF $(NH_4)_2SO_4$ ON $^{14}C$-FIXATION AT LOW AND HIGH pH

| Experimental conditions | | $^{14}C$-fixation (pmoles/$cm^2$/sec.) | % of controls |
|---|---|---|---|
| pH | $(NH_4)_2SO_4$ | | |
| 6 | 0 | 110 ± 2 | 100 |
| 6 | 0.2 mM | 84 ± 4 | 76 |
| 6 | 1.0 mM | 84 ± 8 | 76 |
| 9 | 0 | 4.6 ± .2 | 100 |
| 9 | 0.2 mM | 0.8 ± .09 | 18 |
| 9 | 1.0 mM | 0.48 ±.04 | 11 |

Leaves pretreated with $(NH_4)_2SO_4$ for 2 hours and $^{14}C$-fixation measured for 10 minutes from 1.0 mM $NaHCO_3$. 5.0 mM MES used at pH 6.0 and 5.0 mM TAPS at pH 9.0.

## TABLE 8.2 THE EFFECT OF $(NH_4)_2SO_4$ ON $^{14}C$-FIXATION AT LOW AND HIGH pH - SHORT TERM AND LONG TERM EFFECTS

| Experimental Conditions | | % Change in $^{14}CO_2$ Fixation | |
|---|---|---|---|
| pH | $[(NH_4)_2SO_4]$ | A | B |
| 6.0 | 0 | 100 | 100 |
| " | 0.1 mM | 127 | 120 |
| " | 0.5 " | 128 | 112 |
| " | 1.0 " | 115 | 114 |
| 6.0 | 2.0 mM | 118 | 109 |
| 10.0 | 0 | 100 | 100 |
| " | 0.1 mM | 63 | 4 |
| " | 0.5 " | 29 | 3 |
| " | 1.0 " | 18 | 2 |
| 10.0 | 2.0 mM | 11 | 1 |

Leaves pretreated for 2 hours in $(NH_4)_2SO_4$ in B and $^{14}C$-fixation measured on the addition of $(NH_4)_2SO_4$ in A. $^{14}C$-fixation was measured for 20 minutes - 0-20 (A), 120-140 (B). 5.0 mM MES buffer used at pH 6.0, and 5.0 mM CAPS at pH 10.0.

TABLE 8.3 THE EFFECT OF $(NH_4)_2SO_4$ ON THE $Cl^-$ INFLUX AT LOW AND HIGH pH

| Experimental Conditions | | $Cl^-$ Influx ($pmoles/cm^2/sec$) | % Of Controls |
|---|---|---|---|
| pH | $[(NH_4)_2SO_4]$ | | |
| 6.0 | 0 | 0.90 ± .06 | 100 |
| 6.0 | 0.1 mM | 0.81 ± .06 | 90 |
| 6.0 | 0.5 " | 0.98 ± .08 | 109 |
| 6.0 | 1.0 " | 1.0 ± .07 | 111 |
| 6.0 | 2.0 mM | 0.90 ± .05 | 100 |
| 6.0 DARK | 0 | 0.37 ± .05 | 41 |
| 9.0 | 0 | 1.03 ± .1 | 100 |
| 9.0 | 0.04 mM | 0.34 ± .02 | 33 |
| 9.0 | 0.1 " | 0.33 ± .02 | 33 |
| 9.0 | 0.5 " | 0.18 ± .01 | 17 |
| 9.0 | 1.0 " | 0.34 ± .01 | 33 |
| 9.0 | 2.0 mM | 0.04 ± .005 | 3.5 |
| 9.0 DARK | 0 | 0.47 ± .04 | 46 |

Leaves given 2 hour pretreatment in the experimental solutions. 50 mM MES was used as buffer at pH 6.0 and 5.0 mM TAPS was used at pH 9.0. Leaves grown in indoor tank No. 2 - low $Cl^-$ influx (see Table 3.1).

**TABLE 8.4** THE EFFECT OF $(NH_4)_2SO_4$ ON THE $Cl^-$ INFLUX AT LOW AND HIGH pH

| Experimental Conditions | | $Cl^-$ Influx (pmoles/cm²/sec) | % Of Controls |
|---|---|---|---|
| pH | $[(NH_4)_2SO_4]$ | | |
| 6.0 | 0 | 11.9 ± 1.0 | 100 |
| " | 0.05 | 12.6 ± 1.2 | 106 |
| " | 0.1 | 11.2 ± .6 | 95 |
| " | 0.5 | 10.2 ± .9 | 85 |
| 6.0 | 1.0 | 10.0 ± .5 | 84 |
| 10.0 | 0 | 7.2 ± .5 | 100 |
| " | 0.05 | 7.6 ± .4 | 106 |
| " | 0.1 | 4.9 ± .7 | 68 |
| " | 0.5 | 0.07 ± .01 | 1 |
| 10.0 | 1.0 | 0.08 ± .01 | 1 |

Leaves given 2 hours pretreatment in the experimental solutions. 5.0 mM MES was used at pH 6.0, and 5.0 mM CAPS at pH 10.0 Leaves grown in indoor tank No. 3 - high $Cl^-$ influx (see Table 3.1).

TABLE 8.5 EFFECT OF $(NH_4)_2SO_4$ ON $Ca^{++}$ UPTAKE IN PRESENCE OF 1.0 mM $NaHCO_3$ AT LOW AND HIGH pH

| Expt. A | Experimental Conditions pH | $[(NH_4)_2SO_4]$ | $Ca^{++}$ Uptake (pmoles/cm$^2$/sec) | % Of Controls |
|---|---|---|---|---|
| | 6.0 | 0 | 2.39 ± .14 | 100 |
| | " | 0.1 mM | 2.28 ± .18 | 95 |
| | " | 0.5 " | 3.04 ± .33 | 127 |
| | " | 1.0 " | 2.96 ± .20 | 124 |
| | " | 2.0 mM | 2.60 ± .23 | 109 |
| | 9.0 | 0 | 2.43 ± .16 | 100 |
| | " | 0.1 mM | 2.30 ± .26 | 95 |
| | " | 0.5 " | 2.76 ± .28 | 114 |
| | " | 1.0 " | 2.82 ± .36 | 116 |
| | " | 2.0 mM | 3.09 ± .24 | 127 |
| Expt. B | 9.0 | 0 | 2.43 ± .26 | 100 |
| | " | 0.02 mM | 2.44 ± .30 | 100 |
| | " | 0.05 " | 2.25 ± .25 | 93 |
| | " | 0.1 " | 2.13 ± .21 | 88 |
| | " | 0.2 " | 2.82 ± .21 | 116 |
| | " | 0.5 " | 2.71 ± .30 | 112 |
| | " | 1.0 " | 2.68 ± .24 | 110 |

Leaves pretreated for 2 hours, as described in Tables 8.1 to 8.4.

TABLE 8.6 EFFECT OF $(NH_4)_2SO_4$ ON $Ca^{++}$ UPTAKE IN $N_2$ BUBBLED SOLUTIONS AT LOW AND HIGH pH

| Conditions | $[(NH_4)_2SO_4]$ | $Ca^{++}$ Uptake | % |
|---|---|---|---|
| pH 6.0 | 0 | 5.31 ± .5 | 100 |
| " | 0.1 mM | 4.49 ± .5 | 85 |
| " | 0.5 " | 3.46 ± .4 | 65 |
| " | 1.0 " | 4.41 ± .6 | 83 |
| " | 2.0 mM | 3.99 ± .4 | 75 |
| pH 9.0 | 0 | 4.07 ± .4 | 100 |
| " | 0.1 mM | 4.45 ± .3 | 109 |
| " | 0.5 " | 4.34 ± .4 | 107 |
| " | 1.0 " | 3.23 ± .2 | 79 |
| " | 2.0 mM | 4.77 ± .4 | 117 |

Experimental conditions are the same as in Table 8.5 except that no $NaHCO_3$ was present in the solutions, which were continuously bubbled with $N_2$.

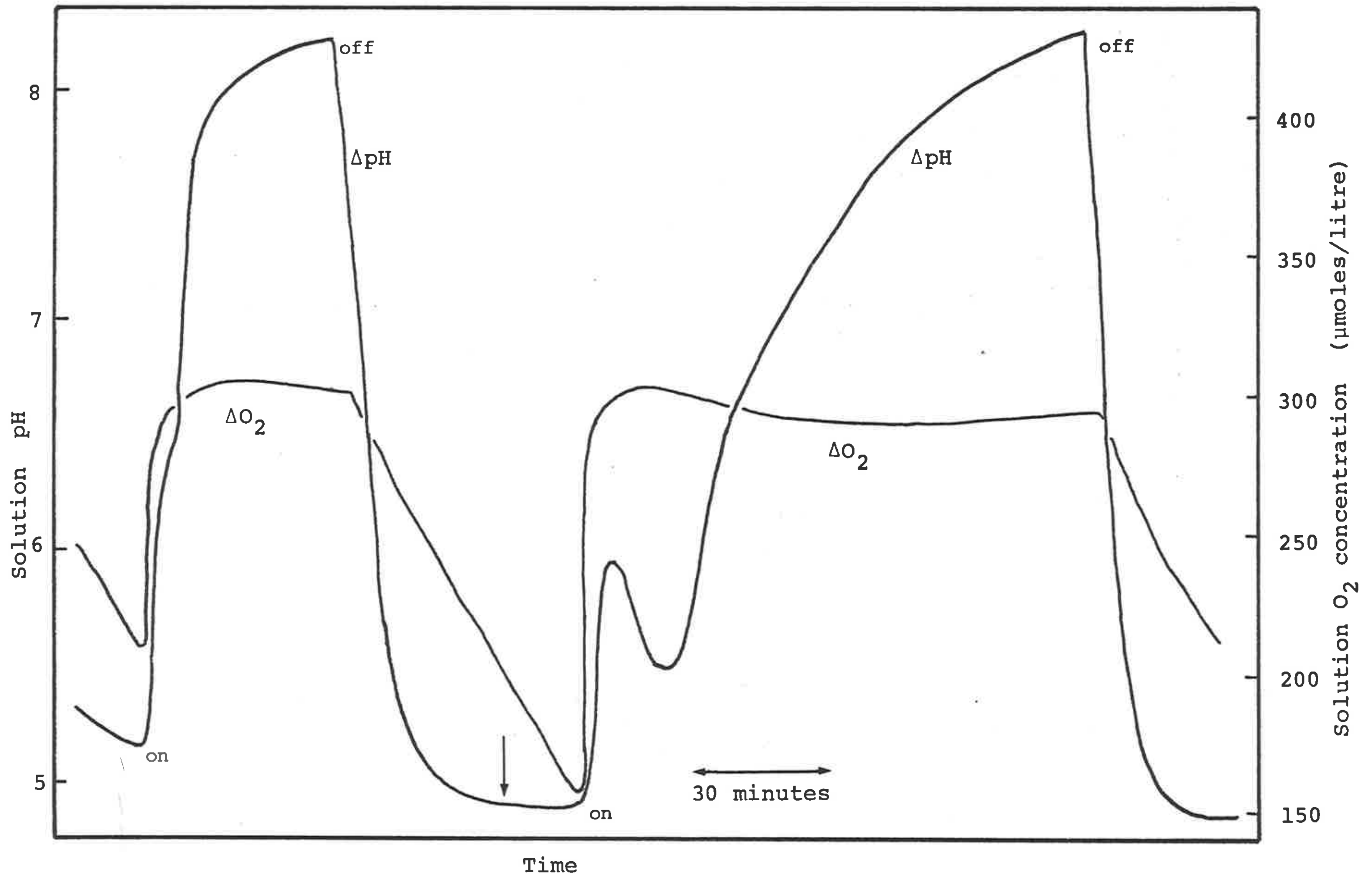



FIGURE 8.1 Solution pH and $O_2$ concentration changes under the effect of 0.1 mM $(NH_4)_2SO_4$. Arrow indicates the addition of the $(NH_4)_2SO_4$. For full explanation see text.

