

A STUDY OF SOME FACTORS AFFECTING THE
DISTRIBUTION OF AQUATIC MACROPHYTES

Patrick Denny

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1966

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A Study of Some Factors Affecting
the Distribution of Aquatic Macrophytes

by

Patrick Denny B.Sc. (St. Andrews)

A thesis submitted to the University
of St. Andrews for the degree of
Doctor of Philosophy

Department of Botany
St. Salvator's College
University of St. Andrews

December 1966



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Certificate

We certify that Patrick Denny has spent nine terms of research work under the direction of ourselves, (Professor J. A. Macdonald at the University of St. Andrews, and Professor D.H.N. Spence at the University of East Africa) and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying Thesis in application for the degree of Doctor of Philosophy.

Declaration

I hereby declare that the following Thesis is based on the record of the work done by me, that the Thesis is my own composition, and that it has not previously been presented for a Higher Degree.

The research was carried out in the Department of Botany at Makerere University College of the University of East Africa under the direction of Professor D.H.N. Spence; and at the Department of Botany at St. Salvator's College of the University of St. Andrews.

16th December 1966

Acknowledgements

I wish to record my gratitude to Professor D.H.N. Spence of the Department of Botany, Makerere University College, and St. Salvator's College for supervising the work presented in this Thesis, and for the stimulation he has given me, and the deep interest he has shown throughout the investigations.

I am also indebted to the members of staff at the Department of Botany, Makerere University College, who offered me full facilities for my investigations and especially to Mr. T. R. Milburn, Reader in Plant Physiology, who trained me in many laboratory techniques.

For my training in electro-physiological techniques and for many subsequent discussions in this field I am very grateful to Dr. D. C. Weeks of the Department of Botany, St. Salvator's College.

Finally, I should like to thank the Leverhulme Trust who awarded me a Leverhulme Research Scholarship (Overseas) for two years, and facilities for travelling within East Africa; and the Science Research Council for a grant without which the investigations could not have been completed.

Career

I graduated from the University of St. Andrews in June 1963 with a second class honours in Botany.

In July 1963 I was admitted as a Research student in the University of St. Andrews under Ordinance 16 and 61 and immediately travelled to Makerere University College, Uganda where I studied until July 1964. I returned to St. Andrews in August 1964 for a short study leave until December, and then resumed my investigations in East Africa until April 1966. In May of this year I finally returned to St. Andrews where I completed my studies.

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I N T R O D U C T I O N

Introduction

Studies on the factors affecting the distribution of aquatic macrophytes have mainly been confined to deductions from correlations between their distribution and change in chemical and physical factors in the environment. Spence (1964) in his analysis of the aquatic macrophytic vegetation of Scotland and in his more recent paper (1966) makes us very much aware of the limitations of such correlations in this multifactorial system. However, if field data were compared and combined with accurately controlled experimental data, a more comprehensive explanation might be achieved.

Of the many factors which are presumed to influence the distribution of water-plants this thesis concentrates on those which may broadly be classified as nutritional.

The experiments on which this thesis is based were carried out in East Africa using plants of three Potamogetons, the nomenclature of which was taken from Dandy (1937).

- 1) Potamogeton schweinfurthii A. Benn. in Dyer, Fl. Trop. Afr. viii, 220 (1901); a totally submerged broad-leaved species closely resembling the British P. praelongus Wulf. (see Clapham, Tutin and Warburg, 1962 pp. 948-950). It was collected from Lake Bunyoni in Southwest Uganda.

- 2) Potamogeton richardi Solms in Schweinf. Beitr. Fl. Aethiop. i, 194 in obs. (1867); a floating-leaved species resembling P. polygonifolius Pourr. and collected from some old brick-pits 4 miles from Kampala.
- 3) A putative hybrid between the two which has both floating and submerged leaves and collected from Lake Bunyoni.

Emphasis is laid on controlled experiments which, for convenience are assembled into four chapters as follows:--

- I Ionic Uptake into the Plant.
- II The Ionic Balance between Leaf Tissue and Bathing Medium with Reference to the Electrochemical Potential Gradient.
- III Carbon Dioxide Requirements of the Plant.
- IV Carbon Dioxide as a Factor in Varying the Cell Membrane Potential and its Possible Ecological Implications.

Each chapter is subdivided into the appropriate Review of Literature, followed by Experiments, Discussions and Conclusion.

CHAPTER I

IONIC UPTAKE INTO THE PLANT

Introduction

The occurrence and distribution of water plants must depend to some extent upon the availability of nutrients; but differing from land plants they have two potential sources of supply, one from the water and the other from the substrate. Whether rooted hydrophytes are able directly to utilise both these sources of nutrition, will obviously affect their distribution.

Mortimer (1941, 1942) and Hutchinson (1957) showed that oligotrophic lakes always have an oxidized mud layer covering the surface of their substrates; eutrophic lakes, later in the growing season, have a very impoverished epilimnion over an oxidized layer in the littoral zone. In both cases the water phase can be very low in nutrients and the substrate phase rich, as the nutrients, which are often freely available in the reduced mud below the oxidized layer, cannot escape through this barrier to the water.

A rooted aquatic plant growing in this environment therefore would have its shoots in a nutrient-poor solution and its roots, if penetrating into the reduced mud, in a nutrient-rich solution. Plants growing in the hypolimnion of eutrophic lakes or in other nutrient-rich waters would have an excellent nutrient supply surrounding its shoots and leaves and yet it might be rooted in sand or poor substrate.

However opinions vary as to whether the function of the roots of aquatic plants are mainly organs for anchorage (see for example Olsen (1953) Sutcliffe (1962 p. 21) and many present-day text-books) or organs for nutrient absorption (see Tansley (1939) Pearsall (1921) Pond (1905)).

A transect across a lake will show a build up of organic matter and silt with increasing depth, there being a consequent increase in fertility and proportion of fine particles. The vegetation over such a region also shows distinctive changes with depth, ranging from emergent forms at the fringe to floating-leaved rooted, and finally to submerged leaved rooted plants alone in the deeper zones.

Pearsall (1920 and 1921) thought that the silting factor was ultimately responsible for the distribution of all the types of vegetation found in the English Lakes and demonstrated, for example, a correlation between texture and potash content of the substrate on the one hand and vegetation on the other.

It cannot be denied that under different vegetation types there are often different substrates and that in a plant succession there is usually a substrate succession also: As Spence (1964) states (referring to a transect at the south end of Lake Croispol, Durness) 'substrate then,

seems to vary with vegetation type'; but this does not imply that the vegetation varies directly because of the substrate.

A correlation between the amount of organic matter in the substrate and the particular vegetation it supported was observed by Misra (1938). By transplanting Potamogeton perfoliatus into different substrates in the same water he was able to show that this species grew best in the mud in which it was naturally found.

Pond (1905) demonstrated that 'Ranunculus aquatilis trichophyllus plants' suspended in a solution over mud or sand grew very little as did those planted in sand, but those planted in mud grew very strongly. This implies that the plants were able to extract something from the mud which was not available in the bathing solution or the sand. However, this does not prove that the roots are the organs of absorption, for nutrients from the mud could diffuse into the bathing solution and be absorbed by the lower leaves of the plant. Bottomly (1920) for example, demonstrated that many water-plants including Lemna would grow better in a nutrient solution if an extract from organic matter was added. But then it would be expected that those plants that Pond suspended over a mud substrate would grow better.

Root hairs are commonly found on aquatic plants so that the

possibility of cation-exchange between root hairs and soil particles as suggested by Gonzales and Jenny (1958), cannot be ignored.

Although there is some circumstantial evidence for the absorption of ions through the roots of water-plants, direct evidence is entirely lacking. This situation is, however, very different in the case of leaves and shoots. Shoots of Elodea when placed in nutrient solutions have been observed to reduce the concentration of ions in the solutions, especially in the light. (see Ingold 1936, Resenfels 1935, and Olsen 1953). There are many other experiments of a similar nature which all support the principle of ionic absorption through the shoots of aquatic plants but the most direct evidence was produced by Arisz.

Arisz (1960, 1964(a), and 1964(b)), Arisz and Sol (1956) took longitudinal strips of Vallisneria leaves and placed them in a chamber divided into three compartments. Part of each strip was in each compartment and passed from one compartment to another via a water-tight seal. By thus dividing the strips into three zones in relation to the bathing solutions and supplying KCl to one of the zones (in his later experiments the Cl^- was labelled) he showed (i) there was diffusion of cations and anions into the

Apparent Free Space (A.F.S.) which Kylin (1957) showed to be between 5 and 7% of total volume of the leaf; (ii) there was light-stimulated active absorption of anions into a central plasm, and secretion of anions and cations from the central plasm into the vacuole and (iii) there was translocation of ions in the central plasm from one cell to the next via the plasmodesmata.

The terms 'active' and 'secretion' as used by Arisz are very loose and do not distinguish between the requirement of energy for movement of a particular ion and the requirement of energy for ion uptake generally; perhaps some ions are pumped inwards whilst others enter passively along the electrochemical gradient.

Arisz's symplasmic theory for translocation in which he proposed that salts are transported from cell to cell in the symplasm is well reviewed by Winter (1961) and leaves no doubt that leaves of *Vallisneria* are not only able to absorb nutrients but also are able to transport them. Further, there is evidence from Sutcliffe and Counter (unpublished, from Sutcliffe 1959) that labelled Rb^{*} is transported down the phloem of *Vallisneria* leaves and from Arisz (1964)(a) that both Cl^{*} and Rb^{*} can travel long distances; from the point of absorption in a leaf to the base of the plant and from there,

to other young shoots. Finally, Hutchinson and Bowen (1950) observed that after a solution containing P^{32} had been added to Linsley Pond, shoots of the littoral vegetation, including Potamogeton crispus became labelled with isotope. As the label was noticed in the shoots soon after its addition to the water, absorption must have taken place through this tissue rather than through the roots.

This type of evidence, together with the fact that some water-plants do not have roots, and most have a reduced xylem conduction system, has led several authors to conclude that all water-plants must absorb most or all their nutrients through their leaves (see Olsen 1953); however Spence (1964 p. 371) reviewed the evidence for both root and shoot absorption and, together with his own data concluded that no such hard and fast ruling could be made.

In this present chapter more evidence is accumulated to support Spence's view. This is accomplished by simple experiments designed to gather information on nutrient uptake into various regions of the plant and to observe differences in growth under different environmental conditions.

In considering the question of absorption regions, comparisons of growth are made between the floating-leaved species P. richardi and the submerged species P. schweinfurthii.

The former might have relatively less surface area for leaf absorption (as half the leaf is exposed to the air) than the latter. The putative hybrid is also used as an interesting intermediate between the two.

The role of roots in ionic absorption is investigated both by varying the substrate and observing subsequent growth, and by root competition experiments between two different species.

. . . .

To Observe the Effect of Substrate on the Growth
of *P. richardi*

Material and Methods:--

Potamogeton richardi was cultured from a single stock - this culture pond providing all the material for future experiments.

An old bath tub was prepared for the experiment in the following manner.

In the bottom of the bath a substrate was prepared to a depth of three inches from equal parts of thoroughly mixed garden loam, silver sand and well rotted compost. The bath was then carefully filled with pond-water (from an old

established pond) and left to settle for a week. During this period 8 eight-inch flower-pots were steeped in the pond, after which 4 of them were filled with silver-sand and the other 4 were filled with the same mixture as was used for the substrate in the bath. (termed 'mud' substrate).

40 approximately equal sized P. richardi shoots were cut from the stock pond each shoot having a terminal bud and one pair of leaves. Five shoots were planted around the perimeter of each pot and the pots were lowered into the bath, their bottoms being embedded in the substrate. They were arranged, equally spaced, in two rows along the bath a 'mud' pot alternating with a 'sand' pot.

The experiment was left for 17 weeks, the water level in the bath being maintained with tap-water. After 17 weeks the pots were removed and the plants cropped.

All the plants from a single pot were massed together and separated into (i) leaves, (ii) stem + petiole, (iii) rhizomes (iv) flowering heads and (v) roots. Each fraction was dried in a forced-draught oven at 100°C for 24 hours, cooled in a desiccator for a further 24 hours, and then weighed.

Considerable difficulty occurred with washing the roots of these plants which grew in mud as organic matter adhered

to them. To overcome this, after a mass of roots had been washed in the normal manner (i.e. by carefully rinsing in many buckets of tap-water) a fraction was separated and every adhering piece of organic matter was removed with fine forceps. This subsample and the organic matter removed from it were weighed separately and thus a correction factor could be applied to all the root masses from the mud substrate.

The dried fractions (i.e. roots or leaves etc.) were powdered and stored, all the material from the 4 'mud' pots in a particular fraction being combined; and likewise with the 'sand' pots material.

Results:-

The data for all the plants grown in 'mud' pots can be compared with that from 'sand' pots:-

TABLE (1), 1

Plant Fraction	Total D.Wt. Plants in MUD (g)	Total D.Wt. Plants in SAND (g)	Difference of Mean p<0.05
Leaves	12.24	2.00	
Stem	20.37	5.90	
Flower	7.36	0.74	
Rhizome	7.19	2.44	
Roots (corrected)	7.20	2.15	
Σ All Fractions	54.36	13.23	SIGNIFICANT
Shoot/Root ratio	6.68	5.49	NON-SIGNIFICANT

Table showing difference in dry weights of Potamogeton richardi grown in mud or sand.

The statistical significance of the difference between the mean of the attributes of those plants grown in mud and in sand was tested using the method of paired comparisons with Student's *t* test at the 95% confidence level. (see appendix p. xxxi)

Wet digestion of the dried plant material and subsequent chemical analysis of the digest (see appendix p. xx) revealed the following total phosphorus content in each fraction.

TABLE (1), 2

Plant Fraction	% Phosphorus	
	MUD	SAND
Leaves	0.253	0.236
Stem	0.277	0.269
Flower	0.307	0.233
Rhizome	0.337	0.315
Roots	0.884	0.960

Table showing g/100g D.Wt. of total phosphorus in the various fractions of F. richardi grown in mud or sand.

(All phosphorus results were the average of duplicate chemical analysis on duplicate digestions. i.e. 3 replica per fraction).

A sample of soil and sand were leached with 0.002N HCl

and analysis of the leachates gave the available phosphate in each sample:-

Sand contained 0.093 mg /100g D.Wt. available phosphate.

Mud contained 0.54 mg /100g D.Wt. available phosphate.

A sample of the bath water was analysed (see appendix p. i-x with the following results:-

Bath-water 0.08 meq/l H_2PO_4^-
2.4 meq/l K^+

Comments:-

The experiment was so designed that there would be a bathing solution rich in all nutrients. The phosphate and potassium analysis of the water confirm that in these two ions at least, the water was rich, the concentration being considerably greater than that found in most natural waters; (e.g. analysis of Lake Bunyoni showed that it contained $<0.2 \mu\text{eq/l H}_2\text{PO}_4^-$ and $175 \mu\text{eq/l K}^+$). It could therefore be assumed that other soluble ions were also present in reasonable quantities.

As the mud in the flower-pots consisted of a mixture of loam and compost it also should be rich in nutrients; an assumption which is confirmed by the phosphate analysis. On the other hand the sand would contain virtually no ions except those in solution in the bathing solution. So in the course

of 17 weeks all the P. richardi cuttings were in a bathing solution rich in nutrients but the roots of half the cuttings were in a poor sand substrate and the other half, in a rich mud substrate.

Results showed that although there was little difference in the phosphorus content per unit weight of the plants grown in different substrates, those plants grown in mud were about 4 times as large as those grown in sand after a 17 week period.

These results confirm Pond's observations (1905) that substrate can have a direct effect upon the growth of rooted water-plants. But, whereas Pond used tap-water for his experiments the present experiment had rich pond-water; therefore the possible objection that ions diffusing from the mud were absorbed by the lower leaves becomes irrelevant.

The analysis of the plant tissue showed that the 'mud' plants and the 'sand' plants contained approximately the same amount of phosphate: perhaps an indication that it was not limiting in either condition.

As the bathing solution was rich in nutrients the inter-particular spaces in the sand pots would also contain nutrients; therefore there would be soluble nutrients available to the sand plants both in the root and leaf region. But the sand would not necessarily contain the less soluble micro-nutrients

which would normally be bound onto soil micelles.

Therefore, perhaps, although the macro-nutrients in solution would be available to both the 'sand' and the 'mud' plants, some micro-nutrients essential for good growth might be much more available to the plants growing in mud where they could readily acquire them by contact exchange between root hairs and soil micelles.

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To Observe the Effect of Substrate on the Growth
of *P. schweinfurthii*

As the last experiment was with floating-leaved plants, in this experiment totally submerged-leaved plants were used in order to be able to compare the two.

Material and Methods:-

Potamogeton schweinfurthii was similarly grown in a culture pond from one original stock.

The experiment corresponded to the previous one but 5 inch flower-pots, each with two cuttings were used. The experiment was stopped, somewhat prematurely, after 10 weeks

as there was considerable contamination of the bath with algae.

The results, therefore, must be treated with much caution.

Results:-

The dry weight of the different fractions were taken as before and a comparison between 'mud' and 'sand' plants was made. In this case there were no flowers. The significance of the difference in mean weights was tested, again using Student's *t* test at the 95% confidence level.

TABLE (1), 3

Plant Fraction	Total D.Wt. Plants in MUD	Total D.Wt. Plants in SAND	Difference of mean $p < 0.05$
Leaves	2.579	2.089	
Stem + Rhizome	1.232	1.194	
Roots (corrected)	0.158	0.205	
Σ All fractions	3.969	3.488	NON-SIGNIFICANT
Shoot/Root ratio	24.55	15.64	NON-SIGNIFICANT

Table showing difference in dry weights of Potamogeton schweinfurthii grown in mud or sand.

Comments:-

The shoot/root ratio is interesting even though the difference is not significant, for it would confirm observations which have frequently been made by both Professor Spence and the present author: i.e. that roots of P. schweinfurthii grown in mud were often reduced and unhealthy looking whereas those in sand looked vigorous. In the present experiment, for example, roots in the sand pots were distributed throughout the pot and had many root hairs whereas those in the mud pots were cramped and only in the top inch of mud. Root hairs were not visible.

There had been growth both in the 'sand' and 'mud' plants and slightly more growth with the mud substrate. Whether this trend would have been amplified, or not, with more healthy plants and a longer time period, cannot be told, and unfortunately the experiment could not be repeated: however, general observations would suggest that in the case of P. schweinfurthii, roots are important in the establishment of the plant but after this their contribution to growth is insignificant as large healthy plants have been observed with only very poorly developed roots, and no root hairs.

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Competition between Rooted Aquatic Plants

Potamogeton hybrid, as it had characteristics of both P. richardi and P. schweinfurthii, was considered useful for root and shoot experiments together with the other two species.

If it could be shown that one species dominated over another whether its roots were free in a substrate, or confined within pots, then the importance of roots for growth could, perhaps, be assessed.

The experiment was subdivided into two sections.

Material and Methods (A):-

Cuttings of all three species (the hybrid, for convenience being considered a separate species), were used.

This preliminary experiment was prepared by planting out cuttings in a 4 ft.² x 1 ft. pond containing 3 inches of loam/sand (30:70) mixed substrate.

The cuttings were roughly equally spaced, there being originally a total of 15 P. richardi cuttings; 26 P. schweinfurthii cuttings and 5 P. hybrid cuttings.

Two fish (Tilapia nilotica) and some snails were added to the pond and it was left for 5 months.

After this period the material was cropped in the usual manner.

Results:-

TABLE (1), 4

Material	Original No. of Shoots	Final No. of Shoots
<i>P. richardi</i> ,	15	0
<i>P. schweinfurthii</i>	26	105
<i>P. hybrid</i>	5	215

Table showing Original No. of shoots of each species planted and the Final No. of each on cropping after 5 months.

TABLE (1), 5

Plant Fraction	Total D.Wt. <i>P. schwein.</i>	Total D.Wt. <i>P. hybrid</i>	Total D.Wt. <i>P. richardi</i>
Leaves	10.90	17.83	0
Stem	2.60	17.27	0
Rhizome	2.80	20.02	0
Roots	2.58	12.66	0
Flowers	0	1.14	0
Σ All fractions	18.91	68.92	0
Shoot/Root ratio	6.32	4.44	0

Table showing the dry weight of the three species in competition after 5 months.

Comments:-

The P. richardi had completely disappeared, and although there were only 5 cuttings of P. hybrid compared with 26 of P. schweinfurthii there is no doubt that after 5 months P. hybrid became dominant in every aspect.

Whether this would be the case or not if the roots were confined to pots can be tested in the following way.

Material and Methods (B):-

Cuttings of P. schweinfurthii and P. hybrid were used.

A concrete pond 4 ft. x 4 ft. had a 4 inch mud substrate of loam : compost : sand mixture (30:30:40) in two sections alternating with silver sand.

In order to get approximately the same weight cuttings of the two species in the original planting, they were roughly balanced on a pan balance before-hand.

In the mud substrate, cuttings of P. schweinfurthii were planted alternately with P. hybrid and in the sand substrate, cuttings of the two species were similarly arranged alternately, but each cutting was in an individual 8 inch flower-pot containing the same loam : compost : sand mixture. The pots were sunk into the sand.

Diagrammatically the planting can be shown thus:-

In the course of 3 weeks the water-level of the pond was slowly raised to the top by adding pond-water (previously, it had been found that cuttings cannot tolerate immediate deep submersion).

After 13 weeks the plants were cropped in the usual manner.

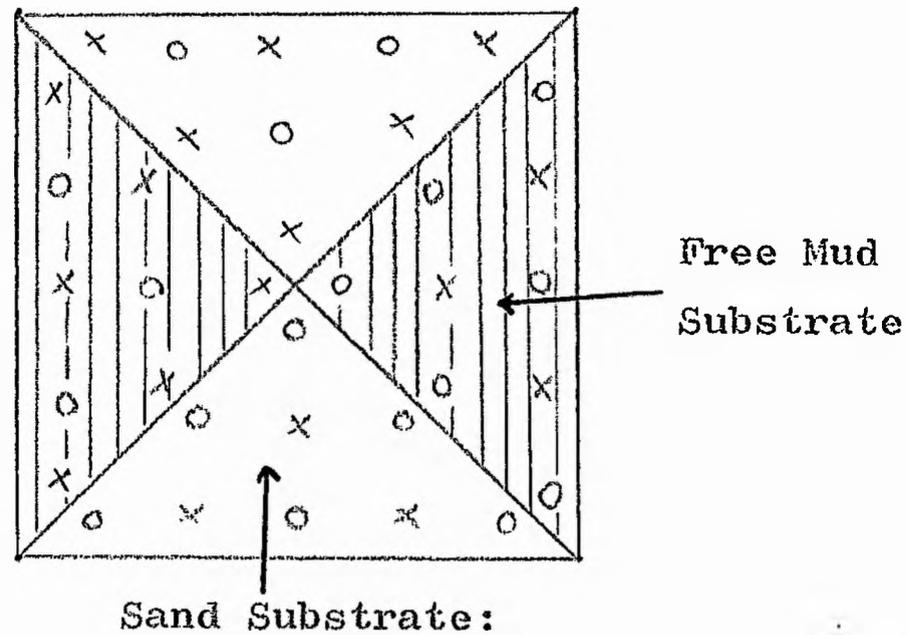
Results:-

TABLE (1), 6

	D.Wt. Whole Plant minus Roots.	
	<i>P. schweinfurthii</i>	<i>P. hybrid</i>
Roots Free	37.59 g	82.01 g
Roots Potted	28.79 g	14.31 g

Table showing dry weight of *P. schweinfurthii* and *P. hybrid* grown together in, or out of, pots.

As the mud contained more fibrous material than usual it was impossible to separate the roots of the plants cleanly so the roots' dry weights were omitted. However, by eye there appeared to be no obvious difference between those of the two species.



Cuttings in Pots containing Mud

x = *P. schweinfurthii*

o = *P. hybrid*

Diagram Showing the Arrangement of the Cuttings in the Pond

Comments:--

Table (1), 6 shows clearly that whereas P. schweinfurthii was only slightly affected by being in pots, the growth of the hybrid was suppressed by them: but where the hybrid was planted free in the substrate then it grew vigorously and considerably more than the P. schweinfurthii. This is of interest in relation to net distribution since repeated observations of the vegetation around Sharp's Island, Lake Bunyoni showed P. richardi to be absent, the shallow water (up to a metre in depth) being occupied by the hybrid, and P. schweinfurthii occurring only in water more than 1 metre deep.

In gross morphological features the hybrid is more similar to P. richardi in having large floating leaves, but it also has small reduced, submerged leaves.

P. richardi was shown to be greatly affected by the type of substrate (see page 9) and it was suggested that in that case the roots could act as organs for absorption. In the present case the hybrid was greatly suppressed by being in pots. If its roots, likewise, absorb nutrients, then the reduction of growth rate could be attributed to the roots being pot-bound, their immediate nutrient supply being limited. In P. schweinfurthii on the other hand, perhaps roots were only important for nutrient uptake in the early stages of plant development and that once

established, the plant absorbed nutrients mainly through its leaves (see page 15). The present experiment supports this further for whereas the hybrid became rapidly 'pot-bound' P. schweinfurthii showed little response from being in pots.

Perhaps then, at this stage the following scheme could be tentatively postulated. The roots of the floating-leaved potamogetons are important to their growth and distribution. As the substrate appears to affect their growth directly, and as the leaves have only one potential absorptive surface (the lower surface) in contact with the water, and as a thin cuticle can be observed covering the epidermis of stems and petioles, then it is probable that the roots have an absorptive function.

P. schweinfurthii on the other hand has delicate, ribbon-like leaves which are totally submerged. As its roots are sometimes very reduced, absorption could be preferentially through the shoots although in the early stages of development the roots might contribute largely to this important role of absorption.

* * * *

To Observe Ion Uptake into Excised Shoots of *P. schweinfurthii*

In the previous experiments it could only be assumed from data on growth that ionic absorption took place through the leaves and/or roots. A more direct method would be to observe the net absorption of ions from a nutrient solution into these two regions.

Using *P. schweinfurthii* this was attempted, but whereas shoot tissue remained healthy during the experiments, the roots quickly died, whether excised or attached to the plant. This problem was never overcome so that only data on absorption of ions by shoots was obtained.

Material and Methods:-

Healthy shoots of *P. schweinfurthii* were cut from the culture pond; had their cut ends sealed with vaseline; and were placed in deionized water in the laboratory. They were illuminated by a 1000 watt water-cooled, tungsten filament bulb placed 2 feet away. In the course of the next 18 hours, the deionized water was changed three times.

Chemical analysis of each change of water showed that by the third change there was virtually no further leakage of K^+ Na^+ or $H_2PO_4^{--}$ ions from the tissue into the water (Table(1), 7).

The tissue was then weighed (35 g F.Wt.), and placed in a perspex tank containing 7 litres of 0.25 meq/l KH_2PO_4 solution, this tank also being illuminated by the bulb. Stirring of the solution was accomplished by a magnetic stirrer placed in the bottom of the tank, a slow steady flow of solution over the tissue being observed.

After 18 hrs. 15 min. the experiment was stopped and a sample of solution was taken. Chemical analysis of the original solution and of the final solution for K^+ and Na^+ (by flame photometry, see appendix p.x) and for H_2PO_4^- (by phosphomolybdate blue colorimetry, see appendix p.i) provided information on the amount of these ions absorbed by the tissue.

In order to observe any net efflux of ions from the tissue it was quickly rinsed in 3 changes of deionized water (5 seconds per rinse), and was replaced in the tank now containing 7 litres of deionized water. The water was changed 3 times in the next 31 hrs. each change of water being analysed for the amount of ions leaked from the tissue.

Finally, the solution in the perspex tank was replaced by filtered, aerated pond-water to see whether the shoots then absorbed ions from natural pond-water. The absorption of ions from the water by the tissue in the light could be observed

by withdrawing samples for analysis and plotting a graph of amount of ions absorbed against time during the next 50 hrs. 20 min. (see graph (1), 1).

As only 5 mls. of sample was generally required for each analysis compared with a total volume of 7 litres, no correction factor for ions removed per sample was necessary, as the error was $< 1\%$.

Results:-

Fresh healthy tissue was placed in deionized water at 8 p.m. The water was changed at 12 midnight (a 4 hr. interval), again at 2 p.m. (a 14 hr. interval) and at 2.30 p.m. (a $\frac{1}{2}$ hr. interval). Analysis of the solutions gave the following results:-

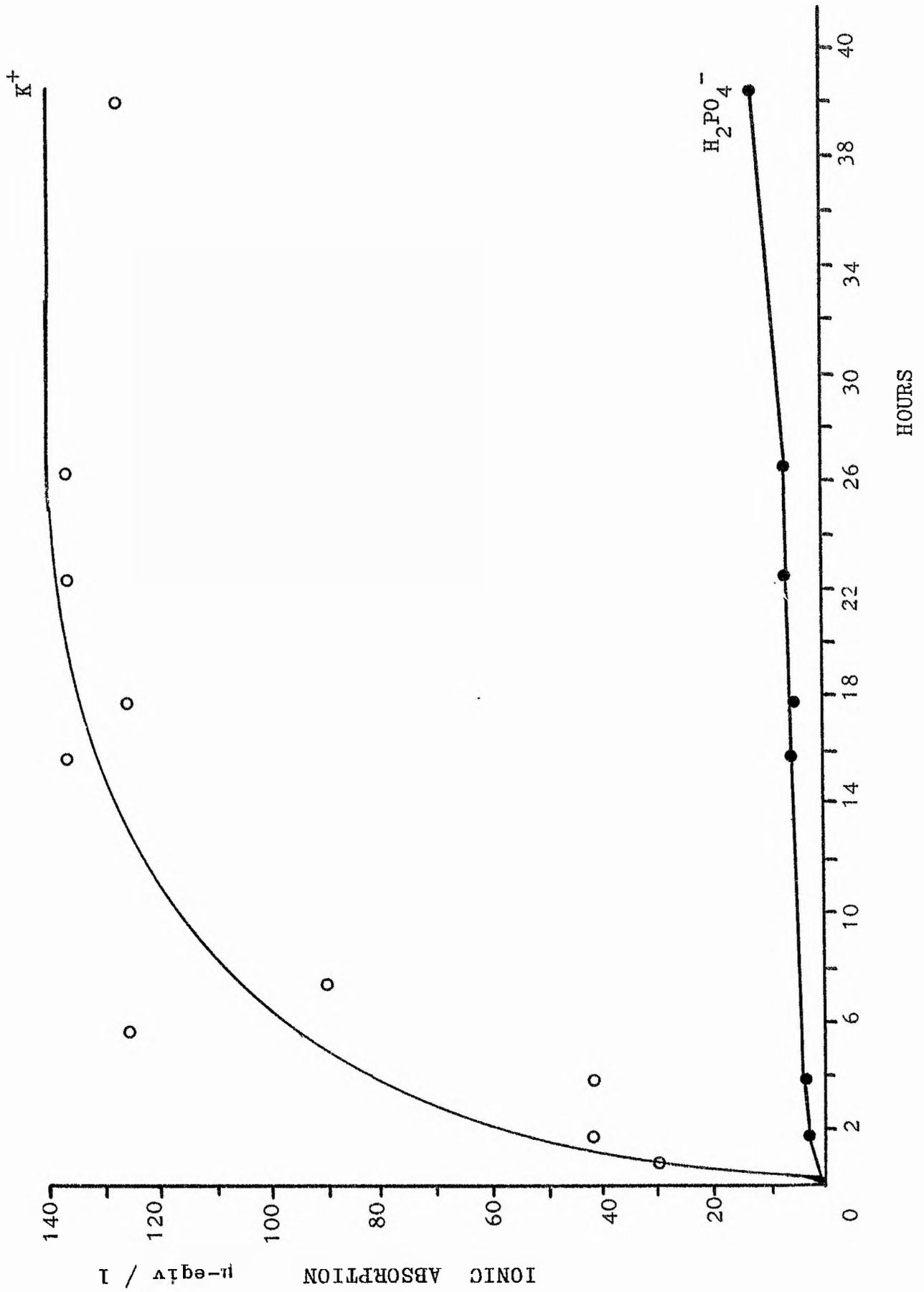
TABLE (1), 7

Time Interval	Ionic Concentration in $\mu\text{eq/l.}$		
	H_2PO_4^-	K^+	Na^+
4 hrs.	8.3	9.0	3.0
14 hrs.	6.25	2.0	4.0
$\frac{1}{2}$ hr.	0.64	0	2.0

Table showing concentration of ions in each change of deionized water.

GRAPH (1). 1

Total amount of phosphate and potassium ions
absorbed from pond-water by illuminated
shoots of P. schweinfurthii, plotted
against time.



As the total volume of each solution was 7 litres the cumulative loss of ions from 35g F.Wt. of tissue can be shown thus:

TABLE (1), 8

Cumulative Time. Hrs.	Total Ion Leaked from Tissue		
	H_2PO_4^-	K^+	Na^+
4	58.1 μeq	63.0 μeq	21.0 μeq
18	102.5 "	77.0 "	40.0 "
18.5	106.98 "	77.0 "	63.0 "

Table showing total amount of ions lost from the tissue in 3 consecutive changes of deionized water after being in pond water.

The tissue was then transferred to a solution of KH_2PO_4 and left for 13 hr. 15 min. Analysis of the solution at the beginning and end gave the following results:-

TABLE (1), 9

Time	Ionic Concentration in $\mu\text{eq}/\text{l.}$		
	H_2PO_4^-	K^+	Na^+
0	216	248	12
13 hr. 15 min.	200	190	8
Uptake /l.	16	58	4
Total Uptake / 7 l.	112 μeq	406 μeq	28 μeq

Table (1), 9 shows the ionic concentrations of the solution at the beginning and the end of an uptake experiment, and the calculated uptake of ions by 35g F.Wt. of tissue in 13 hr. 15 min.

On return to deionized water with three successive changes the loss of ions was:-

TABLE (1), 10

Cumulative Time. Hrs.	Total Ions Leaked from Tissue		
	$\text{H}_2\text{PO}_4^{--}$	K^+	Na^+
4.75	5.6 μeq	35 μeq	56 μeq
10.25	5.88 "	49 "	63 "
31.00	6.94 "	63 "	63 "

Table showing total amount of ions lost from the tissue in 3 consecutive changes of deionized water after being in KH_2PO_4 solution.

Finally, the tissue was placed in pond-water. The amount of ionic absorption with time is shown in graph (1), 1. Table (1) 11 below shows the original and final concentration of the ions in the pond-water after 40 hr. 20 min. of experimentation:-

TABLE (1), 11

Time	Ionic Concentration in $\mu\text{eq/l.}$		
	$\text{H}_2\text{PO}_4^{--}$	K^+	Na^+
0	23.6	1140	498
40 hr. 20 min.	10.8	1014	498
Uptake / l.	12.8	126	0
Total Uptake / 7 l.	89.6 μeq	882 μeq	0 μeq

Table showing ionic concentration of the pond-water before and after an uptake experiment, and the calculated uptake of ions by 35g F.Wt. of tissue in 40 hr. 20 min.

After the experiment several shoots were returned to a pond where they soon rooted and grew well.

Comments:-

If the data is retabulated the total net movement of ions in each section of the experiment can clearly be seen in Table (1), 12.

TABLE (1), 12

Experiment Section	Total Net Movement of Ions (μeq)		
	H_2PO_4^-	K^+	Na^+
Tissue from pond-water Leached in Deionized	106.98	77	63
Tissue after leaching in Deionized water; Uptake in KH_2PO_4 solution.	112	406	28
Tissue from KH_2PO_4 soln. Leached in Deionized	6.94	63	63
Tissue after leaching in Deionized water; Uptake in pond-water	89.6	882	0

Table showing net movement of ions when shoots of *P. schweinfurthii* were transferred from one solution to another.

On transferring the tissue from its original pond-water to deionized water, ions were lost from the tissue and reference to Table (1), 7 shows that by the third change of water the tissue was well leached.

This leached tissue absorbed more phosphate and potassium ions in $13\frac{1}{4}$ hours from a KH_2PO_4 solution than it had originally lost, but this could perhaps, be due to the diffusion of ions into the intercellular and water free spaces of the tissue. However, on transfer again to deionized water only 6.25% of the H_2PO_4^- ion and 15.5% of the K^+ ion were released

although Table (1), 10 shows that the tissue was nearly fully leached.

Finally, graph (1), 1 indicates that in the first 6 hours of the tissue being replaced in pond-water a rapid net uptake of K^+ took place followed by a greatly reduced rate; there was a slow steady uptake of phosphate throughout, and of sodium, none. A control tank with pond-water but no tissue showed no such decreases in ionic concentrations.

The evidence of these experiments would therefore suggest that there was ionic absorption and accumulation by the shoots of P. schweinfurthii in light, the absorption being mainly through the leaves, as the cut stem ends were sealed with vaseline. It is not suggested that the roots cannot absorb nutrients or that the leaves are the major absorption site of the plant, only that they are capable of absorption.

Whether nutrients that were absorbed by the leaves were transported in the conducting system to other parts of the plant was not established but this is investigated by using radio-isotope tracer techniques.

• • • •

Observations on uptake of P³² into roots and shoots
of P. schweinfurthii

3.6 mls High Specific activity phosphorus 32 in dilute hydrochloric acid was obtained from the Radiochemical Centre, Amersham.

This was used in the following series of experiments as a radioactive tracer.

Material and Methods:-

Healthy young P. schweinfurthii plants were carefully selected so that there was sufficient length of stem between roots and first leaves to be able to form a water-tight seal. These plants were washed in distilled water for half an hour.

A porcelain dish was modified so that a whole plant could have its roots in an isotope labelled solution whilst its shoots were in a non-labelled medium. A second plant was placed vice versa.

This was achieved by moulding a wax partition with 2 notches in it across the centre of the dish and the two plants were so arranged that the stem of each passed through a notch.

The notches were filled with silicon grease thus embedding

the stems so that the partition was completely water-tight, the shoots of one plant being one side and the roots the other.

For the isotope solution, 10.75 μ l of high specific active P^{32} was injected from a micropipette into 300 mls of 0.32 ueq/l KH_2PO_4 solution.

By calculation, correcting for decay, this gave a solution containing 0.0117 μ c/ml.

100 mls of isotope solution was poured into one section of the dish and an equal volume of non-isotope solution into the other.

As this was a pilot experiment where appropriate concentrations and suitable environmental conditions were unknown, two variables were taken into consideration. (1) light or dark, (2) the non-isotope solution being either the same PO_4^{3-} concentration as the isotope solution or being just distilled water.

Therefore three dishes containing six plants were arranged in the following manner:- (see Table (1), 13)

The experiment proceeded for 5 hours.

At the finish of the experiment 0.5 ml samples were taken from the non-isotope solutions just where the stems passed through the partition. These were placed on an aluminium planchet and allowed to evaporate. Similar samples were taken from the isotope solutions and from distilled water.

TABLE (1), 13

Dish No.	Soln. in Left Section of Dish	Soln. in Right Section of Dish	Treatment
1	Distilled water	0.32 ueq/litre $H_2P^*O_4^-$	Full Light
2	0.32 ueq/litre $H_2PO_4^-$	0.32 ueq/litre $H_2P^*O_4^-$	Full Light
3	Distilled water	0.32 ueq/litre $H_2P^*O_4^-$	Dark

Table of Isotope treatments of P. schweinfurthii

The planchets were subsequently placed in a lead castle containing an end window counter, and were counted using a Panax scaler and timer.

With both a background count (from the distilled water) and a count for the isotope solution, the samples from the non-isotope solutions would reveal whether there had been any significant leakage of isotope solution through the notches.

The plants were removed from the dishes, rinsed in three successive changes of deionized water and were laid out and pressed between blotting paper. Each internode and the stem just above the roots was cut with a razor before pressing so

that no subsequent translocation could take place.

Where the plant stem had passed through the partition it was noticeable that it had suffered slight damage and was turning brown.

After the plants had completely dried, an X-ray film was laid over them, exposed for a day and then processed to give an autoradiograph.

Result:-

Background count = 0.24 counts/ sec.

Isotope solution = 15.33 counts/sec.

Sample taken by notches:-

TABLE (1), 14

Dish No.	Notch	Counts/sec.
1	a	0.25
	b	0.23
2	a	0.75
	b	2.75
3	a	0.26
	b	-

Table showing amount of leakage of isotope through the notches

All samples were counted for 1000 seconds.

It is clear that no leakage of isotope had occurred through the notches in dishes 1 and 3 but some had occurred in dish 2 especially through one notch.

Autoradiograph:

The autoradiographs of the pressed plants showed that in all cases the part of the plant which had been in isotope solution was heavily labelled.

The plant tissues which were in the non-isotope solution of dishes 1 and 3 were not detectably labelled, but that in dish 2 showed traces of isotope.

Comment:-

No transport of isotope was detectable on the autoradiograph although absorption (or adsorption) in both the shoots and the roots had taken place.

It was observed that although the seal through the wax partition was, on most occasions, leak-proof, it had a detrimental effect on the stem at the point of contact. The damage appeared to be only slight and mainly affected the epidermal cells but if the experiment had continued longer general rotting at that place might have occurred.

Detection of Translocation in *P. schweinfurthii* using
Labelled Phosphorus.

The criticism of the previous experiment can be overcome by labelling a portion of the plant for a short interval only and then leaving the plant for the duration of the experiment in isotope-free solution.

Material and Methods :-

Fresh young plants of *P. schweinfurthii* with healthy roots and shoots were collected from the culture pond.

Two solutions of 0.32 $\mu\text{eq/l}$ $\text{K H}_2\text{PO}_4$ were prepared, one of which was labelled with p-^{32} at 0.01 $\mu\text{c/ml}$.

The plants were first rinsed in deionized water twice. In the treatment an attached section of the plant (either an individual leaf or the roots) was placed in the labelled phosphate solution for a given period of time whilst the rest of the plant remained in water-saturated air. After treatment the section was removed and rinsed 3 times in deionized water to wash off any adhering isotope solution. The whole plant was then transferred to a non-isotope phosphate solution of the same concentration and illuminated for the duration of the experiment. On completion, the plants were rinsed, cut, pressed and dried and autoradiographs were made. Final counts were

made on each of the isotope-free solutions to see if any significant amount of isotope had leaked out of the plant.

Results:-

TABLE (1), 15

Treated Tissue	Time Interval in Isotope K H ₂ P [*] O ₄ Minutes	Time Interval in K H ₂ PO ₄ Hours	Result
Root	5	5	Strong Fogging No movement.
Leaf	5	5	Very slight Fogging No movement.
Root	5	9	Strong Fogging No movement.
Leaf	5	9	Very slight Fogging No movement.
Root	10	9	Very strong Fogging No movement.
Shoot	10	9	Slight Fogging No movement.
Root	5	0	Strong Fogging
Shoot	5	0	Slight Fogging

Table showing Autoradiographic results from labelling
Plant tissue.

The intensity of fogging relates to that section of the X-ray plate which was directly over the region of tissue which had been in the isotope solution. As no other parts of the plates were fogged it was assumed that the isotope had not moved from that region of absorption.

Comment:-

The results suggest that absorption into the roots was rapid but into the leaves was slow.

There was no evidence that there was any translocation of isotope within the plant even 9 hours after treatment.

Absorption and Translocation of P³² from Pond-Water by

P. schweinfurthii

The lack of observable translocation can be interpreted in either one of two ways. Either translocation does not occur in P. schweinfurthii or experimental conditions were unfavourable for it.

Considering the latter, there is no doubt that conditions were unnatural, for the plant was removed from a rich nutrient pond-water and was placed in a very dilute solution of K H₂PO₄ .

The use of labelled and unlabelled filtered pond-water, therefore instead of $K H_2PO_4$ solution would overcome this criticism.

