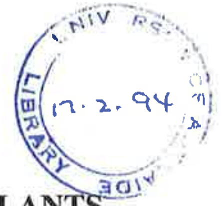

SODIUM AS AN ESSENTIAL ELEMENT FOR C_4 PLANTS

BY

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Awarded 1994

DECLARATION

With the exception of Publications 1 and 3, which formed part of my Ph. D. thesis, this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text.

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ACKNOWLEDGEMENTS

I give my sincere thanks to co-authors, colleagues and associates who have made this work possible. Their stimulation and support have enabled this work to be written up and published. I am particularly grateful to the late Professor J. G. Wood, my Ph. D. supervisor, for suggesting an investigation of sodium metabolism in plants and for leading me to use *Atriplex vesicaria* (Bladder salt bush), a C_4 species, as our experimental material in the early experiments, many years before the discovery of the C_4 photosynthetic pathway! His enthusiasm for the work was infectious and was greatly missed after his untimely death in 1959. During this period, Professor Ray L. Specht gave me generous help, guidance and encouragement. Later, I was particularly fortunate to spend some years in the Department of Agricultural Biochemistry at the Waite Agricultural Research Institute working under the guidance of Professor Donald Nicholas where we studied the involvement of sodium in the nitrogen metabolism of the Cyanobacterium, *Anabaena cylindrica.* I was given much help by Professor Nicholas and my other colleagues and I have continued to enjoy this happy association with my colleagues at the Waite Institute over many years.

At the James Cook University of North Queensland within the Department of Botany, here, the research on the function of sodium was continued when we became aware of the possibility of sodium being essential for plants with the C_4 photosynthetic pathway. Since then, my students and I have been greatly assisted by help and discussions with many colleagues including Dr. M. D. Hatch, Division

of Plant Industry, CSIRO, Canberra, Professor C. B. Osmond, RSBS, ANU, Canberra, Professor J. T. Wiskich, Department of Botany, University of Adelaide, Dr. E. J. Hewitt, formerly of the Long Ashton Research Station, Somerset. The late Dr. John Hawker with whom I was associated as a postgraduate student in the Botany Department, University of Adelaide, gave generous help and suggestions. Finally, the work has greatly benefited from the enthusiasm and questions from my colleagues and students who have made many contributions to our work over the years. In particular, I want to thank Dr. Leone Bielig, Department of Botany and Tropical Agriculture for her constructive criticisms, ideas, strong support and encouragement. I am also indebted to Professor Dilwyn Griffith for reading the thesis and offering constructive suggestions. I wish to thank Mr. Gordon Bailey for preparing the diagrams in Figs. 2, 3, 4, 5, and 6 and Mrs. Dianne McNamara for her assistance in the final compilation.

PREFACE

The initial impetus to undertake the area of the research presented in this thesis came from the question posed by the late Professor J. G. Wood "Do plants require sodium and/or chlorine?" At that time, although there was strong interest in plant nutrition, no careful experiments had been undertaken to exclude sodium and chlorine from the environment of the plant. Professor Wood suggested using the Australian salt bush, *Atriplex vesicaria*, as our experimental material because of the ability of these plants to withstand arid conditions. He thought it possible that the possession of the bundle sheath structure which he had described in 1923 might be involved in their tolerance to drought.

It was a great surprise to discover that sodium was indeed essential for the Australian *Atriplex* species but in such small amounts that it would be unlikely for sodium-deficiency to ever to be encountered under natural conditions. A second surprise was the finding that very few plants exhibited a requirement for sodium. This was quite different from other essential elements which had been found to be generally essential for **all** plants. Eventually, a rationale for this restricted sodium requirement was established when the C_4 photosynthetic pathway was discovered.

We established a correlation between the C_4 pathway and the requirement for sodium. This discovery focussed our attention on the C_4 appendage as the area in which sodium was involved in the nutrition of these plants. We have now defined the mesophyll chloroplast, in which pyruvate is converted to phospho*enol*pyruvate,

as the site of the lesion in sodium-deficient C_4 plants.