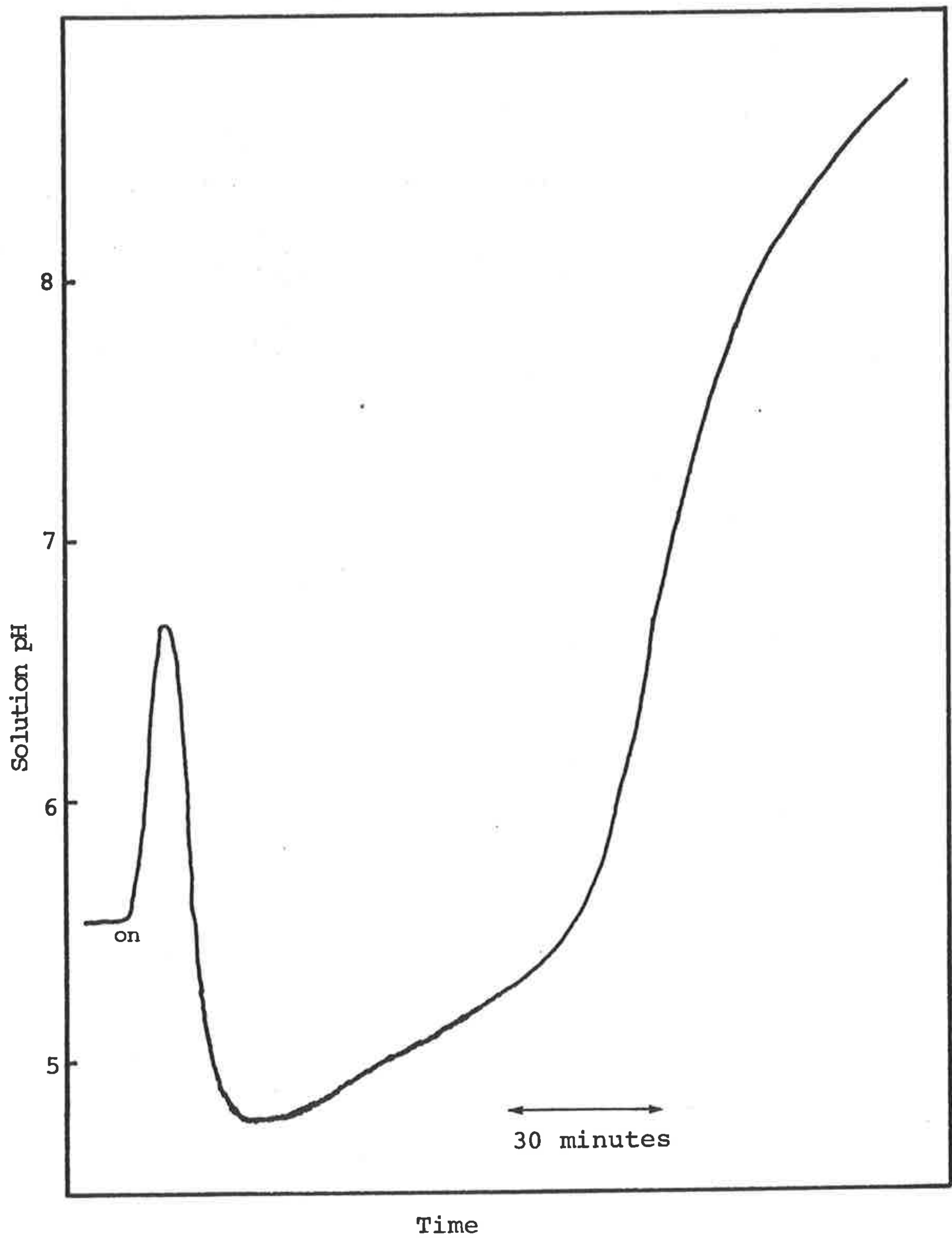



FIGURE 8.2 Effect of 0.3 mM $(NH_4)_2SO_4$ on solution pH changes in the light. For full explanation see text.

# CHAPTER NINE

## GENERAL DISCUSSION

### 9.1 The Calcium Ion

One of the important findings of this Thesis is the role played by $Ca^{++}$. From the experiments, particularly those in Chapter 6, it becomes apparent that $Ca^{++}$ is not actively transported through the cells during movement from the lower surfaces to the upper surfaces of the leaves, as suggested by Lowenhaupt (1956). Rather $Ca^{++}$ moves through the intercellular spaces. $Ca^{++}$ is present in large amounts in these spaces, weakly bound and readily exchangeable. Small amounts of $Ca^{++}$ do enter the cytoplasm but do not account for the active role of $Ca^{++}$ during $HCO_3^-$ fixation.

Since this intercellular $Ca^{++}$ is weakly bound it is possible that during $HCO_3^-$ fixation when there is a net transfer of charge across the leaf with $OH^-$ being released at the upper surface, $Ca^{++}$ diffuses passively through the tissue and maintains charge balance (see Figure 9.1). A similar role could be played by $K^+$ or other cations in *Potamogeton* (Arens, 1933; Helder, 1975), although in *Elodea* it appears that $Ca^{++}$ cannot be replaced by other cations. This model does not contradict the findings of Arens (1933) using *Elodea* because his experiments were only quantitatively able to detect $OH^-$ efflux, using indicator dye. In the experiments described in Chapter 6, although photosynthesis and $OH^-$ efflux were occurring at very low rates with citrate treatment, such rates would have been sufficient to have been observed - see Tables 1.3

and 1.6. Furthermore Arens (1933) may still have had $Ca^{++}$ reserves in the leaves even though he made attempts to remove them.

The active $Ca^{++}$ pump at the lower surface and upper surface proposed by Lowenhaupt (1956) was only deduced from experiments where $Ca^{++}$ exchanges between tissue and solution were estimated by changes in solution $^{45}Ca^{++}$ levels, not cellular levels. His results could thus also be explained by the new model proposed in Figure 9.1.

The existence of small potential differences of 25 mV across *Potamogeton* leaves (Helder, 1975a) is further evidence for an electromotive force which could move cations across the leaf. This is different to the situation in *Chara* where there are localised currents between the acid and alkaline bands and cation involvement is not required.

In *Potamogeton*, Steemann Nielsen (1947) was able to show that photosynthesis ($O_2$ evolution) proceeded at 58% of the control values, when $HCO_3^-$ was supplied to the upper surfaces only. A similar observation was made in preliminary experiments with *Elodea* (not shown). In both these cases it is suggested that $HCO_3^-$ can enter the intercellular spaces from the upper surfaces and migrate to the cellular uptake sites. Under these conditions, there would be no net movement of $Ca^{++}$.

Such a model also explains why citrate treatment which removes $Ca^{++}$ from the intercellular spaces has no effect on $CO_2$ fixation, during which there is no charge redistribution across the leaf. The effect of citrate treatment on $HCO_3^-$ fixation may also be accounted for to some extent by the decreased diffusion of $HCO_3^-$

through the negatively charged intercellular spaces. A similar explanation could be used to account for the inhibition of the $Cl^-$ influx by citrate treatment, and its subsequent restimulation by 0.1 mM $Ca^{++}$ (see 3.5).

The fact that CCCP and CMU have no effect on cellular $Ca^{++}$ influx is taken as further evidence that intracellular $Ca^{++}$ plays no part in the control of photosynthesis or other ion transport processes.

## 9.2 The $Cl^-$ Influx of *Elodea*

The $Cl^-$ influx and $O_2$ evolution showed remarkably similar responses to CCCP treatment in that both were stimulated by low concentrations of CCCP and maximally inhibited at 5 μM CCCP. These were in solutions at pH 6.5. These observations suggest that the source of energy could be NADPH which would be produced in greater quantities when electron transport is uncoupled by CCCP. However the differential effect of CMU on the $Cl^-$ influx in $N_2$ and when carbon fixation is occurring suggest that when NADPH is not available, the $Cl^-$ influx derives its energy from ATP produced, say, in cyclic photophosphorylation (Jeschke 1967). However these interpretations do not consider what effects these treatments have on other ion transport processes, control of cytoplasmic pH, nitrogen metabolism, and carbon assimilation itself, as discussed below.

## 9.3 Ion Transport and Carbon Assimilation

As already mentioned, studies of ion transport - not only active ion transport but also passive transport - cannot really be

successful without extensive knowledge of photosynthesis - both reactants and products. An obvious example of this is the transport of $HCO_3^-$ during photosynthesis. As discussed before, photosynthesis may be controlled by the presence or absence of cations ($Ca^{++}$) which may not even enter the cells. This is due to charge distribution at the tissue level. However, when charged molecules enter the cells or move between cells as a result of photosynthesis, which involves hydroxylation, oxidation and reduction reactions, the problem becomes a dynamic one. Smith and Raven (1976, 1979) have looked at these questions in relation to $H^+$ and the control of intracellular pH.

If $HCO_3^-$ is being assimilated then an uncoupler of photosynthesis may exert its effects on a cation/$HCO_3^-$ symport rather than a supposed energy source. This may also happen if the $Cl^-$ influx is coupled to the $OH^-$ efflux (antiport) - see Smith, 1970 - which is itself dependent on assimilation of $HCO_3^-$. Such an antiport would be light stimulated but the $Cl^-$ influx would not derive its energy specifically from any one of the light reaction products - ATP or NADPH. In this case it would not then be necessary to propose a shuttle mechanism for the transport of energy to the ion transport site at the plasma membrane (see 1.9). Similarly, if any other ion transport systems were coupled to $HCO_3^-$ transport, then any inhibition of photosynthesis reactions would also affect this transport. Furthermore $HCO_3^-$ uptake should have a noticeable effect on the membrane potential and this again would influence other ion exchanges.

However, a different interpretation is possible when $CO_2$ is assimilated. In such a case, diffusion of $CO_2$ into the leaf cells is not coupled to any symports (or antiports) and should have no influence on the membrane potential. However, if the products of fixation are organic acids or salts, then fixation may be accompanied by the transport of $H^+$ or other cations (e.g., $K^+$) respectively. Hence, for example, inhibition of the $Cl^-$ influx at low pH by CCCP may or may not indicate a direct influence on the source of energy for the $Cl^-$ pump.

In barley leaf tissue, it has been shown that light-stimulated salt uptake can alter organic acid synthesis (Kholdebarin and Oertli, 1970). In contrast to the situation in roots (Hiatt and Hendricks, 1967), salt uptake was generally followed by an increase in organic acids, with larger increases when cation uptake was in excess of anion uptake. However, when there was an excess anion uptake, organic acid synthesis was reduced. Hence it should be borne in mind that ion accumulation can also affect the fixation of photosynthetic products.

Experiments have suggested that in *Elodea*, $C_4$, $C_3$ and CAM types of carbon fixation are operating (DeGroote and Kennedy, 1977; Brown et al, 1974; Browse et al, 1977; Hocking et al 1975); that at low external pH, $C_4$ acids are incorporated first and at higher pH, PGA is incorporated. Browse et al (1977) explained these differences as being due to the higher levels of exogenous $CO_2$ at the higher pH (see 1.4.3).

Similarly Raghavendra and Das (1977) found that in $C_4$ plants, incorporation into $C_4$ acids, sugar phosphates sugars and starch

showed considerable variation depending on such parameters as light quality and intensity. Blue light favoured the retention of carbon as malate and aspartate and less carbohydrates were formed. Hence, with *Elodea*, the various differences found in different laboratories may be explained by the differences in the experimental conditions. In addition, evidence has accumulated in the course of this Thesis, that differences in ion transport and photosynthesis do exist under different experimental conditions.