Material and Methods:-

One large healthy plant of P. schweinfurthii was dug up and carefully washed in pond-water. It had one good thick runner 80 cm long and several shorter ones: long healthy roots were growing from the nodes and well developed shoots were growing from alternate nodes.

With much care, to avoid buckling the runners or shoots, the plant was placed in a large glass aquarium (18" x 36" x 18" deep) containing filtered pond-water.

500 mls of the same pond-water was activated with P^{32} and transferred to two 250 ml beakers. These were lowered into the tank at opposite ends so that the water level in the tank was approximately equal to that in the beaker.

The plant was then arranged so that one leaf was suspended in one beaker of labelled pond-water and a mass of roots at the other end of the runner was suspended in the other beaker. By this means the minimum amount of stem or leaf was exposed to the atmosphere. These exposed regions were covered as much as possible with moistened tissue paper, to avoid desiccation.

The plant was left for 8 hours under tungsten lamps producing 3 Kilowatts.

After 8 hours the plant was removed, washed, cut and pressed and autoradiographs were made.

Results:-

Both the leaf and the roots which were in isotope solution were heavily labelled. The growing leaves above and below the labelled leaf, and the shoot apex were labelled, but the mature leaves were not. The intensity of labelling tended to decrease with distance from the labelled leaf but all nodes on the rhizome and the growing points of the off-spring showed labelling.

No roots except those immersed in isotope solution showed any labelling.

Comments:-

8 hours was considered an appropriate time to be able to observe whether the isotope absorbed was transported from the leaf and/or roots. Unfortunately the exposure time was too long and isotope had moved almost all around the plant. However the intensity of fogging of the plant was greatest in those regions nearest the treated leaf which would strongly suggest

that the major part of the isotope distribution in the plant came from the labelled leaf. A contribution however could have come from the root also.

The possibility that the isotope solution contaminated the rest of the pond-water may be discarded, for if this had occurred, all parts of the plant would have been labelled equally, not just the growing areas.

It is interesting that the roots showed no labelling at all even though they appeared healthy and were presumably growing. This is difficult to interpret without further information. It could be that by digging them up from the mud they were damaged and were therefore not functioning normally. Or perhaps there is no transport system into the roots from the rhizome so that the roots must obtain their nutrient supply from the surrounding medium.

Discussion and Conclusion

Chemical evidence strongly supports the view that leaves are able to absorb nutrients from the surrounding medium but root absorption was not established.

In a nutrient-rich solution floating-leaved P. richardi plants growing in a nutrient-rich substrate as compared with a nutrient-poor substrate grew considerably better, whereas

submerged-leaved P. schweinfurthii plants did not appear to show such a difference. When P. hybrid (mainly a floating-leaved variety) and P. schweinfurthii were grown in the same pond and the same substrate, either in pots or in free substrate, the growth of P. hybrid in pots was inhibited whereas that of P. schweinfurthii was virtually unaffected.

The inference from these results is that whilst both the species probably absorb some nutrients through both their leaves and their roots, the roots are much more important for this function in the floating-leaved types than the submerged-leaved types.

With the aid of radioisotope tracer techniques it appears that both leaves and roots absorb labelled phosphate from the surrounding medium, the roots absorbing more quickly than the leaves. There is the possibility, especially in the case of the roots, that the isotope was adsorbed onto the cells but this could not be investigated.

By labelling only one leaf and a few roots of a large intact plant it was shown that the isotope after 8 hours became generally distributed through all growing regions of the plant except the roots.

The variation of intensity of fogging on the autoradiographs would suggest that the major fraction of the distributed isotope

had come from the labelled leaf rather than the labelled roots. If this is the case it must be assumed that not only are leaves able to absorb at least some nutrients but also that the nutrients are translocated away from the leaves, up and down the plant, to regions where they are required.

Whether the roots also function in this manner is not known.

CHAPTER II

THE IONIC BALANCE BETWEEN LEAF
TISSUE AND BATHING MEDIUM WITH
REFERENCE TO THE ELECTROCHEMICAL
POTENTIAL GRADIENT.

Introduction

Having established that under some conditions ions are absorbed by and transported away from the leaves of *Potamogeton*, the mechanism controlling these movements can now be studied.

This is investigated at the cellular level by asking the question: what are the forces acting upon an ionic species which cause the ions to move through the cell membranes?

Using *Nitellopsis obtusa*, MacRobbie and Dainty (1958 a) were amongst the first to enquire into this in plants and it is their approach which forms the foundation for this chapter.

If it can be shown that the ions move by diffusion across the cell membrane along an electrochemical gradient then the process can be considered passive but if they move against the electrochemical gradient then energy must be expended by the plant and the process must be active transport. The same reasoning is applied to molecules but then only the chemical gradient is pertinent.

For a particular ionic species diffusing passively it is possible to calculate the expected electrical potential difference between the inside and the outside of a cell, if the chemical concentrations inside and outside for that ion are known and if that ion has come to flux equilibrium across the cell membrane.

If the calculated potential is equal to the actual measured potential then the ionic distribution inside and out would be passive.

Rephrasing the above, it can be stated that a passively diffusing ion which is in flux equilibrium on the two sides of a membrane has no net driving force acting upon it. Thus, for that ion there is no difference in electrochemical potential between the solutions. (see Dainty 1962 p. 381-382)

$$\text{i.e. } \bar{\mu}^i = \bar{\mu}^o$$

where $\bar{\mu}$ is the electrochemical potential for an ion species j , and i is inside a cell (or one side of a membrane) and o is outside a cell (or the other side of a membrane).

Let chemical potential = μ equiv/ml

Electrical potential = ψ volts

Valance of ion = z

The Faraday = F 96,500 coulombs/equiv

Chemical activity = a equiv/ml

Gas constant = R 8.3 joules/°C/equiv

Temperature absolute = T 273 + °C

Now, electrochemical potential is the sum of the electrical and chemical potential for an ion

$$\text{i.e. } \bar{\mu} = \mu_j + z_j F \psi$$

$$\text{and } \mu_j = R T \ln a_j$$

Therefore substituting in $\bar{\mu}_j^o = \bar{\mu}_j^i$

$$\begin{aligned} RT \ln a_j^o + z_j F \psi^o &= RT \ln a_j^i + z_j F \psi^i \\ \therefore \psi^i - \psi^o &= \frac{RT}{z_j F} \ln \frac{a_j^o}{a_j^i} \quad \text{mv} \end{aligned}$$

Let $\psi^i - \psi^o$, the electrical potential difference between inside and outside equal E and assume that the chemical activity is approximately equal to the chemical concentration C .

Then for ionic species j , $E_j = \frac{RT}{z_j F} \ln \frac{C_j^o}{C_j^i} \quad \text{mv}$

Where C_j^o is the concentration of ion j in the external medium and where C_j^i is the concentration of ion j in the cell

If the temperature is 25°C and the ion is univalent then

$$E_j = 59 \log_{10} \frac{C_j^o}{C_j^i} \quad \text{mv}$$

This is known as the Nernst Equation.

Clearly, it can be seen that E_j the electrical potential difference between the solutions on either side of the membrane, can be calculated from the chemical concentrations of a particular ion j which moves passively to equilibrium in the two solutions.

If an electrode is placed in each solution and the circuit completed by joining the terminals to a millivoltmeter drawing no electrical current the potential difference measured, which is the membrane potential E_m , will be equal to the calculated

potential difference, E_j .

If the system is not in equilibrium for a particular ion then,

$$\bar{u}^o \neq \bar{u}^i$$

and E_j will not equal E_m .

If, when a cell is in flux equilibrium with its external medium, i.e. when net flux = 0

$$E_j \neq E_m$$

then there must be an active process, (an ionic pump) for the transfer of that ion from one side of the membrane to the other.

MacRobbie and Dainty (1958 a) exploited this principle. Using radioisotope techniques, they studied distribution, rates of exchange and permeabilities of ions in single cells of Nitellopsis obtusa. They then calculated E_j for Na^+ , K^+ and Cl^- ions by substituting in the Nernst equation $\frac{C^o}{C^i}$ for each ion.

From their isotope results they demonstrated that the net flux of each ion between external solution and cell sap was zero. Hence for any ion moving passively through the membrane its calculated electrical potential should be equal to the membrane potential.

The measured potential difference between vacuole and external solution was -120mv and the calculated potentials

$$E_{\text{Na}} = -15 \text{ mv}, E_{\text{K}} = -130 \text{ mv} \text{ and } E_{\text{Cl}} = +45 \text{ mv}$$

They concluded that while the potassium in the vacuole was in

electrochemical equilibrium with the external potassium, since $E_k \approx E_m$, the sodium and the chloride were very far from their passive equilibrium distribution. Therefore sodium and chloride must be undergoing active transport sodium being transported out and chloride inwards.

A similar approach was used in the present set of experiments on Potamogeton. After measuring the concentrations of various ions inside the cell and in the external solution, values for E_j were calculated. By inserting a micro-electrode into single cells of a leaf bathed in the same external solution the potential difference across the membrane E_m was then measured. There are, however, some major differences between the work of Dainty and MacRobbie and the present study owing to the relatively large size of the cells of Nitellopsis as compared with Potamogeton.

Dainty and MacRobbie isolated single cells of Nitellopsis and maintained them in a healthy condition. Then, as the cells are large (4 - 10 cm long and 500 - 800 μ diameter), they were able to extract the entire sap, measure its volume (usually from 5 - 10 μ l) and accurately determine the concentrations of K^+ , Na^+ and Cl^- . A distinct boundary between protoplasm and vacuole could be observed under the microscope and the microelectrode could be inserted at will into either of these two regions.

The cells in the leaves of Potamogeton are part of a more complex tissue than those of Nitellopsis and in the present study it was not possible to isolate them. The small size of the cells, those of the epidermis being approximately 50 μ across, made it impossible to measure single cell volumes and ionic contents by MacRobbie and Dainty's method. Since no clear boundary between vacuole and protoplasm was visible, even the successful insertion of a microelectrode into a cell involved considerable chance.

Although direct chemical analysis of cell contents was impossible, various attempts (described below) were made to obtain an accurate assessment of the ionic concentrations within the cell.

For convenience this section is subdivided into two parts; the first part covers the determination of ionic concentrations in the cells and bathing solutions and thus the calculation of E_j 's from the Nernst Equation; the second part covers the measurement of membrane potential of the cells, followed by a general discussion and conclusions.

PART IDETERMINATION OF IONIC CONCENTRATIONS WITH CELLS

The principle underlying this determination is that: assuming all the cells in the leaf tissue have more or less the same ionic composition, then from the chemical analysis of tissue the vacuolar and protoplasmic composition can be extrapolated.

For the determination of this composition from the chemical composition of the whole tissue two adjustments have to be made.

First, the results of total digestion of tissue followed by the determination of various ions in the solution are usually expressed as milliequivalents per unit dry weight tissue. However, if the water content of the tissue is known they can be expressed as milliequivalents per unit volume tissue water. If it is assumed that all the ions are in solution then this would be an indication of the concentration of ions in the cell sap,

Second, the ions will not be evenly distributed throughout the tissue water but will be in at least three compartments; the vacuole, the protoplasmic non-free space and the Apparent Free Space (A.F.S.) including cell wall.

The cell sap volume, or Apparent Osmotic Volume (A.O.V.) was considered as the total volume of tissue water minus that in the A.F.S. Therefore in order to obtain cell sap contents, the A.F.S. must be determined and the appropriate adjustment made.

A. Determination of water content of Leaves

Leaves of Potamogeton were carefully blotted and weighed. They were then transferred to a drying oven at 105°C for 24 hours, cooled in a desiccator, reweighed and placed for a further 24 hours in the desiccator. A final weighing was then taken to confirm that the tissue had been completely dried.

Results:-

TABLE (2), 1

Fresh Weight	Dry Weight	Diff.	% H ₂ O
1.2855	0.1978	1.0877	84.6
0.9362	0.1642	0.7720	82.5

Table showing calculation for % water in tissue.

The result is not critical for the present purposes and 85% was taken as the approximate water content of the tissue.

B. Determination of Ionic Contents in the A.F.S. and A.O.V. Phases of Potamogeton Schweinfurthii Leaves.

In their ionic studies on Rhodymenia and Nitellopsis, MacRobbie and Dainty (1958 a, 1958 b) equilibrated cells with an external isotope solution in order to label the internal ion contents and then proceeded to 'wash out' the labelled ions.

The washing out was achieved by transferring single cells at definite time intervals from the isotope solution through a series of dishes containing non-isotope solution. By subsequently counting the solution in each dish, the logarithm of the rate of loss of radioactivity from the cell could be plotted against time; a plot in which a uniform exchange of labelled with non-labelled ions in a single system gives a straight line.

Plotting the results from Nitellopsis produced three consecutive straight lines each with a different slope. From this MacRobbie and Dainty recognised three compartments in the cell each with a distinct efflux rate i.e. the A.F.S., the protoplasmic non-free space and the vacuole.

The same principle, in which rate of exchange of ions between inside and outside of the cell is plotted against time is the basis of the following series of experiments, but isotopes were not available. Instead therefore, solutions of weakly toxic

reagents were used in attempts to elute those cellular ions in the A.F.S. sufficiently faster than those in the A.O.V., to allow kinetic discrimination between the ionic contents of these phases of the cells.

Weighed samples of fresh tissue were eluted for known time periods with successive aliquots of reagent and each aliquot was then analysed for eluted ions. The total amounts of each ion per sample were added together and the \log_{10} of the sum was plotted against time. For each kind of ion a graph of two or more straight lines of differing slope would be expected if:

a) elution from the A.F.S. was much easier than from the A.O.V. and b) the rates of elution of that ion from each phase depended on the amounts of ion remaining to be eluted. That is, elution was expected to be exponential, with rate constants differing significantly for each phase of the cell. Also, the ratio of the \log_{10} sum total amounts for any two ions from one phase would produce a straight line when plotted against time, thus:- $\sum \log_{10} \frac{K}{Na}$ at time $t_0 + t_1 + t_2$ etc. where K and Na are concentrations of ions in eluent at different time intervals.

If there are two phases and the ratio of ionic concentrations of the two ions is similar, even though their absolute concentrations might have altered, no change would be observed in the \log_{10} ionic ratio progress curves. But if the ratios of

concentrations or permeabilities to elution should differ for the two ions between the two phases, then the ratio of the two ions in the second phase will be different. Therefore, from continuous elution the ionic ratios may be seen to alter at the beginning of a new phase. Plotting the \log_{10} ionic ratios against time would be expected to produce, in these circumstances, a change of slope at the onset of a new phase.

Where a cell cation is partly in free solution and partly electrically adsorbed to insoluble anionic cell constituents, the free cations may be eluted faster than the bound cations owing to consumption of the entering eluent by adsorption. Thus for cell wall protoplasm phases the strongly adsorbed calcium ion might be eluted more slowly than the predominantly free potassium ion. This could possibly confuse the distinctions between cell phases interpreted from the elution method described here.

This approach was experimentally tested, using dilute $\text{Zn}(\text{NO}_3)_2$ and acetic acid as weakly toxic eluents intended to displace from the cell both adsorbed ions and those in free solution in each phase.

* * * *

Estimation of Ionic Concentration in Different
Phases of the Cell using $\frac{N}{10} \text{Zn}(\text{NO}_3)_2$.

Method:-

Freshly cut leaves of Potamogeton were quickly dipped in distilled water and then blotted to remove the surplus pond-water. After blotting they were weighed and transferred to a 250 ml separating funnel, 50 mls $\frac{N}{10} \text{Zn}(\text{NO}_3)_2$ was added and the funnel shaken. At given intervals the solution was run out for analysis and was replaced by a fresh 50 ml of extract solution. At first, samples were taken every few minutes but over the course of 42 hours the intervals became progressively longer, 20 samples being taken in all.

K^+ , Na^+ and Ca^{++} determinations (by flame photometry) were subsequently carried out on each sample and on the original pond-water. (Colorimetric method for Ca^{++} could not be used as Zn interfered).

For each ion a progress curve for \log_{10} total amount extracted was plotted against time (see graph (2), 1). Also $\log \frac{\text{K}}{\text{Na}}, \frac{\text{Ca}}{\text{K}}$ and $\log \frac{\text{Ca}}{\text{Na}}$ progress curves were plotted against time (see graph (2), 2)

Simultaneously with this extraction, a second funnel containing leaves which had been leached in many changes of deionized water for 4 days and pH-controlled deionized for 3 days

(see Ch. 3 pp109-110), was being treated in a similar manner.

Results:-

In both the leached and the fresh material the shape of the curves are similar and in many respects unsatisfactory.

Comments:-

Neither the plots of \log_{10} total amounts against time (graph (2), 1) nor those of \log_{10} ratios for two ions (graph (2), 2) showed clearly distinct phases. The calcium curve suggested two phases but the first phase was so quick (within the first minute) that there was only one point on the curve at that stage.

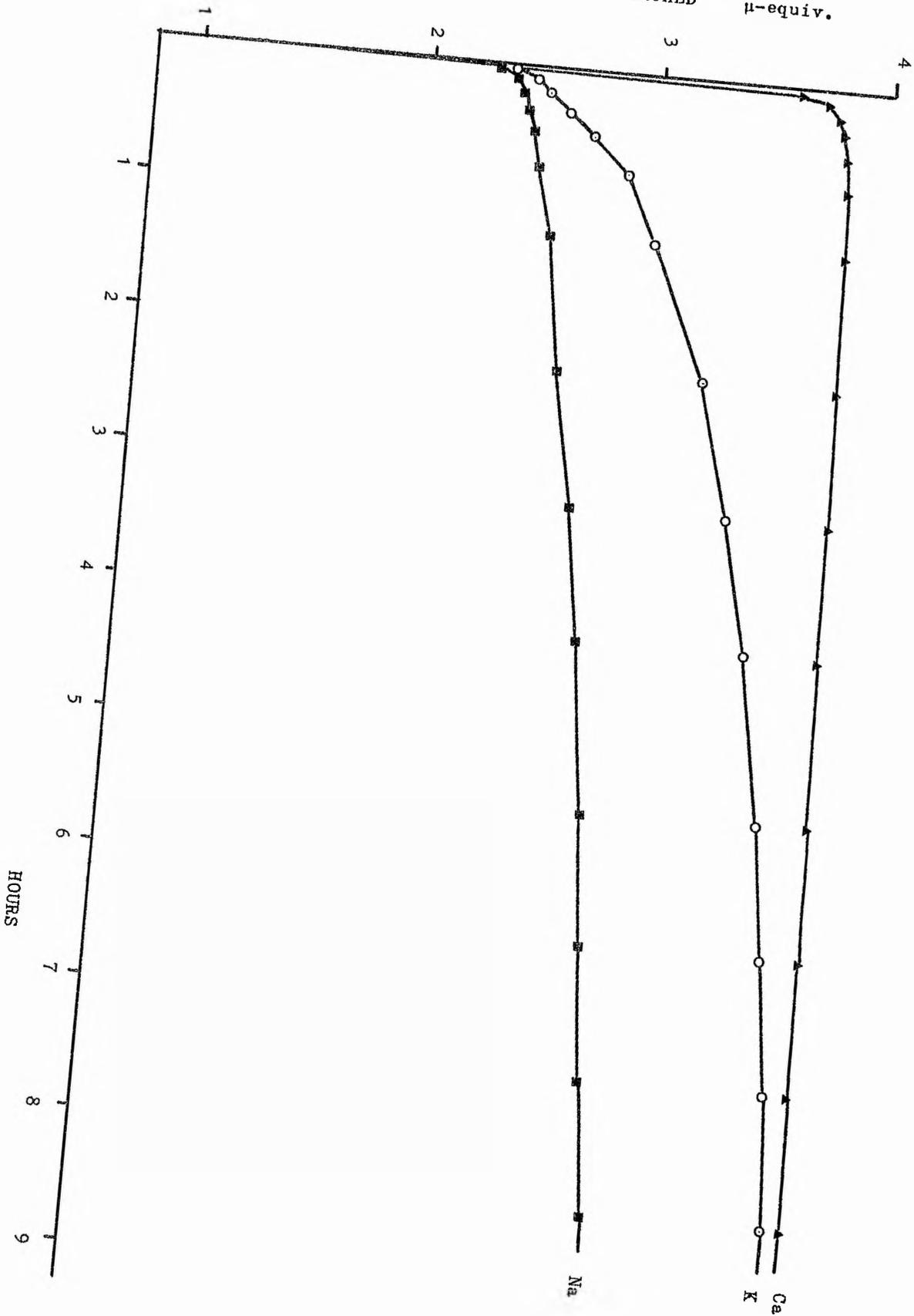
Clearly, some modifications had to be made in order to get better separation of the phases and the experiment was therefore modified.

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GRAPH (2), 1

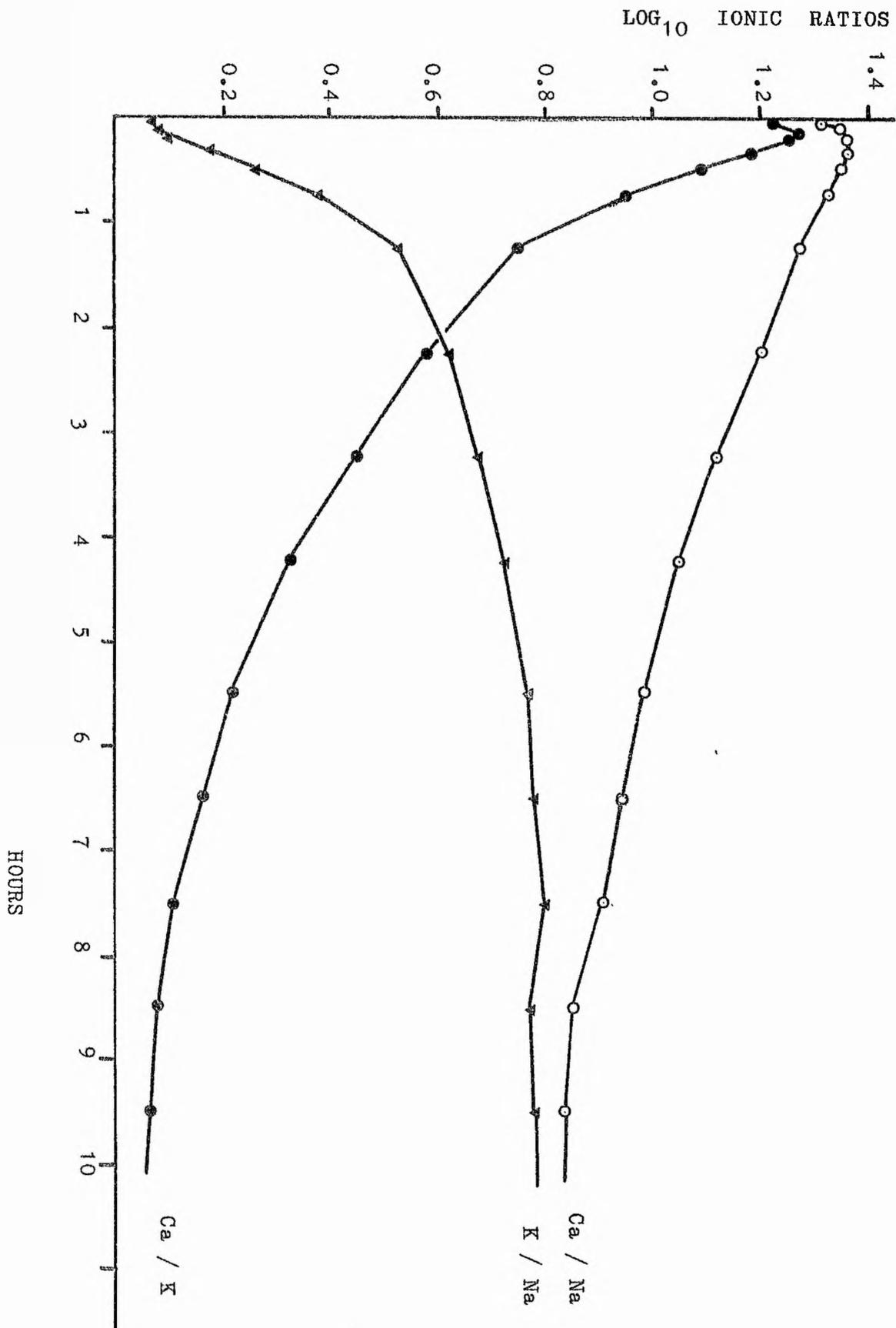
Log progress total amounts of Calcium,
potassium and sodium ions leached from
P. schweinfurthii tissue when placed in
 $\frac{N}{10}$ zinc nitrate extraction solution,
plotted against time.

TOTAL IONS LEACHED μ -equiv.



GRAPH (2), 2

Results from graph (2), 1, plotted against
time as log ratios; $\log \frac{\text{Ca}}{\text{K}}$, $\log \frac{\text{K}}{\text{Na}}$ and
 $\log \frac{\text{Ca}}{\text{Na}}$.



Repeat Experiment of Estimation of Ionic Concentration
in Different Phases of the Cell using $\frac{N}{10} \text{Zn}(\text{NO}_3)_2$.

Method:-

This experiment was conducted in the same manner as the previous one but (1) the leaves had the midribs removed and the lamina only was returned to pond-water for a further 24 hours before extraction; (2) 50 ml samples were taken more frequently in the first stages of extraction and; (3) after 48 hours of extraction (27 samples) five further extractions in 50 ml boiling $\text{Zn}(\text{NO}_3)_2$ were obtained.

The samples were analysed for the following ions: Na^+ , K^+ , Ca^{++} , Cl^- and phosphate, and the results treated as before.

Results:-

(see graph (2), 3).

Comments:-

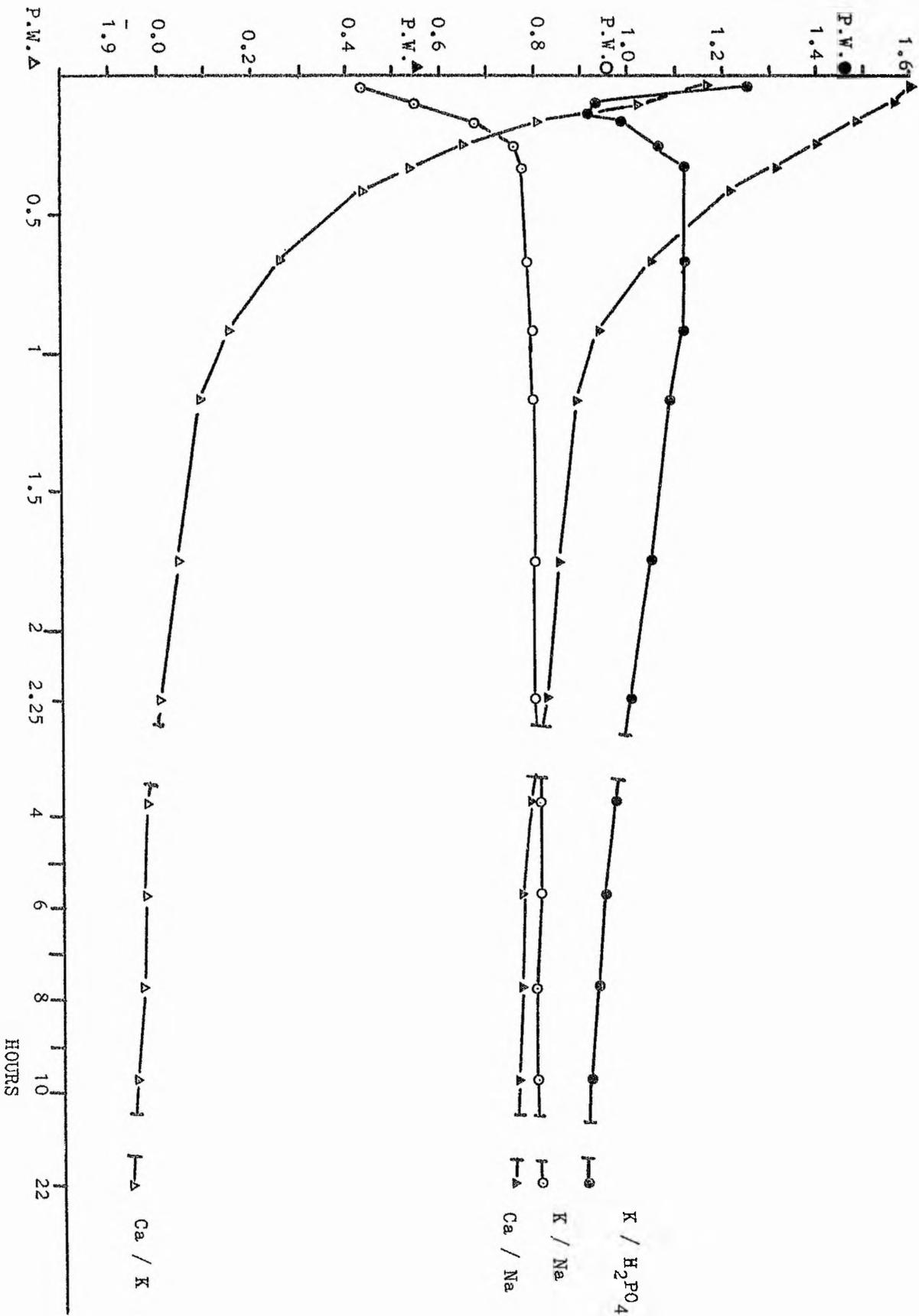
On close inspection the graphs were no improvement on the previous ones. Certainly, different phases (especially when the ratios were plotted) were apparent but the overlap between phases was so great that it was impossible to extrapolate the

GRAPH (2), 3

Log ratios; $\log \frac{K}{H_2PO_4}$, $\log \frac{K}{Na}$, $\log \frac{Ca}{Na}$,

$\log \frac{Ca}{K}$ progress curves of ions leached
from P. schweinfurthii shoots when placed
in $\frac{N}{10}$ zinc nitrate extraction solution,
plotted against time.

LOG₁₀ IONIC RATIOS



line and assume change in phase.

The ratios of ions in pond-water would be expected to be the same as those in the Water Free Space (W.F.S.) but even the first sample (after 2 minutes) was frequently very different from that of pond-water which suggests the W.F.S. phase had been missed.

By the second boiling no ions were detectable in the sample, therefore, at this stage the cell contents must have been completely extracted.

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Extraction of Cell Sap with 0.02 N Acetic Acid and
Constant Flow Stirring

Perhaps the inadequate stirring caused the indistinct phase separation in the last experiment, therefore in this experiment a totally different stirring procedure was used.

Secondly, extractions tried by Dr. D. Weeks on Pistia roots indicated that dilute acetic acid might be more suitable than zinc nitrate. As this had the added advantage that Ca^{++} and Mg^{++} could be estimated colorimetrically rather than by flame analysis a 0.02 N acetic acid extraction solution was used.

Method:-

Leaf tissue was stripped of its midribs 2 hours before use and kept in aerated pond-water until required.

The apparatus was so designed that the tissue, when placed in a vertical glass chamber, was just covered with 50 mls of extracting solution. The solution was continually percolated through the tissue mass by being withdrawn at the bottom of the chamber and being circulated by a pump back into the top of the chamber. The rate of flow through the pump was approximately 200 mls/minute.

When a sample was required the drain tap was opened at the bottom and compressed air was forced through the system thus emptying the apparatus of solution in 10 seconds. The apparatus was then refilled with a further 50 mls of extraction solution from the graduated funnel at the top.

Result and Comment:-

The results of this experiment were similar to the previous ones in that the phases were not clearly defined.

General Comments on Estimation of Ionic Contents of the Cells

For success, this method depends upon rapid permeation of eluent throughout the tissue, slow progressive toxicity of the

eluent, and a rapid and thorough stirring of the extraction medium to carry away immediately all ions that leak out. The elution rates are then controlled by the permeability of the contents from a certain phase rather than by their slow diffusion away from the cell into the extracting medium.

The poor results were first suspected of being a function of inadequate stirring but with even more efficient stirring there was no noticeable improvement. Could there be another reason?

Whereas in single cells the diffusion pathway for ions inside to outside is virtually constant, ions from cells in the centre of tissues will have a much longer diffusion pathway into the external medium than those from epidermal cells. Even with fast percolation of extractant over the tissue it appears that there is mixing of different phases from different cells, a result which rather than being due to inadequate stirring could be explained by slow penetration of the extractant into the interstitial spaces and inner cells.

This could probably be overcome if one was able to strip the epidermis or separate cells more without too much damage but no method was found satisfactory.

Without better separation of the phases it was impossible to extrapolate the amount of a particular ion in any one phase as a percentage of the total amount of that ion in the tissue.

C. Determination of A.F.S. Using Mannitol

Several workers have been able to measure A.F.S. of tissue by placing it in a solution of high density which is harmless to the plant and which does not penetrate into the cell. From the density of the solution and from the increase in weight of the tissue the amount of solution (and thus the volume of the A.F.S.) can be calculated.

One of the more common solutions used is sucrose but in some cases sucrose is metabolised by the plant and thus gives a false result. Mannitol, perhaps, is more satisfactory. The method outlined in Briggs, Hope and Robertson (1961 pp. 79-81) was therefore used to estimate A.F.S. in leaves of Potamogeton.

Method:-

Whole leaves of Potamogeton schweinfurthii float in water but if the central vein is removed the remaining tissue sinks. Leaf tissue stripped of the midrib was therefore used. The weighing pan of a balance (sensitivity 0.1 mgs) was modified for weighing stripped leaf tissue under water. The tissue was weighed in air and then placed on the pan under water in the dark until the weight became constant. The water was then exchanged for a 0.25 M mannitol solution (s.g. 1.015 at 25°C)

and the increase in the weight of tissue with time was noted. A graph was plotted of the weight of tissue in water, and in mannitol, against time.

Results:- (see graph (2), 4.

The mannitol curve was extrapolated back to time zero; i.e. when water was exchanged for mannitol.

Weight of tissue in air	= 1.0862 g
Weight of tissue in water	= 0.241 g
Final wt. of tissue in mannitol	= 0.259 g
Density of mannitol	= 1.015

The final volume of tissue in mannitol

$$= \frac{\text{Weight air} - \text{weight in mannitol}}{\text{Density mannitol}}$$

$$= 1.28$$

Apparent Osmotic Vol.

$$= \frac{\text{Wt. in water} - \text{wt. in mannitol}}{\text{Density mannitol} - \text{Density water}}$$

$$\% \text{ A.O.V.} = \frac{\text{Vol. of tissue in soln.}}{\text{A.O.V.}} \times 100\%$$

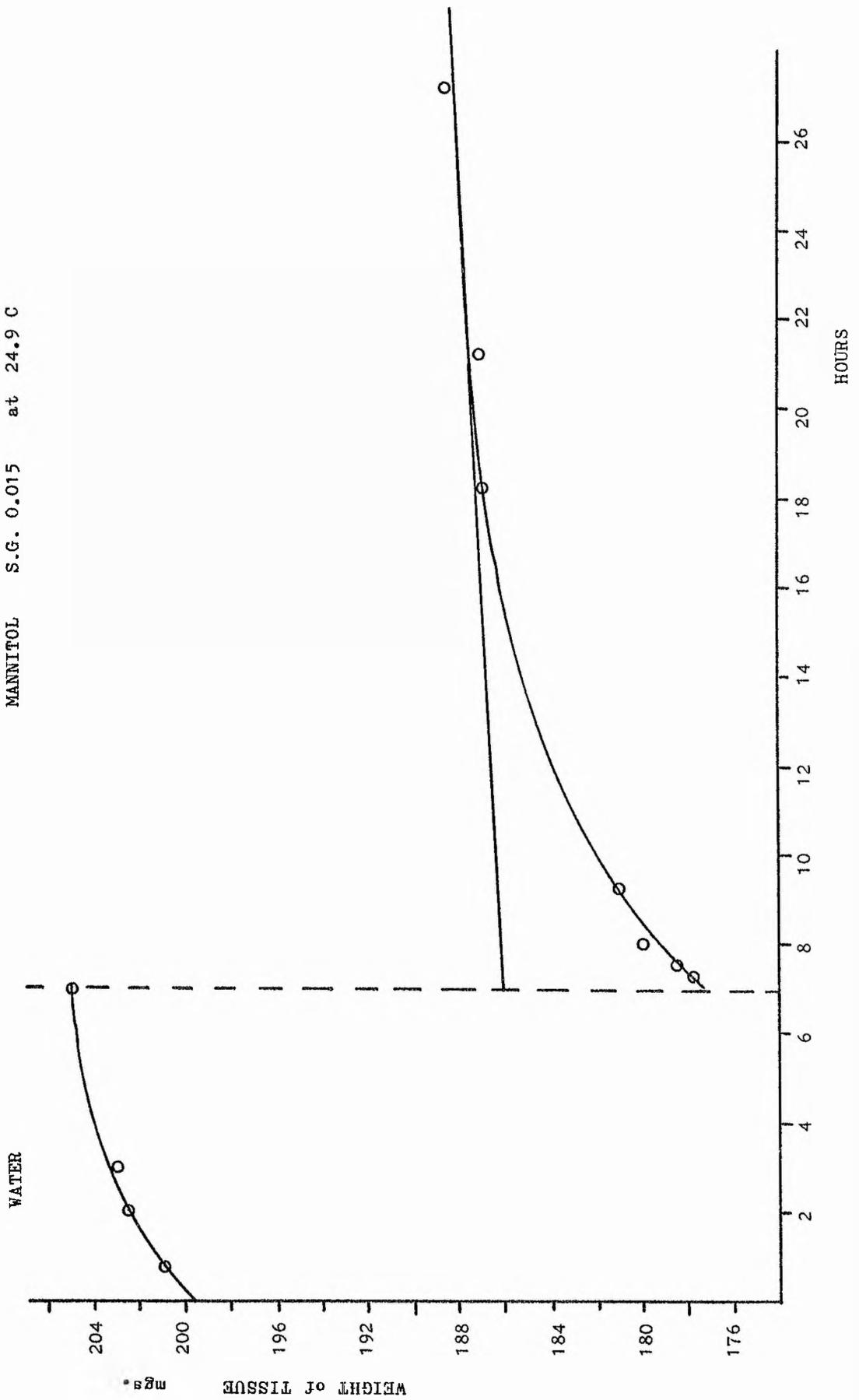
$$= 78\%$$

$$\therefore \text{A.F.S.} = 22\%$$

GRAPH (2), 4

Weight of P. schweinfurthii leaf laminae
in water and in mannitol (s.g. 0.015),
plotted against time.

MANNITOL S.G. 0.015 at 24.9 C



The experiment was repeated in exactly the same manner and the result was once again 22% but this seemed rather high as the estimated A.F.S. for Vallisneria is between 5% and 8% (Kylin 1957). An attempt was therefore made to fill the free space with mannitol first and then to measure chemically the amount of mannitol leached out when the tissue was returned to water. The method for chemical estimation of mannitol was that proposed by S.A. Barker outlined in Paech and Tracey's 'Modern Methods of Plant Analysis' (1955, vol II pp 55-63). This however produced no results as the concentrations of mannitol leached out were below those for chemical detection.

D. Estimation of E_j For Cells in Potamogeton
schweinfurthii

Having obtained the water content of the leaf tissue and an approximate value for the A.F.S. we are now in a position to estimate the concentration of ions within the cells and hence obtain E_j values.

Estimation of ions per unit fresh weight leaf tissue was obtained by the following different methods.

(i) From the zinc nitrate and acetic acid time course

extractions data mentioned previously.

- (ii) Total digestion in hot concentrated Nitric Acid.
- (iii) Killing tissue in a little ether and then extracting in cold $\frac{N}{10}$ Zn (NO₃)₂.

In each case the pond-water from which the tissue came was analysed immediately prior to removal of the tissue. The determinations were by the following methods given in full in the appendix pp i-xix.

Na ⁺ K ⁺	Flame photometry
Ca ⁺⁺	Flame photometry or Colorimetric, with murexide indicator and E.D.T.A. as titrant.
Mg ⁺⁺	Colorimetric by difference (Ca ⁺⁺ + Mg ⁺⁺) using eriochrome black T as indicator with E.D.T.A. as titrant.
H ₂ PO ₄ ⁻	Colorimetric, using Phosphomolybdic acid.
Cl ⁻	Electrochemical, with silver nitrate as titrant.
SO ₄ ^{- -}	Barium precipitation; volumetric estimation of BaSO ₄ using E.D.T.A. solution and solochrome black indicator for back-titration.

All results were firstly expressed in μ eq/g fresh weight tissue.

Assuming the water content of the tissue to be 85% the above can be expressed as μ eq/ml cell solution by the following equation:

TABLE (2), 2

Date	ueq/g FW	meq/l C ¹	meq/l C ⁰	$\frac{C^0}{C^1}$	$\log \frac{C^0}{C^1}$	E _j	Ion
7th Aug	100.8	152	0.46	3.6×10^{-3}	$\bar{3}.557$	-144	K ⁺
13th Aug	173.3	261	0.55	2.5×10^{-3}	$\bar{3}.398$	-153.5	
28th Aug	173.7	262	0.55	2.5×10^{-3}	$\bar{3}.398$	-153.5	
17th Sept	140.8	212	0.37	2.08×10^{-3}	$\bar{3}.316$	-158.0	
4th Sept	138.8	209	0.45	2.56×10^{-3}	$\bar{3}.408$	-153.0	
4th Sept	159.6	241	0.45	2.26×10^{-3}	$\bar{3}.35$	-156.0	
9th Nov	156.0	253	0.26	1.2×10^{-3}	$\bar{3}.08$	-172.0	
17th Nov	165.7	250	0.25	1.09×10^{-3}	$\bar{3}.04$	-175.0	
17th Nov	172.7	261	0.25	1.05×10^{-3}	$\bar{3}.021$	-175.8	
7th Aug	12.6	19	0.68	42.7×10^{-3}	$\bar{2}.63$	-80.8 X	Na ⁺
13th Aug	19.3	29	0.55	22.5×10^{-3}	$\bar{2}.35$	-97.0	
28th Aug	28.3	43	0.55	15.4×10^{-3}	$\bar{2}.187$	-107	
17th Sept	19.55	29	0.39	15.8×10^{-3}	$\bar{2}.199$	-106	
4th Sept	24.9	38	0.53	16.8×10^{-3}	$\bar{2}.225$	-105	
4th Sept	29.3	44	0.53	14.3×10^{-3}	$\bar{2}.155$	-109	
9th Nov	29.6	45	0.30	7.3×10^{-3}	$\bar{2}.135$	-110	
17th Nov	30.3	46	0.33	7.8×10^{-3}	$\bar{2}.104$	-112	
17th Nov	41.2	62	0.33	5.78×10^{-3}	$\bar{2}.24$	-103	
7th Aug	45.7	69	0.14	2.42×10^{-3}	$\bar{3}.384$	-77	Ca ⁺⁺
13th Aug	96.4	145	0.52	4.27×10^{-3}	$\bar{3}.630$	-70	
28th Aug	147.2	222	0.22	1.18×10^{-3}	$\bar{3}.072$	-86	
17th Sept	111.6	168	0.56	3.96×10^{-3}	$\bar{3}.598$	-71	
4th Sept	111.2	168	0.54	3.81×10^{-3}	$\bar{3}.581$	-71	
4th Sept	119.6	180	0.54	3.55×10^{-3}	$\bar{3}.550$	-72	
13th Aug	47.1	71	0.25	4.20×10^{-3}	$\bar{3}.623$	-70	Mg ⁺⁺
17th Aug	47.0	71	0.14	2.4×10^{-3}	$\bar{3}.380$	-77	
4th Sept	51.7	78	0.19	2.94×10^{-3}	$\bar{3}.468$	-75	
28th Aug	136.2	205	0.256	1.488×10^{-3}	$\bar{3}.173$	+167	Cl ⁻
4th Sept	40.8	62	0.08	1.63×10^{-3}	$\bar{3}.212$	+164	
28th Aug	439.3	663	1.0	1.80×10^{-3}	$\bar{3}.255$	+162	SO ₄ ⁻⁻⁻
TOTAL DIGEST							
13th Aug	15.2	23	0.016	0.83×10^{-3}	$\bar{4}.919$	+182X	H ₂ PO ₄ ⁻⁻⁻
28th Aug	23.0	35	0.016	0.55×10^{-3}	$\bar{4}.770$	+192	
17th Sept	23.7	36	0.012	0.41×10^{-3}	$\bar{4}.61$	+200	
4th Sept	30.8	46	0.017	0.435×10^{-3}	$\bar{4}.638$	+204	
4th Sept	33.0	50	0.017	0.408×10^{-3}	$\bar{4}.61$	+200	

Table showing calculations of E_j Potentials for various ions where j is the ionic species.

$$\text{Concentration} = \mu\text{eq/g F.W.} \times \frac{100}{85} \mu\text{eq/ml}$$

The C^i for a particular ion is then obtained by the further adjustment for 22% A.F.S. fraction.

$$C^i = \mu\text{eq/ml} \times \frac{100}{78} \mu\text{eq/ml A.O.V.}$$

Both C^i and C^o are finally expressed in meq/l. The ratio of $\frac{C^o}{C^i}$ and $\log \frac{C^o}{C^i}$ is calculated and the E_j can be calculated from the Nernst equation. (see page 45.)

$$E_j = \frac{59}{z} \log \frac{C^o}{C^i} \text{ at } 25^\circ\text{C}$$

(where z is the valency of the ion and E_j is the electrical potential for an ion species j).