There are still many questions to be answered but it now seems that sodium is needed for the active transport of pyruvate into the mesophyll chloroplast and/or the maintenance of the integrity of the light harvesting machinery, the source of energy to drive the pyruvate transport and/or the high energy demanding reaction in which pyruvate is converted into phospho*enol*pyruvate. Present work is aimed at determining whether the active transport of pyruvate into the chloroplast or the maintenance of the integrity of the light harvesting system are the sole sodium-requiring components ot the metabolism.

Since I initiated the work on sodium nutrition in higher plants, interest in this field has developed in other laboratories. Two groups in Japan are now working in this general area. One group including T. Matoh, S. Murata, D. Ohta, J. Sekiya, and E. Takahashi, of the Kyoto University, published papers suggesting effects of sodium nutrition on nitrate assimilation and has established that sodium stimulates the regeneration of phospho*enol*pyruvate in mesophyll chloroplasts of *Amaranthus tricolor*. The second group, N. Aoki, R. Kanai, and J. Ohnishi at the Saitsama University, has demonstrated that sodium is required for the uptake of pyruvate into mesophyll chloroplasts in **some** C_4 plants. This latter group has recently published a most interesting paper in which they suggest that the members of the tribes, Andropogoneae and Arundinelleae which are NADP-ME type C_4 plants, may not require sodium as a nutrient. We are currently testing species of these tribes for their sodium requirement.

POSTGRADUATE THESES SUPERVISED BY THE AUTHOR

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CONTRIBUTION TO THE PUBLICATIONS CITED

It is difficult to provide an accurate assessment of my contribution to the publications cited. In the table below, my contribution has been estimated under two headings, "conceptual" and "practical". The conceptual component consists of ideas, initiatives, general strategies and supporting facilities whereas the practical component would include the specific planning of experiments and the execution of the processes involved.

Publication*	Contribution (%)	
	Conceptual	**Physical**
1	80	95
2	85	95
3	95	80
4	75	95
5	95	95
6	95	85
7	95	85
8	20	5
9	100	not applicable
10	95	80
11	20	5
12	40	20
13	95	50

*The pulications are listed on pp. 5-8.

14	40	35
15	75	60
16	90	10
17	50	30
18	90	50
19	40	20
20	40	40
21	90	20
22	90	40
23	70	80
24	10	20
25	95	85
26	100	not applicable

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SODIUM AS AN ESSENTIAL ELEMENT IN C_4 PLANTS

1. SUMMARY OF FINDINGS DESCRIBED IN THE PUBLICATIONS LISTED BELOW (pp 5-8)

1. Sodium was shown to be an essential micronutrient element for *Atriplex vesicaria* Heward ex Benth. This was the first report of sodium being essential for a higher plant (Publications 1 and 2).

2. Even with extremely careful exclusion of sodium from the plants' environment (Publication 9), it was found that of thirty species examined, only ten Australian species of *Atriplex* required sodium. Other species including non-endemic species of *Atriplex* and some halophytes, grew normally without the addition of salts of sodium to the cultures (Publication 5).

3. Following the discovery of the C_4 photosynthetic pathway (Hatch and Slack 1966), we were able to demonstrate a clear correlation between the presence of the C_4 pathway and the requirement for sodium as a micronutrient (Publications 6 and 26).

4. Significant responses to sodium by the Crassulacean acid metabolising (CAM) plant, *Bryophyllum tubiflorum*, were obtained under conditions which favoured the CAM photosynthetic pathway but not under conditions favouring the C_3 photosynthetic pathway (Publications 7 and 26).

5. A rapid increase in the respiration rate of leaves of sodium-deficient *Atriplex nummularia*, a C_4 species, was obtained following the application of sodium salts to the culture solution. The response was sodium-specific; no other group

1 element was effective (Publication 3).

6. The Cyanobacterium, *Anabaena cylindrica*, has a small but specific requirement for sodium (Allen and Arnon 1955) and the possibility was considered that the nutrient might have a similar function in the Cyanobacterium as in *Atriplex.* Cyanobacteria, because of their shorter generation times and ease of manipulation, have advantages over higher plants as experimental material in studies of metabolism. Nitrate reductase activity increased markedly in sodium-deficient cells resulting in a dramatic increase in nitrite to levels which were toxic to the cells. Sodium appeared to have a role in controlling nitrate reductase activity. $^{15}N_2$ incorporation into cell protein suggested that sodium is required for the reduction of N_2 to NH_3 (Publication 4).