Similar differences in carbon incoporation have been demonstrated in other aquatic angiosperms *Thalassia testudinum* (Benedict and Scott, 1976) incoporates $C_4$ acids, whereas *Halophila spinulosa* and *Thalassia lemprichii* (Andrews and Abel, 1977) and *Potamogeton pectinatus* (Winter, 1978) incorporation is into $C_3$ compounds. Winter showed that $C_3$ compounds were formed at low and high pH, even when levels of free $CO_2$ were very low.

Davies (1973a, 1973b) proposed a scheme whereby there was an interaction between cytoplasmic pH and the formation of carbon assimilates. He proposed that at high pH, PEP carboxylase activity is increased, causing the formation of malic acid; and that at low pH, the activity is decreased and malate is converted to pyruvate with release of $OH^-$. Such a model does explain how under different conditions the patterns of ion transport and photosynthesis may alter.

Before any models for photosynthesis and ion transport can be proposed, consideration should be made of the anatomy of the *Elodea* leaf. The leaf has two layers of cells with quite different anatomy. For example, the lower layer contains small cells with large numbers of small chloroplasts, whereas the cells in the upper layer

are much larger and contain large chloroplasts with extensive reserves of starch, as do the mid-vein cells (Hocking et al, 1975). Hence, it is quite possible that different types of carbon fixation may occur in each layer. A simple experiment (not described elsewhere in the Thesis) using phenol red also indicated that the lower cell layer has a more alkaline cytoplasm than the upper layer. (The experiment involved placing a leaf in a solution at pH 6.5 containing phenol red and observing the colour change within the cells under the microscope). This again suggests that PEP carboxylase would be the more active enzyme in this layer of cells (Hiatt, 1967a, 1967b).

It is therefore proposed that under different conditions, different types of carbon assimilation occur. At low exogenous $CO_2$ concentrations where $CO_2$ is primarily fixed, it is proposed that carboxylates are fixed and stored in the vacuoles, as shown in Figure 9.2. This varies from CAM-type incorporation in that light is required for it to proceed. Incorporation proceeds mainly in the lower layer of cells where PEP-carboxylase is the active enzyme. No fixation (or very little) proceeds in the upper layer of cells. This is supported by the evidence that PEP-carboxylase has a much lower Km than RuBP-carboxylase (Van, Heller and Bowes, 1976). A primary reaction may be an $H^+$ efflux resulting in the alkalinization of the cytoplasm which activates PEP-carboxylase. To maintain charge balance $K^+$ (or another cation) exchanges with the $H^+$ and is transported to the vacuoles where carboxylate salts are stored. Such a system would account for the observed external pH fall under these conditions.

At higher exogenous $CO_2$ concentrations malate (malic acid) formed by the lower cell layer is now transported to the upper layer and $C_4$ type incorporation occurs (e.g. $C_4$ in the lower layer and $C_3$ in the upper). This results in the formation of sugars and starches in the chloroplasts in the upper layer of cells. Again a primary reaction is the $H^+$ efflux from the lower layer that provides the alkaline medium of PEP-carboxylase. $H^+$ may then re-enter through the lower layer and is transported to the upper layer to ensure maintenance of steady pH levels (Smith and Raven, 1979). Such a system results in only a small increase in pH as $CO_2$ is removed from the solution.

When $HCO_3^-$ is being assimilated, modification of this scheme is proposed - see Figure 9.4. Again there is a primary $H^+$ efflux that re-enters the lower cell layer with $HCO_3^-$. Decarboxylation of the $C_4$ acids in the upper cell layer results in $OH^-$ production and the observed pH increase at the upper leaf surface. Charge and pH balance are maintained between the upper and lower layers of cells.

These models are consistent with the following data from other studies:

(1) that starch was found in greater quantities in the chloroplasts in the upper cell layer (Arens, 1933 - see Table 1.7)

(2) that there are approximately equal activities of PEP-carboxylase and RuBP-carboxylase in *Elodea* at pH 6.5 (Degroote and Kennedy, 1977).

Various other observations made during the course of this Thesis can be explained in terms of these models. These are discussed more fully below.

As shown in Chapter 5, under conditions of low $CO_2$, the pH of the external solution falls. This was taken as evidence of an $H^+$ efflux pump and furthermore the data suggested that this $H^+$ efflux persisted during $HCO_3^-$ fixation although masked by the large $OH^-$ efflux from the upper leaf surfaces. Although the upper and lower leaf surfaces of *Elodea* were not isolated in these experiments, pH changes at the upper and lower leaf surfaces of other aquatic angiosperms have been studied. Steemann Nielsen (1947) and Helder and Zanstra (1977) observed a pH decrease at the lower surface of *Potamogeton* during $HCO_3^-$ fixation (and subsequent $OH^-$ efflux at the upper surface). Similarly in *Chara* there are large areas where there is light promoted $H^+$ extrusion during $HCO_3^-$ assimilation (Lucas and Smith, 1973).

Membrane hyperpolarization in the light during $HCO_3^-$ fixation has also been taken as evidence for an electrogenic $H^+$ efflux (Jeschke, 1970; Spanswick, 1973). This $H^+$ electrogenic pump is favoured as a major pump in plant cells and its re-entry into the cell with associated ion antiports and symports has been well discussed by Smith and Raven (1979) and Poole (1978).

The stimulation of this $H^+$ efflux by $(NH_4)_2SO_4$ - see 8.3 and Figure 8.2 - may be explained by a more rapid $NH_4^+/H^+$ exchange than $K^+/H^+$ exchange, with accumulation of ammonium carboxylates in the vacuoles. In addition, increased fixation of $NH_4^+$ may account for some of the increase in $H^+$ efflux.

One of the most puzzling observations of this work, was the lack of inhibition of $O_2$ evolution by potassium citrate treatment (see 6.7 and Table 6.8). Citrate treatment inhibited $H^{14}CO_3^-$ fixation and $OH^-$ efflux at concentrations that had no effect on $O_2$ evolution. Similar imbalances between $O_2/H^+/CO_2$ exchanges have been observed by Hope et al (1972) when CCCP was added to the experimental solutions. Lüttge, Kramer and Ball (1974) also found that in greening barley and maize leaves $O_2$ evolution proceeded at higher rates than $CO_2$ fixation and attributed this to a faster development of the photosystem. However such imbalances are hard to explain. If $CO_2/HCO_3^-$ fixation stops, then there would be a build up of NADPH and ATP in the chloroplasts and presumably in the cytoplasm (NADH) and once the NADP (NAD) pool was completely reduced, then $O_2$ evolution should be inhibited.

However it is possible that in the experiments of Chapter Six, citrate was exerting an indirect effect on photosynthesis or subsequent carboxylation. As shown in 6.5, Table 6.5, citrate treatment has no effect on the rate of $^{14}CO_2$ fixation at low pH, indicating that it has no effect on primary carbon incorporation. Citrate itself is a carboxylate salt and as such may inhibit conversions between carboxylate salts in the cytoplasm as shown with oxalate in *Digitaria sanguinalis* (Rathnam and Edwards, 1977). Such a reaction could well be the malate to oxaloacetate interconversion which involves oxidation/reduction steps. Such a suggestion could only be tested once the pathways of carbon metabolism for *Elodea* have been clearly worked out.

## 9.4 Future Work

From this general discussion it becomes quite obvious that the patterns of carbon fixation under different experimental conditions need to be established, and correlated with the models proposed (Figures 9.2 and 9.3). Such experiments include conditions of citrate treatment when there is an $O_2/CO_2^-$ fixation imbalance, conditions when $CO_2$ or $HCO_3^-$ are the major sources of exogenous carbon, conditions when exogenous carbon is low, and where there is an observed $H^+$ efflux. The use of inhibitors such as oxalate (NADP-malic enzyme inhibitor), 3-MPA-3-mercaptopicolinic acid (PEP-carboxylase inhibitor) and the phytotoxin fusicoccin which has been shown to increase the $H^+/K^+$ antiport (Pitman, Schaeffer and Wildes, 1975a, 1975b) may also provide useful information.

Isolation chambers that separate the upper and lower surfaces of the leaves need to be developed. Such chambers would be difficult to build for *Elodea* as the leaves are small and fragile. Such work may have to be done on larger and less fragile tissue, such as *Potamogeton lucens*.

Autoradiography may be useful in confirming the model shown in Figure 9.1 where it is proposed that $Ca^{++}$ entering the cytoplasm plays only a minor role during high rates of $HCO_3^-$ fixation compared to $Ca^{++}$ in the intercellular spaces.

## 9.5 Conclusions

The initial aims of this Thesis were essentially achieved, although in some aspects the experiments proposed more questions than answers, but such is the case with a lot of experimental work. The experiments confirmed the crude observations from the earlier part of this century and put their interpretation into line with current ideas on photosynthesis and ion transport. The models proposed explain the important role of calcium during $HCO_3^-$ fixation as opposed to $CO_2$ fixation. *Elodea*, and possibly other aquatic angiosperms, unlike *Chara*, is able to assimilate carboxylates under certain conditions. Otherwise the experiments show that there is a remarkable similarity between the single celled giant algae and the multicelled leaf tissue of aquatic angiosperms - particularly in that they both have acid and alkaline areas where similar ionic processes are occurring and that they both are capable of fixing $HCO_3^-$.

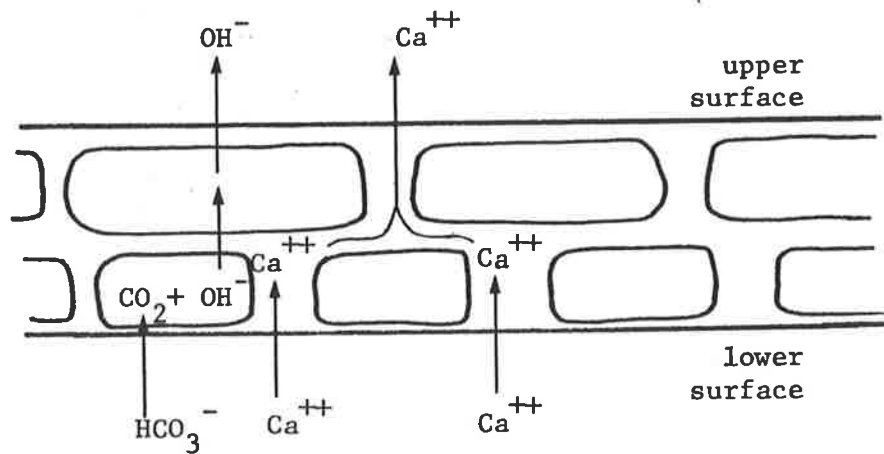



FIGURE 9.1 The role of $Ca^{++}$ during $HCO_3^-$ fixation in leaves of *Elodea densa*. $Ca^{++}$ is weakly bound in the intercellular spaces and can easily move from the lower surface to the upper surface to maintain charge balance during $HCO_3^-$ fixation. Details of carbon fixation are shown in Figures 9.2 to 9.4. Intercellular spaces are grossly exaggerated for clarity.