From Table (2), 2 the results can be summarised thus:-

TABLE (2), 3

	K^+	Na^+	Ca^{++}	Mg^{++}	Cl^-	$H_2PO_4^-$	SO_4^{--}
E_j (mv)	-153	-106	-75	-74	+166	+199	+162
November E_j	-174	-	-	-	-	-	-

Table summarising results from Table (2), 2

These results are not considered absolute as several assumptions had to be made in obtaining them.

To take an example; if it is assumed that the A.F.S. is only 14% rather than 22% and that the water content is 92% rather than 85% both these figures would tend to lower the final E_j value. With a tissue ionic concentration of say, 100 ueq/g F.W. and an external solution concentration of 0.5 meq/l, the final E_j would be 142 mv as compared with 146 mv.

A second inaccuracy arises from the substitution of concentrations (C) rather than chemical activities of ions in the cell sap and external solution, for only at infinite dilution does activity equal concentration. Hinke (1961) for example, by using sodium glass microelectrodes to measure pNa in squid axon was able to estimate the sodium and potassium activity coefficients at 0.605 assuming no binding of K^+ in the axon. By calculation he further estimated that 24% of the total Na^+ in the axon was bound. As the external solution is very dilute it is reasonable to let activity equal concentration, but the cell sap is much more concentrated and ionic activity readings might therefore be appreciably lower than concentrations.

The net result of using concentrations is a tendency towards higher E_j values than if the more correct activities had been used.

However, again the error would only be a few millivolts. A much larger error occurs in the actual chemical determination of concentration of ions in the tissue and pond-water. But in the case of K^+ , Na^+ , Ca^{++} and $H_2PO_4^-$ several independent analyses were made using different methods and the difference in determinations would only cause a variation of ± 9 mv.

In the case of potassium there does appear to be a discrepancy in E_K , earlier results giving an average of -153 mv and later results -174 mv. These will be discussed again later.

PART IIMEASUREMENT OF MEMBRANE POTENTIAL (E_m) OF LEAF EPIDERMAL CELLSIntroduction

Some of the difficulties in obtaining a steady membrane potential across the leaf cells of *Potamogeton* have been mentioned earlier, the smallness of the cell being the major one. The apparatus and techniques required for making these observations possible are described below.

The basic circuit required for the measurements of potentials is as follows:-

A 'microelectrode' consisting of a 3M KCl salt bridge in the form of a hard glass micropipette was inserted into a cell mounted in a bathing medium and a reference electrode of 3mm bore hard glass tubing filled with 3M KCl in agar gel was placed outside in the outflowing solution. Silver wire completely coated with fused silver chloride was inserted into each salt bridge and these were connected via coaxial cable to a millivoltmeter. This completed the circuit. If the millivoltmeter had a very high impedance, the electrical potential difference between the inside and the outside of the cell could be measured without causing its discharge.

A microelectrode often has a tip potential generated from unequal diffusion of K^+ and Cl^- ions out from it and of cellular ions into it, or of mass flow of sap from the cell down the micropipette. This potential can sharply change if the tip becomes damaged, or blocked with charged cell matter such as protein, thus perhaps altering the relative mobilities of the diffusing ions and inducing liquid junction potentials and generating electrokinetic potentials from the outflowing sap. For an exact measurement of cell p.d. the tip potential and resistance should be a constant factor throughout the operation of insertion and withdrawal of the microelectrode through the cell wall. (See Adrian 1956,)

To reduce this error to a minimum, with the electrode in the bathing solution a compensating voltage circuit was so adjusted that the tip potential was nullified, and then only those results were accepted in which the variation of tip potential before penetration and after withdrawal was not greater than ± 5 mv.

The electrodes.

The microelectrode was made from borosilicate glass drawn out to a fine tip (ca. 0.5μ or less) in an electrode

puller and then filled in turn with methanol, then distilled water and finally with 3M KCl solution to form the salt bridge to the cell interior (see appendix pp. xxix).

The reference electrode was 3mm (ID) pyrex glass tubing cut to 6 cm length and filled with 3M KCl set in 7% agar gel.

Into the shank of each electrode was inserted a length of silver wire completely coated with silver chloride which acted as electrode and terminal for the completion of the circuit.

Choice of Voltmeter.

It was essential for the voltmeter to have a sensitivity of ± 2 mv and, in order not to discharge the cell, to have a very high input impedance.

The E.I.L. Vibron Electrometer had both these requirements with an input resistance of approximately 10^{13} ohms and a full scale range of 0 - 1000 mv. In earlier work model 33B was used but mains stability was not good, even at night, so that zero drift was sometimes appreciable (± 10 mv). For later work the more stable 33B-2 model was acquired. A 'Medistor' electrometer of 5×10^6 ohms input resistance was used instead in some experiments.

An automatic recorder was at first attached to the Vibron but this proved unreliable.

Reliability of Electrodes.

As mentioned above, data were used only from micro-electrodes with tip potentials in the bathing solutions showing less than 5 mv. variation from the initial value, after withdrawal from the cell.

Most of the electrical resistance and the tip potential arise in the very fine tip of the micro-electrode. Penetration of tough plant cell may break off this tip, or partially plug it with cell matter, or both, thus altering any electrokinetic or diffusion potentials in the tip, and in turn giving unreliable readings for cell membrane potentials. Measurements of tip resistance therefore gave a second sensitive measure of micro-electrode reliability.

To detect tip resistance variation in a series of experiments an electrometer amplifier and probe (Model A.35 Medistor Instrument Co. Seattle) were substituted for the Vibron electrometer. Incorporated in this instrument is a circuit which can drive a standard small current through the microelectrode when desired.

By passing this current through the microelectrode circuit when it is in the bathing solution before and after cell penetration (and when it is recording in a cell) any change in resistance would be observed.

This instrument which had a grid current of less than 10^{-13} amps had the disadvantage that the input impedance was rather low (5×10^6 ohms) and this would cause slight discharge of the cell; however it had two advantages over the Vibron: a) it was run entirely on battery and b) to reduce electrical interference to a minimum the voltage from the cell was fed via the silver/silver chloride contact directly into a preamplifier probe placed nearby on the microelectrode manipulator, and the output from this was then fed into the main amplifier. The voltage output from the main amplifier could be directly connected to an Avo Universal meter.

The preparation and mounting of tissue.

It is preferable that for the true interpretation of membrane resting potentials, the cell should be in ionic flux equilibrium with the bathing solution so that Nernst's simple equation could be directly applied to the data. Therefore in the preparation of tissue thin transverse strips of leaf approximately 1 mm wide were cut with a razor and left to recover for 48 hours in the bathing solution.

In order to maintain this equilibrium during experiments, a strip was mounted on a microscope slide parallel and near to the edge and was covered lightly by a coverslip.

The slide itself was fixed in a perspex tray so designed that the bathing solution from a large aspirator flowed continuously around the tissue.

The reference of 'indifferent' electrode was placed far from the tissue in the outflow of the bathing medium so that KCl from it could not diffuse backwards and upwards around the tissue.

The tray was clamped to the moving stage of the microscope so that cells could be observed for electrode penetration and yet be continuously washed with fresh bathing solution.

Microelectrode manipulation and cell penetration.

A crude micromanipulator was used to insert microelectrodes into the cells. This was a combination of a Prior micromanipulator plus the fine and coarse adjustment control and the moving stage of a microscope.

In order to reduce external vibrations the microelectrode manipulation and the microscope were aligned together on a concrete paving stone which was supported by an inflated car inner tube. The paving stone and inner tube were placed in the centre of a large water storage tank which had been placed on its side. The tank was resting on two other inflated inner tubes which were on a basement laboratory bench for lack of

vibration. Earthing of the tank and the screening of the coaxial connecting leads provided adequate electrostatic screening for resting potential measurements.

By this apparently rather clumsy setup most external vibrations were eliminated and any residual movements that did occur equally affected both the microscope (thus tissue) and the microelectrode manipulator.

In order to penetrate a cell with the microelectrode an appropriate epidermal cell was first selected along the cut edge of the tissue and brought into focus under the high power lens.

The microelectrode was then manouvered in between slide and coverslip towards the tissue and positioned so that it also was sharply in focus. As the fine tip of the electrode is beyond the power of resolution of the microscope it could not be seen but by focusing on the tapering shaft the tip could be positioned pointing towards the centre of the cell and almost touching it.

A slight adjustment of the moving stage of the microscope (which vibrated less than the micromanipulator) was then sufficient for penetration.

The cell potential was observed on the electrometer and recorded every 5 seconds when the potential fluctuated and every 30 seconds when steady. The e.m.f. was plotted against time

on a graph.

With a good electrode the e.m.f. could be sustained for up to 60 minutes but more usually the cell discharged after 5 - 10 minutes. Results from any cell discharging in under 2 minutes were ignored.

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Measurement of Membrane Potentials (E_m)

Method:-

Potamogeton schweinfurthii shoots were cut and put under mercury discharge lamps for 48 hours in filtered pond-water. The leaves were then transversely cut into strips with a razor and left for a further 24 hours in the pond-water (refiltered).

A sample of tissue was removed for chemical analysis (results shown in Table (2) 2, p. 64) and one strip was mounted on a slide as described earlier, with the filtered pond-water flowing past it.

Results:-

Potentials were measured using the Vibron electrometer for ten upper epidermal cells and ten lower epidermal cells.

<u>Upper Epidermal Cells E_m</u>		<u>Lower Epidermal Cells E_m</u>	
	<u>in mv</u>		<u>in mv</u>
	-185	X	-219
	-180		-155
	-204		-170
	-205		-160
X	-120		-177
	-195		-174
	-173		-170
	-173		-183
	-172		-190
	-160		-180
	<hr/>		<hr/>
	<u>Avr. -183</u>		<u>Avr. -173</u>

Mean = 183 mv (Range 171-195) Mean = 173 mv (Range 165-181)

Two results (those crossed) are not included in the average.
All the ranges are calculated at the 95% confidence limit for
the mean (see appendix pp. xxxi)

Comment:-

It is apparant that there is considerable overlap of the
ranges of E_m for upper and lower epidermal cells, and the
difference between the means is not statistically significant
at the 95% confidence limit.

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Measurement of E_m with Micro-electrodes of Known
Tip Resistance.

As a check on the previous results, the resistances of micro-electrode tips were measured before penetration into the cell and after withdrawal, using the Medistor Electrometer and Probe rather than the Vibron.

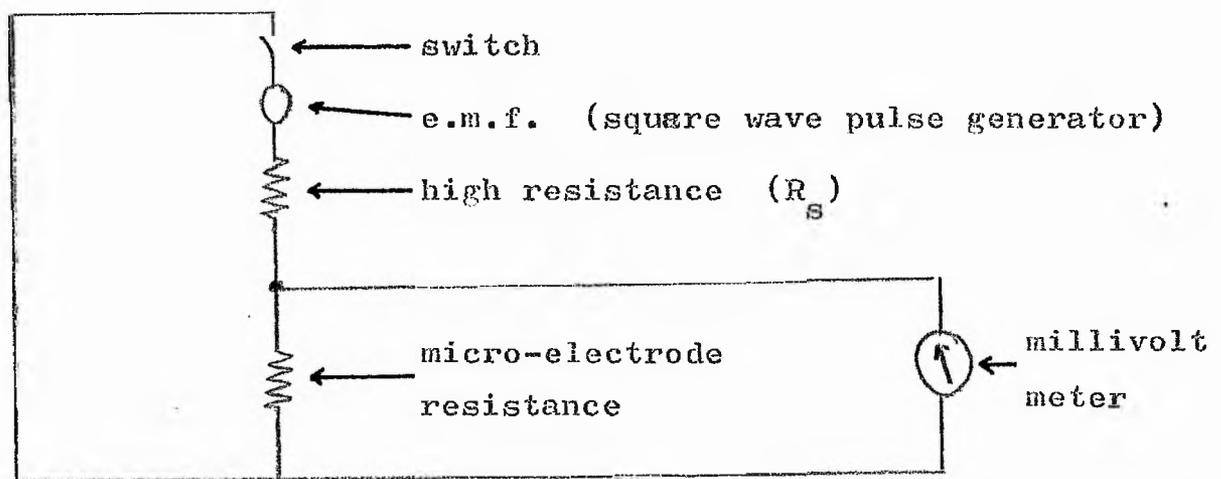
Tips with a change in resistance of more than 10% were considered faulty and were therefore discarded.

Principle:-

The Medistor incorporated a voltage gain which could be calibrated to step up the signal by a factor of 10 ($\pm 1\%$).

With both the micro-electrode and the reference electrode in the bathing solution the resistance of the micro-electrode could quickly be measured by passing a small current which when actuated, produced an amplified output proportional to the electrode resistance (R_e), 1 mv being equivalent to 1 M Ω .

The basic potential divider circuit is :-



If the standard resistance (R_s) is very high, say 10,000 M Ω then

$$\text{Potential across } R_e = \left(\frac{R_e}{R_e + R_s} \right) \cdot \text{e.m.f.} \quad \text{mv}$$

But as R_e is $\lll R_s$,

$$\text{Potential} \cong \left(\frac{R_e}{R_s} \right) \cdot \text{e.m.f.} \quad \text{mv}$$

With the micro-electrode in the cell the cell membrane resistance could theoretically be observed also by actuating the resistance check switch even whilst recording a cell membrane potential, for the membrane resistance would be in series with the micro-electrode resistance so that

$$\begin{array}{l} \text{Potential across cell} \\ \text{membrane and micro-} \\ \text{electrode} \end{array} = \left(\frac{R_e + R_m}{R_s} \right) \cdot \text{e.m.f.} \quad \text{mv}$$

(where R_m = Membrane Resistance)

But this assumes that R_e was not changed by penetration and that the meter was sufficiently accurate to detect R_m which was commonly only a fraction of R_e . The results (p. 78) show that, in fact, this assumption could not be made.

Method:-

Leaf strips were illuminated in aerated filtered pond-water

for 24 hours before the experiment; the tissue was mounted in the normal manner, and cell membrane potentials and electrode resistances were recorded.

TABLE (2), 4

Results:-

Cell No.	p.d. E_m -mv	$R_e(1)$ M Ω	$R_e(2)$ M Ω	$R_e(1):R_e(2)$ % Diffr.	Obs.p.d. on Actuation (- mv)	R_e+R_m M Ω	R_m M Ω
2	190	390	400	2.5	+210	400	0
4 _i	200	390	400	2.5	+200	400	0
5	180	400	400	0.0	+220	400	0
16	195	145	145	0.0	54	141	-4
24	200	60	75	20.0*	135	65	-10
28	175	90	93	3.2	90	85	-8
30	195	75	70	6.7	130	65	-5
31	197	70	75	6.7	117	80	5
44	190	84	85	0.0	100	90	5
45	208	90	105	14.3*	108	100	-5
56	200	80	80	0.0	105	95	15
56 _i	213	80	110	27.3*	92	121	11
57	203	110	115	4.3	64	139	24
57 _i	208	115	117	1.8	78	130	13
58	200	135	140	3.6	45	155	15

Table showing micro-electrode resistances in the bathing solution before $R_e(1)$ and after penetrating the cell, $R_e(2)$; Calculated cell membrane resistance R_m and cell membrane potential E_m .

Mean observed $E_m = -194.4$ mv (Range -188 to -201 mv at the 95% confidence level with 11 degree of freedom).

(% differences marked * are not included in the mean as the differences between the electrode resistances before and after penetrating the cells were greater than 10%).

Comment :-

Many readings (not shown in the table) were eliminated on either grounds of too great a change in tip resistance or an unsteady cell potential.

The electrodes varied considerably in their resistances. Those with resistances below $40 M\Omega$ tended to cause deflation of the turgid cells by leakage, and those with high resistances tended to produce unsteady potentials and high tip potentials, so that electrodes with resistances between $40 M\Omega$ and $100 M\Omega$ were usually selected.

As can be seen, some of the tip resistances were extremely high and most were high in comparison with those normally employed ^{by} /electro-physiologists. This could be caused by several factors, the most likely being the poor preparation of the electrodes. But even with these high resistance tips the recorded cell membrane potentials were not necessarily useless because the grid current of the

Medistor electrometer was 10^{-13} amps. Assuming Ohm's Law, for a 400 M Ω tip resistance the following error would be incorporated:-

$$\begin{aligned} E &= \text{Current} \times \text{Resistance} \\ &= 10^{-13} \times 1000 \times 400 \times 10^6 \text{ mv} \\ &= 40 \times 10^{-3} \text{ mv} \end{aligned}$$

The calculated cell membrane resistance results were so variable as to be meaningless (see page 78) but the observed mean E_m of -194.4 mv (-188 to -201) would appear to be quite satisfactory.

.

Discussion and Conclusion

By reference to the E_j calculated from the Nernst Equation and the observed E_m , we are now in a position to interpret the results in terms of movements of individual ionic species through the epidermal cell membrane.

To summarise the data:-

TABLE (2), 5

DATE	MEAN OBSERVED E_m	REFERENCE PAGE NO.
3rd Sept.	-178 mv Range 171-185 mv	75
20th Nov.	-194 mv Range 188-201 mv	79

Table showing mean observed E_m in September and November.

The difference between the mean E_m in September and November is significant at the 95% confidence level.

TABLE (2), 6

DATE	MEAN CALCULATED E_k	REFERENCE PAGE NO.
7th Aug - 7th Sept	-153 mv Range 148-158 mv	64
9th Nov - 20th Nov	-174 mv Range 169-179 mv	64

Table showing mean calculated E_k in the periods August to September and November.

The difference between the calculated E_k for August to September and November is significant at the 95% confidence level.

TABLE (2), 7

DATE	E_{Na}	E_{Ca}	E_{Mg}	E_{Cl}	E_{PO_4}	E_{SO_4}	REFERENCE PAGE NO.
Aug - Sept	-106	-75	-74	+166	+199	+162	64

Table showing Average calculated E_j for other Cations and Anions.

It can be seen that the only E_j at all approaching the membrane potential is E_k so this will be considered first.

In the calculation of E_k two means were obtained

(1) the mean E_k for plant material and water analysed in August - September and (2) the mean for analysis in November. The difference between these means is statistically significant, the result for the latter being higher than that for the former. What could cause this difference?

If the data for the composition of pond-water (i.e. C^0 for a particular ion) is referred to, it is noticeable that for both potassium and sodium and presumably for other ions also the concentration is nearly twice as high in August - September as compared with November.

TABLE (2), 8

DATE	MEAN K ⁺ meq/l	MEAN Na ⁺ meq/l	REFERENCE TABLE
Aug - Sept	0.47	0.54	(2), 2
November	0.25	0.32	(2), 2

Table showing Mean K⁺ and Na⁺ concentrations in pond-water in August-September and November.

This dilution in November coincided with the onset of the rain season in October. Therefore, although the tissue came from the same plants in the same pond it is reasonable to treat the data separately as the tissue may have had different fluxes in these two seasons.

Therefore comparing E_m with E_k :-

TABLE (2), 9

DATE	E_m in mv	E_k in mv
Aug - Sept	-178 (171-185)	-153 (148-158)
November	-194 (188-201)	-174 (169-179)

Table showing comparison of E_m with E_k .

It can be seen that the ranges in E_m and E_k do not overlap; by calculation the difference between the mean E_m

and E_k is significant at the 95% confidence level, in both the August-September and November readings.

This result would suggest that K^+ was not quite in passive electrochemical equilibrium and if activity had been considered, even less so (see page 65). It would appear therefore that it was being actively pumped out of the cell. But if this is true P. schweinfurthii would stand alone. In all data from other sources, particularly that concerning the Characeae (upon which a considerable amount of similar work has been done) results have shown that K^+ is either in passive electrochemical equilibrium with external K^+ (see for example, MacRobbie and Dainty 1958 (a)) or is actively pumped into the cell (MacRobbie 1965) the pump being linked with a Na^+ efflux pump.

On the other hand there is the possibility, and a more likely explanation, that the discrepancy reflects the inaccuracy of the indirect method of sap analysis as compared with the direct method applicable to the Characeae.

All other calculated E_j 's however, differ very considerably from the observed membrane potential; to such an extent that it cannot be due entirely to analytical inaccuracies.

Barr and Broyer (1964) found in Nitella that the osmotic pressure (O.P.) of the cell sap was mainly caused by the

presence of Na^+ , K^+ and Cl^- ions. If this is the case in *E. schweinfurthii* then the estimated internal concentrations (C^o) of these ions are probably reasonable assessments of their concentrations. In vacuolar solution, and the E_{Na} and E_{Cl} calculations will be a fair approximation to the true value if activities do not differ greatly from concentrations (but see p. 65). Allowing an error of ± 25 mv these E_j 's are still far from the observed E_m . In the case of Na^+ this difference would be further magnified if activity and percentage binding was considered. Therefore, it is probable that there is an active transport system pumping sodium out of the cell and chloride into it.

As the phosphate analysis was performed on the chemical leachate from fresh tissue, the C^i for phosphate would probably be an estimate of free phosphate in the cell sap; so E_{PO_4} of +199 mv (p. 82) would also indicate the phosphate is actively pumped into the cell. The sulphate chemical analysis was carried out on the total plant digest and consequently the amount of free sulphate is not actually known but data would suggest that this ion is also pumped into the cell.

The same line of argument, however, cannot safely be used for Ca^{++} and Mg^{++} for these ions are probably partly bound in

the cell, thus their calculated internal concentrations would not reflect the activities of the ions in solution. But more important, Spanswick and Williams (1965) found that for Ca^{++} in Nitella translucens, the permeability to this ion at the plasmalemma was so small that it was never in flux equilibrium; influx always being greater than efflux. Even though the difference between E_m and E_{Ca} showed a large inward driving force on calcium they suggested a passive flux across the plasmalemma.

With the knowledge of this situation and in the absence of flux data it is not possible to assess from the present E_m and E_{Ca} calculations whether calcium is actively pumped or not in P. schweinfurthii. The same may be said to apply to magnesium as it is similar in many respects to calcium.

CHAPTER III

CARBON DIOXIDE REQUIREMENTS

OF THE PLANT

The diversity of ability of water-plants to utilize the bicarbonate ion could well be of significance to their natural distribution, so in the present study on P. schweinfurthii this aspect is considered in some detail.

According to Ruttner (1953) aquatic plants fall into two physiological groups (a) aquatic seedplants and algae which can utilize bicarbonate and dissolved free carbon dioxide and (b) aquatic mosses and land plants which can only get their carbon for photosynthesis from free carbon dioxide. For example, Steemann Nielsen (1944, 1947) demonstrated that whereas Myriophyllum spicatum grown at optimal light intensity could assimilate either free CO_2 or HCO_3^- , Fontinalis antipyretica was totally unable to use the latter.

Nowadays, however, this classification is not so clear cut. Hood and Park (1962) claimed that in some marine algae, including a species of Chlorella, there was a strong preference for HCO_3^- , and indeed, this behaviour could be universal in all marine plants; but both Steemann Nielsen (1963) and Watt and Paasche (1963), although not disclaiming HCO_3^- utilization by the Chlorella, demonstrated that distinction between HCO_3^- and dissolved free CO_2 uptake is impossible with the method used by these authors.

Osterlind (1951) showed that a young culture of Scenedesmus quadricauda was able to photosynthesize at maximum rates with HCO_3^- concentrations as low as $10 \mu\text{mol/l}$ where the free CO_2 concentration was insignificant ($< 0.2 \mu\text{mol/l}$). For a similar rate of photosynthesis in a free CO_2 solution $100\text{-}200 \mu\text{mol/l}$ free CO_2 was required; yet when the culture became aged it almost entirely lost this ability to utilize HCO_3^- , therefore it could be interpreted that the mechanism for bicarbonate utilization is sensitive to certain factors such as age. Chlorella pyrenoidosa (in Ruttner's classification a bicarbonate utilizer) is able to photosynthesize at optimal rate in solutions of free CO_2 $< 10 \times 10^{-6}$ M. (Stemann Nielsen and Jensen 1958), but is unable to utilize HCO_3^- except under extreme conditions, and then only very slightly.

Although then it is generally accepted that many water-plants are able to use HCO_3^- the extent to which they exploit this ability, and the different mechanisms involved in the utilization of free CO_2 and HCO_3^- is still not clear.

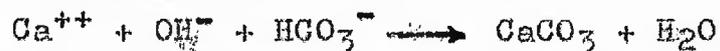
Arens (1933, 1936) first noticed that in the light certain aquatic leaves absorbed HCO_3^- ions with an equivalent amount of cations into their abaxial surface and the cations left through the adaxial surface together with OH^- or CO_3^{--} ions.

Accompanying this there was a rapid increase in the pH of the external medium above the adaxial surface with no change in pH in the medium surrounding the abaxial surface. If the HCO_3^- ions were replaced by Cl^- or NO_3^- ions or if the leaves in bicarbonate solution were put into the dark, then there was no movement of ions through the leaf. Arens suggested that HCO_3^- was assimilated by water-plants during photosynthesis and when the HCO_3^- ions were absorbed cations also had to enter to maintain gross electrical neutrality.

Steemann Nielsen (1947 and 1952 (a)) refined the experiments of Arens and using excised leaves of Potamogeton lucens concluded that the absorption of ions takes place both through the abaxial and adaxial surfaces. The bicarbonate ions are utilized in photosynthesis with the production of OH^- ions which are actively transported with accompanying cations to the adaxial surface only where they are released. On release the OH^- ions combine with HCO_3^- ions in the outside solution to precipitate insoluble CaCO_3 , which would conveniently explain the deposit of CaCO_3 frequently found on plants growing in alkaline waters.

Recently, Steemann Nielsen (1966) suggested an interesting variation of this mechanism found in the marine plankton alga Coccolithus huxleyi, where instead of excreting the OH^-

together with the cations, CaCO_3 is precipitated in the coccoliths according to the equation



If this is the case then only half the amount of HCO_3^- absorbed will be used in photosynthesis, the rest being precipitated.

Using labelled Ca^* on excised leaves of Potamogeton crispus, Lowenhaupt (1956, 1958) produced similar results to Steemann Nielsen and Arens, but whereas they explained the process by an anion pumping mechanism, Lowenhaupt suggested that the ion transport system was the result of two cation pumps, one on the lower surface of the leaf pumping the cations in and the other on the upper surface pumping the cations out. However, neither Steemann Nielsen nor Lowenhaupt base their conclusions on the correct criteria as they give no data on ionic flux nor electrochemical potentials between cell and external medium. Raven (private communication) on the other hand, leaves little doubt that there is an inward directed HCO_3^- pump which is excited by light energy (see general discussion Page 197).

The net result of total CO_2 uptake and photosynthesis by water-plants will be a tendency to raise the pH of the surrounding medium as carbonic acid is removed and as hydroxyl excretion (from bicarbonate uptake) precipitates calcium

carbonate. This increase in pH can be observed often in natural waters during the course of a clear sunny day when rapid photosynthesis is taking place.

With this general scheme in mind carbon dioxide utilization by P. schweinfurthii was investigated.

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Estimation of Rate of Photosynthesis using
the Micro-Winkler Technique

In the following series of experiments the rate of photosynthesis was estimated by measuring the oxygen production using the micro-Winkler technique. (see appendix p. xii).

In this first experiment, apparatus and techniques used were tested for reliability.

Pretreatment :-

Leaves of P. schweinfurthii were cut into discs of 0.5 cm diameter using a cork borer. These discs were placed in aerated deionized water on the windowsill for 24 hours.

Apparatus :-

The apparatus shown on the next page was designed for the experiment.

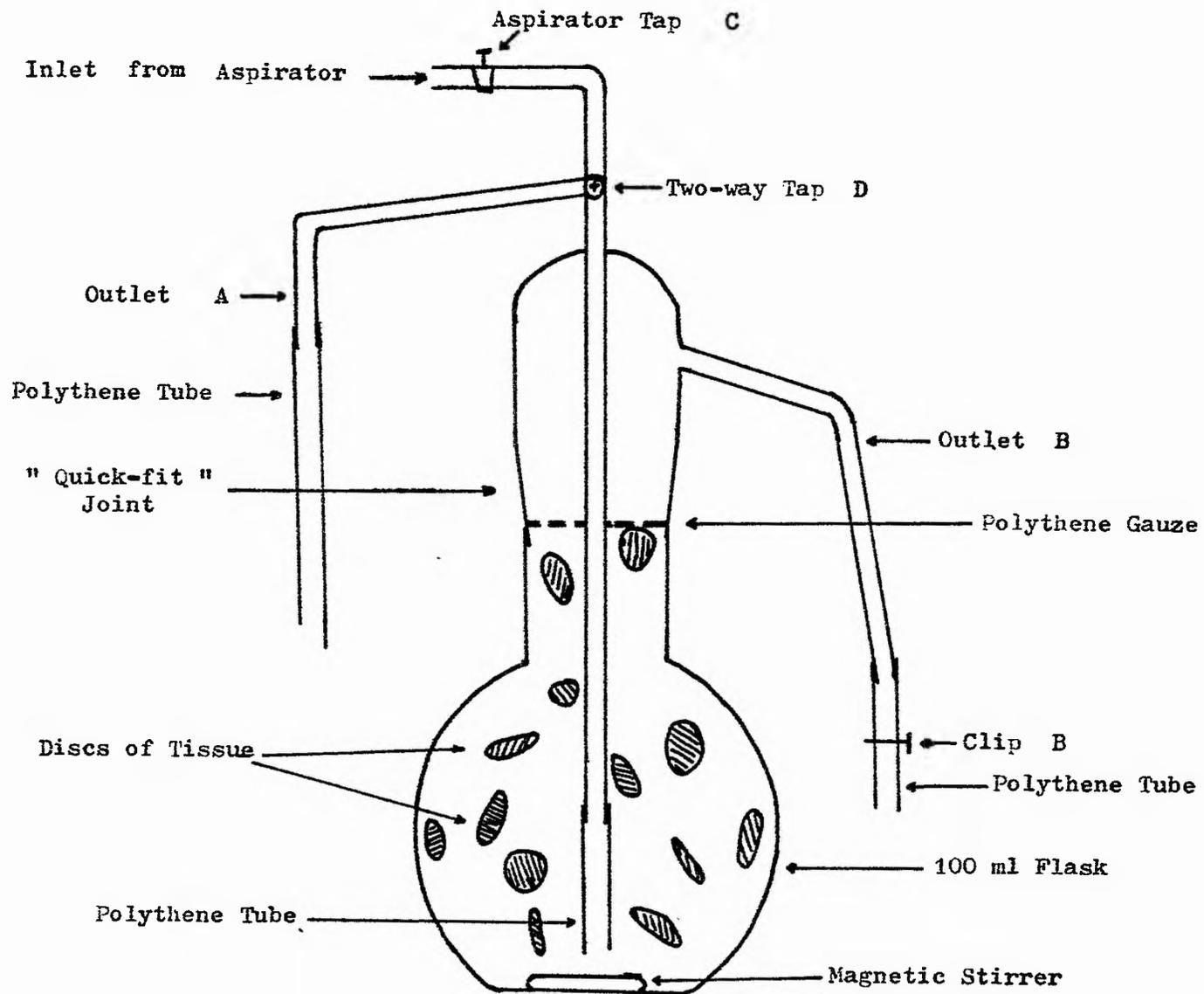


DIAGRAM OF PHOTOSYNTHESIS APPARATUS

Method:-

A 5 litre aspirator was filled with deionized water which had had approximately 40 ml of bottled 'Soda Water' well mixed with it. This provided a solution containing dissolved CO_2 at pH 4.65

A light source was provided from a 1000 watt tungsten bulb which was immersed in a container of flowing water. As a further precaution against heating of the photosynthesis apparatus, a 10 litre perspex tank containing distilled water was placed between the water-cooled light source and the apparatus.

0.45 g F.W. of discs were placed in the 100 ml flask and the solution from the aspirator was allowed to fill the apparatus by opening tap C and D and flood out of outlet B. The discs were prevented from being washed away by a polythene gauze placed at the 'Quick-fit' joint.

After approximately 200 mls had flowed out, clip B was closed, tap D was turned so that the aspirator solution flooded through outlet A, the magnetic stirrer was switched on and the stop-clock started. A blank sample was immediately taken through outlet A so that the solution from the aspirator filled the sample bottle (approximately 35 mls), 'the flooding technique' being used with slow withdrawal of

the nozzle from the bottle. A ground glass stopper was placed in the bottle, extreme care being taken not to trap any air bubbles, and tap D was closed.

After the timed experimental period the stirrer was switched off, clip B was opened and tap D was turned so that the solution from the flask was siphoned out through outlet A and the sample was taken in the same manner as the blank. The filling up process was then repeated and another blank was taken. Samples were withdrawn every 15 minutes and after 1 hour 15 minutes the rate of stirring of the solution was reduced by approximately half.

Results:-

In each case the oxygen content of the sample was compared directly with the blank sample taken immediately prior to the timed interval so that any variation in the oxygen content of the aspirator solution would be compensated.

A graph was plotted of mg oxygen released/g F.Wt. tissue against time. Graph (3), 1.

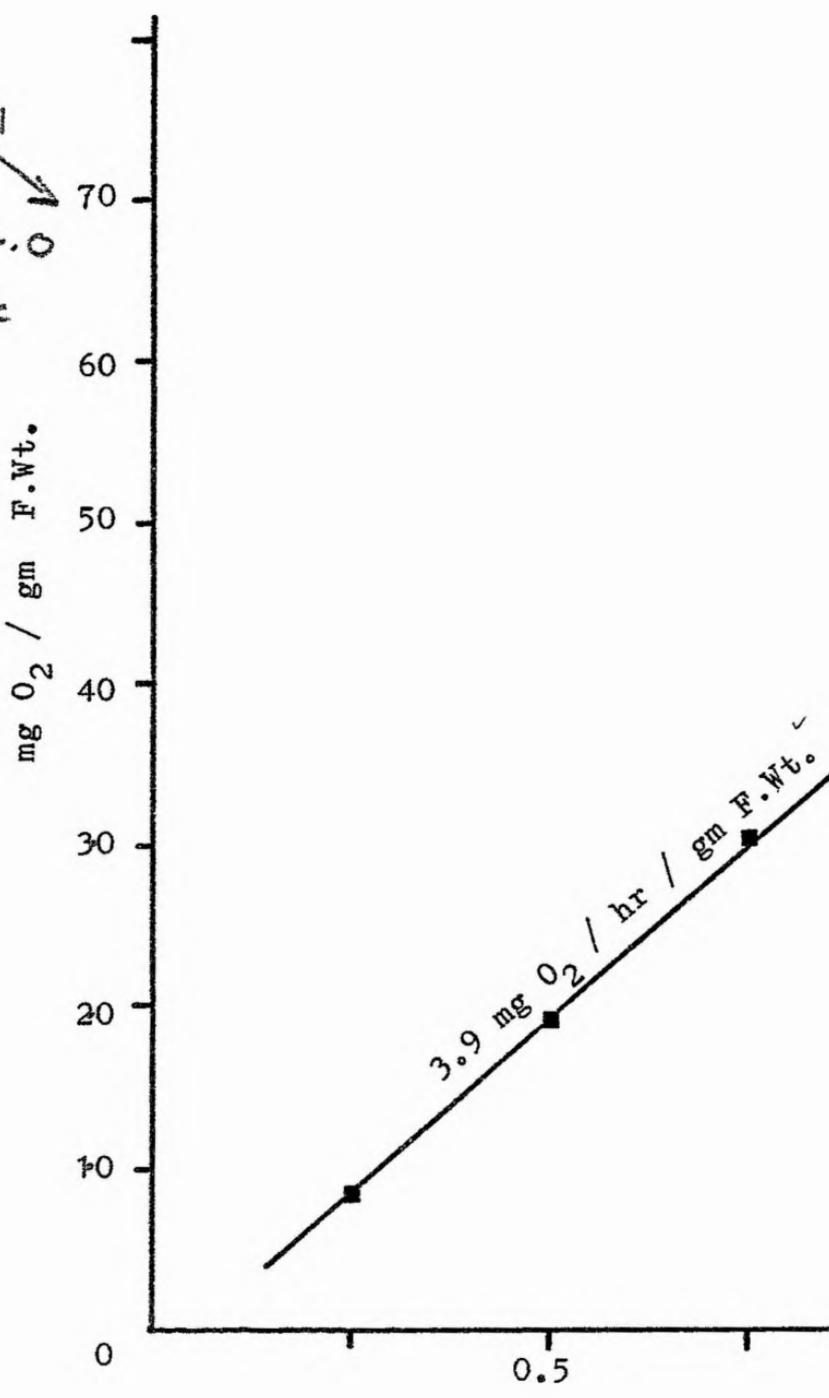
Comments:-

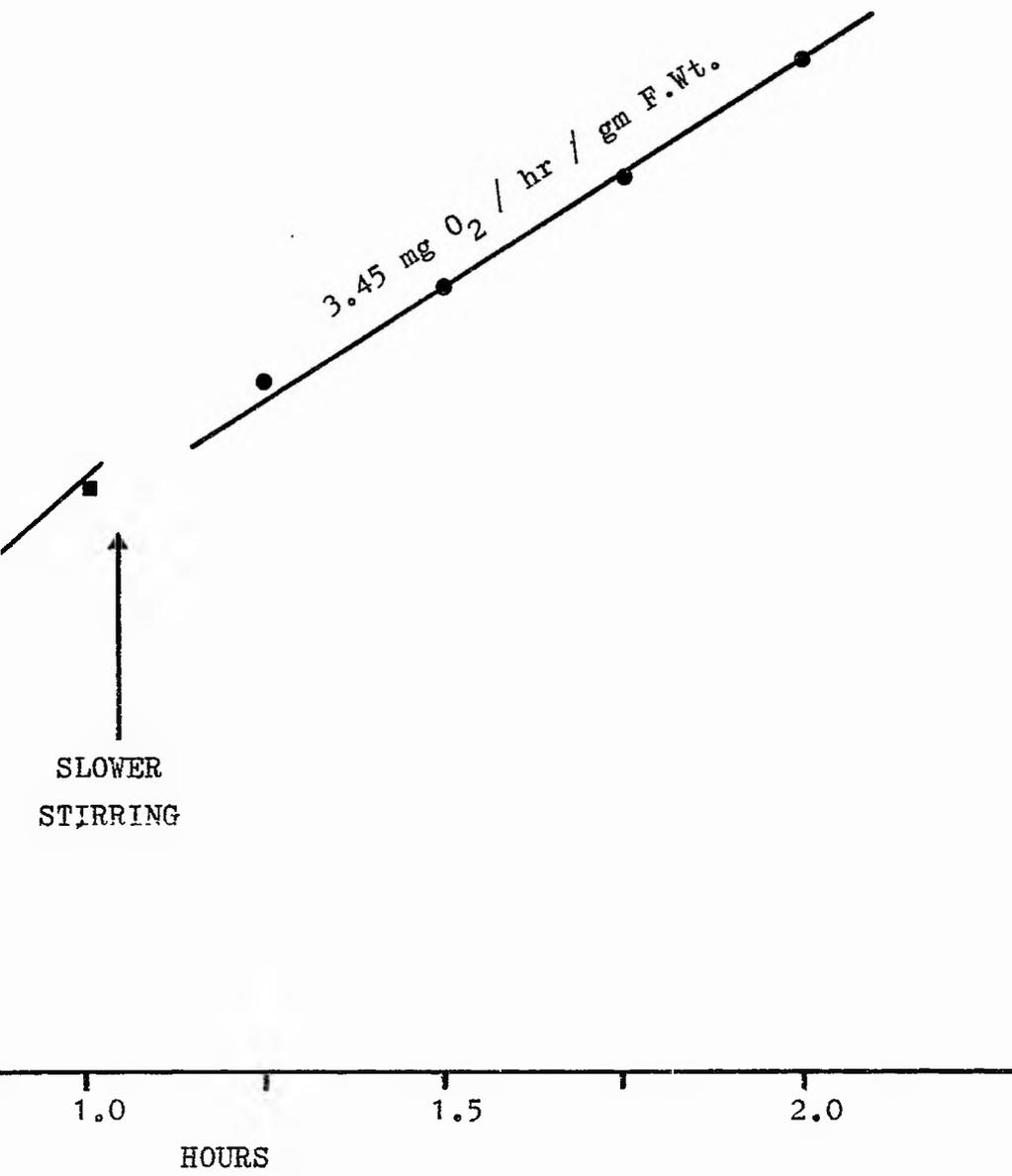
At any one stirring speed the rate of oxygen production was constant which reflects both the ability of the plant to

GRAPH (3), 1

Rate of oxygen release from illuminated
P. schweinfurthii leaf tissue when
placed in deionized water containing
additional CO₂.

X 0.1
is 7.0
etc





utilize free CO_2 (as the solution at pH 4.65 would contain virtually no HCO_3^- ions) and the stability of the conditions.

The faster steady rate of photosynthesis in the more rapidly stirred solution (3.9 mg O_2 released/hour/g F.Wt. as compared with 3.45 mg O_2 in the lesser stirred solution) was probably the consequence of shortening the CO_2 diffusion path into the cells, by reducing the unstirred layers around the tissue.

$$3.9/\text{ml} = 28/\text{DW}$$

• • • • •
 @ approx 0.5 to 1 ml CO_2

(rate. DW).
 + ca. 18 vol. O_2 / cell vol. / hr.

In all the following experiments the faster stirring rate was used unless specifically mentioned otherwise.

To Observe Rate of Photosynthesis of *R. schweinfurthii* in Deionized Water containing Sodium Bicarbonate.

Pretreatment:-

The pretreatment was as in the last experiment.

Method:-

The aspirator contained a solution of 3 mM/l NaHCO_3 , and

0.25g F.Wt. of tissue was used. The procedure was similar to the last experiment but there was a constant stirring rate and a change from 3 mM/l to 9 mM/l sodium bicarbonate solution later in the experiment.

Samples were taken every 15 minutes.

Results:-

*No chemical check on O_2 in
? faulty method of reagents?*

Except for the first sample (where perhaps stability had not been reached) the oxygen content of the solution showed a tendency to fall rather than rise.

i.e. Mean of three samples (1st sample being omitted) equals -1.02 (range -0.05 to -1.99) mg O_2 /hr./g D.Wt.