7. The discovery that sodium was required by plants having C_4 but not C_3 photosynthesis suggested that sodium was needed for the operation of the C_4 component which transports CO_2 to the bundle sheath cells where it is reduced to carbohydrates. This was substantiated by the observation that sodium-deficient plants grown under low CO_2 concentrations (15µL CO_2 L^{-1}) had lower yields and accentuated deficiency symptoms but those grown under conditions of high CO_2 (1500 µL CO_2 L^{-1}), gave increased yields and no deficiency symptoms. On the other hand, the yields of plants receiving sodium were unaffected by the CO_2 concentration (Publications 10, 13, 15).

8. The growth of sodium-deficient C_4 plants was significantly reduced when grown under elevated oxygen concentrations. The growth of sodium-sufficient plants was unaffected by oxygen concentration (Publication 18). This indicated

that under elevated oxygen concentrations, increased amounts of oxygen diffused into the bundle sheath cells causing a reduced ratio of CO_2 /O_2 within these cells and decreasing, in turn, the operation of the photosynthetic carbon reduction cycle relative to that of the photosynthetic carbon oxidation cycle. The overall effect of this would be to increase photorespiration and thus reduce the rate of photosynthesis. This would account for the reduced growth of plants in high concentrations of O_2.

9. A marked accumulation of alanine was observed in leaves of sodium-deficient plants. It was suggested that alanine which is in equilibrium with pyruvate builds up as a result of a block in the conversion of pyruvate to phospho*enol*pyruvate (PEP) in the mesophyll chloroplasts in sodium-deficient plants (Publications 10 and 12). The pool sizes of the other key intermediates of the C_4 pathway provided further evidence for this lesion [Grof *et al.* (1986a) Publication 16; Johnston *et al.* (1988) Publication 21].

10. The major steps in the conversion of pyruvate to PEP involve the transport of pyruvate into the mesophyll chloroplast, its enzymatic conversion to PEP and the provision of energy required for the conversion. Sodium was without effect on the activity of pyruvate orthophosphate dikinase, the enzyme that catalyses the conversion of pyruvate to PEP (Boag 1981; Dorney 1985).

11. The light-harvesting system is the immediate source of energy for pyruvate transport and/or regeneration of PEP from pyruvate in the mesophyll chloroplasts. In sodium-deficient plants, there is evidence of damage to this system as indicated by lower chlorophyll *a/b* ratios (Publications 14, 15, 22) fluorescence yields (Publications 17 and 20) and photosystem II activity

(Publication 21). Furthermore, it has been established that the ultrastructure of mesophyll but not bundle sheath chloroplasts is altered in sodium-deficient plants of *Amaranthus tricolor* and *Kochia childsii* (Publication 19).

12. It was considered possible that photosynthesis in sodium-deficient C_4 plants might be limited by reduced activities of carbonic anhydrase. This enzyme catalyses the interconversion of CO_2 and HCO_3^-, the latter being the substrate for PEP carboxylase in C_4 and CAM plants.

    It was found that carbonic anhydrase was about twice as active in sodium-deficient plants as in control plants when expressed on a fresh weight or protein base and three times as active on a chlorophyll basis (Publication 25). It appears unlikely, therefore, that the activity of this enzyme could be a limiting factor in sodium-deficient plants.

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3. DISCOURSE ON THE PUBLISHED WORK

I. INTRODUCTION

When the work descibed in this thesis was initiated, the mineral nutrition of plants was a subject of intensive research. In the previous three decades, boron, copper, zinc, and molybdenum had been shown to be essential elements for higher plants. These discoveries were not only of great interest scientifically but impacted significantly on agriculture and horticulture. There were suggestions that sodium and chlorine, which had long been regarded as possible macronutrients, might also have a role in the nutrition of plants although their possible status as essential elements was unclear. Increases in dry weight had been reported following the application of salts of sodium to various higher plants growing in low potassium culture solutions (Richards, 1944). This suggested that the sodium could, in part, replace potassium in certain species. There were also suggestions in the literature that the dry weight of some plants, mostly members of the Chenopodiaceae, was higher, in solutions containing adequate potassium following the application of sodium. Harmer and Benne (1945) and Harmer *et al.* (1953) arranged species in groups depending on their responses to sodium with insufficient or sufficient supply of potassium. It was clear that a certain amount of potassium, not replaceable with sodium, was needed by all species. There was no evidence of sodium being an essential element. Its main role appeared to be to substitute for some of the potassium needed for maximum growth. Pfeffer (1899) suggested that sodium might be essential in very small amounts. Little or no attention was given to this possibility until the early 1950's when Professor J. G. Wood, Department of Botany, University of Adelaide, suggested that sodium and/or chlorine might be required by plants in micronutrient quantities. At that time, no