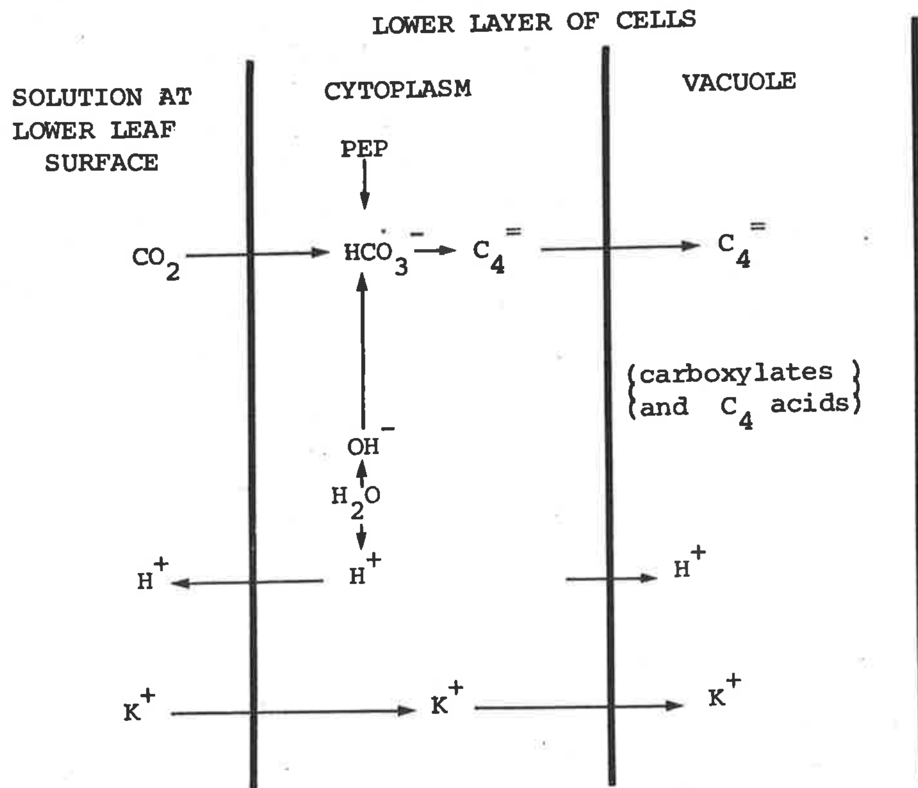



FIGURE 9.2 Carbon metabolism under conditions of low exogenous $CO_2$. PEP-carboxylase in the lower layer of cells incorporates $HCO_3^-$ in the cytoplasm into carboxylates such as malate which move into the vacuoles. From the dissociation of $H_2O$, $H^+$ leaves the cytoplasm in exchange for $K^+$ which moves into the vacuoles causing an external pH fall, or $H^+$ moves into the vacuoles to form carboxylic acid. These latter alternatives are controlled by the cell 'pH stat'.

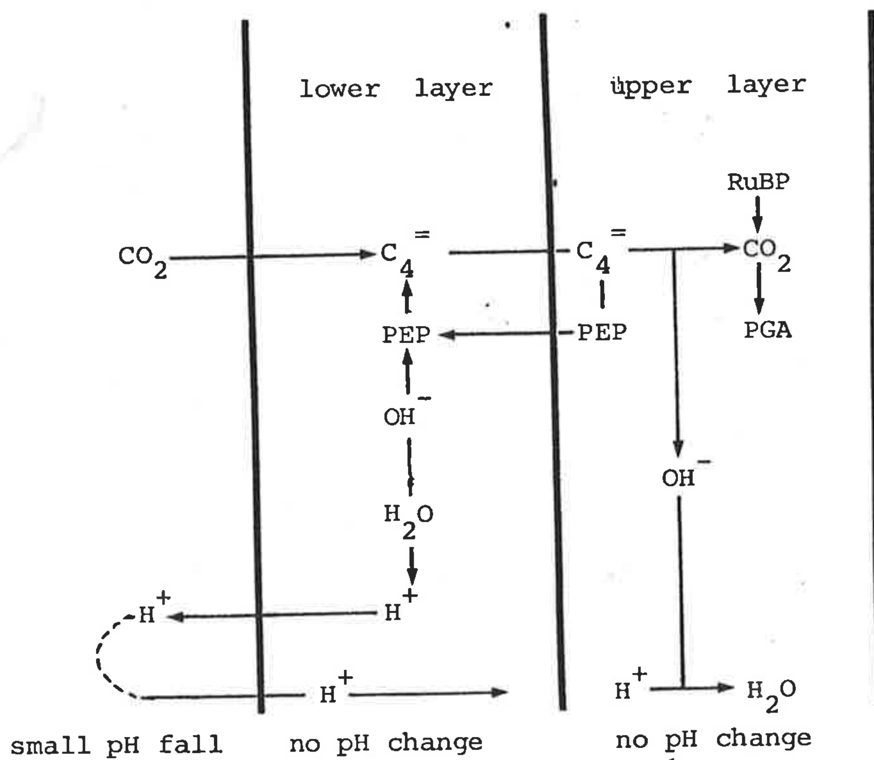



FIGURE 9.3 Carbon metabolism under conditions of high exogenous $CO_2$. $C_4$ acids, incorporated in the lower layer of cells, are transported to the upper layer where it is decarboxylated (PEP returning to the lower layer). PGA is formed from the $CO_2$ released and RuBP in the presence of active RuBP-carboxylase. pH is maintained by the movement of $H^+$ from the lower cell layer to the upper layer. There is no $OH^-$ efflux from the upper surface.

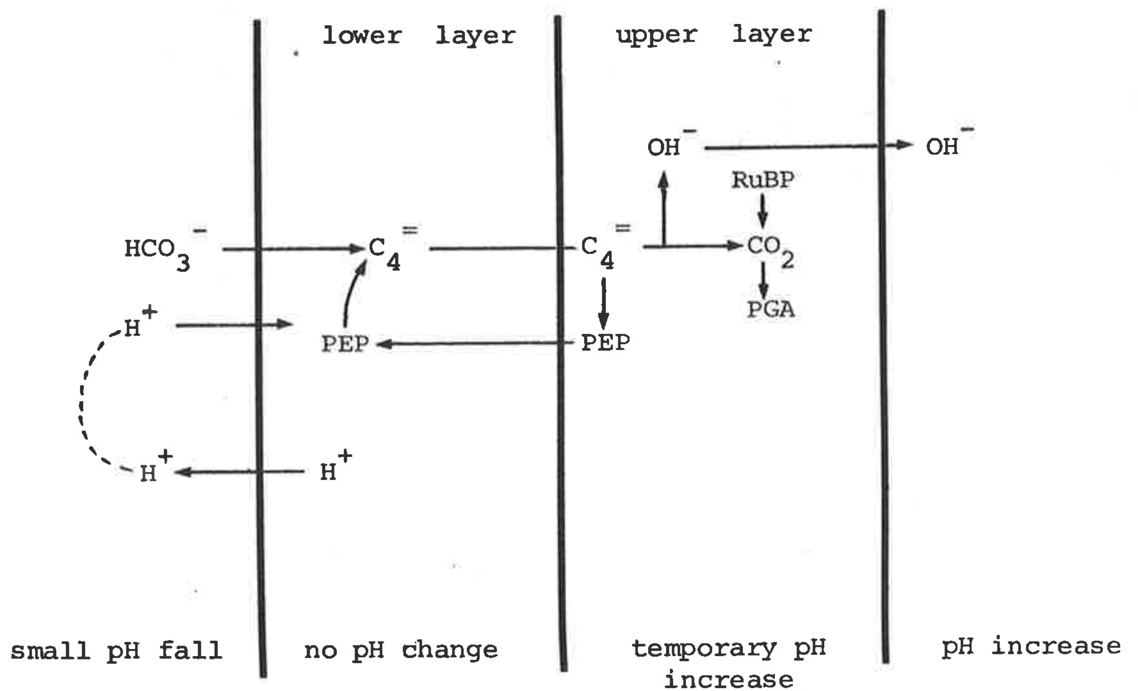



FIGURE 9.4 Carbon metabolism under conditions of $HCO_3^-$ fixation. There is $C_4$ incorporation in the lower cell layer and $C_3$ formation (PGA) in the upper layer. There is a net charge transfer from the lower surface ($HCO_3^-$ uptake) to the upper surface ($OH^-$ efflux). This charge imbalance causes $Ca^{++}$ to move in the intra-cellular spaces from the lower surfaces to the upper surfaces (see Figure 9.1).

# APPENDICES

During the course of this Thesis work, various studies were carried out on *Elodea* which proved to have no direct relationship with the theme of the Thesis. Such studies included the effects of such Herbicides as Copper, BTB-60 (S-Decyl iso-thiouronium bromide) and acrolein on *Elodea*. The latter study was taken out in cojunction with the CSIRO Griffith, NSW, and the *Elodea* Co-ordination Sub-Committee. Both these Organisations were concerned at the time, with the uncontrolled spread of *Elodea* in the irrigation channels around Griffith. Studies were undertaken to study the effects of these compounds on the physiology of *Elodea* in the eventual hope of finding more competent ways of eradicating the *Elodea* infestation in these areas.

In the first two appendices I have described the results of the work with two herbicides - copper and BTB-60.

The apparently non-specific effects of both BTB-60 and $Cu^{++}$ on photosynthesis, respiration and ion transport made the details of their effects unsuitable for inclusion into the main body of the Thesis.

In the final appendix, I have included some programs for the Hewlett Packard HP25 pocket calculator that quickly calculate ion fluxes, c-fixation and other estimates used in this Thesis work.

# APPENDIX A

## THE EFFECTS OF COPPER ON PHOTOSYNTHESIS AND RESPIRATION IN *Elodea densa*

### Introduction

Inorganic copper has been used for a long time as a fresh water algicide. However, its physiological effects on photosynthesis and respiration have not been extensively studied. Most work has been carried out using *Chlorella* and only a few studies have been made on higher plants (Das Gupta and Mukherji, 1977). The present work reports the effects of $Cu^{++}$ on photosynthesis, respiration and the light-stimulated $Cl^-$ influx in *Elodea densa*.

McBrien and Hassal (1967) observed that when *Chlorella vulgaris* absorbed $Cu^{++}$ under anaerobic conditions, respiration, photosynthesis and cell growth were all severely inhibited but this inhibition was absent if the cells absorbed $Cu^{++}$ under aerobic conditions. However this lack of inhibition during aerobiosis was reversed if the cells were subjected to a subsequent period of anaerobiosis. In contrast, Steemann Nielsen, Kamp-Nielsen and Wium-Anderson (1969), found that *Chlorella pyrenoidosa* required light for an effect on photosynthesis of $10^{-7}$M $Cu^{++}$ to be observed and that such an effect required 24 hours to reach its maximum. Increasing the solution pH and external potassium concentration reduced this $Cu^{++}$ inhibition. Gross, Puguo and Dugger (1970) observed an increase in respiration with $10^{-4}$ $\mu$M $CuSO_4$, which was prevented by glutathione and partially prevented by the addition of $MnCl_2$

and anaerobiosis. Haberman (1969) also found that manganese reversed the copper inhibition of photosynthesis in isolated chloroplasts.

In more extensive studies, Cedeno-Maldonato and Swader (1972) found that $Cu^{++}$ strongly inhibited uncoupled photosynthetic electron transport in isolated spinach (*S. oleracea*) chloroplasts. Increased inhibition was observed after pre-incubation in the light as compared to dark pre-incubation; and photosystem II was more sensitive than photosystem I. The magnitude of these inhibitions was determined by the $Cu^{++}$/chloroplast ratio, rather than just the $Cu^{++}$ concentration as reported by other workers.

In these experiments the effects of $Cu^{++}$ on photosynthesis and respiration after short periods of time were studied using the Rank Oxygen Electrode (see 2.3.1). $Cl^-$ influx was measured using the technique described in 2.3.3.

Copper was added to the experimental solutions as either $CuSO_4$ or $CuCl_2$ to give the required concentrations.