(Range at the 95% confidence level and 2 degrees of freedom - see appendix p. xxxi for method of calculation).

On the further addition of HCO_3^- to 9mM/l readings were very questionable but there appeared to be still no photosynthesis.

Comments:-

The amount of tissue or the time between samples was too small for reliable results but under the conditions stated the results were extremely different from those in the previous experiment where the amount of tissue and sample

times were similar.

Calculations from the Henderson-Hasselbach equation (Manometric Techniques 1957) a 3 mM HCO_3^- solution will contain approximately 0.06 mM/l free CO_2 and a 9 mM solution approximately 0.18 mM free CO_2 . This variation in free CO_2 appeared to have little effect upon the rate of photosynthesis and certainly not a linear relationship. Perhaps therefore two conclusions could be suggested; (a) P. schweinfurthii is unable to utilize free CO_2 at these very low concentrations and (b) P. schweinfurthii is unable to use HCO_3^- in pure solutions of up to 9 mM/l NaHCO_3 .

or tissue dead! -
no control such as
later adding Soda water
or Ca^{++} - KHCO_3
etc

.

To Observe Rate of Photosynthesis in a Bicarbonate Solution containing Varying Quantities of Calcium.

It has been shown by several workers including Steemann Nielsen (1947) that bicarbonate users require the presence of other ions, particularly calcium in order to stimulate HCO_3^- ion uptake; this was tested in P. schweinfurthii.

Pretreatment :-

The cutting of leaf tissue into discs caused considerable wastage, therefore the leaves were cut transversly into thin

strips approximately 1 mm wide and these were pretreated as in the former experiments.

Method:-

Four solutions each containing 5 meq/l KHCO_3 had the following calcium composition (added in the form of CaCl_2)

(1)	5 meq/l KHCO_3	+	0.00 meq/l Ca^{++}	pH 8.05
(2)	"	+	0.5 "	pH 7.97
(3)	"	+	2.0 "	pH 7.80
(4)	"	+	5.0 "	pH 7.65

For each calcium solution three samples were taken at intervals of 30 minutes. 0.3425 g F.Wt. tissue strips were used.

Results:-

See Table (3), 1 overleaf.

TABLE (3), 1

Solution Ca ⁺⁺ meq/l	Mean O ₂ Release mg / hour / <u>g Dry Wt.</u> ✓	
0.0	12.78 (range 7.63 to 17.93)	Lower free CO ₂
0.5	19.10 (range 15.42 to 22.78)	
2.0	18.24 (range 15.78 to 20.70)	
5.0	26.10 (range 15.83 to 36.37)	Higher free CO ₂

Table showing oxygen output from P. schweinfurthii in bicarbonate solutions containing different concentrations of Calcium.

(all ranges were calculated at the 95% confidence level with 2 degrees of freedom).

Comment :-

The calculated ranges about the mean show that the results were rather unreliable but trends can be seen.

Even in the absence of calcium it appears that there was slight photosynthesis in 5 meq/l KHCO₃ solution. Results with the sodium bicarbonate solution in the previous experiment showed no such photosynthesis. Either the readings may be more accurate in the present experiment because of larger samples and longer time intervals, or a genuine difference

may exist because the K^+ rather than the Na^+ salt was used ; or perhaps the tissue was less well leached so that ions, especially cations, present in the free space stimulated the HCO_3^- uptake.

The presence of even small quantities of Calcium ions in the bathing solution increased the rate of photosynthesis. There could be two possible causes for this; either Ca^{++} stimulated bicarbonate uptake, or its presence increased the amount of free CO_2 for photosynthesis in the following manner:-



the pH of the solution being lowered.

To consider the second possibility; if one substitutes in the Henderson-Hasselbach equation the ^{initial} pH and the HCO_3^- concentration for each of the four solutions, the following free CO_2 values are obtained, using a pK value of 6.360 at 26°C (Manometric Techniques 1957). (See Table (3), 2).

If the rates of O_2 evolution are compared with the calculated mean free CO_2 concentrations it can be seen that although the ranges are considerable they are more likely to produce an exponential rather than a linear graph (graph (3), 2).

It has been shown by Osterlind (1951) and others that the rate of photosynthesis is directly proportional to the concentration of free CO_2 in water-plants that are able to

utilize it. This being the case, the observed increase in rate of photosynthesis on the addition of Ca^{++} to a HCO_3^- solution is more likely to be due to a stimulatory effect on HCO_3^- uptake rather than an increase in free CO_2 concentration in the solution as a linear relationship is not apparent. This would confirm Steemann Nielsen's suggestion that the presence of additional cations to a bicarbonate solution enhances bicarbonate uptake.

TABLE (3), 2.

Calcium content meq/l.	<i>initial</i> - <i>? final</i> pH	Conc. Free CO_2 mM/l.	Mean O_2 Release mg / hour / g D.Wt. (from Table 3.1)
0.0	8.05	0.099	12.78 (range 7.63 to 17.93)
0.5	7.97	0.120	19.10 (range 15.42 to 22.78)
2.0	7.80	0.175	18.24 (range 15.78 to 20.70)
5.0	7.65	0.245	26.10 (range 15.83 to 36.37)

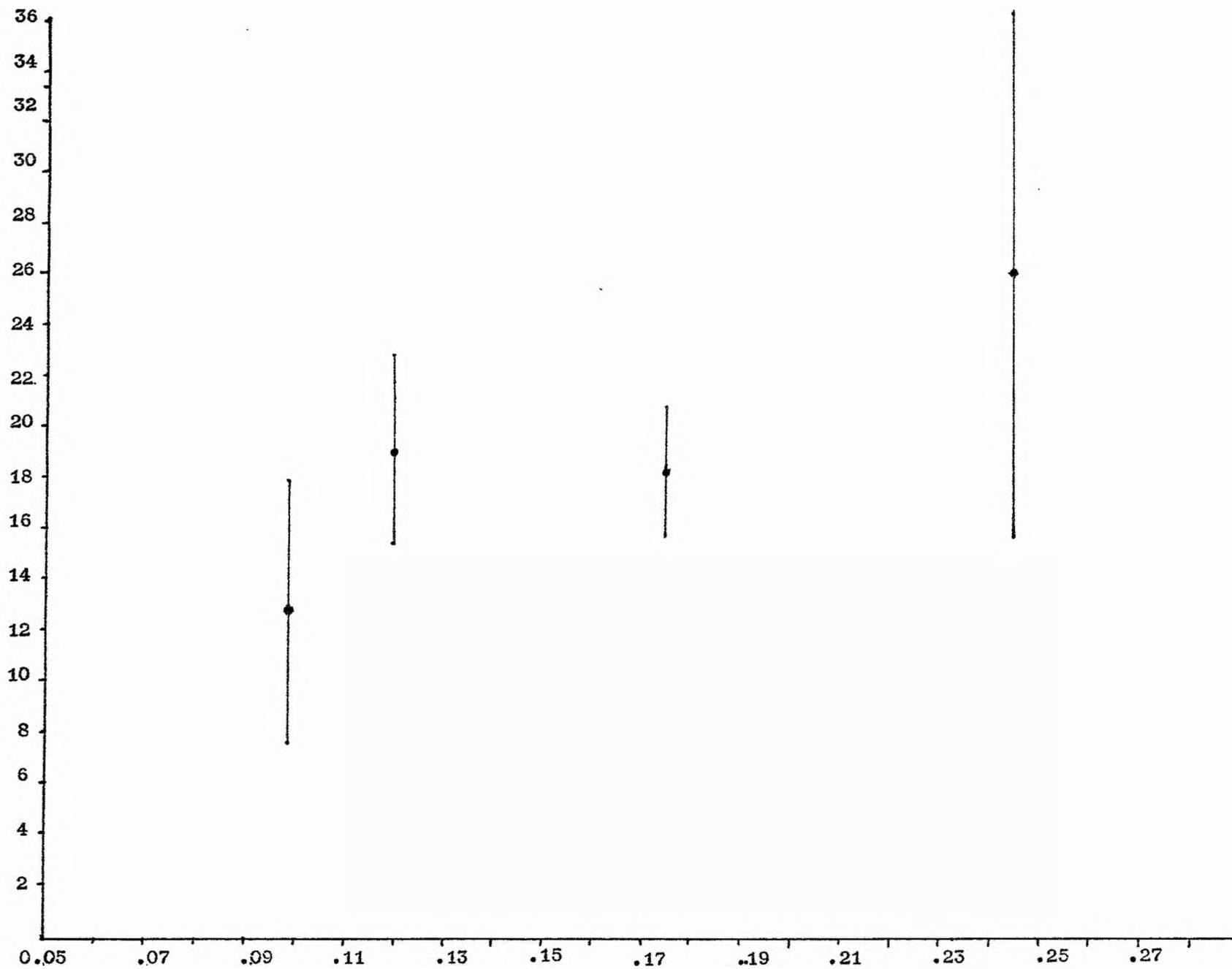
Table giving calculated free CO_2 values in 5 meq/l KHCO_3 solutions containing different amount of Ca^{++}

. . . .

GRAPH (3), 2

Mean rates of oxygen evolution from
illuminated P. schweinfurthii leaf
tissue in bicarbonate solution,
plotted against calculated dissolved
free CO₂ concentrations.

D.V.
Rate of Oxygen Evolution mg / hr / g ~~Wt.~~ Wt.



Concentration of Free CO₂ mM / Litre.

To Measure Rate of Photosynthesis and Ground Respiration of
P. schweinfurthii in CO₂ -free Deionized Water

Pretreatment:-

Leaf tissue was leached in deionized water for 5 days, the water being changed each day, and then was cut into strips and was left as usual for 24 hours.

Method:-

Deionized water was boiled and cooled under a carbon dioxide trap just prior to use; this would give a total CO₂ content of < 0.01 mM/l CO₂.

Oxygen release from the tissue was measured in the normal manner in the light and after several samples had been taken the light was switched off and the apparatus surrounded in total darkness.

This experiment was repeated several times with larger amounts of tissue and longer experimental periods in order to try and get reliable readings. New tissue strips were used for each repeat.

Results:-

TABLE (3), 3

TISSUE FRESH WEIGHT	DATE	TIME INTERVAL BETWEEN SAMPLES	NO. OF SAMPLES	CONDITION	OXYGEN UPTAKE mg O ₂ /hr./g DRY WT. ✓
0.517	30th Dec	30 min	2*	Light	-1.17 (+1.02 to -3.36)
			2	Dark	-2.23 (+18.86 to -23.32)
0.886	31st Dec	45 min	5**	Light	-0.68 (-0.39 to -0.97)
			2	Dark	-1.33 (+5.07 to -7.73)
0.886	1st Jan	2 hrs	4	Light	-0.42 (+0.25 to -1.08)
			1	Dark	-0.71
AVERAGE			11	Light	-0.67 (-0.41 to -0.93)
			5	Dark	-1.57 (-0.24 to -2.89)

* 1st sample excluded as tissue probably not equilibrated (+1.603)

** 2nd sample excluded as readings suspect (+0.019 and -0.032)

Table showing rates of O₂ uptake by P. schweinfurthii in CO₂-free deionized water in conditions of light and dark.

Comments :-

The results showed considerable variation and the difference between the means of the light and dark periods is not significant at the 95% confidence level.

However, in both light and dark oxygen was absorbed from the surrounding deionized water. In the dark more oxygen was absorbed than in the light. This would indicate that there was some photosynthesis in the light which was either obtaining its CO_2 endogenously from respiration, or from the surrounding solution (which contained $< 0.01 \text{ mM/l } \text{CO}_2$) or from both; but then this rate was not sufficient to compensate for the rate of respiration so that there was a net uptake of O_2 .

Potamogeton schweinfurthii has been established as a dissolved free CO_2 and a bicarbonate ion utilizer. According to Steemann Nielsen the pH of the external solution should increase under favourable conditions owing to hydroxyl ion excretion. This prediction was therefore tested in the following series of experiments.

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To Observe the pH of Deionized Water Containing
Cut Shoots in the Light.

Pretreatment :-

Healthy shoots of P. schweinfurthii were cut under water, transferred to the laboratory and were dipped and agitated successively in 3 tanks containing distilled water. Five second agitation was given in each tank. By this procedure the surface film of pond-water on the tissue was presumably removed.

The tissue was then placed in a closed 5 litre aspirator containing deionized water which had just been aerated with compressed air for two days.

Experiment :-

The aspirator contained a magnetic stirrer to ensure a constant circulation of solution around the tissue, and the tissue was illuminated from a 1000 w. tungsten filament electric light bulb.

In the neck of the aspirator was placed a rubber bung through which passed a pair of electrode probes for reading the pH, and a glass tube connecting the aspirator to a large deionized water storage tank. When a sample was drawn from

the tap at the bottom of the aspirator water would siphon from the storage tank and thus retain the level in the aspirator. The volume of water entering the aspirator during the taking of a sample was measured. At regular intervals a sample of solution was run from the tap of the aspirator for chemical analysis and change in pH was recorded. K^+ and Na^+ were estimated by flame photometry and phosphate by colorimetry (see appendix p. i-x)

Results:-

TABLE (3), 4

Time Hrs.	pH	Conc. Total CO_2 mM/l.	Ions leached from 19.6 g F.Wt. tissue		
			$H_2PO_4^-$ μM	K^+ μeq	Na^+ μeq
0	5.65	0.036	0.00	0.00	0.00
$\frac{1}{2}$	8.20	0.021	12.90	0.00	22.00
$1\frac{1}{2}$	9.00	0.021	16.83	0.00	17.06
$3\frac{1}{2}$	9.15	0.021	25.59	49.50	17.52
$5\frac{1}{2}$	9.15	0.027	35.57	78.15	17.90
$7\frac{1}{2}$	9.10	0.033	36.50	91.11	18.32
$9\frac{1}{2}$	9.05	-----	37.20	115.57	18.78

Table showing change in deionized water when photosynthesizing tissue was placed in it, in a closed system.

Total ions lost/100g Dry Wt. tissue

in $9\frac{1}{2}$ hours leaching:-

K^+ = 3.93 meq

Na^+ = 0.64 meq

$H_2PO_4^{--}$ = 1.26 mM

Comments:-

When fresh shoots of P. schweinfurthii were placed in deionized water and illuminated they quickly raised the pH of the water from 5.65 to 9.15 where it remained approximately stable. This was accompanied at first by removal of some CO_2 from the water and thereafter by a fairly steady leaking out of phosphate, K^+ and Na^+ ions.

A Repeat of the Previous Experiment followed by Back Titration
of the Solution to the Original pH.

Pretreatment:-

As in the previous experiment.

Method:-

The procedure was similar to the previous experiment but leaching continued for 16 hours. 24.7 g Fresh Wt. of tissue being used.

The total leachate (6.2 litres) was then back-titrated to the original pH using a Radiometer automatic titrator with $\frac{N}{74}$ HCl.

Results:- See graph (3), 3

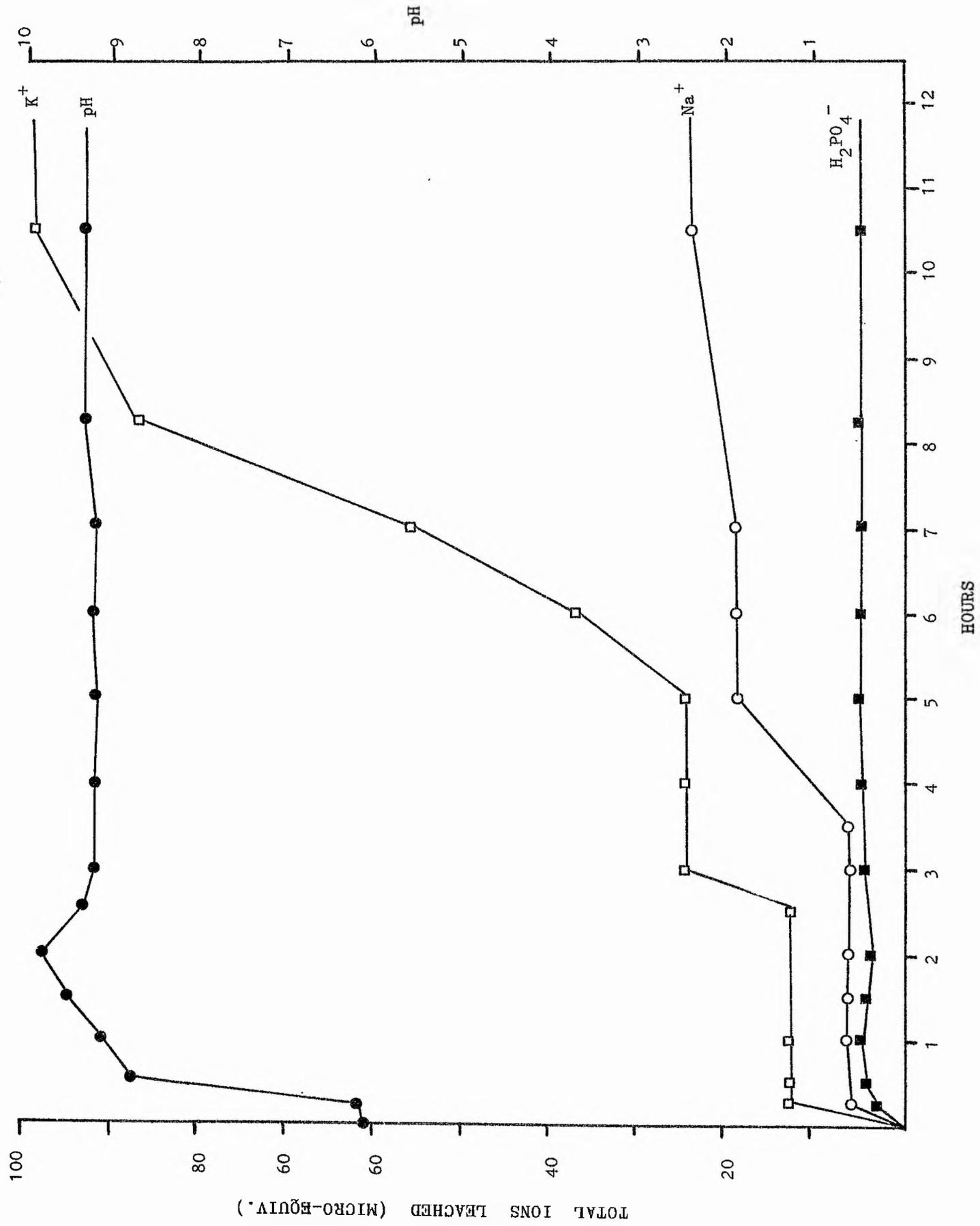
TABLE (3), 5

Time Hrs. & Mins	pH	Σ Ions leached from 24.7 g. F.Wt. Tissue		
		H ₂ PO ₄ ⁻ μM	K ⁺ μeq.	Na ⁺ μeq.
0.00	6.1	0.00	0.00	0.00
0.15	6.2	2.48	12.40	6.20
0.30	8.8	3.78	12.40	6.20
1.00	9.1	3.89	12.40	6.20
1.30	9.5	2.94	12.40	6.20
2.00	9.8	3.00	12.40	6.20
2.30	9.3	4.78	12.40	6.20
3.00	9.2	3.87	24.80	6.20
3.30	9.3	5.68	12.40	6.20
4.00	9.2	4.78	24.80	6.20
5.00	9.2	4.87	24.80	18.60
6.00	9.2	4.44	37.20	18.60
7.10	9.2	4.51	55.80	18.60
8.15	9.3	5.08	86.82	37.20
10.30	9.3	4.67	99.20	24.80
16.15	9.0	5.58	99.20	24.80

Table showing change in deionized water when photosynthesizing tissue was placed in it, in a closed system.

GRAPH (3). 3

Total ions leached from fresh
P. schweinfurthii shoots when
illuminated in deionized water,
plotted against time.



Total Ions lost/100g Dry Wt. tissue

in 16 hours:-

$$\begin{aligned} K^+ &= 2.67 \text{ meq} \\ Na^+ &= 0.67 \text{ meq} \\ Na^+ + K^+ &= 3.34 \text{ meq} \\ H_2PO_4^- &= 0.15 \text{ meq} \end{aligned}$$

Acid used for total back-titration

$$\begin{aligned} &= 0.496 \text{ meq/ 6.2 litres leachate} \\ &= 13.37 \text{ meq/100g Dry Wt. tissue} \end{aligned}$$

Comments:-

It could be suggested that the amount of acid required for the back-titration indicates the amount of OH^- ion excreted by the tissue. If, except for OH^- , the anion leakage is negligible, then the back-titration would also indicate the total cation equivalents leaked out.

Only Na^+ and K^+ were checked and these added up to 3.3 meq/100g Dry Wt. tissue. Since the quantity of acid titrated was equivalent to 13.4 meq/100g Dry Wt. tissue, approximately 10 meq of cations are not accounted for. This may indicate the amount of Ca^{++} and Mg^{++} leaking out of the tissue.

(It will be noticed that in this experiment less phosphate is lost from the tissue as compared with the last experiment,

(0.15 meq/100g Dry Wt. as compared with 1.26 meq) but pond-water analysis showed that whereas this plant grew in a 22 μM /litre solution the previous plant had come from a solution containing 42 μM /litre phosphate.)

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To Observe the pH of Deionized Water containing Cut
Shoots of *P. schweinfurthii* in the Dark.

In the previous experiments a rise in pH of the bathing solution was observed in the light when tissue was placed in it. This could be caused by the excretion of hydroxyl ions during photosynthesis, although the CO_2 level of the solution was very low or by cation exchange between tissue and solution, or by both. This can be checked by performing a similar experiment while maintaining the tissue in complete darkness.

Pretreatment:-

The pretreatment was similar to the last experiment but the deionized water was aerated with CO_2 - free air which had passed through two soda columns.

Method:-

This was similar to the previous experiment but the aspirator was surrounded by black polythene. The pH electrodes were not immersed in the aspirator but were very close so a pH sample could be taken before any appreciable contamination by CO₂ occurred. Sub-samples were also collected and boiled, stoppered tightly and cooled so that any free CO₂ was removed.

The pH of the boiled samples when plotted against time reached a plateau in approximately 3 hours, and the sum of leaching ions in approximately 7 hours, after which the experiment was stopped.

Results:-

TABLE (3), 6

	pH at zero time	pH after 7 hrs.
fresh sample	6.8	6.6
boiled sample	7.8	9.4

Table showing pH of fresh and boiled leachate samples.

Dry Weight of tissue = 3.71 g

Total volume of leachate
= 6.2 litres

Total CO₂ of deionized water at zero time
= 0.011 mM/litre

Total CO₂ of boiled leachate sample after 7 hours
= 0.042 mM/litre

$$\begin{aligned} \therefore \text{CO}_2 \text{ in 7 hour leachate per 100 g Dry Weight tissue} \\ &= 0.042 \times \frac{\text{Total Vol. leachate} \times 100 \text{ mM}}{\text{Total Dry Wt. tissue}} \\ &= 0.042 \times \frac{6.2}{3.71} \times 100 \text{ mM/100g Dry Weight} \\ &= 7.02 \text{ mM CO}_2 / 100\text{g Dry Wt. tissue.} \end{aligned}$$

According to the Henderson-Hasselbach equation, as the pH of the boiled 7 hour leachate was 9.4, the total CO₂ would be 95% HCO₃⁻ and 5% CO₃²⁻

$$\therefore 7.02 \text{ mM CO}_2 \equiv 6.67 \text{ mM HCO}_3^- + 0.35 \text{ mM CO}_3^{2-}$$

$$\therefore \text{Total equivalence HCO}_3^- + \text{CO}_3^{2-} = 7.37 \text{ meq/100g D.Wt. tissue}$$

Total Ions lost/100 g D. Wt. tissue in 7 hours leaching:-

$$\text{K}^+ = 1.7 \text{ meq}$$

$$\text{Na}^+ = 0.2 \text{ meq}$$

$$\text{H}_2\text{PO}_4^- = 0.15 \text{ meq}$$

Total acid used in back-titration

$$= 6.88 \text{ meq/100g Dry Wt. tissue.}$$

Comments:-

The results can be tabulated in the following manner in order to see clearly net ionic efflux.

TABLE (3), 7

Cation Efflux from 100g Dry Wt. tissue (meq)		Anion Efflux from 100g Dry Wt. tissue (meq)	
K ⁺	1.7	H ₂ PO ₄ ⁻	0.15
Na ⁺	0.2	HCO ₃ ⁻	6.67
Total	1.9	CO ₃ ⁻	0.70
		Total	7.52

Table showing net efflux of ions from P. schweinfurthii tissue when placed in deionized water in the dark for 7 hours.

It can be seen that the carbon dioxide fraction produces an equivalence of 7.37 meq anions /100g Dry Wt. tissue and the total acid required to neutralize the leachate solution was 6.88 meq / 100g Dry Wt. tissue; thus the alkalinity was entirely due to the HCO₃⁻ and CO₃⁻ content and the titre was a direct reflection of net cations efflux. As the sum of K⁺ + Na⁺ was only 1.9 meq/100g Dry Wt. the

remaining 5.6 meq cations required for a balanced stoichiometry were probably Ca^{++} and Mg^{++} .

This shows that the rise in pH in the dark was at least to some extent due to cation efflux from the tissue into the bathing solution and therefore pH rise in the light cannot be assumed to be only the result of OH^- excretion from photosynthesis.

. . . .

To Observe Change in pH of Deionized Water Containing
fully leached material in the light.

There is no doubt that there was a pH rise when fresh material was placed in deionized water in the light, but as ions were leaking out from the material one cannot say at this stage that the rise was only due to excretion of OH^- . If however, material is pretreated in many changes of deionized water so that all ions that are going to leak out will do so, then if the leached material is placed in fresh deionized water any pH change in the solution could perhaps be interpreted as a consequence of OH^- excretion.

Pretreatment:-

Shoots were collected in the afternoon, wiped clear of any loosely adhering algae, snail eggs etc. and were placed in a vessel of distilled water. The water was changed hourly for 4 hours and then the vessel was placed under Mercury Vapour lights overnight with aeration. The following day and before commencement of the experiment in the afternoon, there were 3 further changes of water, this time using deionized water previously aerated with CO₂-free air for two days.

Experiment:-

The leached shoots were placed in the 5 litre aspirator containing aerated deionized water and pH probes were sealed in the neck as before. Illumination and stirring were provided as before. No samples were taken until the end of the experiment. After 4 hours no further pH readings were taken. The tissue was left in the light until the following morning (14 hours) and the experiment was then repeated.

Results:-

Dry Wt. of tissue = 2.78 g

pH of deionized water at start
= 5.6

After 1 hour pH = 8.2

After $2\frac{1}{2}$ hours pH = 8.7 (and remained steady for the
next $5\frac{1}{2}$ hours.)

At the finish, after 8 hours there was no detectable K^+ ,
 Na^+ or $H_2PO_4^-$ and only 0.018 mM/l total CO_2 . At pH 8.7
total CO_2 will be almost entirely in the form of HCO_3^- .

On back titration with mixed indicator, 0.3 meq/5 litres of
acid were required.

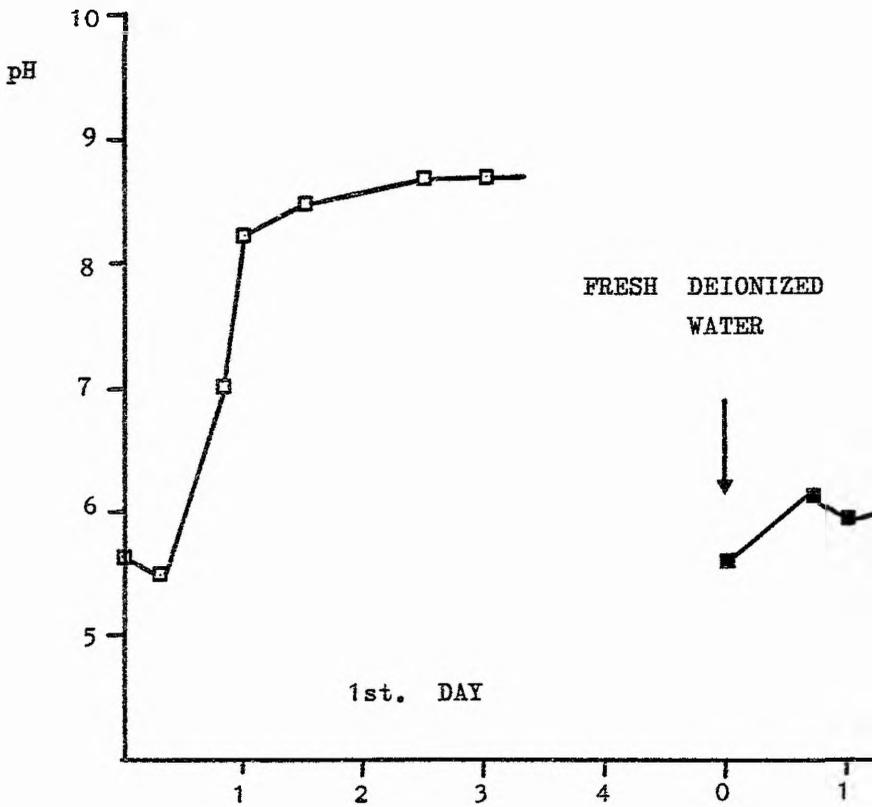
The following day the experiment was repeated with the
same material and a fresh supply of deionized water, but in
this case the pH rose only very slowly; from 5.6 to 7.8
in 8 hours. See graph (3), 4

Comments:-

As there was no detectable Na^+ , K^+ or $H_2PO_4^-$ it can be
assumed that the material was well leached. In the first
day's experiment the pH rose rapidly in the absence of
detectable cations. This rise might be explained by Steemann
Nielsen's theory that uptake of bicarbonate had produced
 OH^- . However, with fully leached material in deionized water
at pH 5.6 any carbon dioxide would mainly be in the form of
dissolved CO_2 and in the absence of cations, a bicarbonate
ion could not be formed.

GRAPH (3). 4

Change in pH of deionized water
containing fully leached, illuminated
P. schweinfurthii shoots plotted
against time.





2nd. DAY

2 3 4 5 6 7 8

HOURS

The utilization of the little dissolved free CO_2 and endogenous CO_2 during the photosynthesis of the tissue could produce OH^- ions and cause the observed rise in pH. Later work, however, showed that dissolved free CO_2 uptake does not cause OH^- excretion from the plant (pp. 122-125).

On the second day, although there was a pH rise it only occurred very slowly. This would suggest that something which was available on the first day had become limiting by the second or that the condition of the material was becoming poor through prolonged experimentation.

To consider the former possibility: It is most unlikely that OH^- was excreted without an accompanying cation. Assuming, therefore, that the total cation concentration amounted to 0.3 meq/5 l. it cannot involve K^+ or Na^+ as these would have been detectable at this concentration, thus, although Ca^{++} and Mg^{++} were not analysed they are likely candidates.

Accepting this, then perhaps by the second day's experiment there was insufficient Ca^{++} left in the tissue to accompany OH^- . Even if OH^- was still being produced it might not be able to leave the cells, at least not as rapidly, and a rise in pH of the outside solution would not be so obvious.

Possibly OH^- is a byproduct from a completely different metabolic process and the slower increase in pH of the solution is due, not to lack of a cation carrier for OH^- but to a decrease in OH^- concentration within the cell. Or perhaps there was a pool of HCO_3^- ions within the cells which slowly became depleted.

The final possibility, that the tissue was becoming poorly is not so likely for after the experiment it remained in several changes of deionized water for a further week and was then transferred to a pond where it quickly started growing vigorously.

. . . .

To Observe Quantitatively OH^- Production from Leached Material by Using a pH-stat Maintained at pH 5.4.

Pretreatment:-

Material was leached for four days with many changes of deionized water as in the previous experiments. The deionized water used in the experiment had been well aerated with compressed air.

Experiment:-

The material was placed in an illuminated aspirator containing deionized water and the pH probes were placed in the neck.

The Radiometer pH-stat was set at pH 5.4, the original pH of the deionized water. The burette contained dilute sulphuric acid so that if the pH of the solution rose the acid would be automatically titrated into the aspirator until the original pH was re-established, the titre volume being recorded on a dial.

A time course for amount of acid used was recorded and at the end of the experiment, after 8 hours, analysis for Na^+ , K^+ , Ca^{++} and CO_2 concentrations were carried out.

Result:-

Dry Wt. of material = 4 g (assuming 85% water content in
fresh material)

Normality of acid = 0.00978 N H_2SO_4

Total acid used = 45.92 ml

= 0.45 meq/ 6.2 litres

= 11.25 meq/ 100g Dry Wt.

Concentration of CO_2 in final solution

$$= 0.16 \text{ mM}/6.2 \text{ litres}$$

$$= 4.0 \text{ meq}/100\text{g Dry Weight}$$

dec. from 0.22 initial
(p. tot)

Concentration of K^+ in final solution

$$= 0$$

Concentration of Na^+ in final solution

$$= 0.019 \text{ meq}/6.2 \text{ litres}$$

$$= 0.475 \text{ meq}/100\text{g Dry Weight}$$

1 litre of final sample was evaporated to 50 ml and Na^+ and K^+ analysis were repeated. Ca^{++} was estimated on this solution by two independent means. (1) by flame photometry and (2) using an E.D.T.A. micro-titration with murexide indicator in an 'Eel' colorimeter.

The colorimetric estimation of Ca^{++} was considered the more reliable (see appendix p. xii)

This gave:-

$$\text{K}^+ = 0.003 \text{ meq}/6.2 \text{ litres}$$

$$\text{Na}^+ = 0.04 \text{ meq}/6.2 \text{ litres}$$

$$\text{Flame } \text{Ca}^{++} = 0.013 \text{ meq}/6.2 \text{ litres}$$

$$\begin{aligned} \text{Colorimeter } \text{Ca}^{++} \\ = 0.087 \text{ meq}/6.2 \text{ litres} \end{aligned}$$

$$\begin{aligned} \text{Therefore total cations} \\ = 0.13 \text{ meq}/6.2 \text{ litres} \end{aligned}$$

The Rate of acid titration was approximately a linear function.

These results can be converted to meq/100g Dry Wt. tissue and tabulated thus:-

TABLE (3), 8

	Net Cation Efflux per 100g Dry Wt. (meq)
K^+	0.075
Na^+	1.00
Ca^{++}	2.18
Total	3.255

Table showing cation efflux from leached P. schweinfurthii when illuminated and kept at constant pH for 8 hours in deionized water.

Comments:-

The stoichiometry is not balanced but it does appear that Ca^{++} is important since twice as much Ca^{++} leaks as $Na^+ + K^+$.

The sum of the concentration for Na^+ , K^+ , and Ca^{++} was 0.13 meq/6.2 litres whereas the acid titre was 0.45 meq/6.2 l. Assuming the total cations should balance. the acid used, then either the chemical methods for cation analysis are not

accurate enough at this extreme sensitivity or there are still other cations to take into consideration.

Is the acid titre value an approximate quantitative estimate of hydrox ion release?

The carbon dioxide concentration decreased during the experiment from 0.22 mM/6.2 litres (see p.105) to 0.16 mM/6.2 l. At pH 5.4 and 26°C, 90% of the total CO₂ would be in the form of dissolved free CO₂ and the removal of this from the solution would cause the pH to rise slightly. But also, if OH⁻ is being produced and there is a Ca⁺⁺ store in the plant then Ca(OH)₂ could be released and this would be neutralized with acid.



Calcium sulphate is relatively insoluble and once it is formed it is unlikely that the plant is able to reclaim the Ca⁺⁺ i.e. calcium is lost to the system. Gradually the whole system would become depleted of free Ca⁺⁺ and the pH would tend not to rise further.

The rate of acid titration is linear with time gradually getting slower until finally it stops. The result therefore would fit with the above suggestion.

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To Observe whether *P. schweinfurthii* in a Solution
Containing free CO₂ at pH 4.5 Produces Hydroxyl ions.

Pretreatment:-

Fresh shoots were cut from a culture pond (pH 8.8) and brought to the laboratory. The same pond-water was filtered and acidified with H₂SO₄ so that the pH was reduced to 4.5 thus forming a stock solution of acidified pond-water.

Experiment:-

One shoot of *P. schweinfurthii* was placed in a 500 ml round flask containing acidified pond-water. The flask was illuminated by a 1000 watt tungsten bulb and aerated from a cylinder containing 5% CO₂ air. After 2 hours a sample of solution was taken and boiled to remove free CO₂ and the pH was noted. The material was then placed in a fresh solution of acidified pond-water aerated with 5% CO₂ and left for a further 3 hours 25 minutes. After this time interval a second sample was boiled and its pH was taken.

This procedure was repeated a third time with a 4 hours interval and a fourth time with a 10 hour interval.

Results:-

TABLE (3), 9

Time Interval	pH of Boiled sample
2 hours	5.35
3 hrs 25 min	5.15
4 hours	4.50
10 hours	4.50

Table showing pH effect of photosynthesising tissue on acidified pond-water when dissolved free CO₂ was provided.

Original pH of pond-water = 4.50

Comments:-

This tissue was taken from a nutrient-rich pond-water at pH 8.8 and placed in the same pond-water at 4.5. The ionic content of the two solutions would be the same except for hydrogen and sulphate ions which increased. The increase in SO₄⁻⁻⁻ as compared with SO₄⁻⁻⁻ ions in the pond-water was negligible.

The results showed that there was an initial rise in pH but after the second change there was no further rise.

What caused this initial rise?

1. Before the tissue was transferred to acidified pond-water there was ample bicarbonate available for it and this, if

utilized, would produce OH^- ions according to Steemann Nielsen. On transferring the tissue to pond-water with a pH of 4.5 the plant might still have an HCO_3^- store which could be used in photosynthesis with accompanying OH^- production. However, if the system could not be replenished with HCO_3^- ions, no further rise in pH would be observed after the initial rise.

2. Because of the free diffusion of ions between the tissue free space and the acid solution the pH of the solution would show an initial rise until the tissue was in equilibrium with the acid solution.

Both these factors could well explain the initial rise in pH.

After several hours it was noted that the pH rose no more and the tissue appeared to be in equilibrium with the acid solution. At pH 4.5 the solution would contain no HCO_3^- ions and as free CO_2 was bubbled through the solution, the only available carbon source to the plant would be dissolved free CO_2 .

During the experiment the tissue was seen to be producing bubbles thus suggesting rapid photosynthesis. If OH^- ions were being produced, as the tissue was in pond-water, there would be ample supply of cations to act as carriers if necessary. Therefore it is concluded that dissolved free CO_2

utilization does not entail hydroxyl production in
P. schweinfurthii.

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To Observe pH Effects on Leached Material Kept at
 Constant pH in the Presence of Ca⁺⁺ in Deionized Water

Pretreatment:-

Shoot material was collected from the ponds and leached for six days in many changes of deionized water, and illuminated under mercury vapour during the night. A ^{low} dilute solution of Ca(OH)₂ was acidified with dilute HCl to pH 4.0

1. At pH 4.0

Experiment:-

The acidified Ca⁺⁺ solution was placed in a 500 ml round flask and 10 whole leaves of leached material were added. 5% CO₂ air was bubbled through continuously for a carbon source and for stirring, and the tissue was illuminated. Samples were taken and boiled (to remove free CO₂) and then their pH noted.

Results:-

TABLE (3), 10

Cumulative Time	Time Interval	Original pH of Boiled sample	Final pH of Boiled sample
0-3 hrs.	3 hrs.	4.0	5.2
3-25 hrs	22 hrs	4.0	5.2
25-31½ hrs	6½ hrs	4.1	4.1

Table showing change in pH with time caused by illuminated P. schweinfurthii in a solution of $\text{Ca}(\text{OH})_2$ acidified to pH 4.0

After each sample was taken the pH was reduced again to \pm 4.0 by adding dilute HCl.

(2) At pH 5.0

Continuing the experiment, the pH was then raised to 5.0 using 5 meq/l KHCO_3 solution and aeration was stopped. The flask however remained open to the air.

Results:-

See Table (3), 11

TABLE (3), 11

Cumulative Time	Time Interval	Original pH of Boiled sample	Final pH of Boiled sample
0-10 hrs	10 hrs	5.0	4.5
10-13 $\frac{3}{4}$ hrs	3 $\frac{3}{4}$ hrs	5.0	4.7
13 $\frac{3}{4}$ -15 $\frac{1}{2}$ hrs	1 $\frac{3}{4}$ hrs	4.9	4.65
15 $\frac{1}{2}$ -17 $\frac{1}{2}$ hrs	2 hrs	5.0	5.05
17 $\frac{1}{2}$ -21 hrs	3 $\frac{1}{2}$ hrs	5.05	5.05

Table showing change in pH with time caused by R. schweinfurthii in a solution of $\text{Ca}(\text{OH})_2$ acidified to pH 5.0

Note - the pH was adjusted to approximately 5.0 by adding 5 meq/l KHCO_3 solution; and for short intervals (<30 sec.) during the experiments 5% CO_2 air was slowly bubbled through so that a carbon dioxide supply for the plant was maintained.

(3) At pH 5.2

Result:-

See Table (3), 12

TABLE (3), 12

Cumulative Time	Time Interval	Original pH of Boiled sample	Final pH of Boiled sample
0 - 12 hrs	12 hrs	5.2	5.4
12 - 13 hrs	1 hr.	5.0	5.4
13 - 17 hrs	4 hrs	5.2	5.35
17 - 22 $\frac{3}{4}$ hrs	5 $\frac{3}{4}$ hrs	5.35	5.8
22 $\frac{3}{4}$ - 34 $\frac{3}{4}$ hrs	12 hrs	5.2	5.5
34 $\frac{3}{4}$ - 39 $\frac{3}{4}$ hrs	5 hrs	5.5	6.1

Table showing change in pH with time caused by P. schweinfurthii in a solution of $\text{Ca}(\text{OH})_2$ acidified to pH 5.2

The solutions were aerated at intervals with 5% CO_2 for a carbon supply and with compressed air. The pH was lowered with dilute HCl.

Comments:-

In the pretreatment the material had been thoroughly leached and on transfer to acidified water containing Ca^{++} , OH^- , H^+ and Cl^- ions at pH 4.0 it still remained healthy.

In section (1) of the experiment there was a tendency for the pH of the solution to rise above 4.0 but after a while this tendency ceased. This initial rise could be an

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equilibration effect in that some cations from the tissue would exchange with H^+ in solution but not in sufficient quantities to affect the health of the plant.

In section (2) when the pH was raised to 5.0 then there was a tendency for the solution pH to fall until equilibration. This would be expected as some H^+ ions from the tissue free space and wall would diffuse out and exchange with cations in the solution.

In section (3) where the pH was raised to 5.2 the opposite was observed. According to the suggestion in section (2) above, a slight fall in pH might be expected but instead it continued to rise. Below pH 5.2 perhaps the only available carbon dioxide supply to the tissue was dissolved free CO_2 even though there might have been some HCO_3^- ions in the solution. As was previously shown (p. 123 & 124) utilization of dissolved free CO_2 does not produce an OH^- byproduct. Above pH 5.0 the HCO_3^- : free CO_2 ratio increases rapidly and HCO_3^- would become increasingly more available to the plant.

Possibly then, under these conditions, the critical pH for commencement of HCO_3^- assimilation lies between pH 5.0 and 5.2.

Of course, if the tissue was beginning to degenerate

then ions would leak out and this would cause the pH of the solution to rise. But the tissue was finally transferred to natural conditions again and observed for a further two weeks. During that time there were no visible signs of degeneration.

. . . .

To Observe the pH of CO₂-free Deionized Water Containing Leached, Illuminated Tissue, Followed by the Addition of Ca⁺⁺.