experiments had been reported in which these elements had been carefully excluded from the plants' environment. Initially, experiments to determine if sodium was an essential element for higher plants were carried out using *Banksia ornata* (broad leaf Banksia), *Avena sativa* (oats) var. "Mulga", and *Atriplex vesicaria* Heward ex Benth. (bladder salt bush). Difficulties were experienced in obtaining a uniform population of *Banksia ornata* seedlings and oats did not respond to sodium in the preliminary experiments.

Atriplex vesicaria was chosen as an experimental plant because it accumulates massive amounts (up to 23% on a dry weight basis) of sodium and chlorine in its leaves (Wood 1925). Furthermore, it possesses bundle sheath leaf anatomy (Wood 1923), now known to be a feature of C_4 plants but considered then to play a role in the ability of the plant to withstand arid environments. To test the hypothesis that Na and Cl might be needed by *Atriplex vesicaria* in micronutrient quantities, a factorial experiment with the following design was conducted:-

Salt addition	x	**Equivalent concentrations** (m. equiv. L^{-1})	x	**Harvests**	x	**Replications**	= 128
NaCl		0.00		2		4	
Na_2SO_4 + KCl		0.35					
Na_2SO_4		0.74					
KCl		6.94					

Ten plants were grown in 4.5-L culture solution. Sodium was partially removed from the solution by recrystallization from several salts of the culture solution. At each harvest, the dry weight production of the plant material was determined. Two important findings arose from this preliminary experiment (Fig. 1). Firstly, the