## Results and Discussion

The effect of $Cu^{++}$ on the net photosynthetic $O_2$-production of *Elodea* is shown in Figure A.1. As the $Cu^{++}$ concentration in the bathing solution increased, photosynthesis decreased such that at 100 µM $Cu^{++}$, it is reduced to 25% of the control value. However the relative effect on the dark $O_2$ consumption - respiration - was much more marked, and at 100 µM $Cu^{++}$ was four times the control rate (Figure A.2). Presumably this increased $O_2$ consumption reflects an uncoupling of respiratory electron flow.

Figure A.3 shows the effect of 80 μM $Cu^{++}$ over a period of time. The effect on respiratory electron flow was very marked over a four hour period, whereas the effect on photosynthetic $O_2$ production was much smaller. With lower concentrations of $Cu^{++}$, these changes occurred over a much longer period of time.

These inhibitory effects of $Cu^{++}$ on photosynthesis occur at much smaller concentrations and were much greater than those reported for *Chlorella* over similar time intervals. Shioi, Tamai and Sasa (1978) found that 2 μM $Cu^{++}$ inhibited photosynthetic electron flow of isolated spinach chloroplasts by 50% and such inhibitions occurred in less than 10 minutes. The very large uncoupling of respiratory electron flow (400 - 500% of control values) has not been reported before.

The effect of 5.5 hours treatment at increasing $Cu^{++}$ concentrations has an even greater effect on the $Cl^-$ influx in the light (Figure A.4). 10 μM $Cu^{++}$ inhibited the $Cl^-$ influx to 14% of the control values in this time. The actual time course of this inhibition by 10 μM $Cu^{++}$ is shown in Figure A.5. 50% inhibition occurred in less then 1 hour and maximum inhibition was attained after approximately 4 hours. Such effects have not been described before. As the $Cl^-$ influx in the light is more sensitive to $Cu^{++}$ than is photosynthesis, $Cu^{++}$ probably does not affect the energy source for the $Cl^-$ influx, but probably the carrier proteins in the plasmalemma.

The site of action of $Cu^{++}$ has not been determined yet. Presumably it has different mechanisms of action in the chloroplasts and mitochondria since in the former organelles electron transport

is inhibited whereas in the mitochondria electron transport is stimulated. Das Gupta and Mukherji (1977) showed that $Cu^{++}$ (10 mM) had effects on the alkali-soluble proteins of rice seedlings but that the effects were different in the embryo and the endosperm. Shioi, Tamai and Sasa (1978) concluded that in isolated chloroplasts $Cu^{++}$ directly interacted with ferredoxin causing inhibition of ferredoxin dependent reactions; and also caused a weak inactivation between the oxidising side of photosystem II and the electron donating site of DPC (1,5-diphenylcarbazide).

Hence, a lot more work is needed to determine the many apparent sites of copper sensitivity in *in vivo* and *in vitro* studies.

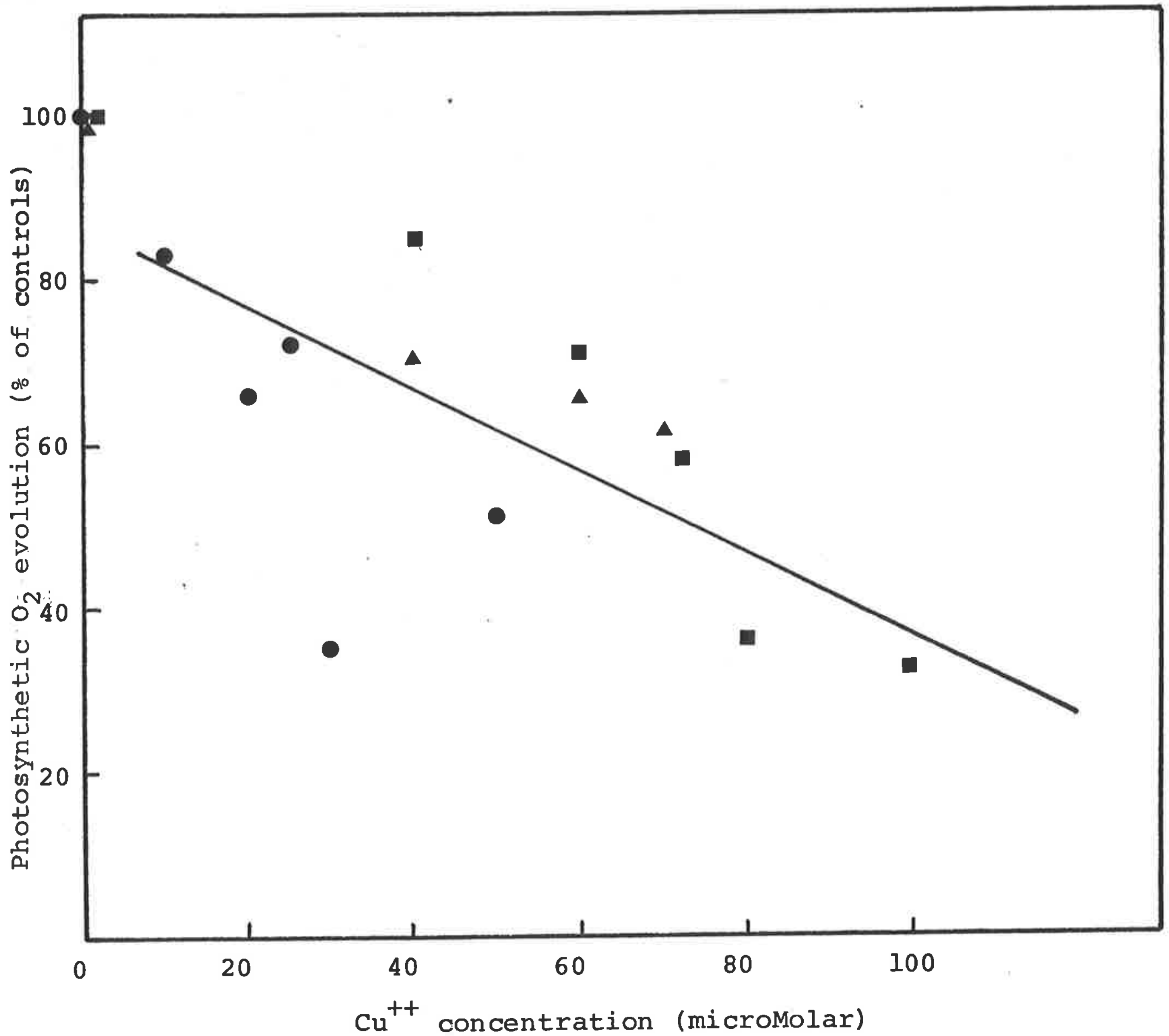



FIGURE A.1 Effect of increasing external $Cu^{++}$ (as $CuSO_4$) on photosynthetic $O_2$ evolution (expressed as a % of control values). Symbols represent data from different experiments. Regression line ($Y = 86.2 - 0.5X$, $R^2 = 0.7$). Solutions buffered with 5.0 mM HEPES at pH 6.0. Pretreatment time 5 hours.

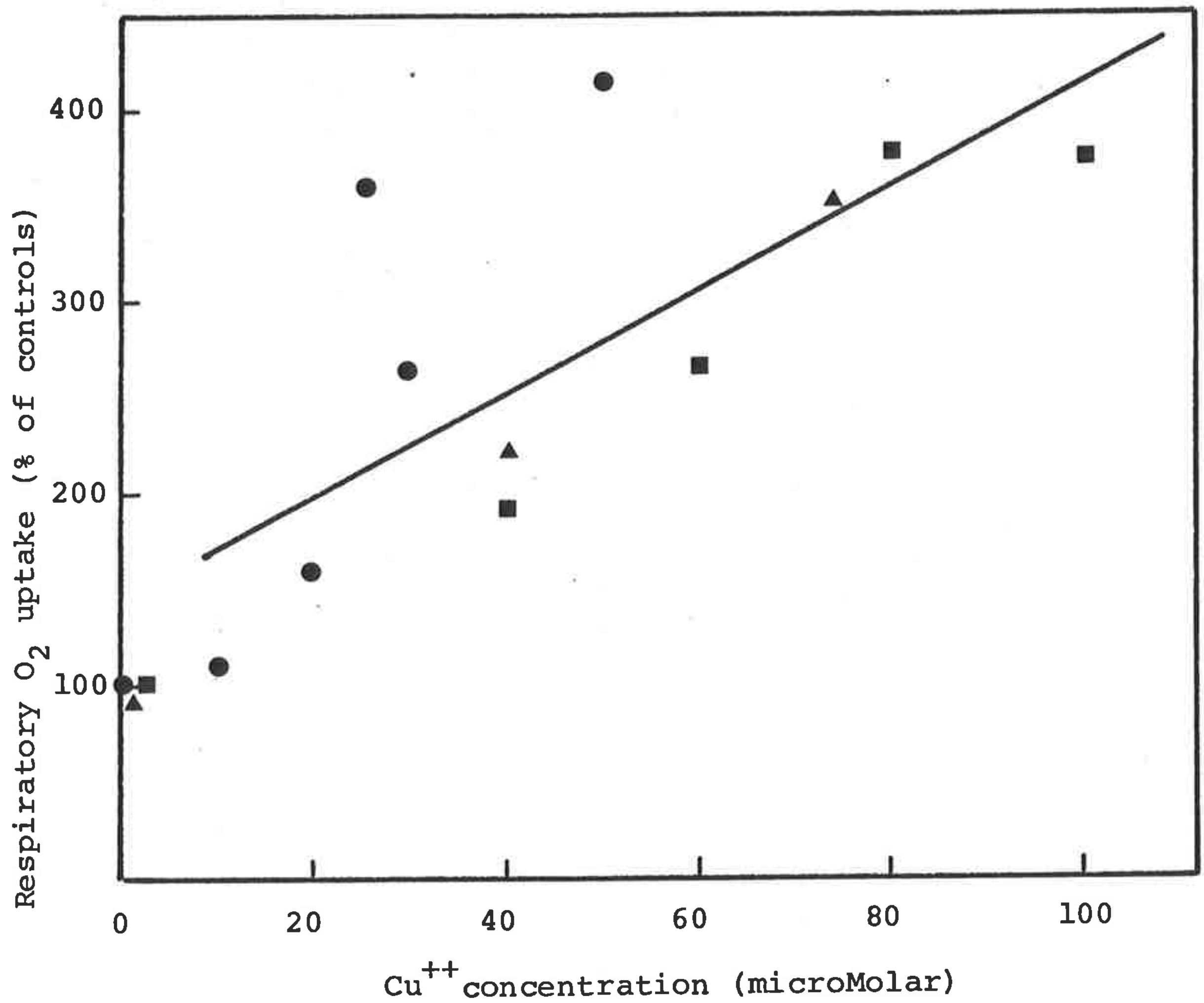



FIGURE A.2 Effect of increasing external $Cu^{++}$ (as $CuSO_4$) on respiratory $O_2$ uptake (expressed as a % of control values). Symbols represent data from different experiments. Regression line ($Y = 145.5 + 2.75X$, $R^2 = 0.74$). Solutions buffered with 5.0 mM HEPES at pH 6.0. Pretreatment time 5 hours.