Pretreatment:-

Fresh shoots of P. schweinfurthii were leached for seven days in deionized water, in daylight and darkness, the water being changed at least three times each day.

Method:-

The leached shoots were placed in an airtight 5 litre round flask containing deionized water that had been aerated with CO₂-free air for 48 hours previously. The solution was stirred with a magnetic stirrer and the tissue was illuminated by a water-cooled 1000 watt tungsten bulb as in previous experiments. The pH was recorded from electrodes sealed into the rubber bung placed in the neck of the flask.

ref. electrode
leaks KCl

At 3 hours approximately 25 ml of 0.5 meq/l CaCl_2 solution was added to the flask.

Results:-

For the first $2\frac{1}{2}$ hours the pH remained steady at pH 5.7 to 5.8. After the calcium was added the pH rose steadily from 5.25 to 7.9 in 6 hours. (see graph (3), 5).

(I am indebted to Mr. T.R. Milburn for the results of this experiment.)

Comments:-

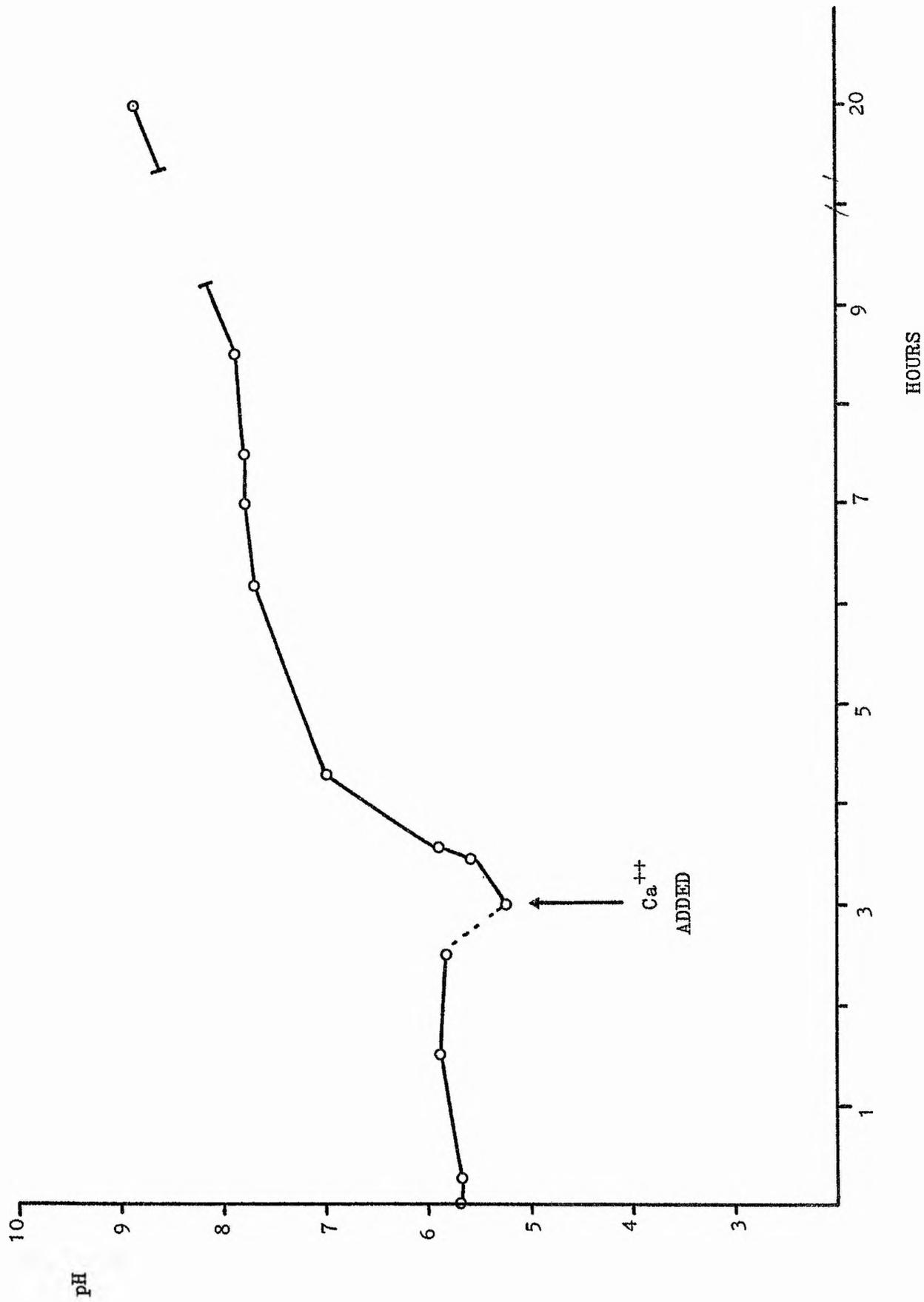
At the beginning of the experiment any CO_2 in solution would be in the form of dissolved free CO_2 since there were no cations. Any photosynthesis in the plant would arise from endogenous CO_2 and this would not raise the pH externally. with pH 5.7!

On adding Ca^{++} as CaCl_2 to the solution the pH fell to 5.3 but at this pH a little CO_2 in solution would be present as bicarbonate. There is evidence (Chapter 3 p. 98) that Ca^{++} at 0.5 meq/l added to a bicarbonate solution stimulated photosynthesis of the plant. This Ca^{++} solution would provide some HCO_3^- ions at pH 5.3 which would be just above the suggested pH threshold for HCO_3^- assimilation.

Assuming dissolved free CO_2 and HCO_3^- uptake can go on

GRAPH (3), 5

Graph showing rise in pH on addition
of calcium ions plotted against time,
when leached shoots of P. schweinfurthii
were placed in deionized water.



simultaneously although mainly free CO_2 would be taken up, a little HCO_3^- uptake would tend to raise the pH, the pH rise then snowballing.

This system is a little difficult to visualize for there was only a very limited supply of carbon in solution. The only available supply of carbon would be from endogenous respiration. For this system to work, presumably endogenous CO_2 would have to go into solution and then be converted to HCO_3^- . This would be a little surprising when it is much easier for CO_2 to pass directly from respiration to photosynthesis without leaving the cell.

Another explanation might be that OH^- was being produced in the pretreatment but could not be released from the cell as there were no cations in deionized water. This OH^- was therefore stored in the cell until a carrier was provided, i.e. when CaCl_2 was added.

. . . .

DISCUSSION AND CONCLUSION

An attempt has been made to get a clearer understanding of the carbon source in the photosynthesis of P. schweinfurthii.

Although many experiments are inconclusive trends are observed leaving little doubt that this plant falls into the

general category of Ruttner's Aquatic seed plants in being a bicarbonate user.

pH Tolerance.

In a cation-free solution acidified to pH 4.0 and with a low CO₂ content, *Potamogeton* soon died but in similar conditions with cations in the solution the tissue remained healthy.

Material which was leached of all mobile ions in many changes of deionized water with or without CO₂ could survive indefinitely.

This would suggest that at low pH and in the absence of cations death resulted not through ion leakage from the tissue or H⁺ ion concentration, but through cation exchange; the bound (immobile) cations in the tissue exchanging for H⁺ in solution. If there were sufficient M⁺ ions in solution at this pH such as in the case of acidified pond-water, H⁺ ion exchange would be reduced to a minimum and the tissue survive.

In natural conditions the plant is frequently found in waters of pH values around 9.0 or 10.0; therefore *Potamogeton schweinfurthii* appears to have a wide pH tolerance in the presence of cations (from 4.0 to 10.0).

CO₂ Balance.

It will be appreciated that over such a wide pH range,

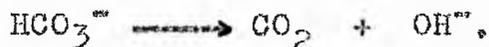
carbon dioxide in the external solution will be in different forms, i.e. only dissolved free CO_2 at pH 4.0 and mainly as HCO_3^- at pH 9.0.

Where there was only dissolved free CO_2 in deionized water, *P. schweinfurthii* photosynthesised readily and the absence or addition of cations in the solution had no effect on the rate. There is strong evidence that hydroxyl ions were not produced during photosynthesis with dissolved free CO_2 as the carbon source.

In a pure 5 meq/l bicarbonate solution *P. schweinfurthii* was able to photosynthesise only very slowly but on the addition of CaCl_2 the rate was increased considerably. This confirms Steemann Nielsen's hypothesis that additional cations in a bicarbonate solution enhance the rate of photosynthesis.

In solutions where there were additional cations and where the pH was sufficiently high for a quantity of HCO_3^- to be available, illuminated tissue raised the pH of the solution further. All evidence suggests that this was the result of OH^- ion excretion and negative evidence points to Ca^{++} as the co-ion.

This pattern conforms with the long standing theory that OH^- is disengaged in the plant from bicarbonate thus:



In the present set of experiments which were run at 25°C it appears that there was a critical pH of 5.0 to 5.25 above which the plant was able to get at least some of its carbon supply from HCO_3^- ions.

There was no evidence to suggest that HCO_3^- and free CO_2 assimilation cannot take place concurrently but there is little doubt that the plant had a preference for dissolved free CO_2 .

Sometimes it is suggested that bicarbonate users may not in fact use bicarbonate as such, for in bicarbonate solution there is always some dissolved free CO_2 . The free CO_2 is taken up by the plant and is replenished from HCO_3^- dissociation.

This cannot be wholly accepted for *P. schweinfurthii* in the light of present evidence for it is shown above that free CO_2 does not disengage OH^- whereas HCO_3^- does. Further, the dissociation takes place in the tissue intracellularly. If the tissue was not in fact taking up HCO_3^- but the little dissolved free CO_2 in a HCO_3^- solution then the tissue would not produce OH^- ions.

CHAPTER IV

CARBON DIOXIDE AS A FACTOR IN VARYING THE
CELL MEMBRANE POTENTIAL AND ITS POSSIBLE
ECOLOGICAL IMPLICATIONS

Introduction

In chapter 2 the steady membrane potential (E_m) of Potamogeton schweinfurthii was shown to be approximately -180 mv when the cell was equilibrated in pond water in the light.

Variation in membrane potential can occur, however, and in the present chapter the factors causing this are investigated.

It is known that in the cell there are anions e.g., proteins, chloride and probably bicarbonate, which cannot easily diffuse out passively, and which thus produce a pool of virtually non-permeating anions. There will be a tendency to balance this anionic charge by the net inward diffusion of passively mobile cations under the influence of both chemical and electrical forces.

Now, as the membrane is selectively permeable different ions will tend to move passively across it at different rates thus producing, at equilibrium, the membrane potential. If it is assumed that the major ions moving passively are K^+ , Cl^- and possibly HCO_3^- , then the membrane potential E_m , would be according to the Goldman Equation

$$E_m = \frac{RT}{F} \ln \frac{P_K [K^o] + P_{Cl} [Cl^i] + P_{HCO_3} [HCO_3^i]}{P_K [K^i] + P_{Cl} [Cl^o] + P_{HCO_3} [HCO_3^o]}$$

where P is the permeability and superscripts o and i are concentrations outside and inside the cell. R is the gas constant, T the absolute temperature and F the Faraday.

As can be seen, E_m will be most strongly affected, and set, by the concentration, inside and outside, of the most permeable ion. In chapter 2 this ion was shown to be K^+ which accords with findings on other cells (see MacRobbie and Dainty et al.)

If the concentration of K^+ outside the cell were increased then there would be less tendency for K^+ to diffuse out of the cell along its concentration gradient, resulting in a decrease in cell potential. This possibility was tested in *P. schweinfurthii* by observing the membrane potential of the cells when placed in pond water and then adding KNO_3 to give 50 meq/l K^+ (i.e. an approximate 100 fold increase in concentration).

The cells were immediately depolarized from -180 mv to -140 mv. By calculation a fall of nearer 100 mv would have been expected but as the time interval was only a few minutes equilibrium might not have been reached, owing to slow and selective permeation through the cell wall.

If $Ca(NO_3)_2$ instead of KNO_3 were added to the pond water to a concentration of 50 meq/l Ca^{++} , then hyperpolarization of approximately 20 mv was observed. This is contrary to expectation according to the Nernst equation (p.45).

A similar response was reported in *Avena coleoptiles*, by Higinbotham et al. (1964), who suggested that Ca^{++} could, perhaps, affect membrane permeability to K^+ and/or stimulate an anion

influx or cation efflux pump. Laties et al. (1964) on theoretical grounds appear to favour the view that the hyperpolarization was due either to calcium permeability being exceedingly low as compared with anion permeability, or to the reduction of the permeability of K^+ outwards. Whatever the reasons there is no doubt that the change in the concentrations of ions outside can have an immediate effect upon the membrane potential which must therefore be an ion-diffusion potential.

A factor, however, which is not so generally appreciated as being able to cause potential variations under some conditions, is light intensity. Barr and Broyer (1964) claimed that in Nitella clavata, ranges in light intensity from 320 to 1800 ergs/cm²/sec did not affect the membrane potential although sodium influx rate depended upon it, and the magnitude of ion fluxes in both directions across the plasmalemma were correlated with it. They found that the ionic composition of the cell sap was also unaffected.

Alternatively, Nagai and Tazawa (1962) observed that on illuminating cells of Nitella flexilis, after a period of darkness the cell e.m.f. increased by about 30 mv with a corresponding increase in ion absorption and osmotic pressure of the cell sap. Further, red light at 148 ergs/cm²/sec. and blue light at 140 ergs/cm²/sec. respectively increased the membrane potential by 80% and 60% of that in white light at 1800 ergs/cm²/sec., whereas green light had no effect on potential.

Hope (1965) found that in Chara australis the effect of light and dark on the e.m.f. and on membrane resistance was negligible in a medium not containing bicarbonate ions. However, in the presence of bicarbonate ions illumination caused cell hyperpolarization and, changing from light to dark or dark to light (from now on being referred to as light/dark or dark/light switches) caused large changes in cell resistance.

In the present study both CO₂ and light intensity are shown to have an effect on the membrane potential and these aspects are studied more closely in this chapter.

The procedure and apparatus used in the following experiments are basically the same as those described in the previous chapter.

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A) The Effect of Light on Cell E.M.F.

To observe the Effect of Light and Dark on Cell Potentials

Pretreatment:-

A few shoots from the same plant of *Potamogeton* were cut and placed in a 5 litre beaker containing their own pond water filtered through Whatman's No. 1 paper. This was aerated and placed on a windowsill for two days before being refiltered and divided into two 250 ml beakers. Two leaves were excised from one shoot and were cut transversely across, a section from each leaf being placed in each beaker.

Both beakers were aerated at constant temperature but one was illuminated and the other was kept in total darkness. The illuminated tissue was used after 24 hours and the darkened tissue after 48 hours.

Prior to the experiment the leaf sections were cut transversely into suitable strips and left for a further 6 hours to recover.

The experiment was divided into two parts; (a) using strips which had been illuminated and (b) using those that had been kept in total darkness.

a) Strips which were illuminated for 24 hours before the experiment

Method:-

A strip with its midrib removed, was mounted on the microscope as described earlier, with filtered pond water flowing past. The Medistor probe and electrometer amplifier was used to record potentials against time. Resistances of the micro electrode tip inside and outside the cell were recorded and results that did not lie within the specifications previously discussed, (p.68), were ignored.

After a steady potential had been obtained for at least two minutes the microscope light was switched off for a period and then switched on again. In some cases this alternation of light and darkness was continued for a considerable time whilst recording

the potential for a single cell. The switching on and off of the ordinary laboratory light at no time made any difference to the results.

Results:-

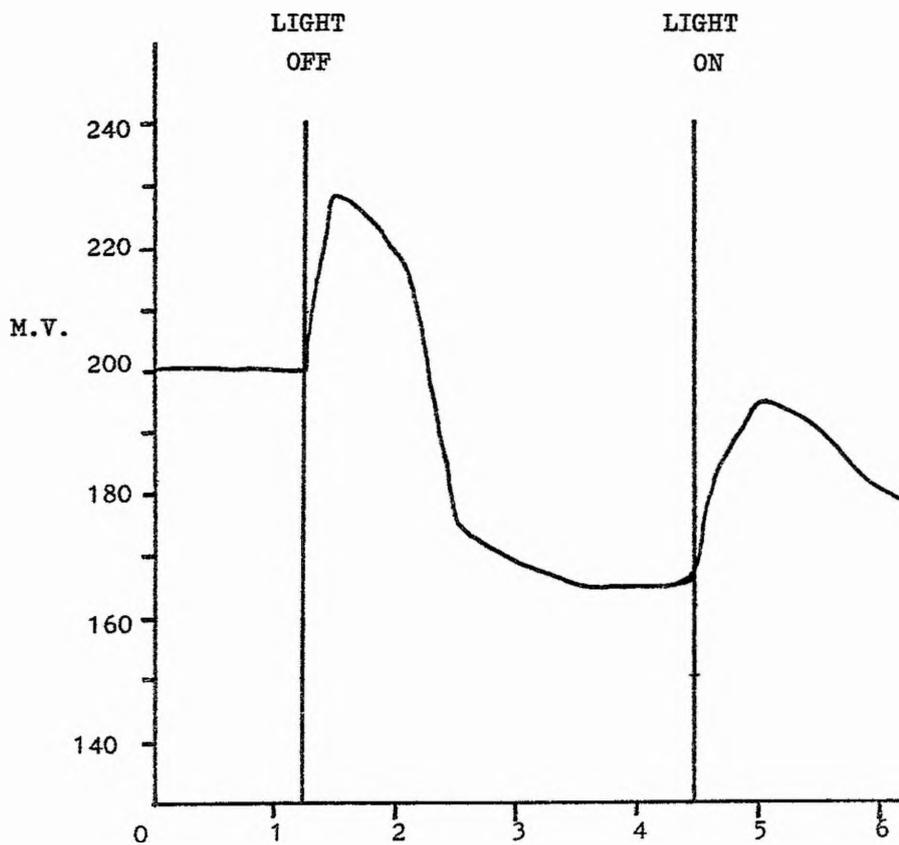
Switching the light either from light/dark or dark/light produced a change in cell potential. With light/dark changes the cell always showed an immediate rapid hyperpolarization generally at the rate of about 20 mv/30 sec. This usually reached a plateau within the first minute or two and then fell again either rapidly or slowly, to about the original potential. (see graphs (4)1 and (4)2). Rises of 80 mv or more were observed but usually the rise was in the range of 15 to 30 mv.

Dark/light changes were not nearly so consistent, on some occasions there was an immediate fall in the membrane potential and on other occasions (more frequently) there was an initial rise followed by a fall in a manner similar to that observed in the light/dark changes.

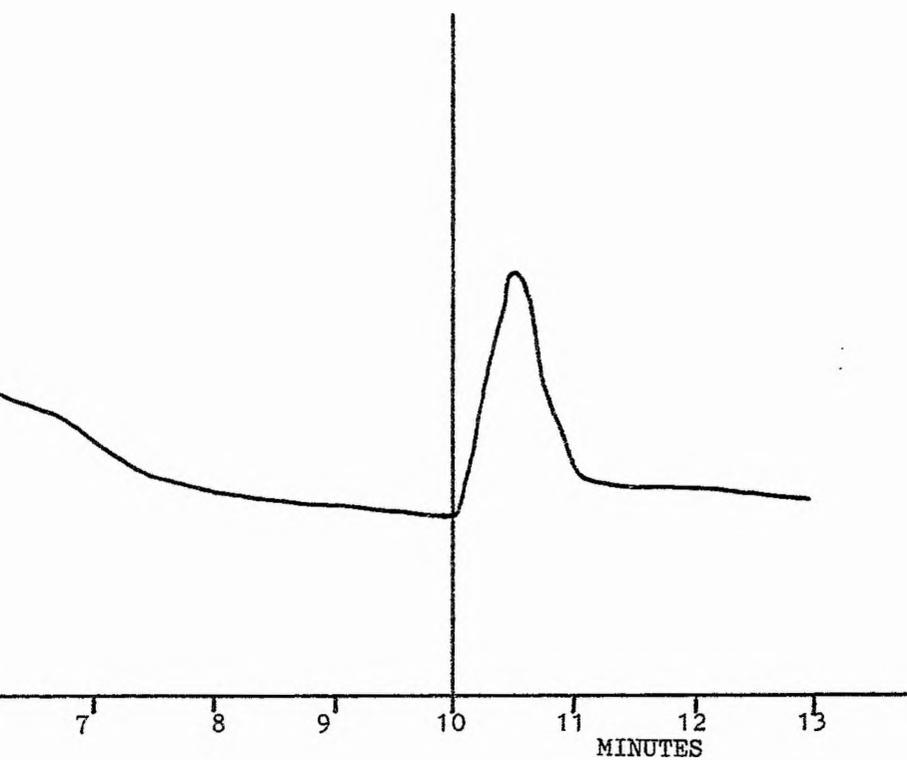
Illuminated tissue which had dark periods of up to 60 minutes produced on reillumination the same results with light/dark, dark/light switches.

GRAPH (4), 1

Observed membrane potential, (mv) of
P. schweinfurthii leaf cells in light or
darkness plotted against time.

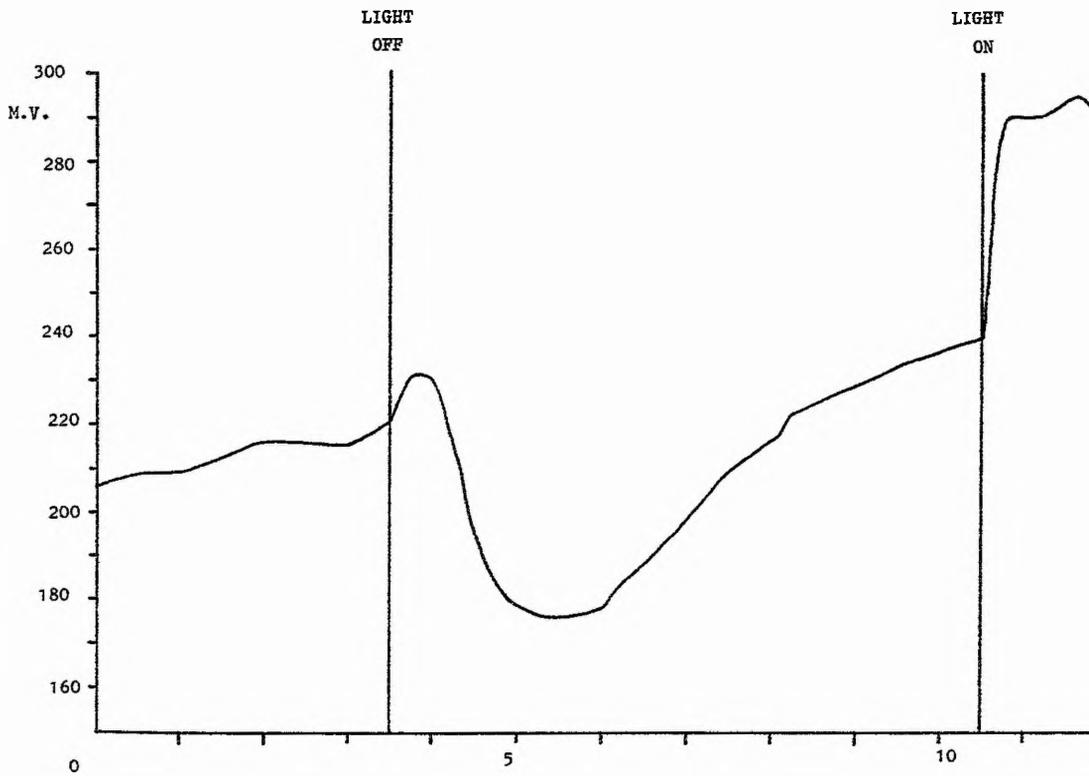


LIGHT
OFF



GRAPH (4), 2

Observed membrane potential (mv) of
P. schweinfurthii leaf cells in
light or darkness plotted against
time.



LIGHT
OFF

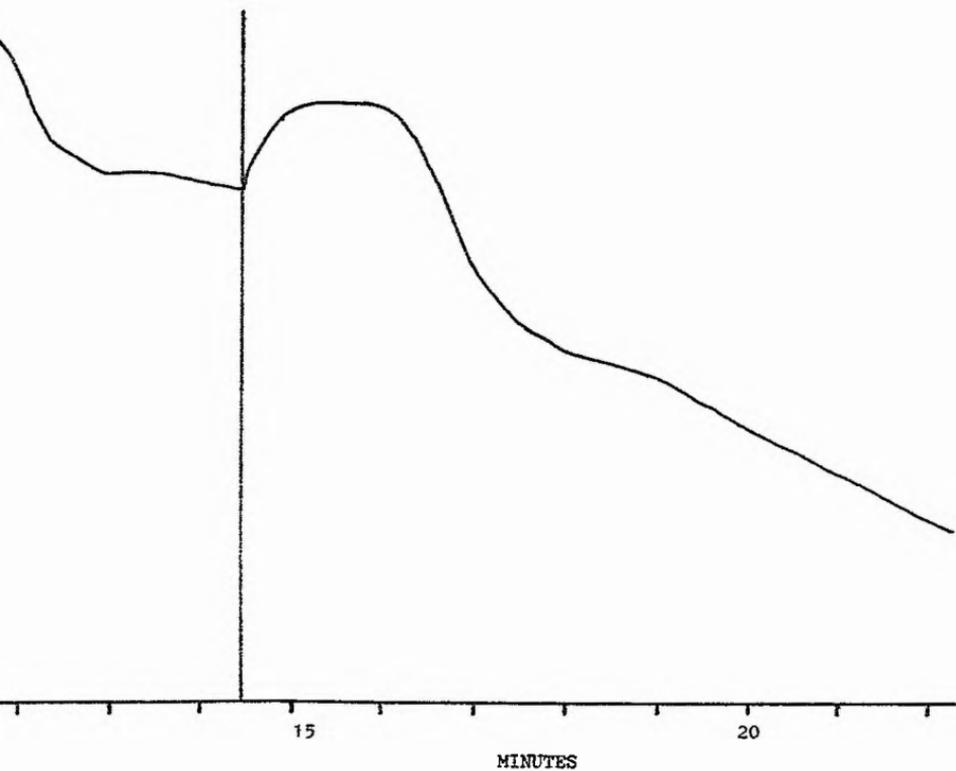


TABLE (4), 1

Switch	Number of incidences of:-		
	Hyperpolarization	Steady e.m.f.	Depolarization
Light/dark	16	0	0
Dark/light	9	0	3

Table showing number of incidences of Hyperpolarization, Steady e.m.f. and Depolarization with light/dark and dark/light switches. The tissue was mounted without a midrib.

Comments:-

Light/dark and dark/light switching caused an immediate rapid change in cell e.m.f., but the cell membrane potentials generated by ionic diffusion are, in theory, temperature sensitive (according to factor T in the Nernst equation and Goldman equation) (p.45). Bright illumination of the cells might, therefore, warm them, and as E is proportional to T there would be the tendency for hyperpolarization in the light, and depolarization in the dark. The opposite was most frequently observed therefore these changes in e.m.f. cannot be attributed to changes in temperature, but to changes in light intensity.

The above results obtained in November contrast with those obtained in July and August where with Dark/light changes depolarization was more normal. See table (4), 2.

TABLE (4), 2

Switch	Number of incidences of:-		
	Hyperpolarization	Steady	Depolarization
30th July			
Light/dark	8	0	0
Dark/light	0	2	5
6th August			
Light/dark	8	0	0
Dark/light	0	0	6

Table similar to (4), 1 above but these experiments were conducted in July and August. The tissue was mounted with a midrib.

However, whereas in the present experiment tissue strips were mounted without the midrib, in the previous two experiments mentioned a midrib was present.

The presence or absence of a midrib in the strip could well have been an important factor in producing this discrepancy. The whole system relied upon good stirring around the cell by having the solution flowing fast through the apparatus over and around the tissue. When a strip was mounted, the midrib, which was more bulky than the rest of the leaf tissue facilitated this flow by keeping the coverslip raised off the tissue. Although electrode

Footnote:- The experiment on the 6th August differed also in that the bathing solution was distilled water but this is unlikely to affect any argument at this stage and will be treated more fully later. See p. 153

penetration was made considerably easier with the midrib removed, the coverslip then rested on the lamina so that the flow of the medium over the cells might be restricted. This might have had the depletion or accumulation of certain ions in the medium around the cell thus directly altering the electrochemical equilibrium. However the difference may rather be directly associated with cell metabolism. For example, without flow over the cells there would be a removal of total CO_2 from the poorly stirred layers in the light and a rapid accumulation in the dark, whereas with a good flow the CO_2 concentration would remain fairly constant. The oxygen tension would vary in the opposite manner. (Graph (4) 9 shows that change in flow does affect the e.m.f.)

If this discrepancy in results with the dark/light switch was caused by the difference in concentration of total $\text{CO}_2(\text{outside})$, i.e. in the external medium, then to be consistent a high concentration of $\text{CO}_2(\text{outside})$ would be expected to produce hyperpolarization and a low concentration, depolarization, when switching from dark to light. Further experiments showed this to be the case.

(See p. 175)

Conclusion:-

- 1) Change in light intensity affected the cell potential.
- 2) Previously illuminated tissue when put in darkness produced an initial rapid hyperpolarization which then drifted back again to approximately the original potential.

- 3) Previously illuminated tissue which was interrupted by a short interval of darkness either produced an initial rise or an immediate fall in membrane potential when exposed to light again.
 - 4) It is suggested that the inconsistency of (3) above was the result of variable rate of flow of solution over the lamina and could, perhaps, be attributed to carbon dioxide or oxygen tensions in the solution immediately surrounding the cells.
 - 5) Most evidence would suggest that, under conditions of good stirring, depolarization was the normal reaction to the dark/light switch.
- b) Strips which were in darkness for 48 hours before the experiment

Method:-

The method was similar to that described for the illuminated tissue but the mounting of the tissue on the slide was carried out in darkness except for one red photographic safe light facing the wall.

Micro-electrode penetration was possible by using a dense chlorophyll extract filter over the microscope light so that the cells and electrodes could just be seen under the high power. Tests showed that this illumination was equivalent to total darkness in respect to cell potential, i.e. there was no change in e.m.f. on changing from this green light to darkness, and vice-versa.

The manipulation proved to be very difficult so that reliable readings were limited to one cell only but in this cell the penetration appeared to be excellent and readings were obtained reproducibly for 50 minutes before the electrode was withdrawn.

Results:-

On penetration (in the dark) in the first 90 seconds the e.m.f. fell from 100 to 70 mv. This was considerably lower than for other cells which showed initial potentials of about -180 mv (but these did not maintain their potentials for more than a few minutes).

After 90 seconds the light was switched on for 30 seconds and the potential rose to -120 mv. The light was then switched off and the e.m.f. immediately increased at a rate of approximately 20 mv/30 sec. to -180 mv after which it started to fall, at first rapidly and then less so. At -140 mv the fall was interrupted by switching the light on. This increased the rate of fall until, in about 2 minutes, the potential steadied to 92 mv.

The light was then switched off again and the same pattern of results was obtained. This procedure was repeated five times and in each case there was a rapid hyperpolarization in the dark followed by an initial rapid and then more steady depolarization. On illumination the rate of fall was again accelerated. The

potential fluctuation from light/dark/light reached a maximum of 170 mv (from -100 mv to -270 mv and back again to -115 mv). See graph (4), 3.

To test whether the effects of light were due to its absorption by chloroplast pigments in the cells a chloroplast pigment extract from P. schweinfurthii leaf tissue was used as a green filter, a cell mounted in the dark being exposed alternately to green light/darkness/green light/white light. Results are shown in graph (4), 4.

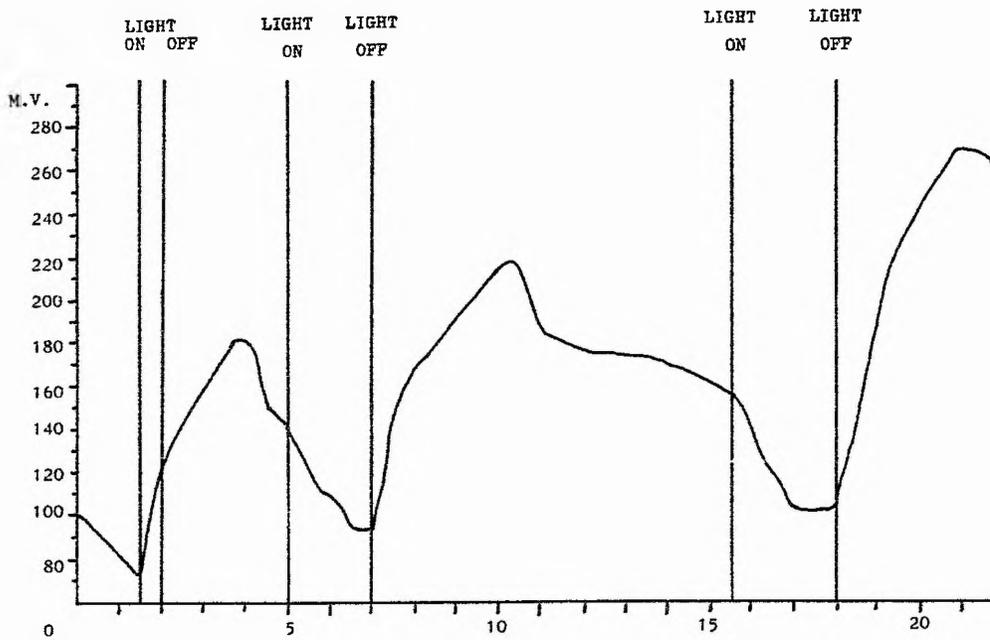
Although there were slight potential changes with the green light/dark switches they were only in the range of 10 mv as compared with the very large fluctuations in the light/dark experiments. The green chloroplast extract therefore appeared to be an effective absorber of the "excitation" wave lengths of light.

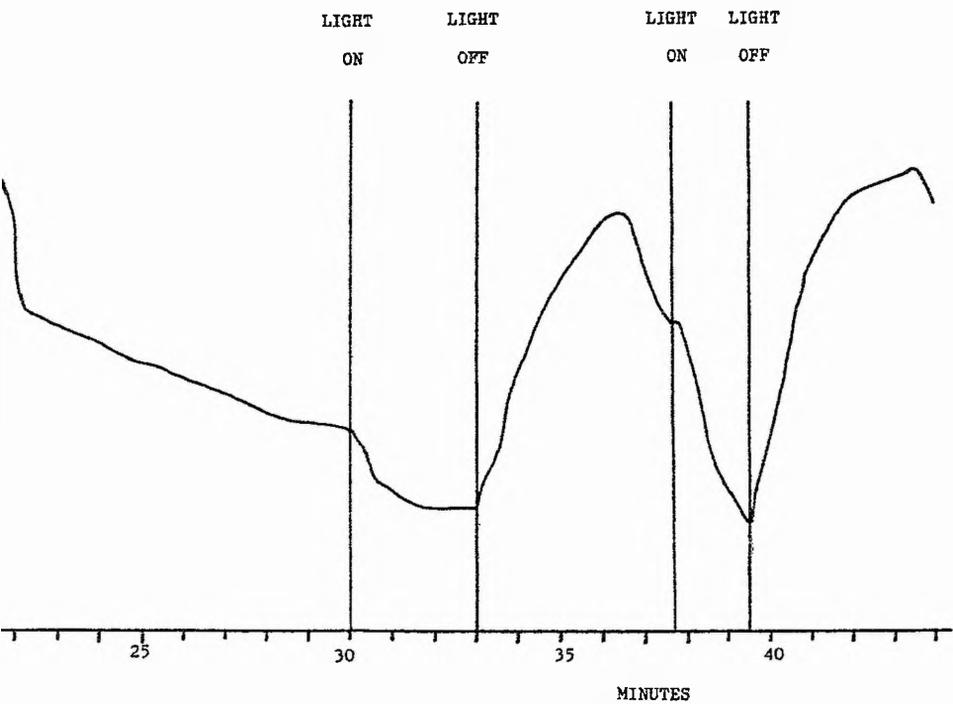
Comments:-

It is difficult to interpret the first 30 sec. light flash which produced some hyperpolarization, since there are unfortunately no data comparable. Apart from this the sequence was consistent and repeatable throughout. Again the tissue was mounted without midrib but, if the lamina had been mounted very near the edge of the slide (and this was always attempted), then the cell would have had sufficient irrigation even in the absence of a midrib. The position of the tissue, however, was not recorded. If it is assumed there were sufficient movement of solution over the cells

GRAPH (4), 3

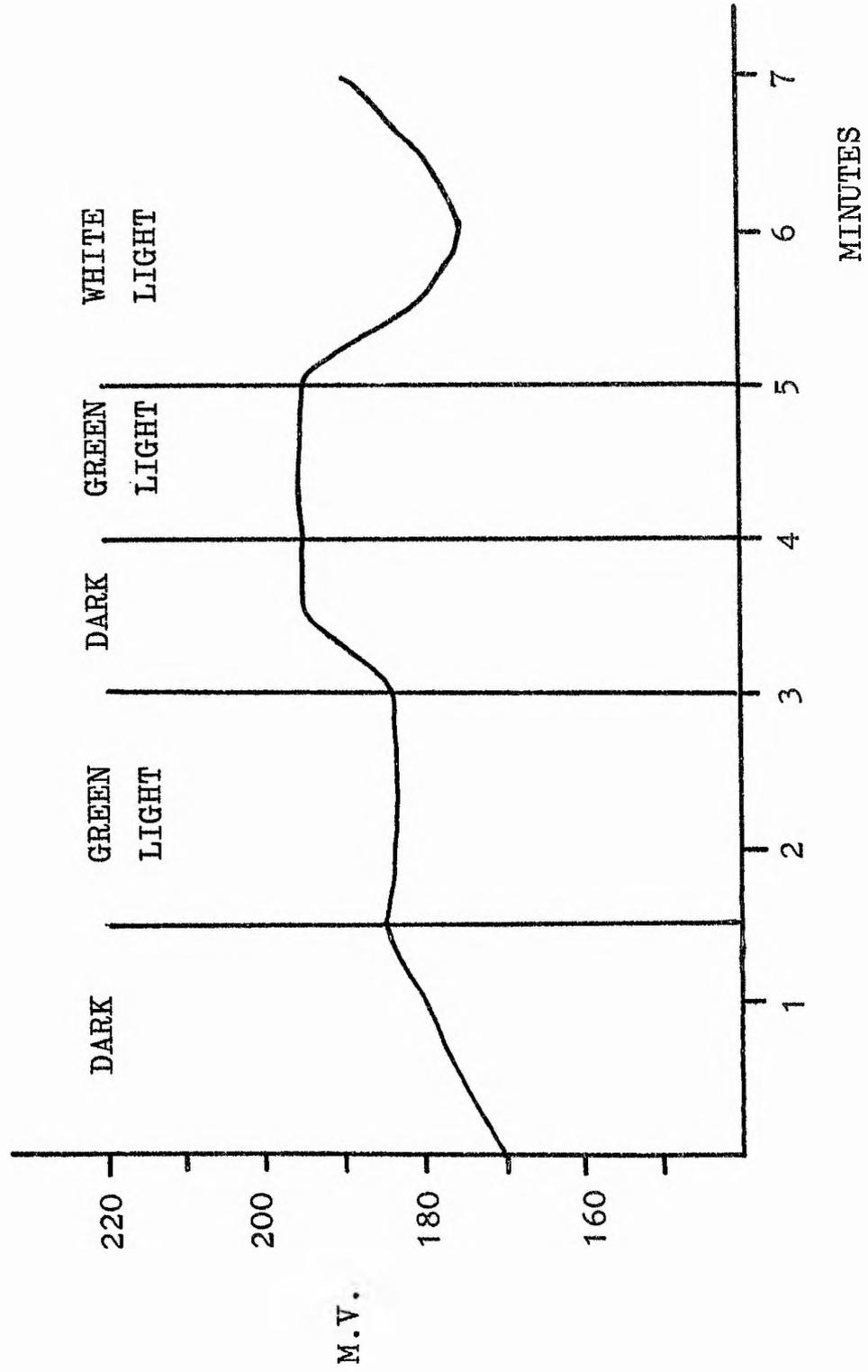
Variation in cell membrane potential of leaf tissue which had been pretreated for 48 hours in darkness. The tissue was in aerated pond-water and flashes of light and dark were given to it whilst observing E_m with time.





GRAPH (4), 4.

Variation in E_m of cells bathed in aerated pond-water plotted against time, whilst darkness was interchanged with green light and white light.



then the results should be similar to those obtained for illuminated tissue with good stirring; This was indeed the case; also, there is a noticeable graphic similarity between the dark stages (light/dark switch) of this experiment and of the experiment with illuminated tissue, (compare graph (4), 2, with graph (4),3), but the amplitude of the potential changes in this experiment appear to be greater.

Conclusion:-

- 1) The results are consistent with those obtained for illuminated tissue which had a midrib (and therefore good flowing of solution over the cells).
- 2) If it is assumed that there was a satisfactory flow of solution over the cells in the present experiment then it can be concluded that, with respect to changes in e.m.f. and light intensity, tissue that had been previously in the dark for 48 hours behaved in a similar manner to that which had been previously illuminated for 24 hours.

. . . .

The change in e.m.f. with changing conditions of light and dark and the suggestion that the concentration of total CO_2 (outside) also plays a part would favour a hypothesis in which photosynthesis directly or indirectly affects the E_m . If red and blue wave lengths of light have the same effect as white light then this would

strengthen the hypothesis as these wave lengths are absorbed by chlorophyll.

If photosynthesis is directly involved and, as concentration of CO_2 (outside) is one of the factors controlling its rate, a change from high to low external concentration of CO_2 would be expected to produce the same responses as a light/dark switch.

Alternatively, perhaps the light provides a source of energy for the active pumping of ions through the membrane and it is this ionic flux which causes the changes in the cell e.m.f. In that case, active transport of anions outwards or cations inwards should depolarize newly illuminated cells previously in darkness.

. . . .

B) The Ionic Effect

To Investigate the Role of Ions in the Change of E_m

If the effects of light or dark on E_m operate through the sudden cessation or operation of active, inward, ion-transporting mechanisms, then these effects should not be observed in tissue bathed in external solutions virtually free of ions.

Pretreatment:-

Shoots were leached for 4 days in many changes of deionised

water and then were placed under illumination in a water filled aspirator. The water was then pH controlled at pH 5.4 using 0.0 1N H_2SO_4 and left for a further 3 days. (This was the experiment discussed in chapter 3, pp 117-121).

At the end of the experiment some of the leached tissue was removed from the aspirator and treated in the normal manner for observations on membrane potential, the solution flowing past the cells being the pH controlled solution.

On analysis this solution contained the following concentrations of ions:-

K^+	=	0.55×10^{-3}	meq/l
Na^+	=	6.50×10^{-3}	meq/l
Ca^{++}	=	14.2×10^{-3}	meq/l
Total CO_2	=	0.125	mM/l
H_2SO_4	=	75×10^{-3}	meq/l

Results:-

This material was used in combination with an experiment using different wavelengths of light and the results are therefore discussed together on p 155-156.

The results relevant to the present discussion are that the dark/light switch produces an immediate depolarization and the light/dark immediate hyperpolarization. i.e. the same results as were observed with tissue in normal flowing pond water.

Comments:-

As was shown earlier (p 104-109) although Ca^{++} , K^+ , and Na^+ are initially lost from the tissue when it is placed in deionized water, the tissue finally comes to passive flux equilibrium. Direct chemical analysis of the tissue after leaching gave the following results shown in Table (4),3. (See also p. 55)

TABLE (4), 3

Ions	Concentration in tissue $\mu\text{eq/g}$ Fresh Water	C^i meq/l	C^o meq/l	$\frac{\text{C}^o}{\text{C}^i}$
K^+	97	123	0.55×10^{-3}	4×10^{-6}
Na^+	7	9	6.50×10^{-3}	7×10^{-4}
Ca^{++}	57	72	14.20×10^{-3}	2×10^{-4}

Calculated Concentrations of Ions in the Cell Sap (C^i) of Leached Material, and Concentrations of the Ions in the Bathing Solution (C^o).