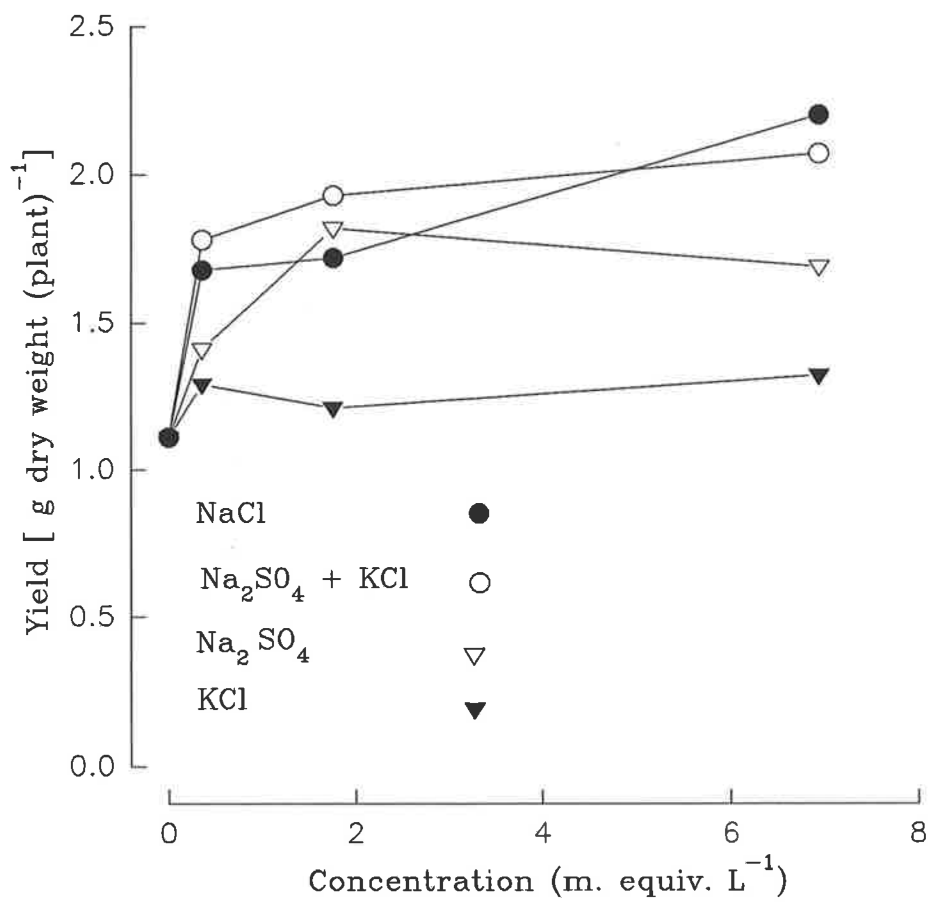


Fig.1. Dry weight yields of *Atriplex vesicaria* (Bladder salt bush) supplied with NaCl, Na_2SO_4 + KCl, Na_2SO_4, or KCl.

dry-weight yield increased significantly, when either NaCl or Na_2SO_4 plus KCl were supplied. Furthermore, the yields from these salt treatments did not differ, significantly, indicating that the dry weight yields were unaffected by the associated ions, SO_4^{2-} and K^+ in the Na_2SO_4 plus KCl treatment. The supply of Na_2SO_4 alone also significantly increased the dry weight yield. It, therefore, seemed likely that sodium was having an independent role as a plant nutrient. Secondly, in this experiment, carried out in a conventional glasshouse, the amounts of sodium and chlorine determined in the plant material and the remaining culture solution were several times greater than that originally present in the culture solution salts, water, and seeds. This suggested that sodium and chlorine had been contributed to the plants by the atmosphere, possibly via dust or in the case of chlorine as a gas (Publication 2, Fig. 1).

At about this time, two important discoveries were made in other laboratories. Broyer *et al.* (1954) at Berkeley University of California, in an attempt to define the status of cobalt as an essential element for tomatoes, went to great lengths to achieve a cobalt-free environment. Their plants developed severe deficiency symptoms which were prevented by the addition of cobalt chloride. However, further work showed that the symptoms were due to chlorine and not cobalt deficiency. Soon after, Allen and Arnon (1955), also at Berkeley University, demonstrated a specific requirement for small amounts of sodium in the Cyanobacterium, *Anabaena cylindrica.* This was the first report of sodium being required for plant growth. The results of the preliminary experiment and the findings of Allen and Arnon (1955) suggested that sodium might also be essential for higher plants. We, therefore, took great care remove sodium from

II. DISCUSSION OF THE MAJOR FINDINGS

Section 1: The growth of plants under low sodium concentrations

It is not possible to specify an exact concentration of sodium at which growth is limited in plants for which it is essential. However, such plants including certain Cyanobacteria, C_4 and CAM plants, appear to require less than about 217 mmol m^{-3} (5.00 ppm) of sodium for optimal growth. In order to obtain decisive visual signs of sodium-deficiency in these plants, it is therefore necessary to eliminate sodium from the culture solution salts (Publication 9, p. 127).

Early in the project, it became necessary to determine sodium at very low concentrations in the presence of high concentrations of interfering ions. Initially, a flame photometer was found to be satisfactory for the estimation of sodium in water, plant material and salts of the culture solution. However, with progressive purification of the salts, interference from other ions became too great to obtain reliable data. At this time, the atomic absorption instrument had just been invented by Sir Alan Walsh, CSIRO. He brought an atomic absorption photometer to our laboratories which enabled sodium concentrations to be determined relatively free from interferences. A critical assessment of the methods used to estimate sodium in water, culture solution salts and plant material is described (Publication 9, pp. 127-32).

Due to the ubiquity of sodium in the environment, its exclusion from experimental areas is difficult and proof of its essentiality in plants necessitated rigorous experimental techniques. The development of a sodium-free environment for plant growth required the double distillation of water, the final stage in a silica still, to

achieve a sodium concentration of less than 0.009 mmol m^{-3} (0.0002 ppm). To obtain sodium-free salts for preparing culture solutions, analytical reagent salts were recrystallised up to six times in silica or platinum vessels. Some components of the culture solution were prepared from reagents distilled in silica. For example, ammonium chloride was made from silica-distilled ammonia and hydrochloric acid. While it is impossible to totally eliminate sodium, the final concentration of sodium in the nutrient solution was less than 0.07 mmol m^{-3} (0.0016 ppm) this is approximately one-hundredth of the concentration of sodium which would have been supplied from untreated analytical reagent salts. It was established that dust which circulates in untreated air is contaminated with sodium, hence plants were grown in an atmosphere filtered to remove sodium-containing dust. These aspects of the work are described more fully in Publications 2, and 9, pp. 133-44.