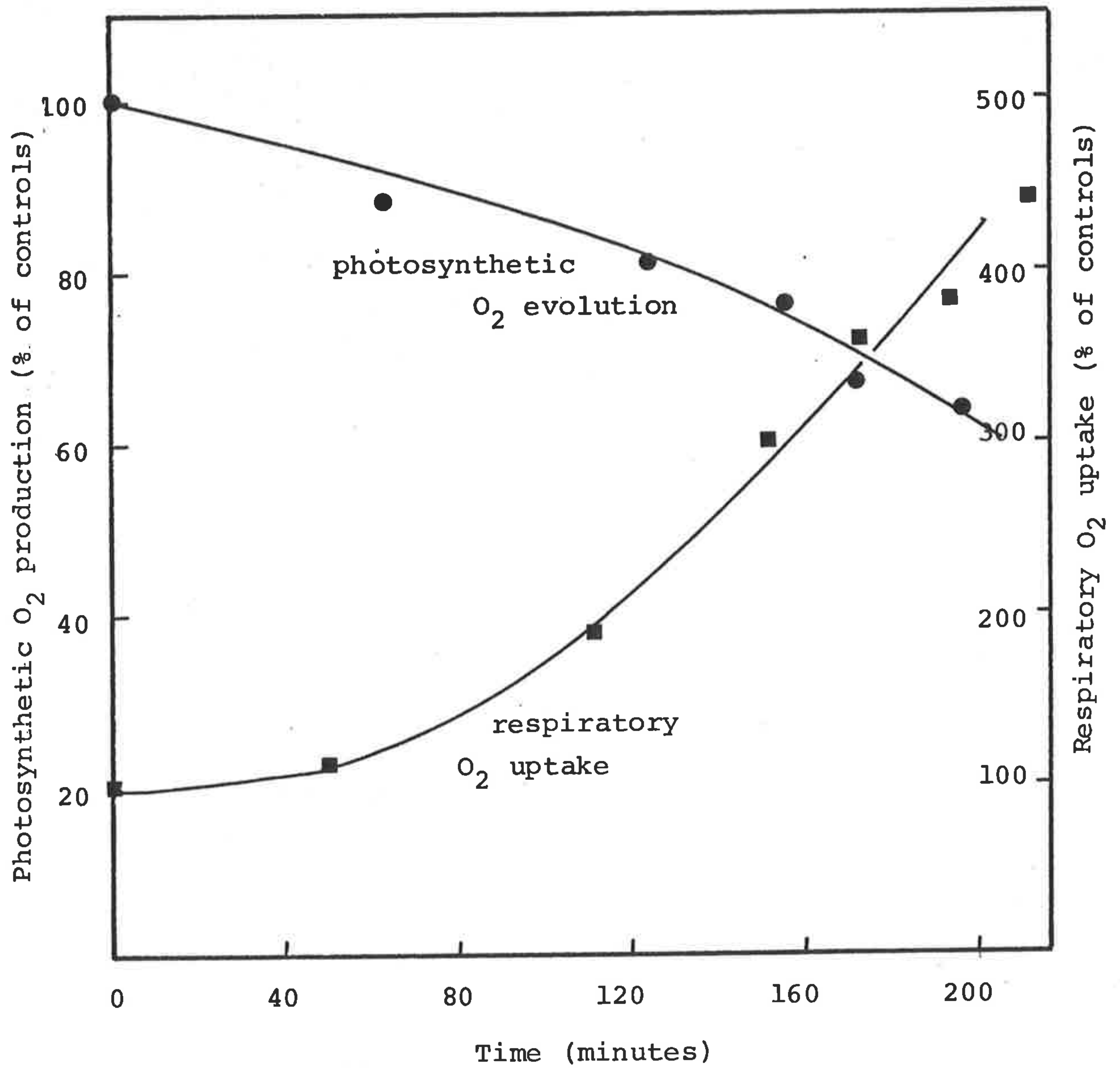



FIGURE A.3 Effect of 80 µM $Cu^{++}$ (as $CuCl_2$) on photosynthetic and respiratory $O_2$ exchange over a period of 4 hours. Solutions as in Figure A.1.

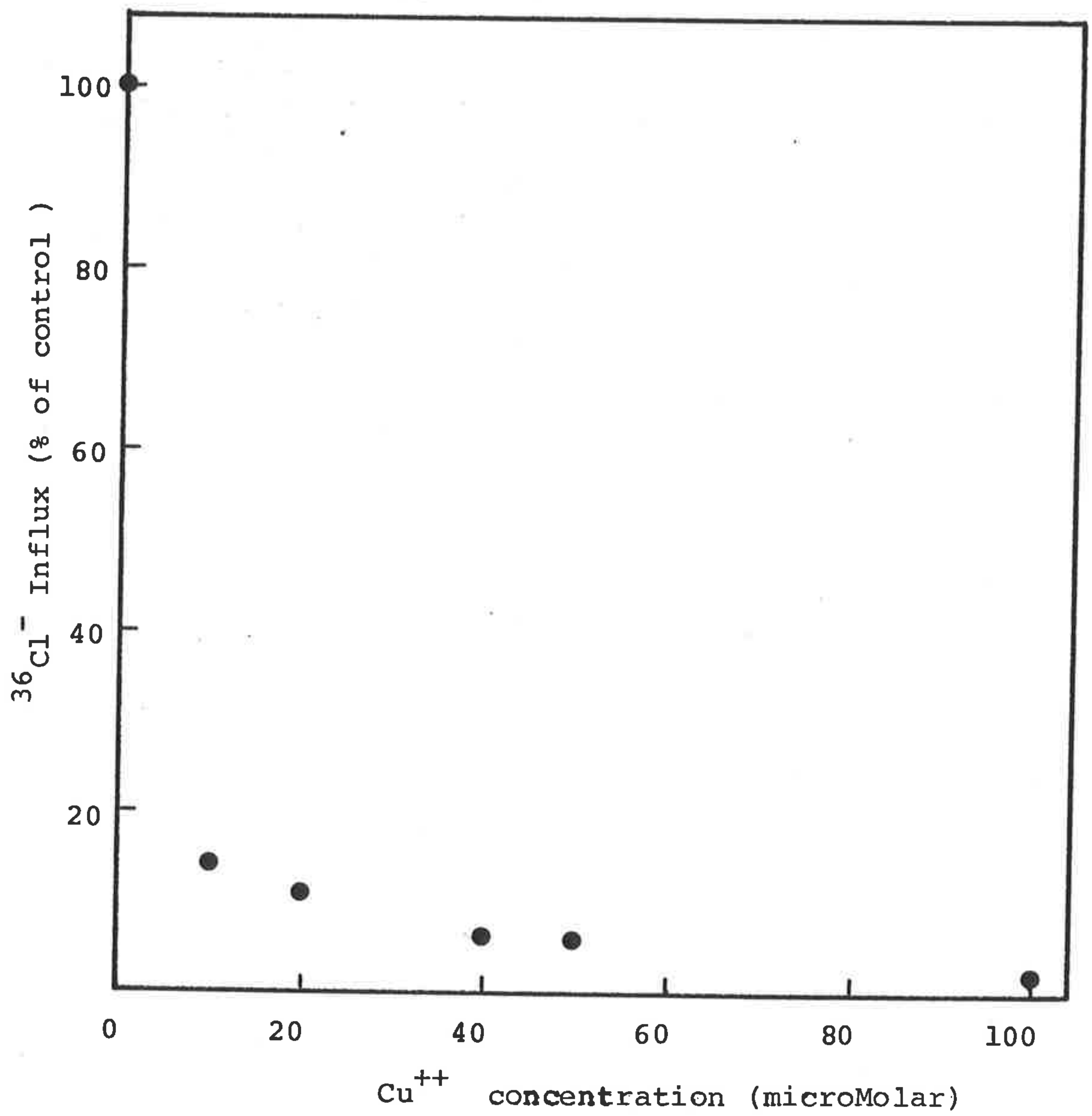



FIGURE A.4 Effect of $Cu^{++}$ ($CuSO_4$) on the light-stimulated $^{36}Cl^-$ influx (expressed as a % of the control value). Pretreatment for 5.5 hours. Solutions buffered with 5.0 mM HEPES at pH 6.0.

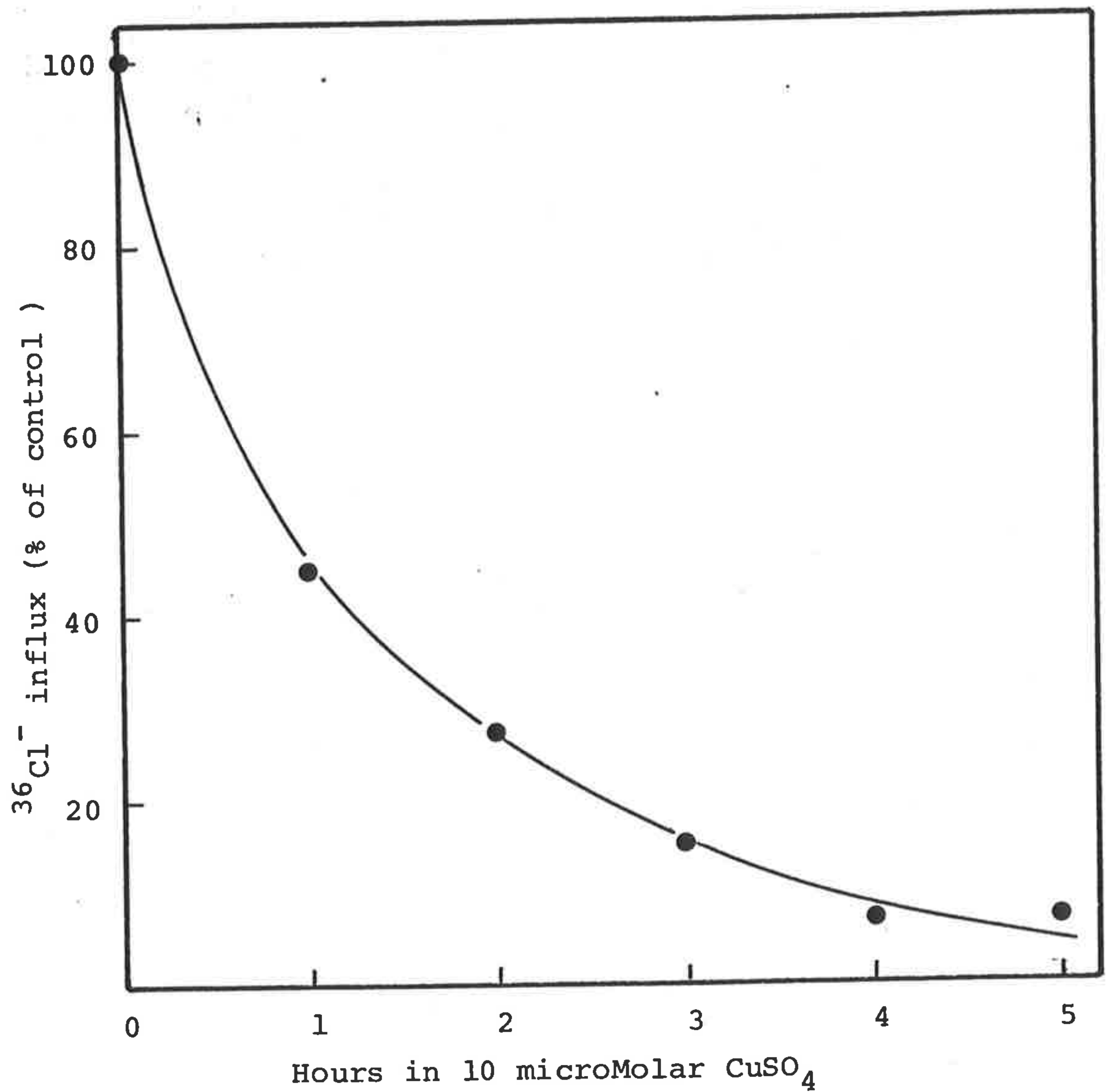



FIGURE A.5 Effect of 10 µM $Cu^{++}$ (as $CuSO_4$) on the light-stimulated $^{36}Cl^-$ influx (expressed as a % of the control value) over a period of 5 hours. Solutions buffered with 5.0 mM HEPES at pH 6.0.