Accepting as correct, the conclusion in chapter 2 that the passively highly mobile K^+ ion is in electrochemical equilibrium (See p. 84) then by substituting in the Nernst Equation

$$E_K = 59 \times -5.398 \text{ mv}$$

$$E_K = -300 \text{ mv}$$

The same material in normal pond water would give an E_K of approximately -150 mv.

From these E_K values one would expect the E_m to be much higher for those cells in deionised water than those in pond water. This is in fact the case; the average observed potential in deionised water being about -230 to -240 mv and in pond water -196 mv (see p. 78). (It is not surprising that the full -300 mv was not realized with those cells in deionised water as this would require an immense electrical resistance of the membrane).

The light/dark or dark/light switches produced, within a few minutes, changes in e.m.f. of as much as 80 mv in the leached cells. Can these changes be explained by the tendency of cations and/or anions to move through the cell membrane?

The light/dark switch produced hyperpolarization which could, perhaps, be the result of quick net efflux of cations. The solution flowing past the cells would wash any excreted cations away and maintain the cation concentration outside, in the external medium around the cell at near zero. But there are objections to this idea.

- i) The waste showed no observable increase in cations. *Expect any fibre*
- ii) A tenfold decrease in the concentration of K^+ within the cell would only produce a 40 mv depolarization. *small if need any way*
- iii) The rate of depolarization was too fast for any pumping mechanism. *whif electrogenic*
- iv) When the light was switched on again the e.m.f. fell to approximately the original value but, unless there was an accumulation in the Free Space, the re-entry of cations could not be involved as they had been washed away. *flux ratio chan not Co, chan*

- v) The total $\text{CO}_2(\text{outside})$ (some of which would be in the form of HCO_3^- at pH 5.4) was extremely low and it is questionable as to whether it was available to the plant.

The data would therefore suggest that these rapid e.m.f. changes in light and dark are directly related to metabolism, not to gross influx of ions or CO_2 .

Conclusion:-

- 1) Evidence suggests that e.m.f. changes in light/dark/light switches cannot be attributed to gross ionic influxes as large changes in cell ionic concentration would be necessary, and even if this were possible the ionic pumps could not work fast enough to bring it about in the time required.
- 2) As the concentration of $\text{CO}_2(\text{outside})$ was extremely low, it is also unlikely to explain the changes in E_m .
- 3) The changes in e.m.f. are, therefore, most likely to be caused by intracellular metabolism.

. . . .

C) The Effect of Light Quality

To observe Changes in Cell Membrane Potential with Different Wave-lengths and Different Intensities of Light

Pretreatment:-

This experiment was performed on leached material in deionized water in combination with the previous experiment (see p. 150).

Method:-

On the microscope the tissue was either treated with light and dark periods or different intensities of light (by varying the light rheostat) or different colours of light by placing filters between the lamp and the tissue.

The Vibron electrometer 33B was in circuit so tip resistances of the micro-electrodes could not be observed.

The four colours used were:-

- i) Green; obtained by extracting chlorophyll from Potamogeton in acetone.
- ii) Red; using a Spekker Wratten Red⁶⁰⁸ filter.
- iii) Far Red; using the red filter plus the Spekker Wratten OVI purple.
- iv) Blue; using the Spekker Wratten H579 + OB2

Intensity of light was judged very crudely by whether it appeared bright, medium or dim when observing cells under high power,

since no measuring equipment was available.

Results:-

It may be recalled that the light/dark switch always produced a rise in potential and the dark/light a fall. (See graph (4))

If the intensity of light was reduced to approximately half, until it was a 'glowing yellow' and the above switches were tried similar results, with the same amplitude of changes in e.m.f., were obtained. On reducing the light intensity further until the cell could only just be seen they behaved as if in total darkness. Similarly, with the green filter obtained from a concentrated extract of chlorophyll, even with the cell easily visible under the microscope the electrical responses were as if the cells were in total darkness. If, however, the chlorophyll extract was rather weak slight changes in e.m.f. were observed with the light/dark/light switches. (see graph (4)),4

Even with fairly high light intensity such that the cell could very easily be seen, the blue filter produced the dark response.

The Far Red produced a similar response.

On the other hand, with a reasonably high light intensity the Red filter produced the white light response and, with a low intensity, the dark response. i.e.,

Bright red/dull red switch,	e.m.f. rise 195 to 221 mv
Dull red/bright red switch,	e.m.f. fall 221 to 190 mv
Bright red/dark red switch,	e.m.f. rise 190 to 205 mv

Comments:-

The normal response of the cells to light and dark is evidence in favour of their not being adversely affected by the pretreatment.

The experiment itself was necessarily rather crude so that only trends of the responses of e.m.f. to light quality and quantity can be suggested, however it appears that white or yellow light did not cause a response below a certain intensity, whereas red light and green light produced the responses expected if the light effect was associated with chlorophyll and photosynthesis. But chlorophyll absorbs in the blue band as well as red and therefore a blue response similar to red and white would be expected also, but none was observed. Unfortunately, as the experiment was not repeated the results could not be confirmed but if it were a reproducible observation it could lead to interesting speculation. E.g. Carotinoids only absorb in the blue waveband, therefore these could either prevent the blue light reaching the chlorophyll, or directly negate the effect of the blue light on the chlorophyll.

Conclusion:-

- 1) E.m.f. of the cells was affected by light and dark changes.
- 2) The red waveband in light was definitely involved in this effect.

Evidence concerning other wavebands was doubtful.

- 3) The negative effect of the blue waveband is particularly interesting but needs to be verified.
- 4) It is suggested that as the red wave-length is involved then these e.m.f. changes could be associated with photosynthesis thus supporting the previous conclusion that they are an intracellular response.

. . . .

gluc extract; frei
pH 6.2
45% HCO_3^-
 $\sim 55\%$ $\text{CO}_2 + \text{H}_2\text{CO}_3$
(+ CO_3^{2-})

PRESENTATION OF A POSSIBLE MECHANISM

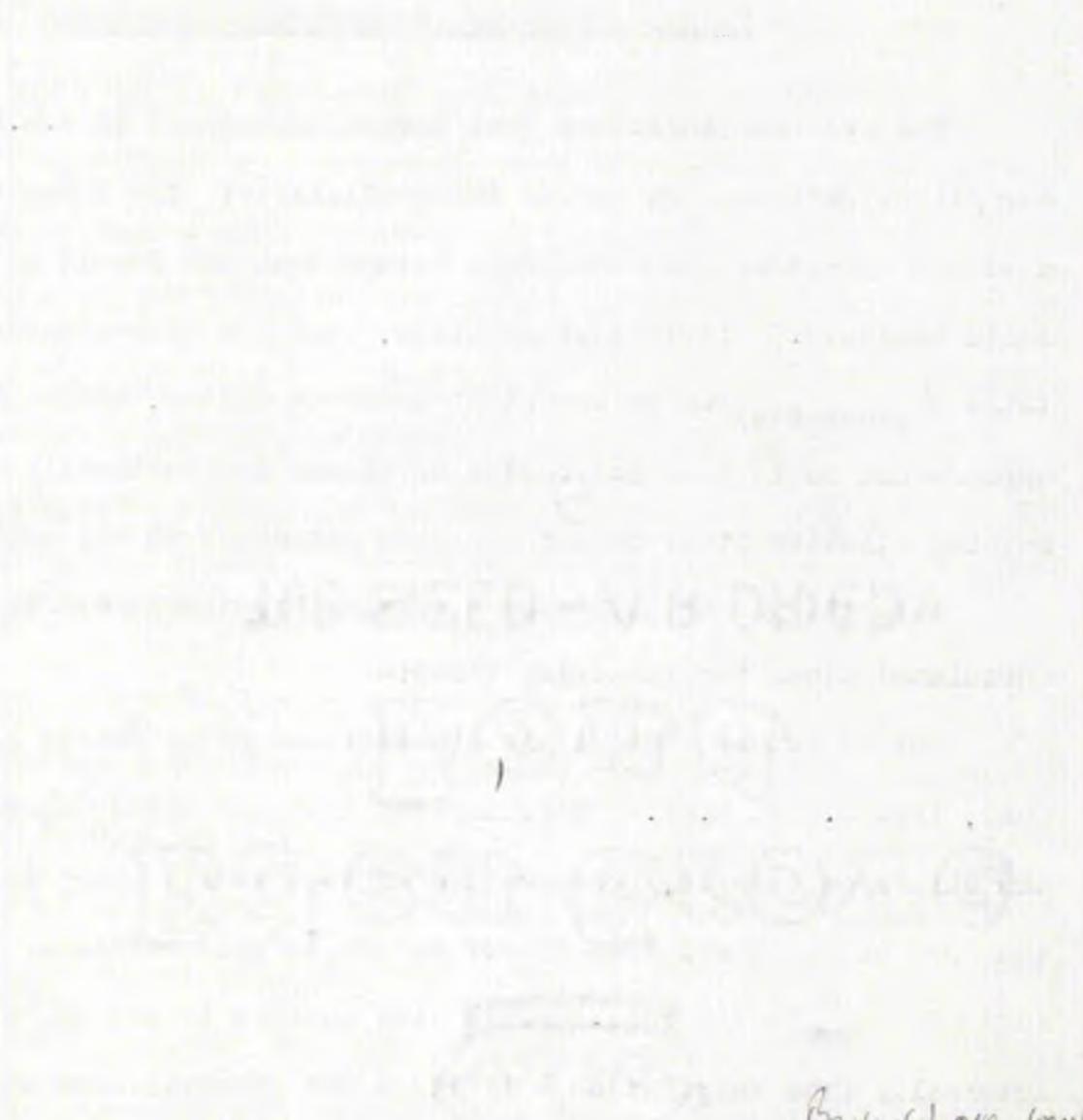
The evidence indicates that the rapid changes in e.m.f. with dark/light switches are caused intracellularly; the changes being a direct effect of photosynthesis rather than the result of gross ionic influxes. It is also possible, that the concentration of total CO_2 (outside) is an important indirect factor, although it appears not to be a direct factor as tissue in practically CO_2 -free bathing solution still showed the same responses to the switches.

With the present evidence a working hypothesis can be formulated along the following lines:-

Let us assume that within the cell the pH is fairly high (say, from 6.5 to 8.0). This implies that any total CO_2 entering the cell from outside, irrespective of whether its entry is in the form of dissolved free CO_2 or as HCO_3^- , will be mainly in the form of HCO_3^- in the cell. This also applies to any CO_2 produced internally from respiration - if it is not directly used up in photosynthesis.

If we accept that HCO_3^- , like other anions, are passively impermeable or only slowly permeable through the plasmalemma, then these ions will be retained unless there is an active outward directed pump.

On the other hand dissolved free CO_2 is known to be freely permeable through the membrane and its distribution depends upon the concentration gradient of dissolved free CO_2 between the inside and the outside of the cell.



But: these would \ominus net
 come from for
 HCO_3^- formation

if $[\text{HCO}_3^-]$ rises, faster efflux rate ($\frac{\text{no rise}}{M_x}$)
 \rightarrow hypernatremia. (Using eq 1)

Let us first take the instance where illuminated cells were bathed in a solution which was very low in total CO_2 . The CO_2 within the cell could have been at the same concentration as the outside, or perhaps higher, thus forming a bicarbonate ion pool. CO_2 from respiration would be fed into the pool, and at the same time some would be removed for photosynthesis.

When the system had stabilized, i.e. when rates of photosynthesis and respiration were steady and compensated, the HCO_3^- pool would also be in equilibrium as there was no external CO_2 supply.

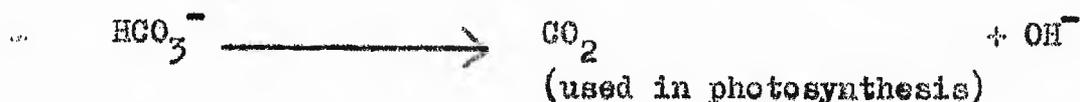
On turning off the light, photosynthesis stopped but respiration continued thus upsetting the equilibrium and initially increasing the free CO_2 within the cell. But the excess free CO_2 from respiration would only have a slightly greater tendency to diffuse out of the cell, if it is well buffered, as the CO_2 would rapidly be hydrated and dissociated into the bicarbonate ion, H^+ which is impermeable. This would produce the observed immediate hyperpolarization of the cell. By increasing the concentration of total CO_2 within the cell the rate of diffusion of dissolved free CO_2 to the outside would also increase for, as the total CO_2 is dissociated ($\text{CO}_2 \rightleftharpoons \text{HCO}_3^-$), free CO_2 as well as HCO_3^- would increase.

Theoretically, in the dark, a second equilibrium should be obtained when the rate of dissolved free CO_2 diffusion outwards was equal to the rate of CO_2 production from respiration: this

equilibrium should produce a more highly polarized cell than in the light because of the accumulation of HCO_3^- . However, this highly polarized state need not be stable as perhaps a change in permeability of K^+ or anions through the cell wall also occurred. In fact, slow depolarization was observed.

Admittedly, depolarization also occurred with deionized water outside where, no cations were available to move in but (i) there might have been sufficient cations in the free space, especially the D.F.S. and (ii) the membrane potentials were relatively higher than in normal cells and, as is mentioned earlier (p. 152) the membrane might not have been able to sustain such high potentials.

When the light was then switched on again the reverse effect could take place, i.e. depolarization; rapid photosynthesis removing HCO_3^- ions and leaving OH^- ions thus:-



If the mobility of OH^- is greater than HCO_3^- (which is the case in water) then OH^- would perhaps diffuse out of the cell (if not, it could be pumped out) and depolarization would occur. (The role of OH^- is considered more fully on pp. 201-202).

This is also consistent with observations.

On the basis of this hypothesis, what would one expect from varying the concentration of total CO_2 in the external medium, other factors remaining constant?

In chapter 3 (pp. 133-134) it is claimed that P. schweinfurthii, during photosynthesis, is able to assimilate both free CO_2 and HCO_3^- but there is a preference for free CO_2 : therefore, my scheme would have to explain both the entry of the undissociated molecule of CO_2 and the entry of the HCO_3^- ion.

At the lower pH's the undissociated molecule would quickly diffuse into the cell along the concentration gradient but as it is not charged it would not directly affect the cell e.m.f. However, its hydration and dissociation into HCO_3^- would cause hyperpolarization.

At the higher pH values where total CO_2 (outside) will be mainly in the form of HCO_3^- an anion pump on the plasmalemma for transferring the ion into the cell must be assumed. The entry of the anion would also produce hyperpolarization of the cell.

Thus, increase of total CO_2 outside, irrespective of the pH of the solution would be expected to cause hyperpolarization.

Transfer from a solution containing a high concentration of CO_2 to one of low concentration would cause the opposite effect: dissolved free CO_2 inside would diffuse along the concentration gradient to the outside thus upsetting the dissociation equilibrium of CO_2 in the cell and HCO_3^- ions would be dehydrated to the CO_2 molecule. Also HCO_3^- ions would be removed for photosynthesis without replenishment, the combined effect producing depolarization. In the dark, transfer from high to low CO_2 might also at first,

produce depolarization as dissolved free CO_2 diffused out of the cell.

To summarize, therefore, a bicarbonate pool within the cell is visualized and this could cause a membrane potential in a similar way to the Cl^- pool found in Nitellopsis (see MacRobbie and Dainty 1958, a) Any change in the bicarbonate pool would consequently cause change in the e.m.f. this perhaps being compensated for by change in membrane permeability to ions, particularly potassium.

If this hypothesis is to hold the expected change in e.m.f. with changing CO_2 concentration in the external solution would be as follows:-

- i) Increase in total CO_2 (outside) would tend to cause initial hyperpolarization of the cell the major factor probably being diffusion of dissolved free CO_2 along a concentration gradient, although a bicarbonate pump inwards would produce a similar effect.
- ii) Decrease in total CO_2 (outside) would produce depolarization this being caused solely by diffusion out of dissolved free CO_2 along the concentration gradient.

In the following set of experiments these are tested.

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D) The Effect of $\text{CO}_2(\text{outside})$ on Cell E.M.F.

To observe Changes in E.M.F. with Changes in $\text{CO}_2(\text{outside})$
Concentration in Conditions of Light and Dark

Pretreatment:-

A few leaves of Potamogeton were cut and placed in filtered pond water which was aerated. After 48 hours light the leaves were cut into transverse strips (including midrib) half the strips being placed in a beaker containing freshly filtered aerated pond water under mercury vapour lamps and the other half having the same conditions but being kept in total darkness. They were kept in this condition for approximately 30 hours before experimentation.

Method:-

15 litres of pond water was collected and filtered through Whatman's No. 1 filter paper. The filtrate was transferred to 3 5l. aspirators.

The first aspirator, labelled 'Aerated pond water' was aerated from the air compressor through sintered glass. The second, labelled 'pond water - CO_2 ' had air from the compressor passed through dilute KOH and two Carbosol columns before bubbling it through the pond water. The third aspirator 'pond water + CO_2 ' was aerated directly from a cylinder containing 95% air and 5% CO_2 . Although the terms '+' and '-' CO_2 were used these are

only relative for the $-CO_2$ solution still contained a considerable amount of CO_2 . Each solution was bubbled for three days. Just prior to the experiment a sample was taken from each aspirator and CO_2 and K^+ concentration, and pH were estimated.

TABLE (4), 4

	Aerated Pond Water	Pond Water $-CO_2$	Pond Water $+CO_2$
K^+	240 μ eq/l	240 μ eq/l	239 μ eq/l
pH	8.2	8.9	6.15
Total CO_2	0.45 mM	0.36 mM	1.73 mM
% Free CO_2	1.45%	0.28%	62.0%
Free CO_2	0.0065 mM	0.0009 mM	1.07 mM
HCO_3^-	0.4435 mM	0.3591 mM	0.66 mM

Composition of the three bathing solutions. See appendix (P. xiv) for calculation of % Free CO_2 at given pH values.

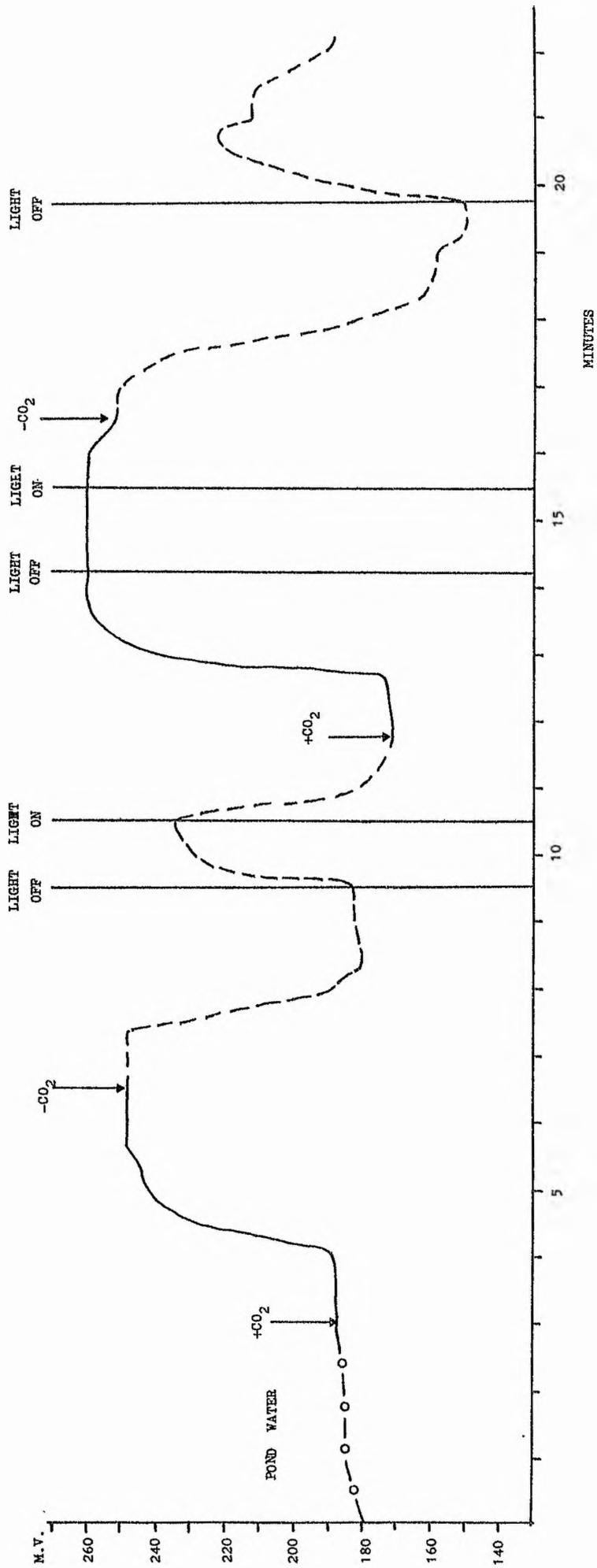
The equality of the K^+ in the three aspirators confirms that no solution had altered through bacterial or algal action.

By connecting the aspirators to a manifold with taps over the microscope assembly one solution or another could be made to flow past the tissue as required. After changing solutions there was a time lag before the new solution passed down the tubing and surrounded the cells.

The Medistor electrometer and probe were in circuit so that any change in tip resistance could be measured.

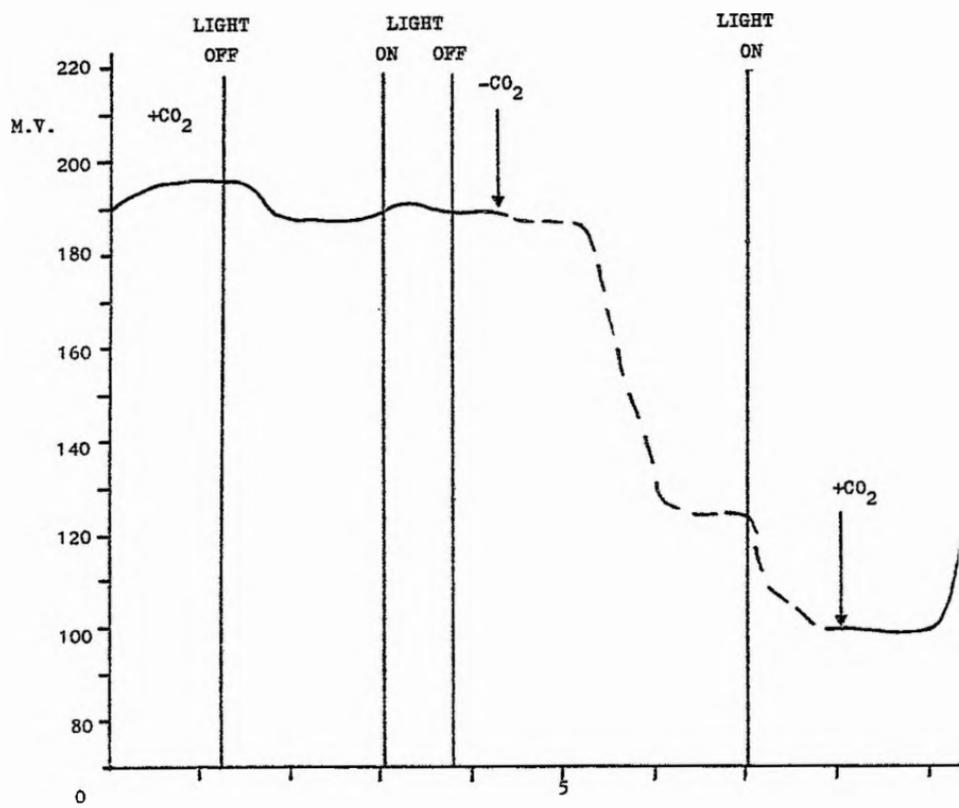
GRAPH (4), 5

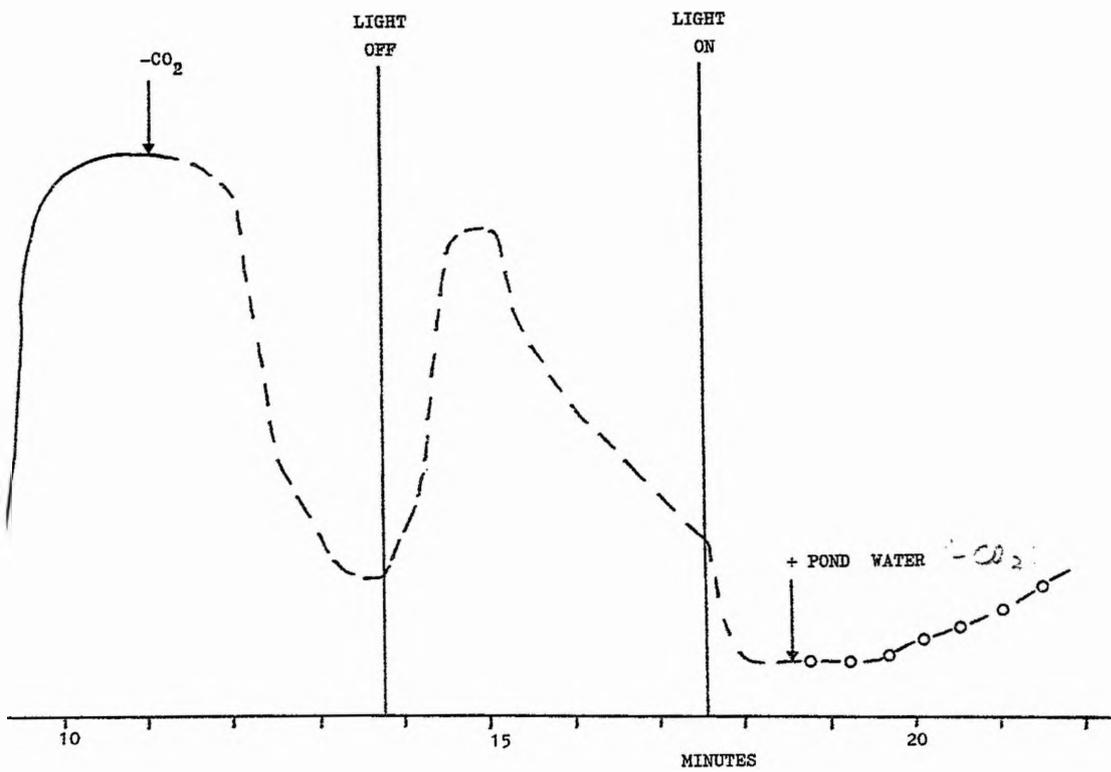
Variation in E_m of cells bathed in pond-water with different concentrations of total CO_2 , plotted against time. The dark/light and light/dark switch was also operated.



GRAPH (4), 6

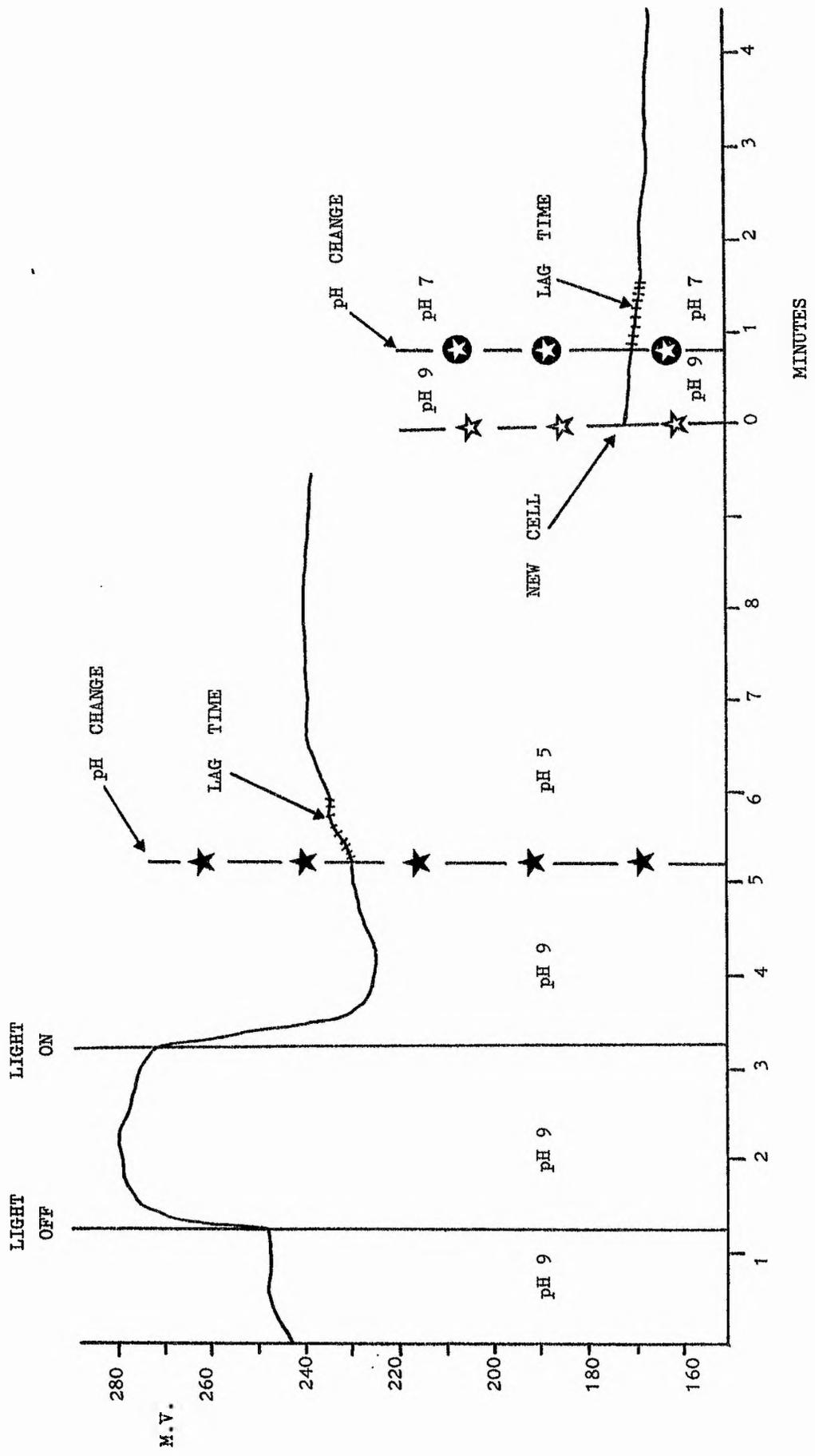
Conditions similar to those in graph
(4), 5, plotting h_m against time.





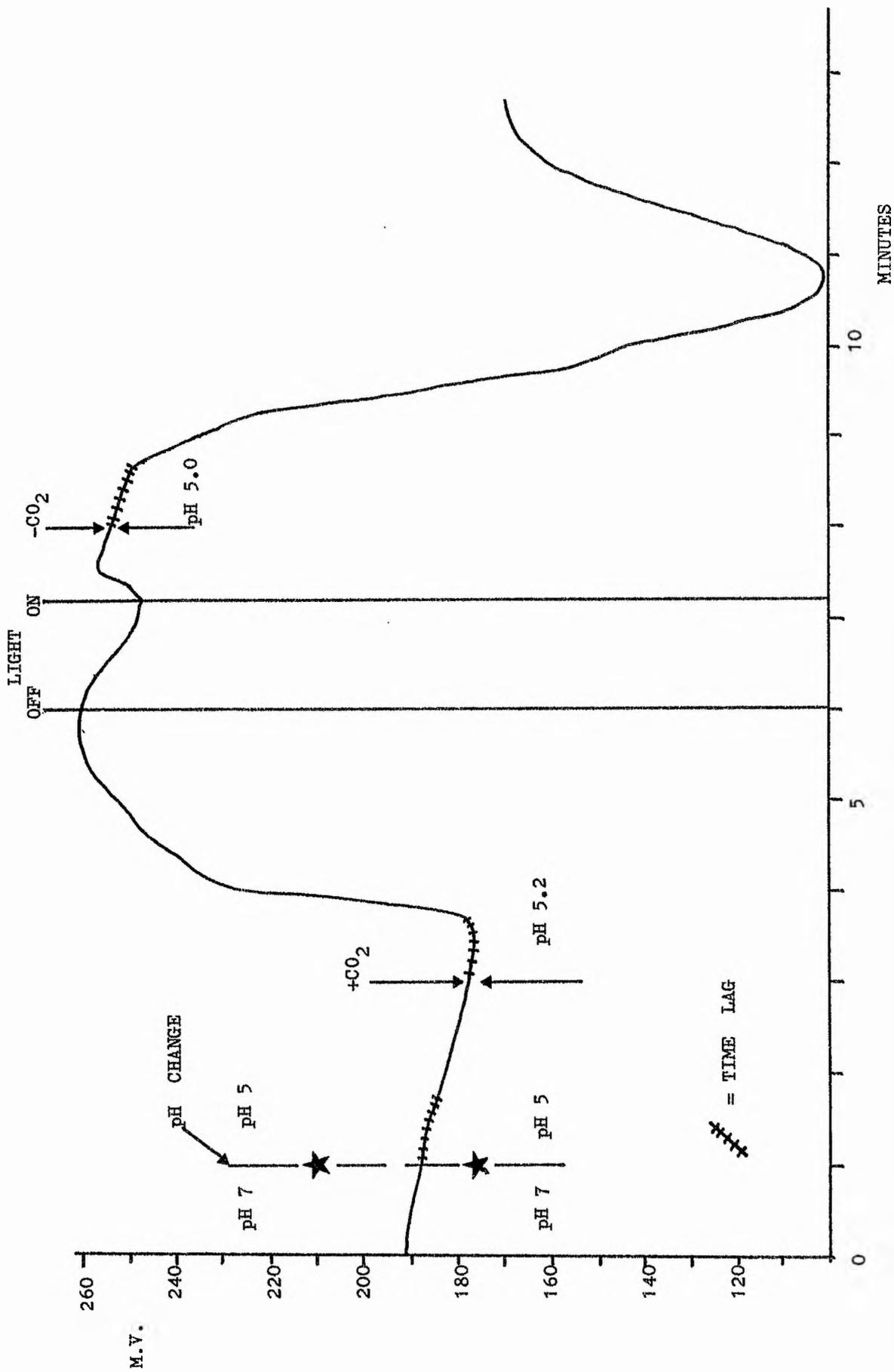
GRAPH (4), 7

Variation in E_m of cells bathed in pond-water maintained at low total CO_2 concentration plotted against time whilst varying the pH of the solution. The light/dark and dark/light switches were also operated.



GRAPH (4), 8

Conditions similar to those in graph (4), 7
plotting E_m against time but finally adding
some CO_2 to the bathing solution.



b) Tissue with dark pretreatment

Results with this tissue were not so numerous as it was a much more tricky manipulation but those obtained showed exactly the same trends as for light tissue.

Comments:-

As would be expected the $-CO_2$ solution had the highest pH and the CO_2 would be mainly in the form of HCO_3^- . The $+CO_2$ solution was probably nearly saturated 1.75 mM. As the pH of this solution was 6.15, by calculation 62% of the total CO_2 would be in the form of dissolved free CO_2 amounting to 1.07 mM/l. (See Table (4),4).

With this as the bathing solution, graphs (4),5 and (4),6 show that the light/dark and dark/light switches did not produce the usual response in e.m.f.; rather it remained steady.

This could be explained thus:-

As the total CO_2 in the bathing solution was high then the total CO_2 (and thus the dissolved free CO_2) inside the cell should have been high also; for dissolved free CO_2 is highly permeable through the cell membranes and would quickly come to equilibrium inside and outside the cell. Under these conditions, when the cell was illuminated photosynthesis and carbon dioxide utilization would be initiated but a change in e.m.f. would not necessarily be expected. This is because there might have been sufficient

available free CO_2 from within the cell and, by diffusion through the plasmalemma, from the bathing solution outside without the HCO_3^- pool being affected. The same would apply when switching from light to darkness.

Changing the external solution from '+' to '-' CO_2 incorporated two distinct variables; total dissolved CO_2 and pH. In the one case there was a high concentration of CO_2 mainly in the form of free CO_2 and in the other case a much lower concentration mainly in the form of HCO_3^- . (This concentration would still be sufficient for some net photosynthesis).

Therefore, the observed changes in e.m.f. might result either from differences in concentration of total $\text{CO}_2(\text{outside})$ or differences in form of CO_2 .

Conclusion:-

- 1) Tissue pretreated in the light and in darkness behaved in a similar manner.
- 2) In conditions of relatively low total $\text{CO}_2(\text{outside})$ concentrations the e.m.f. varied with the light/dark switch in the manner previously observed, i.e. hyperpolarization with the light/dark switch and depolarization with the dark/light switch.
- 3) In conditions of high concentration of $\text{CO}_2(\text{outside})$ this pattern was not repeated but rather, the e.m.f. stayed steady with the light/dark/light switches.

- 4) In conditions of uninterrupted light or uninterrupted darkness, changes from low concentration of $\text{CO}_2(\text{outside})$ to high concentration of $\text{CO}_2(\text{outside})$ produced rapid hyperpolarization of up to 100 mv and changes from high to low concentration of $\text{CO}_2(\text{outside})$ produced rapid depolarization of the cell.
- 5) These changes in e.m.f. could either be correlated with concentration of total $\text{CO}_2(\text{outside})$ or with pH, and thus with the dissociated form of CO_2 , or with both.
-

The Effect of Changing pH on Membrane Potentials
at Constant Total $\text{CO}_2(\text{outside})$ Tension.

Pretreatment:-

To study the effect of pond water at different pH values and yet constant CO_2 it was decided to keep the CO_2 in all solutions as low as possible.

Three solutions of pond water one at pH 5, the second at pH 7 and the third at pH 9 were prepared in the following manner.

15 litres of filtered pond water were boiled and cooled under a CO_2 trap. This would remove most free CO_2 . The solution was then acidified (400 mls of 0.05N H_2SO_4) to reduce the pH to approximately 4, thus converting all HCO_3^- and CO_3^{--} to free CO_2 . This was then reboiled and cooled under a CO_2 trap.

With approximately 45 ml of CO_2 -free 0.05N NaOH the pH of the pond water was raised to 9.2, again avoiding contact with CO_2 as much as possible.

The solution was then separated into three 5 litre aspirators which had been previously flushed with CO_2 -free air. Two of the solutions were acidified with dilute acid to pH 5 and pH 7 respectively, the third remaining untouched.

In this way all three solutions were ionically similar except for a little dilute sulphuric acid.

They were left to stand for three days and were aerated with CO_2 -free air for three hours before use. Just prior to use a sample was taken for analysis from each, and the following results were obtained:-

TABLE (4), 7

Aspirator Reference	Actual pH of Solutions	Concentration CO_2 mM/l	Concentration K^+ ($\mu\text{eq/l}$)
pH 9	8.95	0.11	232
pH 7	6.6	0.05	234
pH 5	4.95	0.03	233

Table showing pH, CO_2 concentration and K^+ concentration of the three bathing solutions.

The tissue was collected in the normal manner and was equilibrated for 48 hours in the solution 'pH 9' under constant illumination.

Method:-

The method was similar to that of the previous experiment but the time lag between changing solutions and the new solution washing the tissue was reduced to about 1 minute.

The Medistor Electrometer was not available so a new Vibron Electrometer, 33B-2, was used. This proved to be very reliable.

Later in the experiment half a bottle of soda water (approximately 100 ml) was added to approximately 3 litres of the pond water which remained in the pH 9 aspirator. This effectively reduced the pH to 5.2 and raised the CO₂ level.

Results:-

Excluding the "soda water solution" each solution produced the normal response to the light/dark switch when it was flowing over the cells, i.e. hyperpolarization in the dark and depolarization in the light. (See Table (4), 8).

TABLE (4), 8

Number of Incidences of:-

Switch	Hyperpolarisation	Depolarization
Light/dark	10	0
Dark/light	0	10

E.m.f. response to light/dark and dark/light switches irrespective of pH of solution.

In permanent light, the change from one solution to another produced the following response. See below, Table (4), 9 and graphs (4), 7 and (4), 8.

TABLE (4), 9

Solution Switch	e.m.f. Response
pH 9/pH 5	no change
pH 5/pH 7	no change
pH 7/pH 5	no change

Table showing e.m.f. response to changes in solution pH at low, approximately constant CO₂ levels in permanent light.

Change from pH 5 solution to the "soda water solution" at ^{in (new) water} pH 5.2, in the light, produced a very steep increase in e.m.f. of 80 mv in just over a minute. Switching off the light then produced a slight fall and switching on a slight rise in e.m.f. (See graph (4), 8).

In continuous light change from Soda water solution to the normal pH 5 solution produced rapid depolarization (approximately 150 mv in 2 minutes).

Change from the pH 7 solution to the Soda water solution produced hyperpolarization. Also, the same sequence of changes in extended darkness rather than light produced the same results. See Tables (4), 10 and (4), 11 below.

TABLE (4), 10

Lighting Condition	Variable	Switch	e.m.f. Response
Constant Light	pH	4.95 / 5.2	Rise
	CO ₂	Low / High	
	pH	6.6 / 5.2	Rise
	CO ₂	Low / High	
Constant Dark	pH	6.6 / 5.2	Rise
	CO ₂	Low / High	
Constant Light	pH	5.2 / 4.95	Fall
	CO ₂	High / Low	
	pH	5.2 / 6.6	Fall
	CO ₂	High / Low	
Constant Dark	pH	5.2 / 6.6	Fall
	CO ₂	High / Low	

Table showing e.m.f. response in conditions of either constant light or constant darkness with switching from high to low CO₂(outside) or vice versa. The change in pH is also recorded.

TABLE (4), 11

pH of Solution	CO ₂ Concentration	Switch	e.m.f. Response
5, 7, or 9	Low	Light/dark	Rise
		Dark/light	Fall
5.2	High	Light/dark	Fall
		Dark/light	Rise

Table showing e.m.f. response to light/dark and dark/light switches in conditions of high and low CO₂ concentrations. pH status is also shown.

Comments:-

There was a slight difference in CO₂ concentrations in the three original solutions but all the concentrations were very much lower than would be normal in pond water.

The negative response with switching from one solution to another not only showed that the cell potential is unaffected by a wide variation in hydrogen ion concentration but also confirms that the difference in the CO₂ concentration in the three solutions was insignificant.

By adding some Soda water to one of the solutions (thus reducing the pH to 5.2) the concentration of total CO₂(outside) was raised whilst leaving the metal ion concentration unchanged.

In substituting this solution for the originally prepared pH 5 solution (which was low in CO_2) there was only 0.25 pH units difference in hydrogen ion concentration but a considerable difference in concentration of total $\text{CO}_2(\text{outside})$.

The sudden change in cell potential observed at this substitution must therefore, be correlated with difference in the total CO_2 concentration. At pH 5 approximately 95% of the total CO_2 would be the undissociated molecule although some HCO_3^- would be present, therefore, the change in e.m.f. is more likely to be an effect of dissolved free CO_2 rather than a bicarbonate effect especially as P. schweinfurthii shows a preference for the former. (See chapter 3, p 133-134).

Conclusion:-

- 1) At low external CO_2 levels the membrane potential was unaffected by changes in pH ranging from 5.0 to 9.0.
- 2) In these conditions of low CO_2 concentration the e.m.f. showed the usual response to light and dark intervals. i.e. hyperpolarization in the dark and depolarization in the light.
- 3) At a steady pH of 5.0, either in permanent light or permanent dark, the e.m.f. was directly affected by change in concentration of total $\text{CO}_2(\text{outside})$; increase in $\text{CO}_2(\text{outside})$ produced hyperpolarization and decrease in $\text{CO}_2(\text{outside})$ produced depolarization.