# APPENDIX B

## THE EFFECTS OF BTB-60 ON PHOTOSYNTHESIS, RESPIRATION AND $Cl^-$ TRANSPORT IN *Elodea densa*

### Introduction

In the CSIRO in Canberra, Australia, BTB-60 (S-Decyl isothiouronium bromide) is being examined as a potential herbicide by studies on its effects on photosynthetic $O_2$ evolution and respiratory $O_2$ uptake with *Elodea canadensis* using an oxygen electrode (Brown, personal communication, 1974).

The initial reports from the laboratory indicated that at high pH, 50 to 200 μM BTB-60 uncoupled respiration and completely inhibited photosynthesis in *Elodea canadensis*. Preliminary work using *Elodea densa*, carried out at the request of Dr. Brown, at pH 5 and 8, indicated that although 50 μM and 100 μM BTB-60 uncoupled respiration ($O_2$ uptake), it had little effect on photosynthesis - inhibiting it by less than 20% of the control values - while higher concentrations (greater than 200 μM) did significantly inhibit $O_2$ evolution.

This work examines in some detail the effects of BTB-60 and (i) photosynthetic $O_2$ evolution and respiratory $O_2$ uptake using the Rank Oxygen Electrode (2.3.1); (ii) $^{14}CO_2$ fixation using the method described in 2.3.4; and (iii) $Cl^-$ influx using the method described in 2.3.3.

Results

The effects of increasing concentrations of BTB-60 on photosynthetic $O_2$ evolution at pH 6.5 and 8.0 are shown in Figure B.1. The control rates at pH 6.5 and 8.0 were 29 and 14.9 nmoles/$cm^2$/GFW respectively. Although these rates at the two pH values were quite different, the effect of increasing BTB-60 concentrations were very similar, producing approximately 50% inhibition of photosynthesis at 300 μM BTB. This was in contrast to the effects observed on respiratory $O_2$ uptake (Figure B.2), where at pH 8.0, uncoupling occurred at much lower BTB-60 concentrations than at pH 6.5. At pH 8.0, 150 μM BTB-60 had increased respiratory $O_2$ uptake to 250% of the control values, whereas at pH 6.5 there was virtually no stimulation. However, at higher concentrations of BTB-60 (300 μM), respiration was uncoupled to a similar extent (300% of controls) at both pH values. The control rates of respiration were approximately 3.4 nmoles/sec/GFW at each pH.

Increasing concentrations of BTB-60 showed a differential effect between $O_2$ evolution and $^{14}CO_2$ fixation, as shown in Figure B.3. $CO_2$ fixation was more sensitive to BTB-60, being reduced to 20% of the control values. At this concentration the $O_2$ evolution is 88% of the control values. As found with $Cu^{++}$ (Appendix A), the $Cl^-$ influx is more sensitive to BTB-60 than both $^{14}CO_2$ fixation and oxygen evolution (Figure B.3), being reduced to 20% of the control value at 50 μM BTB-60. This differential effect suggests that the site of action of BTB-60 is different for photosynthesis and the $Cl^-$ influx - the $Cl^-$ influx being possibly inhibited at the transport site.

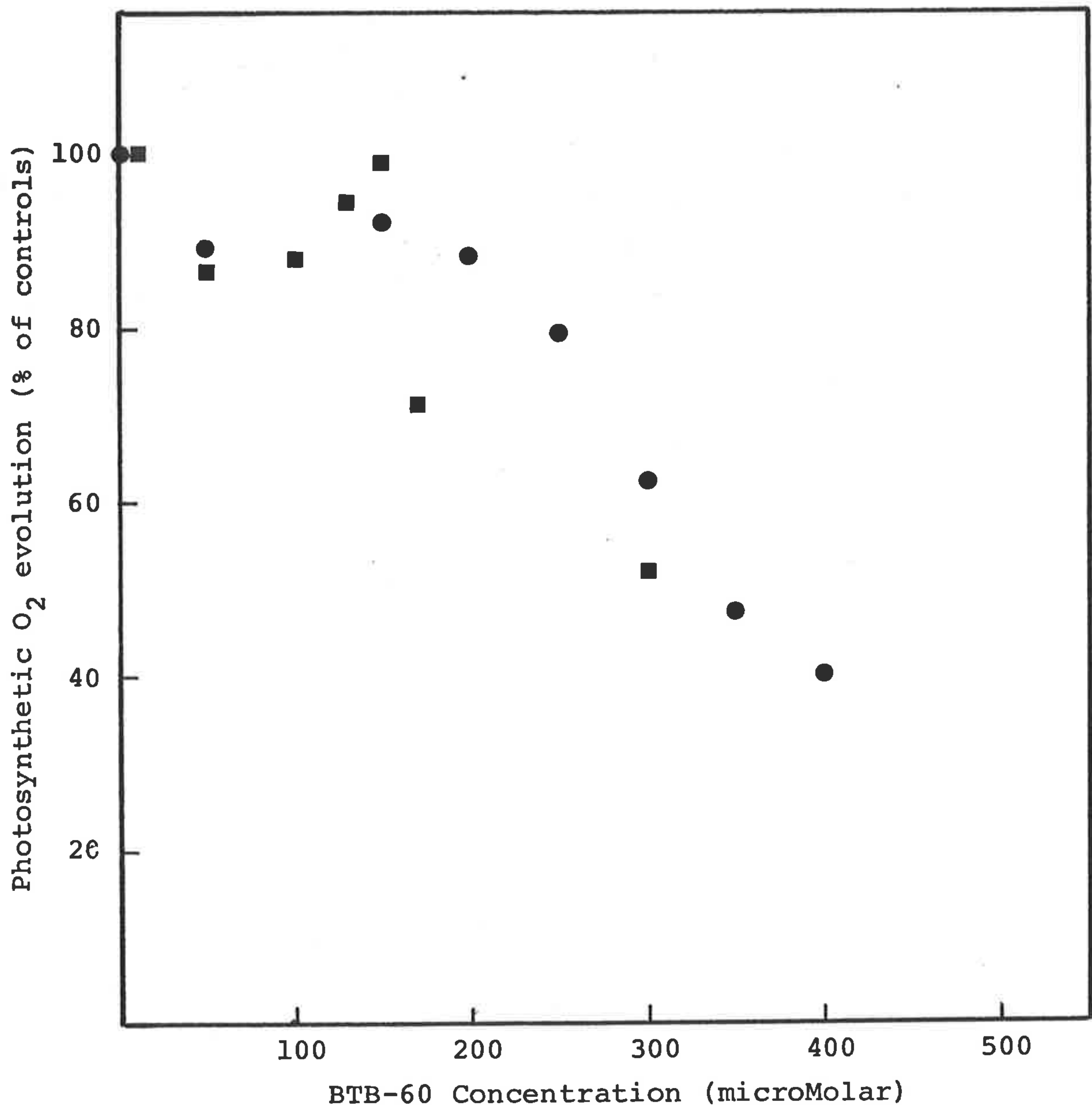



FIGURE B.1 The effect of BTB-60 on photosynthetic $O_2$ evolution at pH 6.5 (●) and pH 8.0 (■). Values are expressed as percentages of control values with no BTB-60 present. Solutions as in 2.3.10.

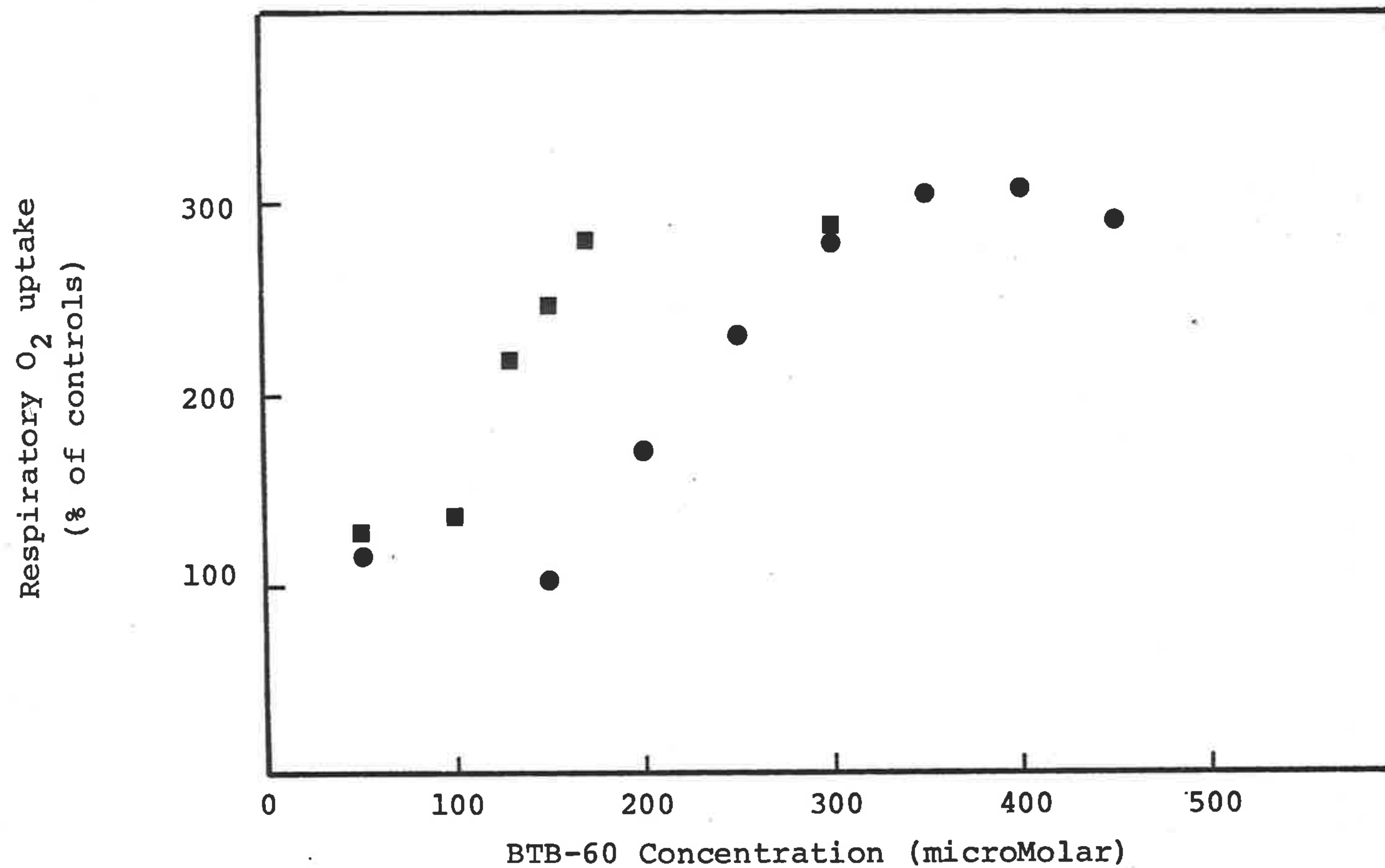



FIGURE B.2 Effect of BTB-60 on Respiratory $O_2$ uptake at pH 6.5 (●) and pH 8 (■). Values are expressed as percentages of control value with no BTB-60 present. Solutions as in 2.3.10.

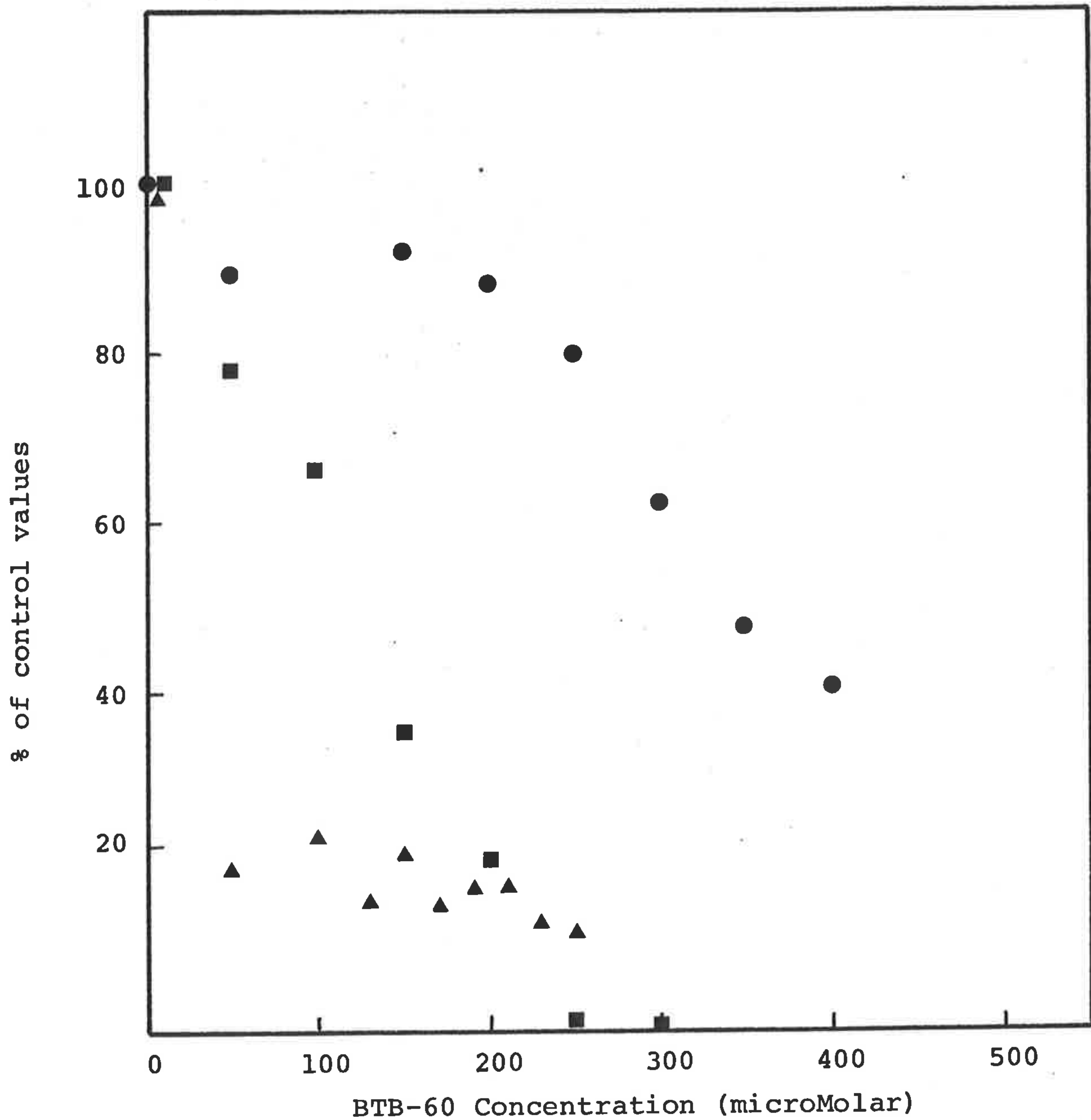



FIGURE B.3 The effect of BTB-60 on photosynthetic $O_2$ evolution (●) and $^{14}CO_2$ fixation (■) and the $Cl^-$ influx (▲) at pH 6.5. Values are expressed as percentages of control values with no BTB-60 present. Solutions as in 2.3.10.

# APPENDIX C

## HP-25 (HEWLETT PACKARD) PROGRAMS FOR CALCULATING INFLUX AND FIXATION RATES

Two programs are presented here that enable the calculation of ion influx (or carbon fixation rates) from isotope experiments depending on the nature of the data. In general both programs require data about the solution activity in order to compute the solution specific activity and then data about the experimental counts in order to compute the influx rates. In both the programs the calculation moves onto the next calculation step at the end of each block of data without direction from the operator. This is done by flagging with zero, and if no number is entered the programs move to the next step in the program.

As specific activity counts are always quite high both programs are designed for the counter to set for a given number of counts so that the time can be measured for this number for any number of replicates. However, as the experimental counts may be high or low, the two programs are designed to compute influx when (1) the experimental counts are fixed and the time for replicate counts is measured - Program 1 or (2) the replicate experimental counts are measured and the time for these counts is fixed - Program II.

Program I - experimental counts fixed, time for replicate counts measured.

Program displays

(a) mean specific activity count time.

(b) the specific activity (1 cpm = x nmoles)

(c) influx ( nmoles/$cm^2$/sec.)

PROGRAM

| DISPLAY LINE | DISPLAY CODE | KEY ENTRY |
|---|---|---|
| 00 | | |
| 01 | 1571 | gx=0 |
| 02 | 1308 | GTO 08 |
| 03 | 235106 | STO+6 |
| 04 | 01 | 1 |
| 05 | 235107 | STO+7 |
| 06 | 00 | 0 |
| 07 | 1300 | GTO 00 |
| 08 | 2406 | RCL 6 |
| 09 | 2407 | RCL 7 |
| 10 | 71 | ÷ |
| 11 | 74 | R/S |
| 12 | 21 | X ⇄ Y |
| 13 | 71 | ÷ |
| 14 | 2400 | RCL 0 |
| 15 | 41 | − |
| 16 | 2401 | RCL 1 |
| 17 | 21 | X ⇄ Y |
| 18 | 71 | ÷ |
| 19 | 2305 | STO 5 |
| 20 | 00 | 0 |
| 21 | 2306 | STO 6 |
| 22 | 2307 | STO 7 |
| 23 | 2405 | RCL 5 |
| 24 | 74 | R/S |
| 25 | 1571 | gx=0 |
| 26 | 1332 | GTO 32 |
| 27 | 235106 | STO+6 |
| 28 | 01 | 1 |
| 29 | 235107 | STO+7 |
| 30 | 00 | 0 |
| 31 | 1324 | GTO 24 |
| 32 | 2406 | RCL 6 |
| 33 | 2407 | RCL 7 |
| 34 | 71 | ÷ |
| 35 | 2402 | RCL 2 |
| 36 | 21 | X ⇄ Y |
| 37 | 71 | ÷ |
| 38 | 2400 | RCL 0 |
| 39 | 41 | − |
| 40 | 2405 | RCL 5 |
| 41 | 61 | x |
| 42 | 2403 | RCL 3 |
| 43 | 71 | ÷ |
| 44 | 2404 | RCL 4 |
| 45 | 71 | ÷ |
| 46 | 74 | R/S |
| 47 | 1320 | GTO 20 |

REGISTERS

$R_o$ Background

$R_1$ Specific activity (µL x mM)

$R_2$ Sample counts

$R_3$ Uptake time (secs)

$R_4$ Leaf area ($cm^2$)

User Instructions

| Step | Instructions | Input Data/Units | Keys | Output Data/Units |
|---|---|---|---|---|
| 1 | Key in Program | | | |
| 2 | Key in background (cpm) | | STO 0 | |
| 3 | Key in S.A. 1 (vol. (µl) x conc (mM)) | | STO 1 | |
| 4 | Key in sample counts | | STO 2 | |
| 5 | Key in uptake time (secs) | | STO 3 | |
| 6 | Key in leaf area ($cm^2$) | | STO 4 | |
| 7 | Initialize | | fENG 2<br>fPROG | |
| 8 | Key in first S.A. time (mins) | x | R/S | 0 |
| 9 | Repeat 8 n times | ....$x_n$ | R/S | 0 |
| 10 | Obtain mean S.A. time | | R/S | mean S.A. time |
| 11 | Key in S.A. counts and compute S.A. | | R/S | S.A. |
| 12 | Key in first sample time | x | R/S | 0 |
| 13 | Repeat 12 n times | ....$x_n$ | R/S | 0 |
| 14 | To calculate influx | | R/S | Influx |
| 15 | For next set of influx estimates Key then repeat steps 12 to 14 | | R/S | S.A. |

1 This number is the product to the volume (µL) sampled from the labelled solution and the concentration (mM) of the ion to be measured.

Program II - time for replicate counts fixed.

Program displays

(a) mean specific activity count time

(b) the specific activity (1 cpm = x nmoles)

(c) influx (nmoles/$cm^2$/sec.)

PROGRAM

| LINE | DISPLAY CODE | KEY ENTRY |
|---|---|---|
| 00 | | |
| 01 | 1571 | gx=0 |
| 02 | 1308 | GTO 08 |
| 03 | 235106 | STO+6 |
| 04 | 01 | 1 |
| 05 | 235107 | STO+7 |
| 06 | 00 | 0 |
| 07 | 1300 | GTO 00 |
| 08 | 2406 | RCL 6 |
| 09 | 2407 | RCL 7 |
| 10 | 71 | ÷ |
| 11 | 74 | R/S |
| 12 | 21 | X ⇌ Y |
| 13 | 71 | ÷ |
| 14 | 2400 | RCL 0 |
| 15 | 41 | - |
| 16 | 2401 | RCL 1 |
| 17 | 21 | X ⇌ Y |
| 18 | 71 | ÷ |
| 19 | 2305 | STO 5 |
| 20 | 0 | 0 |
| 21 | 2306 | STO 6 |
| 22 | 2307 | STO 7 |
| 23 | 2405 | RCL 5 |
| 24 | 74 | R/S |
| 25 | 1571 | gx=0 |
| 26 | 1332 | GTO 32 |
| 27 | 235106 | STO+6 |
| 28 | 01 | 1 |
| 29 | 235107 | STO+7 |
| 30 | 00 | 0 |
| 31 | 1324 | GTO 24 |
| 32 | 2406 | RCL 6 |
| 33 | 2407 | RCL 7 |
| 34 | 71 | ÷ |
| 35 | 2402 | RCL 2 |
| 36 | 71 | ÷ |
| 37 | 2400 | RCL 0 |
| 38 | 41 | - |
| 39 | 2405 | RCL 5 |
| 40 | 61 | x |
| 41 | 2403 | RCL 3 |
| 42 | 71 | ÷ |
| 43 | 2404 | RCL 4 |
| 44 | 71 | ÷ |
| 45 | 74 | R/S |
| 46 | 1320 | GTO 20 |

REGISTERS

$R_o$ Background

$R_1$ Specific activity (µL x mM)

$R_2$ Sample Count Time

$R_3$ Uptake Time (secs)

$R_4$ Leaf Area ($cm^2$)

User Instructions

| Step | Instructions | Input Data/Units | Keys | Output Data/Units |
|---|---|---|---|---|
| 1 | Key in Program | | | |
| 2 | Key in background (cpm) | | STO 0 | |
| 3 | Key in S.A. 1 (vol. (μL) x conc (mM)) | | STO 1 | |
| 4 | Key in sample count time (mins) | | STO 2 | |
| 5 | Key in uptake time (secs) | | STO 3 | |
| 6 | Key in leaf area ($cm^2$) | | STO 4 | |
| 7 | Initialize | | f ENG 2<br>f PROG | |
| 8 | Key in first S.A. count time | x | R/S | 0 |
| 9 | Repeat 8 n times | ....$X_n$ | R/S | 0 |
| 10 | To obtain mean S.A. count time | | R/S | mean S.A. time |
| 11 | Key in S.A. counts and compute S.A. | | R/S | S.A. |
| 12 | Key in first sample count | x | R/S | 0 |
| 13 | Repeat 12 n times | ....$X_n$ | R/S | 0 |
| 14 | To calculate influx | | R/S | Influx |
| 15 | For next set of influx estimates Key then repeat steps 12 to 14 | | R/S | S.A. |

1 This number is the product to the volume (μL) sampled from the labelled solution and the concentration (mM) of the ion to be measured.

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