- 4) At the high concentration of total CO_2 (outside) and pH 5.2, it is suggested that as the dissolved free CO_2 molecule rather than the HCO_3^- ion is pre-dominant, and as the tissue appeared to show a preference for the former rather than the latter, hyperpolarization could be the result mainly of diffusion of the molecular form through the membrane rather than the inward pumping of HCO_3^- .
- 5) In conditions of high external total CO_2 the e.m.f. showed the opposite response to light and dark switches. i.e. depolarization in the dark and hyperpolarization in the light.

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The Effect on Membrane Potential of External CO_2 Concentrations
in the Presence and Absence of Cations

Pretreatment:-

Previous experience had shown that pure deionized water was not suitable in microelectrode work as it was not a good conductor, therefore, 10 litres of water was acidified with 5 ml of 0.05N H_2SO_4 .

This was placed in two 5 litre aspirators, one of which was aerated in CO_2 -free air and the other with air containing 5% CO_2 .

Analysis of these solutions gave the following results:-

TABLE (4), 12

Aspirator Reference	pH	Concentration Total CO ₂ mM
- CO ₂	5.0	0.04
+ CO ₂	6.1	0.93
Pond water - CO ₂	4.95	0.03

Table showing the pH and the CO₂ concentration of the three bathing solutions.

A third aspirator containing the 'pH 5' pond water from the previous experiment was also assembled. (Referred to as Pond water -CO₂ in table (4), 12 above)

Tissue was leached in many changes of deionized water for 7 days, in daylight. It was then cut into strips and placed in 'acidified' deionized water under mercury light for 2 hours. After this period the strips were transferred to a fresh solution of acidified water which had been aerated with CO₂-free air, and left for a further 2½ hours in the light.

Method:-

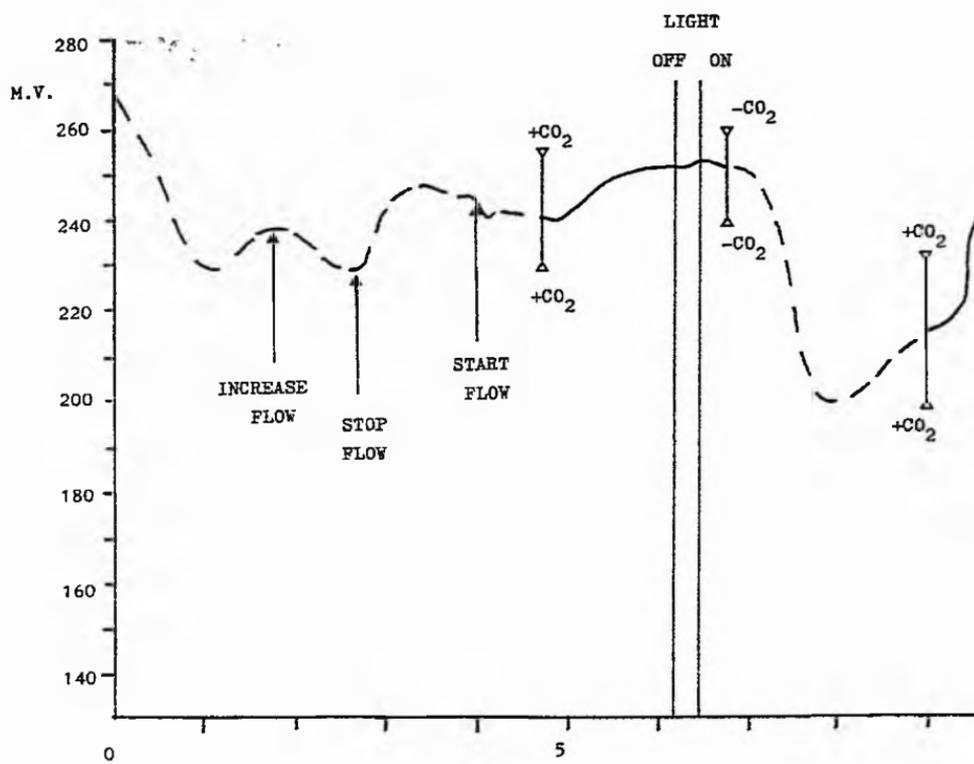
The method was as in the previous experiment with the addition that flow rate was also varied.

Results:-

The results are tabulated below and shown in graph (4), 9.

GRAPH (4), 9

Variation in E_m of fully leached cells
in deionized water plotted against
time whilst varying the CO_2 concentration,
the rate of flow of the bathing solution,
light and dark, and interchanging
deionized water with pond-water.



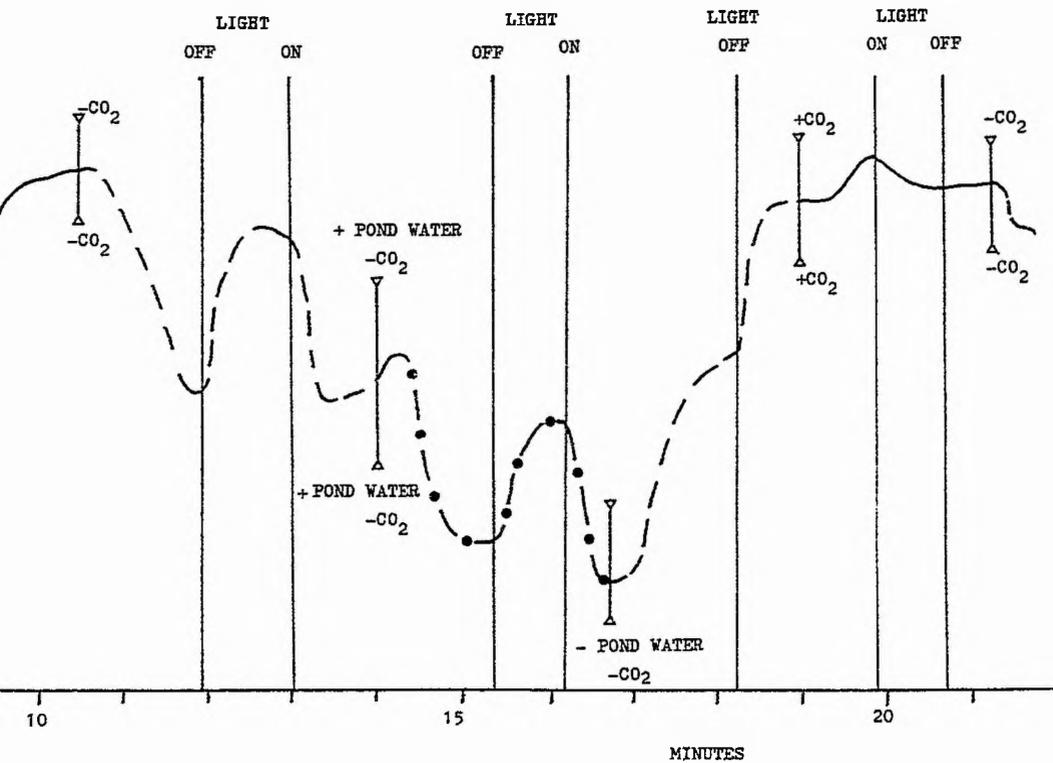


TABLE (4), 13

Light Condition	Switch	e.m.f. Response
Light	$-CO_2/+CO_2$	Rise
Dark	$-CO_2/+CO_2$	Rise
Light	$+CO_2/-CO_2$	Fall
Dark	$+CO_2/-CO_2$	Fall
Light	$-CO_2/P.W. -CO_2$	Fall
Light	$P.W. -CO_2/-CO_2$	Rise

Table showing e.m.f. response in conditions of either light or dark, with change in total CO_2 concentration in deionized water bathing solution. Also response to changes from pond water (P.W.) to deionized water, both minus CO_2 , are shown.

TABLE (4), 14

	Bathing Solution	Switch	e.m.f. Response
(1)	Deionized Water -CO ₂	Light/dark	Rise
(2)	P.W. -CO ₂	Light/dark	Rise
(3)	Deionized Water +CO ₂	Light/dark	Steady
(1)	Deionized Water -CO ₂	Dark/light	Fall
(2)	P.W. -CO ₂	Dark/light	Fall
(3)	Deionized Water +CO ₂	Dark/light	Rise or Steady

Table showing e.m.f. response to dark/light or light/dark switches with (1) deionized water -CO₂ (2) pond water -CO₂ (3) deionized water +CO₂, as the bathing solution.

If one examines graph (4), 9 it can be seen that in the light, with the -CO₂ solution, and increased flow rate slight depolarization was produced; stopping the flow produced hyperpolarization; and restarting the flow again, immediate depolarization.

Comments:-

The three solutions used in this experiment differ only slightly in pH (See Table (4), 12) so that any response observed can be considered to be independent of hydrogen ion concentration. This conclusion is supported by the data from the previous experiment.

The difference between the two deionized water solutions was one of CO_2 concentration (0.04 mM as compared with 0.93 mM) whereas the difference between the $-\text{CO}_2$ deionized water and the $-\text{CO}_2$ pond water was on the contrary, not CO_2 concentration, nor pH, but the presence or absence of cations.

The tissue was thoroughly leached before the experiment and the two deionized water solutions were devoid of cations: yet the e.m.f. responses to light and dark switches and to high and low concentrations of total CO_2 (outside) were exactly the same as in the previous experiment where there was an abundance of cations. Further, the responses also correspond to those observed with the leached material discussed on pages 152-153. Therefore, this must re-enforce the suggestion that e.m.f. responses to light and dark, to different qualities of light, and to changes in concentration of total CO_2 in the external medium are independent of ion influx.

On the other hand, variation in e.m.f. of illuminated tissue when changed from $-CO_2$ deionized water, to $-CO_2$ pond water, and then back again, must be explained by the tendency for cations to move through the cell membranes, and not in terms of CO_2 concentration or pH.

With pond water flowing past the cells the responses to light and dark switches again corresponded to those observed with deionized water.

The change in e.m.f. with the change in rate of flow of $-CO_2$ deionized water supports the suggestion made on page 144 , that the absence of a midrib in mounted tissue, by affecting the free flow of solution over the cells could cause e.m.f. variation. But if the hyperpolarization, caused by stopping the flow of solution over the tissue, is to be explained in terms of forms and concentrations of CO_2 alone, then the bicarbonate pool within the cell should be increasing. This would be surprising as the tissue is photosynthesizing and the bathing solution contains only 0.04 mM total CO_2 (96% of which is dissolved CO_2). There would be more chance of replenishing the pool from a flowing solution than an unstirred solution as the diffusion pathway would tend to be shorter. It is also improbable that this hyperpolarization is due to an outwardly directed cation pump for this should operate whether the solution were flowing or static.

Bright illumination of the cells without the flow of the medium, which was at constant temperature, might warm the cells. As the cell membrane potential, E , generated by ion diffusion is temperature sensitive, E varying as the temperature, T , then the observed slight hyperpolarization on stopping the flow might be the result of temperature variation. This can be tested in the following way:-

Assuming hyperpolarization is caused by temperature change, one can use the Nernst equation, (see p 45)

$$E = \frac{2.3RT}{zF} \log_{10} \frac{C^o}{C^i}$$

R, z, F , and $\log_{10} \frac{C^o}{C^i}$ have the normal meaning and can be substituted by (K) as they are assumed constant in this case.

E_1 , the initial membrane potential was 228 mv when the flowing solution was at 25°C , T_1 and E_2 , the final membrane potential was 248 mv at temperature T_2 when the flow was stopped. Therefore substituting in the formula

$$K \cdot \frac{E_1}{T_1} = K \cdot \frac{E_2}{T_2} \text{ and calculating } T_2$$

the temperature would have to have risen by 26°C to cause the observed change in e.m.f. assuming no changes in ionic permeability. This is impossible in the circumstances, and the hyperpolarization cannot be attributed to possible change in temperature.

Until further experiments are conducted this problem will remain unexplained.

Conclusion:-

- 1) E.m.f. responses to light, dark, and different qualities of light and to changes in concentration of total CO_2 (outside) are independent of cation movement through the plasmalemma.
 - 2) E.m.f. responses are obtained with changes in cation concentration (outside) in the direction expected from the tendency for K^+ to diffuse along an electrochemical gradient. These changes are independent of concentration of CO_2 (outside) light and dark, and hydrogen ion concentration.
 - 3) The results of the previous experiments described in this chapter are confirmed: i.e. the light/dark and dark/light switches produces hyperpolarization and depolarization respectively, when the CO_2 concentration of the external medium is low. When the external CO_2 concentration is high little or no change in cell e.m.f. is observed.
 - 4) It was noted that variation in the rate of flow of the bathing solution over the tissue caused changes in cell e.m.f. This phenomenon was discussed, and although it appeared not to be related to concentration of CO_2 in the bathing medium, nor to cation flux, nor to temperature variation of the tissue, no explanation could be given.
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General Discussion

The present membrane potential studies on leaf epidermal cells of P. schweinfurthii have shown that the e.m.f. of the cell was strongly negative, as has also been found in the Characeae (see, for example, MacRobbie and Dainty 1958a) and Avena (Higinbotham et al 1964). P. schweinfurthii cell membrane potential, however, is directly affected by change in intensity of illumination upon the cell. In conditions of medium or low total external CO₂ concentrations the usual response to change from light to darkness was hyperpolarization, and vice-versa from darkness to light, but with high total CO₂ no such change was observed.

The e.m.f. was particularly sensitive to the red waveband of light and insensitive to the blue and green wavebands.

This immediate rapid change in cell e.m.f. was shown to be independent of any ion influx.

Under constant light or darkness, at pH 6.6, a change in concentration of total CO₂ of the bathing solution caused immediate change in cell e.m.f.; i.e. hyperpolarization with increasing CO₂ and depolarization with decreasing CO₂ concentrations. When the concentration of CO₂ was exceedingly low, a change in pH of the external solution had no effect upon the membrane potential.

These results can be interpreted in terms of a bicarbonate pool within the cell, thus:-

Fresh tissue in pond water, on illumination, initially used up some HCO_3^- from the anion pool thus decreasing the pool and causing depolarization. This loss is gradually compensated for by influx of HCO_3^- from the external solution and/or diffusion of dissolved free CO_2 into the cell where it is hydrated and hydrolysed to HCO_3^- . The extent of hydrolysis will depend upon the pH of the cell sap.

The hyperpolarization observed by changing to darkness could be the result of upsetting the total CO_2 influx/utilization ratio. In darkness, as HCO_3^- ions would not be extracted from the pool for photosynthesis it would initially increase and cause a rise in e.m.f.

By raising the total CO_2 concentration in an external medium of moderate pH, value of say, 7.0, the internal HCO_3^- pool of illuminated or darkened cells could increase through the passive diffusion of free CO_2 inwards. Internally, this is converted to HCO_3^- and hyperpolarization would result. If on the other hand the solution with the high total CO_2 concentration also had a high pH value of, say, 9.5, then there would be an exceedingly low concentration of free CO_2 outside. In this case when changing from a solution of low total CO_2 to one of high CO_2 and high pH, hyperpolarization of illuminated tissue could result from increased HCO_3^- influx and not from free CO_2 inward diffusion.

In the dark, however, addition of HCO_3^- ions to the external medium might not cause hyperpolarization as Hope (1965) states that HCO_3^- ions are very impermeable and, to gain entrance into the cells of Chara australis, are most probably pumped. Evidence from MacRobbie (1965) and Raven (1966, private communication) would favour a light-activated influx pump for HCO_3^- ions in Nitella. If a similar mechanism exists in P. schweinfurthii, in the dark there could be no HCO_3^- influx and therefore no hyperpolarization.

With a high external total CO_2 concentration at pH 6.15 the change from light to darkness and again to light caused no corresponding change in e.m.f. But as, at this pH, 62% of all the CO_2 would be in the form of free CO_2 there might be sufficient free CO_2 in the cell (in passive equilibrium with the external free CO_2) without the proposed HCO_3^- pool being affected; then the e.m.f. would remain unaltered.

On several occasions it was observed that cells in a high total CO_2 bathing solution depolarized slightly on changing from light to darkness. This phenomenon need not necessarily be answered in terms of CO_2 for Hope (1965) and Hope and Walker (1960) both have data indicating that light and dark can also affect the membrane resistance in the Characeae and thus the passive movement of ions. If, for example K^+ permeability in P. schweinfurthii is affected by light then its e.m.f. will be also.

Evidence for a bicarbonate pool is now examined.

In the appendix p. xiv the osmotic pressure (O.P.) of the cell sap from leaf tissue taken in August was estimated to be equivalent to 0.48 M sucrose solution. By two separate analyses from different tissue, the sum of the concentrations in the cell for K^+ , Na^+ , Cl^- and $H_2PO_4^-$ amounted to 545 mM and 391 mM. It was therefore concluded that the osmotic pressure was mainly due to the ionic content of the sap. But in both cases the sums of the cations $Na^+ + K^+$ were greater than the sum of the anions $Cl^- + H_2PO_4^-$ (Mg^{++} and Ca^{++} can be ignored as they would be bound mainly in the cell wall). See Table (4), 15.

TABLE (4), 15

Date of Analysis	Concentration	Concentration	Difference
	Cations $Na^+ + K^+$ (mM)	Anions $Cl^- + H_2PO_4^-$ (mM)	
28th August	305	240	65
4th September	279	112	167

Table showing cell contents of P. schweinfurthii showing difference between sum of anions and cations.

A gross imbalance is impossible so there must be either other anions involved or an over estimation of the cation concentration. The latter is rather improbable as various techniques for cell extraction were used and they all produced similar results but the existence of other balancing anions cannot be excluded.

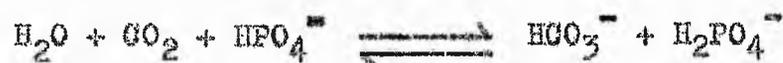
A total digest of leaf tissue gave a sulphate content of 0.878 mM/g Fresh Weight (See Table (2) 2.) and although it is unlikely that all the sulphur existed as the free sulphate ion there might be sufficient to account for some of the imbalance. If it is assumed for instance, that no SO_4^{--} was bound then it would have a concentration of approximately 100 mM.

The cells probably have about 45% of their total volume as protoplasm and in this, immobile anions such as phospholipids and proteins would occur but these were also not included in the anion estimations. Neither were the immobile anions of metabolic acids but these are not thought to be a large contributing factor as an attempted estimation of organic acids in *P. schweinfurthii* leaves was below the detectable concentration.

With the possibility of all these other anions besides chloride and phosphate the imbalance between cations and anions cannot necessarily be attributed to HCO_3^- anions.

Rabinowitch (1945, p.189) pointed out that in nearly all plants investigated the absorption of CO_2 in the cell sap, whether from a photosynthesizing zone or not, is well in excess of that explainable by solubility of CO_2 in water. This he attributed to a conversion of CO_2 to HCO_3^- by alkalizing agents, the most important of which are alkaline earth carbonates and dissolved phosphates.

Smith, (1940) using sunflower leaves demonstrated that the excess CO_2 was absorbed irrespective of any metabolic process in the plant, and the absorption was a reversible reaction. He concluded that the excess CO_2 was in the form of HCO_3^- and that its production was mainly from two independent absorption processes; i) from the buffer components in the cell sap especially phosphate, in the following manner,



and ii) from alkaline earth carbonates, found in the insoluble leaf residue i.e.



The presence of CaCO_3 in a solution can increase its absorptive capacity for CO_2 by a factor of 500 but carbonate is only stable at pH values above about 8.5. A more comprehensive review of this topic can be found in Rabinowitch (1945).

In *F. schweinfurthii* it was shown that leaf tissue contains from 10-33 $\mu\text{M/g}$ F.Wt. total phosphate. (See chapter 2. p.22.) Cold water extraction from dried leaf tissue revealed that 61% of the phosphate was soluble. (See appendix ix)

On analysing *Pisum* epicotyls, Palmer and Loughman (1964) found that inorganic phosphate comprised $\frac{33}{97}$ or 34% of total phosphorus content. The rest of the phosphorus was either acid-insoluble or bound in organic compounds.

With this data from Palmer and Loughman and the data from P. schweinfurthii analysis, the presence of inorganic phosphate in the cell of P. schweinfurthii is probable; thus, the equilibrium equation (p.188) between phosphate and bicarbonate is a possibility, and would be dependent upon the pH of the cell sap.

The presence of the second absorbent, the alkaline earth carbonate (p.188) in the cell sap is unlikely as the pH of the cell sap would have to be rather high. Therefore a HCO_3^- pool in the vacuole is possible but would depend upon the pH of the cell sap.

Finally the results of the present research and explanation must be examined in the light of relevant published work. This has mainly been on Algae of the family Characeae, with but little on higher plants (particularly *Avena coleoptiles*) and none on higher water-plants.

Nagai and Tazawa (1962) observed that on transferring Nitella flexilis from nutrient culture solution in the light via a dark pretreatment period of several hours, to 10^{-4}M KCl or 10^{-4}M NaCl solution in the dark, the cell e.m.f. did not change over a 48 hour period.

The cells were then transferred to the light in a fresh solution of KCl or NaCl. Over the next 24 hours the e.m.f. rose by 24 mv in the K^+ solution and 63 mv in the Na^+ solution. They concluded that cells in the light have a higher resting

potential than those in the dark.

But the resting potential varies with the flux ratio and Nernst's equation is applicable only at flux equilibrium. In these experiments they transferred cells from a full nutrient solution in the light to NaCl or KCl solutions of different ionic strength in the dark. They left them in the latter solution for several hours before recording potentials but "several hours" would be insufficient for equilibration of ions inside and outside: many days would be better in the light of MacRobbie's data (1962) on rates of vacuolar ion exchange with ions in the external medium. Variation in resting potential being related, perhaps, to change in free CO_2 and/or HCO_3^- concentrations was overlooked. In the dark experimental period there was no mention of aeration or stirring of the solution thus CO_2 accumulation would, presumably, occur.

After 48 hours darkness the cells were incubated in small glass tubes containing 10^{-4} KCl or NaCl solution under continuous illumination, changes in e.m.f. being recorded. Transferring the cells to small glass tubes almost surely led to gross changes in CO_2 supply which would soon become limiting in the light in such a small volume.

On the other hand, Barr and Broyer (1964) with Nitella clavata found that cells maintained in the light in the nutrient solution in which they were cultured did not show variation in e.m.f. with various relative changes in light intensity. It is

probable that their cells were near flux equilibrium with the bathing solution. In the present study also, when cells of P. schweinfurthii were maintained in either darkness or light until flux equilibrium was obtained, there was no obvious difference in cell e.m.f. It is therefore suggested that when equilibrated at a given light intensity similar cells in the same bathing solution should have a similar membrane potential. However, when changing from light to darkness or vice-versa, some ion flux ratios might be upset, this causing a change in e.m.f.

Increase in ion absorption in the light has been demonstrated by many workers, e.g. Scott and Hayward (1953). They found that after an interval of darkness net K^+ uptake in Ulva lactuca was exceedingly rapid on initial exposure to light.

Barr and Broyer (1964) demonstrated with radio-sodium that in Nitella sodium influx increases with an increase in light intensity; a process that is limited by absence of CO_2 or HCO_3^- . As they found that the total sodium in the cell did not increase, and as there was no change in e.m.f. in increasing light intensity they concluded that Na^+ influx was controlled not by e.m.f. but by membrane permeability and cell metabolism.

Tazawa and Nagai (1960) observed that when Nitella cells were illuminated an increase in the rate of net K^+ ion uptake occurred. Later, (1962) they found that by switching from dark to white light, after a 20 minute time lag, cells in a 10^{-4} M KCl

solution, became suddenly and rapidly hyperpolarized. A similar time lag occurred before K^+ uptake commenced.

On switching the light off again there was both immediate depolarization and cessation of ion absorption. Similar responses were observed with blue and with red light, and with different single salt solutions. They therefore suggested, as opposed to Barr and Broyer, that hyperpolarization probably caused the absorption of K^+ and Na^+ .

There is considerable evidence that salt absorption is inhibited by shortage of total CO_2 in the light, e.g. Scott (1954) found that Rhodymenia palmata would not take up caesium in the light unless there was also a carbon source present. Tazawa and Nagai with this knowledge, further suggested that, perhaps, on illumination an anion pump requires CO_2 for activation: then the active influx of anions, producing hyperpolarization, could be accompanied by a passive influx of cations. This is in keeping with MacRobbie and Dainty's generalization (1958) for the Characeae that perhaps in the light chloride is actively pumped inwards against an electrochemical gradient, accompanied by the passive influx of cations.

But do Nagai and Tazawa have direct evidence that the e.m.f. rises in the absence of CO_2 when cells are illuminated? Tazawa (1961), showed ion absorption (in the same species) to be inhibited in the absence of CO_2 and yet Nagai and Tazawa (1962) claimed that cells in 10^{-4} M KCl in the light absorbed ions during

hyperpolarization. Their KCl solution must therefore have been contaminated with HCO_3^- . If the solution had been HCO_3^- -free (so that no cation absorption took place) then perhaps hyperpolarization would not occur.

The importance of carbon dioxide is also evident from the studies of Hope (1965) on Chara australis and from the present studies on P. schweinfurthii.

Cells of Chara in constant illumination and bathing in an all-chloride medium produced immediate hyperpolarization on substitution of some Cl^- by HCO_3^- . The effect was apparent with HCO_3^- concentrations ranging from 0.05 mM to 1.0 mM and was not influenced by the presence or absence of Ca^{++} , Sr^{++} or Mg^{++} . Removal of HCO_3^- reversed the effect. Further, the HCO_3^- also increased the cell membrane resistance.

A similar response was shown in cells of P. schweinfurthii (see pp 164 & 182), hyperpolarization with increased external total CO_2 being independent of any cation absorption and of pH, but whereas Hope related the effect specifically to HCO_3^- ion the present author suggested that in Potamogeton dissolved free CO_2 is the major factor (see page 175) although HCO_3^- is not entirely excluded.

Hope observed that if Chara cells were in a chloride medium containing HCO_3^- , then, on illumination, there was an immediate slow drift from -168 mv to -178 mv in about 10 minutes followed

by a sudden much steeper increase to -21.5 mv in about one minute.

For this phenomenon he suggests the following mechanism:-

HCO_3^- ions stimulate an inwardly directed anion pump upon illumination of the cells. A passive flux of cations follows. Briggs (1962) has shown that such an anion pump could increase the o.m.f. of the cell, by the following equation:-

$$K_o + \alpha \text{Na}_o = -(RT/EF) (\phi/P_K) (1 - \exp. EF/RT) + (K_i + \alpha \text{Na}_i) \exp. (EF/RT)$$

α = permeability ratio $P_{\text{Na}}/P_{\text{K}}$

ϕ = active transport flux

Therefore, this hyperpolarization could be the result of either HCO_3^- being pumped in, or of HCO_3^- stimulating active Cl^- influx. Further, Hope argues that as the permeability of potassium (P_K) decreased during hyperpolarization, Brigg's equation could be modified by making P_K a negative function of E, (as in animal nerve cells). Plotting theoretical curves (see Hope fig: 7, p.799) from this modified equation he demonstrated that with active transport (i.e. the presence of HCO_3^- in the light) and decreasing P_K a time course of cell potential first shows a steady rise followed by a steep hyperpolarization: i.e. a similar curve to the one he observed:

The presence of (up to 0.5 mM) O-phenanthroline in a solution of 0.1 mM KHCO_3 + 1.0 mM NaCl caused slight depolarization in Chara cells which is consistent with the view that hyperpolarization

is caused by the anion pumps. (This inhibitor has a similar effect to Dichlorophenyldimethylurea (D.C.M.U.) and on MacRobbie's and Raven's principle would therefore cut out photosystem II thus inhibiting the HCO_3^- and Cl^- influx pumps but not affecting the Na^+ and K^+ cation pumps. (See p 196)

The necessity of photosynthesis, that is the presence of HCO_3^- and light together, to cause hyperpolarization is demonstrated by Hope in Chara where, in the absence of HCO_3^- , no hyperpolarization occurred in the light, nor did membrane resistance alter. Further support can be gained from Higginbotham et. al. (1964) who observed that Avena coleoptiles (a non-photosynthesizing tissue) showed no change in e.m.f. when 0.5 mM HCO_3^- was added to the bathing solution in the light, and from Hope's observations that in the dark no hyperpolarization occurred on addition of HCO_3^- to Chara.

This reasoning would also explain the time lag observed by Nagai and Tazawa before hyperpolarization, as long as it was assumed that HCO_3^- too was present in their solutions. Hope, however, assumed that the solutions used by Nagai and Tazawa did not contain HCO_3^- ions and suggested that their observations could possibly be the result of an electrogenic chloride pump. This possibility is supported by the system of Raven (1966 private communication) that chloride influx does not require HCO_3^- but, then, Tazawa (1961) should have observed ionic absorption under the same conditions.

Rather than trying to explain the difference in the observations of Hope and of Nagai and Tazawa by two entirely different mechanisms in two closely related genera, it would be reasonable to accept the same mechanism for both, assuming with evidence, some HCO_3^- contamination in the experiments of the latter.

According to MacRobbie (1962) the nature and site of active transport pumps in Nitella translucens are similar to those found in Nitellopsis obtusa (a brackish-water alga) MacRobbie and Dainty (1958.(a)) namely, an active transport of K^+ and Cl^- into the cell and Na^+ out. The pumps in Nitella translucens are non-active in the dark and are not therefore powered by the respiration cycle, their energy being obtained only from the photochemical system. Evidence suggested that the K^+ and Na^+ pumps are on the plasmalemma and are coupled. Whilst ouabain inhibited the cation pumps it had no effect on the chloride pump and therefore MacRobbie concluded that there were two independent light-activated processes, one for cation pumping and the other for chloride influx.

Further investigation of the processes (MacRobbie 1965) revealed that the cation transport required A.T.P. from either cyclic or non-cyclic phosphorylation. (It can work on photosystem I alone where light reduced NADP and oxidizes chlorophyll a, and by a cyclic electron flow produces A.T.P.). The chloride pump, however, requires photosystem II (where light leads to oxidation of water and reduction of plastoquinone; and the production of A.T.P. by dark electron flow from plastoquinone, through cytochrome,

to chlorophyll a, i.e. non-cyclic phosphorylation). As chloride does not require A.T.P. for transport it must be directly linked with the electron transfer system in photosystem II.

Arisz, (1952) concluded that Vallisneria spiralis leaves treated with 2,4-dinitrophenol (D.N.P.) were able to absorb Cl^- ions into the cytoplasm but could not accumulate them in the vacuole. MacRobbie (1965) suggested that although the initial entry of Cl^- through the plasmalemma is not dependent upon A.T.P. its accumulation in the vacuole requires A.T.P. at the tonoplast. Arisz's data would be consistent with this opinion as D.N.P. is an effective uncoupler of oxidative phosphorylation.

Using Hydrodictyon africanum Raven (1966 personal communication) confirmed MacRobbie's observations that 10^{-7} M. D.C.M.U. (dichlorophenyldimethylurea), which inhibits photosystem II, inhibited HCO_3^- and Cl^- influxes but did not affect cation fluxes, and 5×10^{-6} M. C.C.C.P. inhibited active cation pumps but did not affect the HCO_3^- or Cl^- fluxes. (By deduction from Arisz's work this might only refer to uptake and not to accumulation as C.C.C.P. is also, like D.N.P., an oxidative phosphorylation uncoupler). He therefore concluded that the HCO_3^- pump is activated in a manner similar to the Cl^- pump and that although all the pumps require light they do not require CO_2 .

An obvious discrepancy exists between the findings of MacRobbie and Raven and those of Hope, Tasawa and Nagai, Scott, Barr and Broyer, and others.

Considering the anions only, the former claim that the active influx of chloride does not require CO_2 , the ions passing through the plasmalemma by electron transfer from photosystem II although the possibility that A.T.P. may drive Cl^- transport across the tonoplast cannot yet be excluded. The latter authors, however, find that anion absorption is inhibited in the light by shortage of CO_2 , thus an anion pump may exist which depends upon CO_2 and light for operation.

Hope expresses the belief that hyperpolarization of Chara cells in the light is the result of active Cl^- and/or HCO_3^- influx. In the absence of HCO_3^- there is no hyperpolarization.

It must therefore be concluded that either HCO_3^- influx alone causes hyperpolarization or that HCO_3^- stimulates Cl^- influx, the combination of the two producing the effect.

Nagai and Tasawa hold that Cl^- influx causes the hyperpolarization: Hope, on the other hand, appears to favour the HCO_3^- pump with accompanying passive flux of cations.

If the chloride influx causes hyperpolarization then contrary to the beliefs of MacRobbie and Raven, CO_2 must be required at some stage to energize it. As all potential measurements are between vacuole and exterior and as MacRobbie has no conclusive evidence for a tonoplast chloride pump, then perhaps it is this pump that requires CO_2 and light.

We are now in a position to make a comparison between observations made on the algae, particularly the Characeae, and P. schweinfurthii in respect to the effect of CO_2 and light on membrane potentials and the possible mechanisms causing these. The treatments and results can be summarized in the following table:-

TABLE (4), 16

Treatment	<u>Characeae</u>	<u>P. schweinfurthii</u>
$+\text{HCO}_3^-$ or CO_2 in steady light	sharp increase in e.m.f. increase resistance	sharp increase in e.m.f. - - - - -
$+\text{HCO}_3^-$ or CO_2 in steady dark	steady e.m.f. (or slow rise)	sharp increase in e.m.f.
Dark/light switch with HCO_3^- or CO_2	drift. then sharp increase. in e.m.f. decrease in P_K increase in P_{Na}^K	immediate decrease in e.m.f. - - - - - - - - - -
Dark/light switch + High CO_2 concentration	- - - - -	steady e.m.f.
Dark/light switch - CO_2	steady e.m.f. steady resistance	sharp decrease in e.m.f. - - - - -
Variation in external pH	- - - - -	steady e.m.f.
$+\text{CO}_2$ in deionized water	- - - - -	increase e.m.f.

Table comparing responses of the Characeae and P. schweinfurthii to different treatments involving light, CO_2 and pH.

Note:- where no observations were made a blank - - - - - is printed.

It will be noted that only one treatment gives the same response in both the Characeae and P. schweinfurthii that being the addition of HCO_3^- or dissolved CO_2 to the outside medium in the light, producing rapid hyperpolarization.

Hope interprets this reaction as being caused by the pumping of anions, probably HCO_3^- into the cell.

The present author suggests that although, in P. schweinfurthii, HCO_3^- could be pumped in, this cannot be the only cause of hyperpolarization, for it also occurred in the dark and in the presence of dissolved free CO_2 when the HCO_3^- content was negligible. It is therefore proposed that dissolved free CO_2 will also enter the cell passively along a concentration gradient, and once in the cell will be hydrated and hydrolysed to some extent, the HCO_3^- ions thus formed causing hyperpolarization.

Can this mechanism work in Chara?

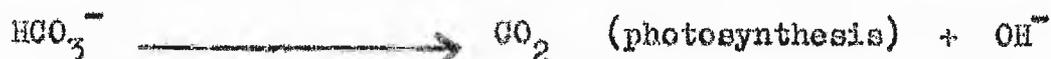
The Characeae exist predominantly but by no means invariably in alkaline waters where the total CO_2 fraction is mainly as the HCO_3^- ion, but in Hope's experiments there would be some dissolved free CO_2 present as his nutrient solution were around neutrality, and this would be available to the plant. Excepting that the lower pH's might be detrimental there is no evidence, to suggest that the dissolved free CO_2 mechanism could not operate in Chara although the HCO_3^- pump might be preferred.

The table shows that all other similar treatments give different responses in Characeae and P. schweinfurthii.

For example, in the presence of CO_2 or HCO_3^- , when the light is switched on there is immediate rapid depolarization in P. schweinfurthii whereas in Chara there is a slow upward drift period followed by rapid hyperpolarization. To explain these opposite responses a different mechanism in each plant must be assumed.

As mentioned earlier Hope explains the hyperpolarization in Chara by means of an inward directed anion pump pumping chloride or bicarbonate ions.

His suggested mechanism is reasonable for chloride ions but if it is a HCO_3^- pump then another factor which has been ignored must be considered. The HCO_3^- ions in the cell will immediately be used in photosynthesis leaving behind hydroxyl ions (assuming that the rate of CO_2 uptake in photosynthesis is at least as fast as HCO_3^- entry into the cell) i.e.



He must then assume that OH^- ions accumulate in the cell; but provides no evidence for this.

It is well known that many Characeae form crusts of CaCO_3 on their outer surfaces, this being caused by excretion of OH^- from the plant reacting with HCO_3^- and Ca^{++} in the water. This rise in pH takes place in the light and is evident within a few minutes of switching the light on.

P. schweinfurthii, for example, when placed in a HCO_3^- solution containing phenolphthalein produces the pink colouration (indicating rise in pH) within 5 minutes of illumination.)

Bearing this in mind, it is unlikely that hyperpolarization in Characeae can be explained solely in terms of an inward directed HCO_3^- pump. It is more conceivable that there are two inward directed anion pumps; one for chloride and one for bicarbonate. The bicarbonate ion provides CO_2 for photosynthesis the hydroxyl ion produced being pumped, or moving passively, out of the cell, (the net movement of HCO_3^- inwards and OH^- outwards causing no change in e.m.f.) and the chloride uptake being stimulated by photosynthesis, and causing hyperpolarization. It would be interesting to observe e.m.f. changes in Chara with light/dark switches in the presence of HCO_3^- and absence of Cl^- .

In P. schweinfurthii the observed depolarization in the light is interpreted as being caused by removal of CO_2 for photosynthesis from a HCO_3^- pool within the cell. The OH^- ions thus formed either move passively, or are pumped actively out. Active pumping of HCO_3^- and/or diffusion of dissolved CO_2 into the cell gradually replenishes this. Absence of all ions and dissolved CO_2 in the external medium do not affect this phenomenon; depolarization therefore cannot be caused by any inward directed pump or any inward passive movement of ions. It is of significance that this mechanism relies upon the movement of hydroxyl ions outwards whereas Hope's mechanism must mean their retention.

Both the depolarization of cells in the light in CO_2 -free medium and the steady e.m.f. observed with P. schweinfurthii during the light/dark switch with high concentration of CO_2 outside supports the view that there is a HCO_3^- pool, (which, in the latter remains virtually unchanged, the plant using directly the dissolved free CO_2 supply) and contrarily, the observation in Chara that e.m.f. does not change during the light/dark switch in the absence of HCO_3^- outside, would suggest that in this plant there is no such reserve. This is further supported by evidence suggesting that the osmotic pressure of the sap (in Nitella) is mainly due to the presence of Na, K, and Cl (see Barr and Broyer 1964).

The suggestion that there are at least these two independent physiological mechanisms for carbon uptake into waterplants could explain the observations and subsequent conclusions of Ruttner, Steeman Nielsen and others, that waterplants fall into two distinct physiological groups; free CO_2 users and bicarbonate users, (see chapter 3, p. 87). But it also favours the proposal made on page 88 that these groups are by no means rigid. Potamogeton schweinfurthii for example, responds physiologically, and by insinuation metabolically, to changes in concentration of external dissolved free CO_2 , but this does not preclude the possibility that HCO_3^- ions also have a similar effect. In fact, entirely independent experiments described in chapter 3 led to the same conclusion, (see pp 134-135). On the other hand, Hope has

shown convincingly with Chara australis, that external concentration of HCO_3^- ions affect the cell physiology but, this likewise, does not preclude the possibility of dissolved free CO_2 entry and assimilation.

Perhaps waterplants have both mechanisms built in but whereas some plants have developed one type more, others have developed the other type and in extreme cases only one mechanism is functional. This could be of great ecological importance and be a major factor in controlling their distribution, for, not only will the quality of CO_2 directly affect photosynthesis but also, as data on electropotential gradients between the inside and the outside of cells has shown, it will affect ionic fluxes as well.

Conclusion

It is apparent that the e.m.f. of the leaf cells of Potamogeton schweinfurthii does not only respond to change in ionic composition of the bathing medium but also to variation in total CO_2 outside, and to illumination.

There is no other comparable work on higher plants but in the Characeae variation in e.m.f. has been observed also with changing total CO_2 and light conditions.

However, the responses to the same treatment by P. schweinfurthii and the Characeae are, in many instances, directly opposed; so different mechanisms must be responsible.

From data for Chara, provided mainly by Hope (1965), it appears that hyperpolarization does not occur in the dark on addition of HCO_3^- outside, or in the light without HCO_3^- , but only in the light and in the presence of HCO_3^- .

Hope interpreted these results by suggesting that hyperpolarization is caused by the active influx of anions against an electrochemical gradient, the pumps being activated through photosynthetic metabolism. The anion pumps involved are HCO_3^- and/or Cl^- but whereas Hope considered it more likely to be HCO_3^- influx the present author suggests that it is probably the influx of both, but only the chloride causing hyperpolarization.

Results obtained by Nagai and Tazawa (1962) on Nitella are open to criticism, but if it assumed that their bathing media are contaminated by HCO_3^- , then there is agreement with Hope, but evidence would again favour chloride influx causing the upward change in potential.

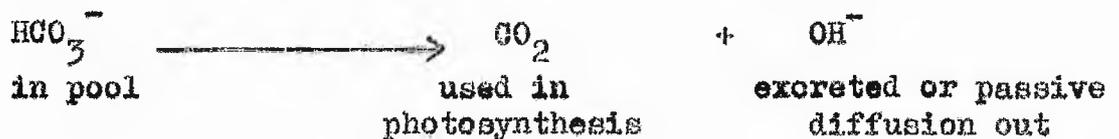
MacRobbie and Raven both suggest that the anion pumps are excited by a photochemical reaction rather than by a product of photosynthesis, so CO_2 is not required to activate Cl^- influx. Even though this might be the case for the plasmalemma pump it is proposed that CO_2 is required at some stage for Cl^- accumulation in the vacuole.

The mechanism suggested by Hope for Chara is clearly sound in principle but it is not appropriate for P. schweinfurthii.

In P. schweinfurthii, hyperpolarization occurs in the dark on addition of CO₂ outside; depolarization takes place in the light in the presence or absence of CO₂; and in high concentrations of CO₂ little change in potential occurs with the dark/light switch.

To explain these reactions a bicarbonate pool is visualized within the cell which can provide an immediate source of CO₂ for photosynthesis, where there is not a sufficiently high dissolved free CO₂ concentration outside.

Depolarization occurs during photosynthesis through the disappearance of HCO₃⁻ ions from the pool and the excretion or passive movement of OH⁻ ions out of the cell thus:-



The bicarbonate pool is replenished both from the active influx of HCO₃⁻ ion and from the passive diffusion of dissolved CO₂ along a concentration gradient into the cell where it is hydrated. The latter process is thought to be dominant in normal conditions.

It is therefore concluded that there are at least two different mechanisms causing changes in the electrical properties of the green cell with varying light and CO₂ conditions; one in operation in the Characeae and another in Potamogeton schweinfurthii.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Investigations on floating and submerged-leaved aquatic plants using the two species Potamogeton richardi and P. schweinfurthii respectively reveal that these plants are able to absorb nutrients both through their shoots and through their roots.

Absorption through their shoots was demonstrated by two independent methods:-

- 1) By observing, chemically, the loss of ions from a nutrient solution over a given time period when excised shoots alone were in the solution.
- 2) By labelling a single leaf of a whole plant with radio-phosphorus solution and observing, by autoradiograph technique, uptake of the isotope into the leaf and its subsequent transfer within the plant to all growing regions except the roots.

Absorption through the roots could only be demonstrated indirectly by growing 'paired' whole plants in either nutrient-rich or nutrient-poor substrata, the shoots of both being in a nutrient-rich solution. Subsequent dry weights of P. richardi plants showed that those in a nutrient-rich substratum grew considerably better than those in a poor substratum.

P. schweinfurthii had only slightly better growth in the rich substratum, as compared with the poor substratum.

With competition experiments and the above results it is suggested that whereas floating-leaved Potamogetons rely more upon ion absorption through the roots, the submerged-leaved type absorb more through the shoots.

Further investigations on P. schweinfurthii shoots showed that they tolerate a wide range in pH in their bathing solutions, (from pH 4 to 10), as long as sufficient cations are present. This pH range provides two possible carbon sources for photosynthesis; dissolved free CO_2 at the lower pH value and dissolved HCO_3^- ions at the upper pH value.

Rates of photosynthesis, assessed by the rates of oxygen increase in the nutrient solutions, showed that P. schweinfurthii has a preference for dissolved free CO_2 which it can utilize in the absence of any ions in the external solution; however, as long as there are sufficient cations present, especially calcium, it is also able to utilize HCO_3^- ions.

Utilization of HCO_3^- ions also involve secretion, or passive diffusion outwards, of OH^- ions; a phenomenon which does not occur with dissolved free CO_2 utilization.

Studies on the electrochemical gradients between the inside and outside of single leaf epidermal cells of P. schweinfurthii

provides evidence that when the cells are in flux equilibrium they have an average observed potential gradient of -194 mv with respect to the outside medium.

Comparison of this result with the electrical potentials calculated in the Nernst equation by substitution of the ionic concentrations inside and outside the cell, revealed that whilst potassium was very near passive electrochemical equilibrium other ions were far from it. Even though some doubt is expressed about the results of internal ionic concentrations of the cells it would appear that sodium is being actively pumped out of the cell and chloride, phosphate, and perhaps sulphate, are being actively pumped inwards. As it was impossible to separate 'bound' calcium and magnesium from the 'free' ions no conclusion could be made concerning these two.

Cell membrane potential studies directly relating to 'carbon' supply in the nutrient solution confirmed the chemical results that *P. schweinfurthii* is able to assimilate both dissolved free CO_2 and dissolved HCO_3^- ions. However, membrane potential observations were, in many instances, the reverse of those found in the Characeae by Hope and others. Therefore, although the mechanism relating 'carbon' uptake with change in membrane potential suggested by Hope (1965), may be feasible in the case of *Chara*, it cannot be applicable to *P. schweinfurthii*. An alternative mechanism is suggested for the latter involving the necessity of a small bicarbonate pool within the cell.

It is not claimed that this is the only possible mechanism or that it is necessarily the correct one but until further information is forthcoming it is put forward as a working hypothesis.

The results of these studies form a basis for a clearer understanding of factors limiting the distribution of rooted aquatic macrophytes as two fundamental requirements for successful plant growth are available nutrients and an appropriate 'carbon' supply.

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APPENDICES

APPENDIX IThe Colorimetric Estimation of PhosphateA. Estimation of Micro-quantities.

(Modified from Mackereth (1963)).

Principle

The principle depends upon the phosphate in solution forming a molybdophosphoric acid complex and the selective reduction of this complex to form a molybdophosphoric blue coloration.

The phosphomolybdate complex was extracted from the sample into a small volume of organic solvent and reduced in the solvent to molybdenum blue.

Since the phosphate was extracted from a relatively large sample into a small volume of solvent the sensitivity of the method was very high and had the added advantage that the interference, especially from the coloration of the water sample was reduced.

Reagents (All reagents were analytical grade)1) Wash Acid.

1.2 N H_2SO_4 saturated with Tert-amyl alcohol.

2) Acid Ammonium Molybdate

30 g of ammonium molybdate was dissolved in 300 ml of distilled water and 150 ml of concentrated H_2SO_4 was added. The mixture was diluted to 600 ml with distilled water.

3) Reducing Agent

Stannous chloride solution.

2 g of hydrated salt ($SnCl_2 \cdot 2 H_2O$) was dissolved in 100 ml of 10% HCl.

The solution was stored in a brown bottle with a few additional pieces of tin, under 1 inch of oil.

4) Solvent

Analar Tertiary amyl alcohol.

5) Standard phosphate solution

2.195 g of $K H_2PO_4$ was dissolved in 500 ml H_2O giving a standard solution of 1 g / litre phosphorus.

Method

A sample ranging from 2 to 50 ml was added to a 250 ml separating funnel and diluted to 50 ml if necessary. 10 ml of acid ammonium molybdate was added whilst swirling the solution and the mixture was left for approximately 5 minutes.

Exactly 15 ml of amyl alcohol was added, the funnel was stoppered tightly and then shaken on a mechanical shaker for 5 minutes.

The layers were allowed to separate and the lower aqueous layer was discarded. The solvent layer was washed twice, both times with approximately 25 ml of wash acid, which was then discarded.

10 ml of distilled water (previously saturated with amyl alcohol) was added to the solvent together with 6 drops of stannous chloride solution.

The mixture was shaken for exactly 3 minutes using the mechanical shaker. The layers were allowed to separate and the lower aqueous layer was discarded. The solvent layer was again washed with 25 ml of wash acid which was discarded, the remaining solvent layer having 1 ml of iso-propyl alcohol added to clear it.

A drop of the solvent layer together with any remaining water droplets was run out and the remainder was run into a dry test-tube. This solution (which if it contained phosphate would be blue in colour) had its optical density measured on an 'Optica' spectrophotometer at 730 m μ , in a 1 cm. cell.

The phosphate content could be assessed by reference to a standard curve prepared in a similar manner using a series of standard phosphate solutions.

The sample was always compared with a blank treated in the same way but containing distilled water.

All spectrophotometer readings had to be made within 12 minutes of colour formation as after this period the intensity began to fade.

The standard curve was linear and the results were reliable over a range from 0.5 μg to 8.0 μg -P. (0.0162 μM to 0.258 μM).

As samples from 2 to 50 ml could be used this gave a working range of 0.32 $\mu\text{M}/\text{l}$ to 129 $\mu\text{M}/\text{l}$.

The standard curve was checked every three months or whenever any fresh reagents were made up.

A typical standard curve is shown in graph A 1 (i).

Comment

The main modifications as compared with Mackereth was

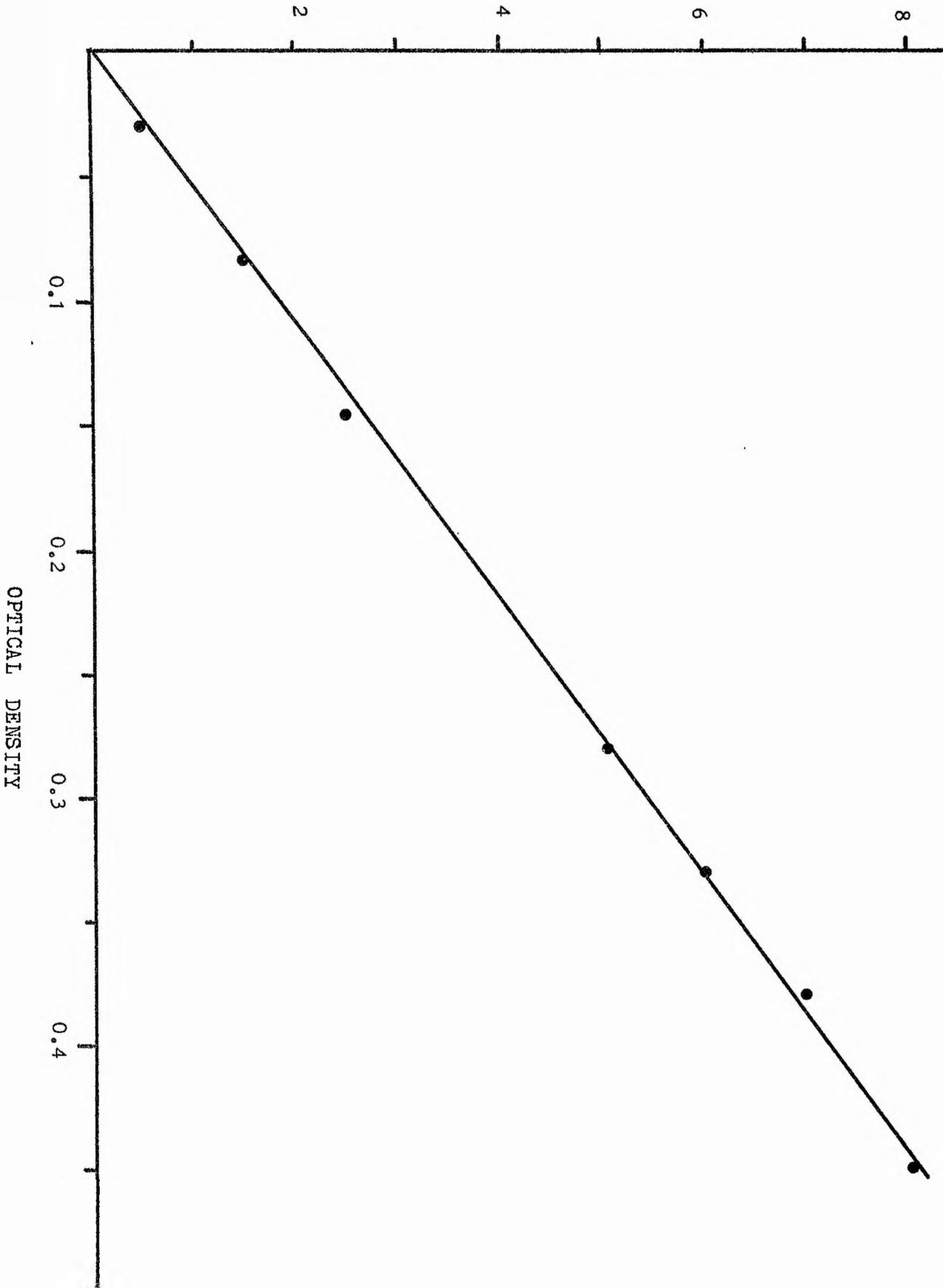
1. The use of tert-amyl alcohol rather than n-hexanol (which was not available).
2. It was found essential to make the sample volume up to a standard volume; in this case 50 ml, otherwise variation in results occurred.
3. Longer shaking times using a mechanical shaker was more reliable than Mackereth's hand shaking method.

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GRAPH (A) 1 (1)

Standard curve for phosphate using the amyl alcohol extraction technique; micrograms of phosphorus plotted against optical density.

MICROGRAMS PHOSPHORUS



The Colorimetric Estimation of Phosphate

B. Estimation of non-micro quantities.

(Modified from Fogg and Wilkinson (1958)).

Principle

The principle is similar to the previous method in forming a molybdenum blue coloration but the complex was not concentrated into a solvent as phosphate levels were higher, and the reduction was achieved by boiling with ascorbic acid.

Reagents

1. Molybdate-sulphuric acid.

10 g of ammonium molybdate was dissolved in 100 ml distilled water. 150 ml of concentrated H_2SO_4 (s.g. 1.84) was diluted to 300 ml with distilled water and cooled. The molybdate and acid solutions were then mixed.

2. Ascorbic acid crystals.

Method

An aliquot of sample not greater than 30 ml was pipetted into a 250 ml conical flask, a drop of phenolphthalein was added and the sample neutralized with either dilute acid or NaOH. The volume of solution was adjusted to approximately 40 ml.

4 ml of molybdate-sulphuric acid was added, followed by approximately 0.1 g ascorbic acid (a few crystals on the tip of a spatula), and the solution stirred.

It was then boiled for exactly 1 minute, cooled, transferred to a 50 ml volumetric flask and made up to the mark.

Using a 1 cm. cell the optical density of the solution was measured in the 'Optica' spectrophotometer and the result read off on a standard curve prepared in a similar manner using a series of known phosphate standards.

A blank sample was always taken.

The standard curve was linear and the results were reliable over a range from 10 μg to 80 μg -P. (0.32 μM to 2.58 μM).

As samples from 2 ml to 30 ml could be used this gave a working range from 0.01 mM/l to 1.29 mM/l.

A typical standard curve is shown in graph A 1 (ii).

Comment

This method was developed by Fogg and Wilkinson as it was less affected by interfering ions than the more normal Denigès method, and had the advantage that the colour formation was stable for several months.

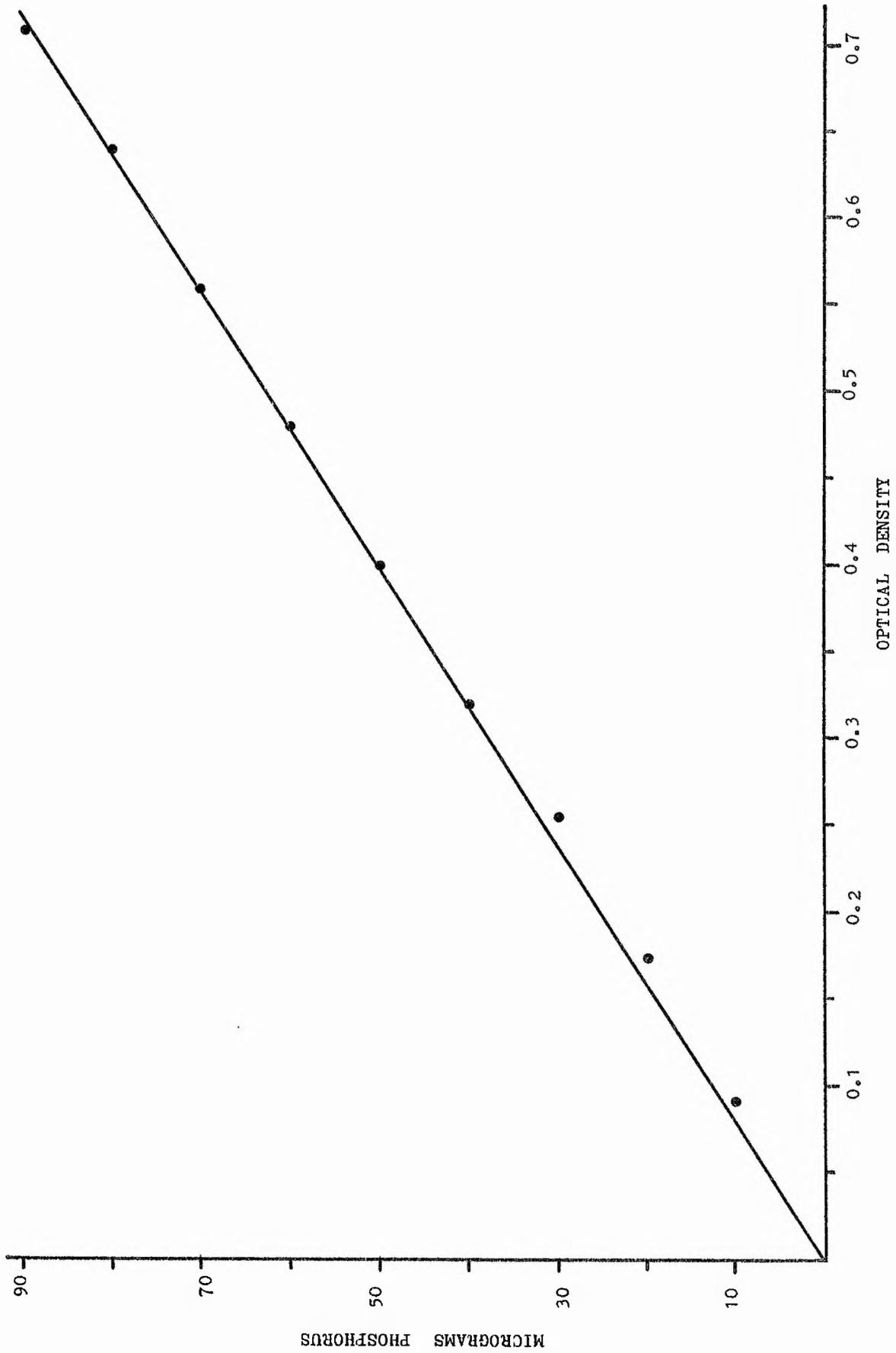
Although designed for commercial use the present author found it highly successful for measuring the phosphate concentrations in plant or soil extracts.

Its accuracy and simplicity was such that it became a standard technique used in the training of undergraduates.

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GRAPH (A) 1 (ii)

Standard curve for phosphate using the
modified Fogg and Wilkinson technique;
micrograms of phosphorus plotted
against optical density.



APPENDIX IIEstimation of Cold Water Soluble Phosphate in DriedP. schweinfurthii LeavesMethod

A weighed aliquot of dried powdered leaf material was shaken with 35 ml of deionized water in a 250 ml stoppered conical flask for 30 minutes. The mixture was then filtered through Whatman's 542 filter paper into a 100 ml volumetric flask and the conical flask was rinsed out with deionized water. The volumetric flask was made up to the mark and the contents analysed for phosphate concentration using the amyl alcohol extraction technique. (p. i)

The powdered material retained on the filter paper was washed into another conical flask with deionized water for a second cold water extraction and phosphate analysis.

After the repeat extraction, the powdered material was washed into a keijeldahl flask and digested using concentrated sulphuric and nitric acids. (p. xx). After being made up to standard volume, aliquots of the digestion were taken for phosphate analysis (p. v) of the cold water insoluble fraction.

A second weighed aliquot of dried powdered leaf material

was directly digested for total phosphate.

From the results, percentage of total phosphate in the tissue soluble in cold deionized water could be calculated. (See Table A 2).

. . . .

Sample Dry Weight g	Cold water soluble P g/100 g Dry Weight	1st extraction	2nd extraction	In soluble P in tissue g/100 g Dry Weight	Total P soluble + insoluble g/100 g Dry Weight	% P cold water soluble
0.3491	0.158	0.000	0.0602	0.2236	62.95	
0.0898	0.151	0.000	0.0724	0.2256	60.16	
0.3145	---	---	---	0.251 (direct digestion)		

TABLE A 2

Table showing percentage of cold water soluble

phosphate in leaves of P. schweinfurthii

APPENDIX IIIEstimation of Sodium and Potassium using Flame Photometry

The normal technique using an 'Eel' flame photometer was applied for both these ions.

Method

Standard solutions containing both salts were made.

The flame photometer with either the K^+ or the Na^+ filter in place was adjusted so that by spraying distilled water through the flame the galvanometer registered zero, and by spraying the standard solution it showed 100% deflection. On return to distilled water zero should once again register. The unknown sample was then sprayed through and the deflection compared with a graph constructed in a similar manner from known standard solutions.

Two standard curves were prepared for each ion, one with 250 $\mu\text{eq/l}$ full deflection and the other with 100 $\mu\text{eq/l}$.

The 250 $\mu\text{eq/l}$ concentration did not produce a linear curve but as the instrument was set at a lower sensitivity results for duplicate samples were consistently $\pm 2\%$.

The 100 $\mu\text{eq/l}$ concentration produced a nearly linear curve but readings tended to be slightly variable so that

most samples were assessed in triplicate. See Table A 3 and graph A 3.

TABLE A 3

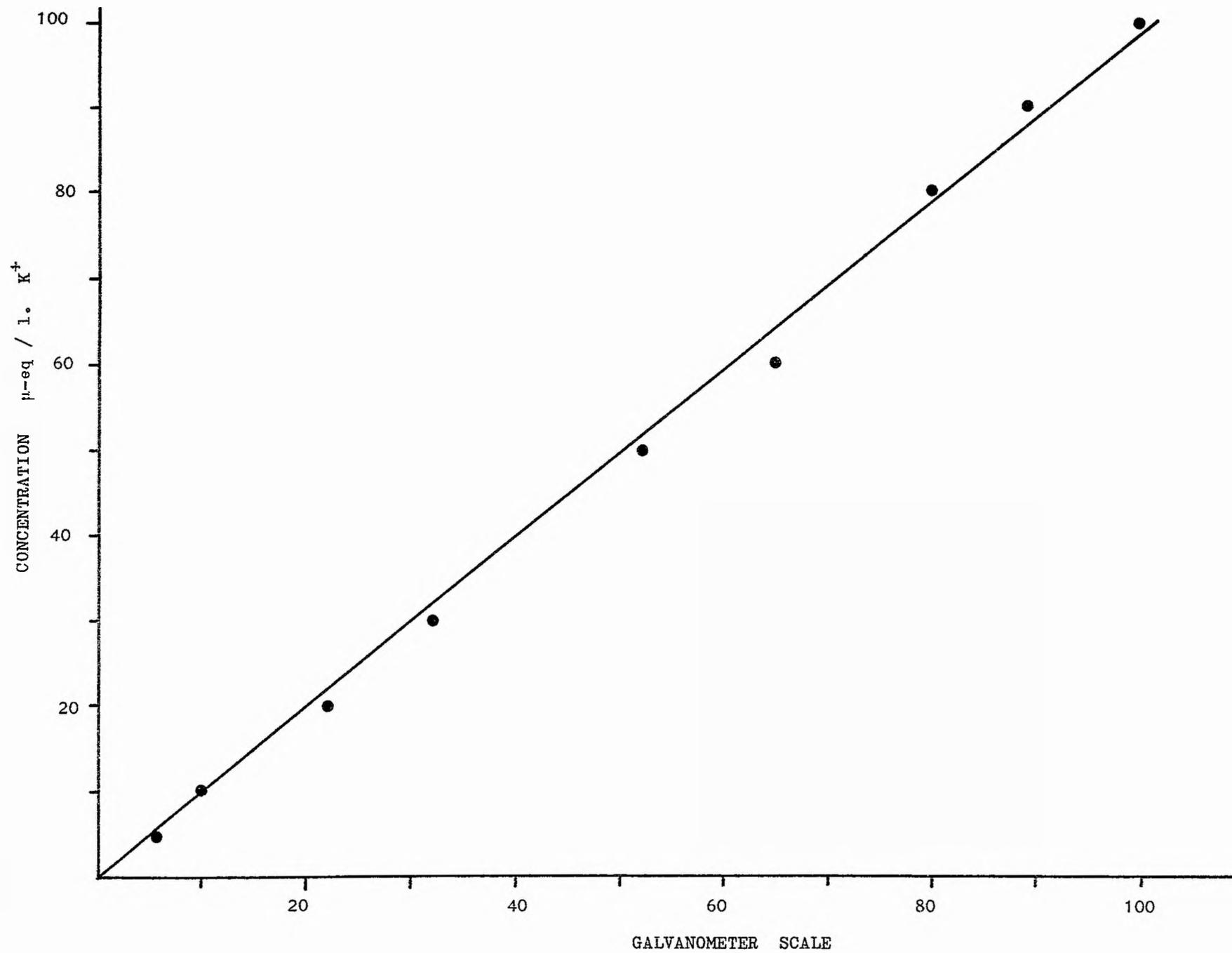
Standard $\mu\text{eq/l.}$	Galvanometer Readings				
	Na ⁺			K ⁺	
5	5	4	5	6	6
10	10	10	10	10	11
20	18	18	20	24	20
30	34	32	30	30	29
50	60	56	56	52	52
60	60	64	60	66	65
80	83	80	-	80	80
90	96	96	-	88	91
100	100	100	-	100	100

Table showing galvanometer readings for standard solutions of Potassium and Sodium.

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GRAPH (A) 3

Standard curve for potassium using flame
photometry; $\mu\text{eq/litre K}^+$ plotted against
galvanometer scale.



APPENDIX IVEstimation of Calcium

Calcium was, on occasions, estimated using flame photometry the procedure being the same as for K^+ and Na^+ (see p. x), but the calcium filter was used.

However, this method was rather insensitive (full deflection equivalent to 2 meq/l Ca^{++}), and interfering ions, especially phosphate, produced variable results.

A more accurate method, although no more sensitive was using the versinate complex and indicator and measuring the end-point with an 'Eel' Auto-titrator incorporating a photo-electric cell, a 606 olive-green filter and a galvanometer.

Reagents

1. E.D.T.A. (di-sodium ethylene-diamine-tetraacetic acid)
4.77 g were dissolved in 1 litre of distilled water.

2. 9 N NaOH solution

3. Murexide indicator

A few milligrams were dissolved in 5 ml of distilled water, this being made up fresh daily.

4. Standard $CaCl_2$ Solution

2 meq/l.

Method

In a 4 ml beaker was placed 1 ml of sample + 1 drop of 9 N NaOH and 1.5 ml of distilled water.

4 to 5 ml of murexide indicator was added and the beaker, containing a magnetic stirrer, was placed in the auto-titrator. This was then titrated with the E.D.T.A. solution from an 'Agla' micro-syringe burette.

The galvanometer showed a change in potential on the addition of E.D.T.A. until the end-point was reached. At the end-point three further turns of the 'Agla' showed no further change in galvanometer reading. The first 'Agla' reading which produced no change was the titre for that sample.

A standard known concentration of Ca^{++} was treated in the same manner and as the titre was linear with concentration the slope could be calculated.

The unknown sample titre was directly multiplied by the slope to give the concentration of Ca^{++} in the sample.

e.g. See Table A 4 overleaf.

TABLE A 4

Standard Ca ⁺⁺	Titre Value in Agla Units			Blank Agla Units	Actual Titre	Slope Conc./ Agla Unit
2 meq/l	4.00	4.00	3.95	0.09	3.91	0.51 meq/l
5.2 meq/l	9.92	10.11	10.03	0.09	9.93	0.52 meq/l

Table showing titre values for a standard Ca⁺⁺ solution and the calculated concentration per Agla Unit.

∴ Concentration of Ca⁺⁺ in sample = titre (in Agla Units) x
0.515 meq/l

APPENDIX VEstimation of Magnesium

The Magnesium was estimated in a similar way to Ca^{++} using E.D.T.A. and the 'Eel' auto-titrator, but Eriochrome Black T (E.B.T.) was used as the indicator.

The titre then was proportional to the $\text{Ca}^{++} + \text{Mg}^{++}$ in the sample.

By estimation of the Ca^{++} separately (using murexide) the magnesium could be calculated by difference.

Reagents

1. E.D.T.A. as for Ca^{++} analysis.
2. Indicator

A stock solution of E.B.T. was made by dissolving 0.4 g E.B.T. in 100 ml of methanol and then adding 4.0 ml of 0.88 s.g. Ammonia.

The indicator was then prepared by mixing together 50 ml of distilled water + 0.5 ml of ethanolamine (redistilled) and 0.8 ml of the stock E.B.T.

Method

1 ml of sample was pipetted into a 4 ml beaker and 2.5 ml

of indicator was added. This was titrated as with Ca^{++} , using E.D.T.A. but filter No. 607 in place of filter 606.

TABLE A 5

Standard meq/l.	Titre Value Agla Units	Blank Agla Units	Actual Titre	Slope Conc./ Agla Units
5.2 Ca^{++}				
+	11.55 11.50	0.05	11.5	0.52
0.8 Mg^{++}				

Table showing titre values for standard $\text{Ca}^{++} + \text{Mg}^{++}$ solution and the calculated concentration per Agla Unit.

$\text{Ca}^{++} + \text{Mg}^{++}$ in sample = titre (in agla units) \times 0.52 meq/l.

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APPENDIX VIEstimation of Chloride

The chloride estimation was by the electrometric principle, two clean silver wire electrodes in the acidified sample solution being connected to a 'Vibron' electrometer via coaxial cable, and the fall in potential on the addition of AgNO_3 to the sample, being observed.

Reagents

1. 0.3 N HNO_3 solution.
2. 0.01 N AgNO_3 standard solution.
3. Standard KCl solution.

Method

1 ml of sample had 2 ml of 0.3 N HNO_3 added to it in a 5 ml beaker. The beaker was placed so that the electrodes were immersed in the solution and the solution was stirred using a magnetic stirrer.

Standard AgNO_3 solution was added from an 'Agla' micro-syringe burette until the potential was lowered to 140 mv. and was stable for 5 seconds.

This was repeated for a blank sample with deionized water.

A standard graph with known concentrations of chloride was constructed (see graph A 6) and by reference to this graph the titre value for the unknown sample indicated the concentration of chloride in solution.

Data for standard curve:

TABLE A 6

Concentration Cl ⁻ µeq/l.	Replicate Titre in Agla Units			Avr. Blank Agla Units	Avr. Titre minus Blank
100	1.095	1.090	-	0.695	0.398
200	1.590	1.425	1.525		0.818
300	1.935	1.905	-		1.240
500	2.940	-	-		2.245
1000	5.30	5.22	-		4.565
1500	7.42	7.45	-		6.745

Table showing replicate titre values with known concentrations of chloride for the construction of a standard curve.

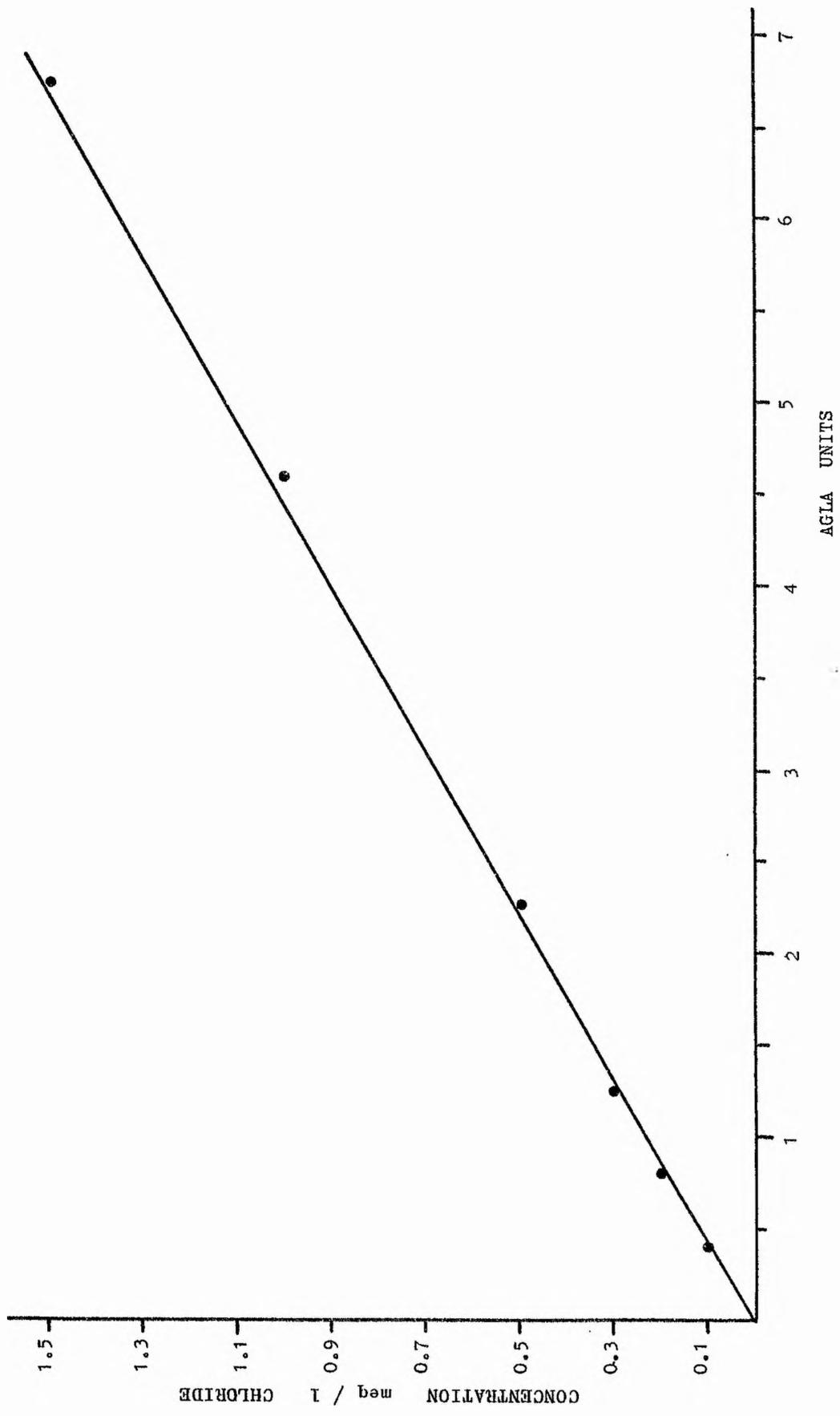
Comment

Frequently, a considerable amount of galvanometer drift occurred and good duplicate results were difficult to obtain.

Under these conditions, up to 8 replicates for a single sample were made each time with reference back to a blank and checking with a standard sample; but the cause of these drifts was never located.

GRAPH (A) 6

Standard curve for chloride using the
electrometric technique; meq/litre Cl^-
plotted against x 'Agla' units.



APPENDIX VIIEstimation of Sulphate

The technique was exactly according to that described in the Agricultural Research Council Report (1963) p. 26.

Method

The sample was acidified with 1 ml of 5 N HCl and boiled with 15 ml of 96% ethanol. 2 ml of 2.4 % barium chloride was added and the mixture was simmered for 5 minutes. It was then cooled and left overnight for the precipitate of barium sulphate to settle. The precipitate was washed with distilled water and dissolved with heating in excess of 0.02 M E.D.T.A. made ammoniacal with 5 ml of 9 N ammonia.

After cooling 10 drops of Solochrome Black indicator and 0.5 ml of 9 N ammonia was added and the excess E.D.T.A. was determined by titration with 0.02 M magnesium sulphate solution.

A blank titration was carried out and the amount of sulphate present in the sample was calculated from the difference between the blank and sample titres.

All the sulphate analysis was carried out by Dr. D.C. Weeks, to whom I am indebted.

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APPENDIX VIII

Digestion of Plant Material

For the analysis of ionic concentrations in plant material the wet digestion technique was used.

Reagents

1. Concentrated H_2SO_4
2. Concentrated HNO_3

Method

0.5 g of dried powdered plant material was digested with 5 ml concentrated H_2SO_4 and successive portions of about 10 ml concentrated HNO_3 in a long necked Kjeldahl flask of approximately 100 ml capacity.

The digest was boiled in a micro-Kjeldahl digestion rack with electric heating mantles.

When the solution had become clear and colourless it was cooled and transferred to a 250 ml conical flask. The Kjeldahl flask was washed three times with deionized water and the washings were added to the conical flask.

The contents of the conical flask were diluted to approximately 75 ml with deionized water, were boiled for

15 minutes, rinsed into a 100 ml volumetric flask with boiling deionized water, cooled and made up to the mark.

Aliquots of this were used for chemical analysis.

The exact same procedure of digestion was used on standard samples of $K H_2PO_4$ ranging from 20 to 80 mg/l -P the phosphate content, after digestion being analysed using the ascorbic acid technique (see page A v).

Mean percentage recovery of phosphate after digestion was 99.96 (range 97.84 to 102.10).

(Range calculated at the 95% confidence level where the number of samples was 10).

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APPENDIX IXEstimation of Oxygen in Solution

The Micro-Winkler technique.

Reagents1. Manganous sulphate

50 g MnSO_4 was dissolved in 100 ml deionized water.

2. Winkler's Reagent

15 g KI + 70 g KOH was dissolved in 100 ml. deionized water.

3. Orthophosphoric Acid

Concentrated 'Analar'.

4. Standard Potassium Iodate

A solution containing 0.82mg/l KIO_3 was equivalent to 184 mg/l O_2 .

5. Standard Sodium ThiosulphateMethod

The water sample was collected in a 35 ml sample bottle by the 'displacement flooding' technique. 0.51 ml of manganous sulphate and 0.51 ml of Winkler reagent were quickly injected into the bottom of the sample with hypodermic

syringes and the bottle was carefully stoppered to exclude air-bubbles.

The bottle was then well shaken and the precipitate of manganeous hydroxide formed was allowed to settle for five minutes.

0.3 ml of orthophosphoric acid was then introduced from a syringe by quickly removing the stopper and replacing it, and on shaking the precipitate was redissolved.

5 ml of this solution was pippetted into a micro-beaker containing a magnetic stirrer, and was titrated with standard sodium thiosulphate from an 'Agla' micro-syringe burette.

When the end-point was near (i.e. when the yellow iodine colouration had nearly disappeared) one drop of starch indicator was added to sharpen it.

The sodium thiosulphate was standardized against standard KI solution so that one Agla unit of thiosulphate used in titration was equivalent to 0.83 mg/l O_2 .

The thiosulphate was restandardized every day but as it was stored in the refrigerator it retained its normality for a long period.

All samples were estimated in duplicate and where change in O_2 level with time was being observed in an experiment,

duplicate blank samples were taken immediately before the
timed interval.

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APPENDIX X

Estimation of Total Ionic Concentration of Cell Sap
Using the Principle of Plasmolysis

Method

Strips of leaf tissue of Potamogeton schweinfurthii were put into sucrose solutions of varying concentrations.

After 20 minutes the epidermal cells of the tissue were observed under the microscope and approximately 100 cells were scored for plasmolysis, or not, for each sucrose concentration.

The equivalent ionic concentration of the cell sap was considered to be that molarity of sucrose solution which produced 50% plasmolysis.

Results

TABLE A 10

Sucrose Molarity.	Cell Plasmolysis %
0.450	17
0.475	43
0.500	78

Table showing % plasmolysis of P. schweinfurthii leaf epidermal cell in different sucrose solutions.

∴ Total Ionic concentration of cell sap was between 0.47 - 0.48 M.

APPENDIX XIDetermination of Total Carbon Dioxide.

The technique was exactly according to that described by Milburn and Beadle (1960)

Principle

All gases in solution were removed by acidifying a 10 ml sample with concentrated orthophosphoric acid using van Slyke and Neill's (1924) extraction technique. The gases were then transferred without contact with the atmosphere to a standard volume of 0.005 N NaOH solution with known electrical resistance at 30°C.

Within a given range of CO₂ content, the change in resistance of the absorbent after equilibration with the gas bears a strictly linear relationship to the total CO₂ content of the sample.

The apparatus was calibrated using a series of standard Na(CO₃)₂ solutions.

The coefficient of variation for samples within the range 0 to 50 mg/l CO₂ was approximately 0.2 %.

Many of the carbon dioxide analyses were carried out by Mr. T. R. Milburn to whom I am indebted.

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APPENDIX XIICalculation of Free CO₂ and HCO₃⁻ Levels at Given Total CO₂ Concentrations and Given pH Values.

Substituting in the Henderson-Hasselbach equation one is able to calculate the percentage of HCO₃⁻ and free CO₂ at any pH for,

$$\text{pH} = \text{pK} + \log \left[\text{HCO}_3^- \right] - \log \left[\text{free CO}_2 \right]$$

where pK is $-\log K$

$$K, \text{ the dissociation constant} = \frac{(\text{H}^+)(\text{HCO}_3^-)}{\text{CO}_2}$$

at 25°C pK = 6.365 (Manometric technique p. 23)

TABLE A 12

pH	pK	Assumed HCO ₃ ⁻ Conc. (mM)	Calc. CO ₂ Conc. (mM)	Therefore % CO ₂
8.9	6.365	0.36	0.001	0.28
6.15	-	1.73	2.84	62
8.2	-	0.45	0.0065	1.45

(See p 164)

As a check on the calculation, using a pK value of 6.578 at 0°C the results are within 2% error of the Fairholt Calculations at 0°C. (1924)

Note the second dissociation $\text{HCO}_3^- \longrightarrow \text{CO}_3^{--}$ is not taken into consideration but at pH 8 contributes about 0.5% and at pH 9.0 about 5.0% of total CO₂.

APPENDIX XIIIPreparation of Micro-electrodesPreparation of Glass

Pyrex melting-point borosilicate tubing (Corning 7740) of 1.7 mm outside diameter and 0.8 mm inside diameter was cut into 4 inch lengths.

These were washed in strong detergent, rinsed in distilled water; soaked in filtered 50% HNO₃ and again rinsed in dust-free distilled water. They were then rinsed in 50% methanol and dried in the oven.

From this stage all processes were carried out in as near as possible dust-free conditions, and all solutions used were filtered through Whatman's No. 542 filter paper.

Pulling of Micro-pipettes

A tube was mounted in an electrode puller (designed and loaned by Dr. D.C. Weeks) such that the tube was rigidly clamped at one end and attached to a sliding trolley at the other end. The trolley was attached to an extended elastic band so that the tube was under slight pulling tension. Around the mid-point of the tube was coiled a 'Brightray' flat tungsten alloy ribbon approximately 1 mm wide and 0.02 mm thick.

By passing a small electric current through the alloy thus heating it, the glass tubing was melted at that point. The trolley, under tension from the elastic band, caused the tubing to be drawn out to very fine points thus forming two micro-pipettes.

The size of the tip and the taper of the shank would depend upon the tension of the elastic band and the heating from the coil; both of which were adjustable.

Filling the Micro-pipettes

A group of micro-pipettes were supported in a carrier and immersed in a solution of filtered absolute methanol. The container with the methanol was then gently evacuated for about one hour until the methanol had completely replaced the air in the micro-pipettes.

They were transferred to filtered distilled water for from 2 to 4 hours and then to a 3 M KCl solution for at least 3 days.

They could be stored in the KCl solution for nearly 2 weeks but after this, the tips became notably corroded.

Before use the micro-pipettes (which form a KCl salt bridge for the micro-electrodes) were rinsed in distilled water and their tips checked under the high power of a microscope.

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APPENDIX XIV

Statistical Analysis of the Data

x = Observed measurement

n = Number of sample

\bar{x} = Mean

s^2 = Estimated variance

s = Standard Deviation

t = Student's t .

f = Numbers of degrees of Freedom

1. Calculation of Standard Deviation and Range for the Mean at the 95% confidence level when the number of samples is small.

Standard Deviation is calculated by substituting in the following:-

$$s = \sqrt{\frac{1}{n-1} \cdot \left(\sum x^2 - \frac{1}{n} (\sum x)^2 \right)} \quad \text{---(1)}$$

From this the Range is calculated substituting in the following:-

$$\bar{x} + \frac{ts}{\sqrt{n}} \quad \text{to} \quad \bar{x} - \frac{ts}{\sqrt{n}} \quad \text{---(2)}$$

t is taken at the 95% confidence level

In order to test whether the difference between two sample Means is significant at the 95% probability level, the following formula is used:-

where, the first sample contains n_1 observations and has a mean \bar{x}_1 and a variance s^2_1 ; and the second sample has n_2 observations and has a mean of \bar{x}_2 and a variance of s^2_2
t is:-

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad \text{--- (3)}$$

where s is an estimate of the standard deviation based on both samples jointly.

s is calculated from the following:-

$$s = \sqrt{\frac{1}{n_1 + n_2 - 2} \cdot \left(\sum_1 x^2 - \frac{(\sum_1 x)^2}{n_1} + \sum_2 x^2 - \frac{(\sum_2 x)^2}{n_2} \right)} \quad \text{--- (4)}$$

TABLE A 14

CALCULATION OF MEAN, STANDARD DEVIATION, AND RANGE AT 95% CONFIDENCE LEVEL

Substituting in formulae (1) and (2)

Description of Data	Page Ref. N ^o .	No. of Samples n	$\sum x$	$\sum x^2$	\bar{x}	$\sum x^2 - \frac{(\sum x)^2}{n}$	s	t at 'f' degrees of freedom	$\frac{ts}{n}$	Range
Observed E _m Upper Epidermis 3rd Sept.	74	9	1647	303333	183	1932	15.5	2.306	11.9	183 (171-195)
Observed E _m Lower Epidermis 3rd Sept.	74	9	1559	271019	173	996	11.0	2.306	8.46	173 (165-181)
Average E _m Upper + Lower	78	18	3206	574352	178	3328	14.0	2.110	6.95	178 (171-185)
Observed E _m 20th November	78	11	2151	422081	196	1463	12.0	2.228	8.1	196 (188-204)
Calculated E _k August-September	78	6	919	140877	153	117	4.8	2.571	5.09	153 (148-158)
Calculated E _k November	78	3	523	91185	174	9	2.1	4.303	5.2	174 (169-179)