Section 2: The demonstration of the essentiality of sodium for *Atriplex vesicaria* Heward ex Benth.

When plants of *Atriplex vesicaria* were grown under sodium-free conditions, they showed greatly reduced growth, chlorosis, and necrosis of leaves. In some cases, yield of sodium-treated plants was over twenty-times that of untreated plants at 45 days from germination. The response was specific to sodium as no other group 1 elements were effective. These experiments are described in Publications 1, and 2.

Section 3: Species requiring sodium as a micronutrient

Two major questions arose from the demonstration of the essentiality of sodium for *Atriplex vesicaria:* do other higher plants require sodium as a micronutrient element

and what is the function of sodium in *Atriplex vesicaria*? We were puzzled when we attempted to answer the question of whether or not there is a general requirement by higher plants for sodium. From the pattern observed with other essential elements, it was expected that sodium would be required by all higher plants. Surprisingly, of the thirty species examined, including halophytes, other Chenopods and other nonendemic species of *Atriplex,* only the ten Australian species of *Atriplex* were found to require sodium (Publication 5). At this time, these differences in response could not be correlated with any other obvious differences between the species studied. It still seemed possible that all higher plants might require sodium but that those plants which grow normally without added sodium might require only very small amounts compared with the Australian *Atriplex* species. However, soon after the discovery of the C_4 photosynthetic pathway by Hatch and Slack (1966), it seemed likely that only plants having the C_4 pathway had a requirement for sodium. We tested this possibility with C_4 plants from different families and found they all responded to small amounts of sodium as did the Australian species of *Atriplex* which were all C_4 plants (Publications 6, and 26). This discovery was significant because it gave a clue to the possible function of sodium in these plants. It suggested that sodium is required for the operation of the C_4 mechanism in transporting CO_2 to the bundle sheath cells where it is reduced to carbohydrates.

Due to the similarity in their photosynthetic pathways, it seemed likely that plants having Crassulacean acid metabolism (CAM) might also require sodium for their growth. When experiments with *Bryophyllum tubiflorum* were carried out under conditions which favoured the CAM option, their growth was substantially increased

in response to small additions of sodium. Plants growing under conditions favouring the operation of the C_3 pathway did not respond (Publications 7, and 26; Boag 1976). In a later experiment, plants of *B. tubiflorum* grown in untreated air did not respond to sodium but when grown in CO_2-free air in the light and untreated air in the dark responded to sodium treatment. Under the latter conditions, the plants could only photosynthesise by using the CAM system (Brownell, unpublished data).

Section 4: The function of sodium in plants for which it is essential

The functions in plants of the essential transition elements including iron, zinc, manganese, and molybdenum which can take part in oxidation/reduction reactions have generally been discovered incidentally during other metabolic studies. For example, from research on photosystems, a function for copper became obvious when it was shown to be a component of plastocyanin. Similarly, a function for molybdenum became apparent from studies of nitrogen metabolism when this element was found to be a component of the nitrate reductase and nitrogenase enzymes. Our knowledge of the function of the other essential elements, boron, chlorine and sodium has lagged behind as no direct effects have been observed on enzyme systems.

The almost complete lack of information on the involvement of sodium at low concentrations in any biological system made it difficult to find effective approaches to defining a role for sodium in those plants for which it was essential. One approach has been to look for an early response to sodium in sodium-deficient plants preceding the more obvious signs of recovery like the greening of chlorotic leaves or increased growth. A metabolic response to sodium detected near the beginning of this period,

it was argued, may represent a primary step in the recovery and hence give a clue as to the function of sodium. The cascade of measurable responses which follow in the longer term ranging from changes in soluble and insoluble nitrogen fractions and increases in carbohydrate content are probably indirect effects of the sodium treatment. An early response detected in the C_4 species of *Atriplex* was an increased rate of respiration which occurred within a few hours of applying sodium whereas dry weight increases were observed only after six days (Publication 3).

A further approach was to study metabolic responses to sodium in *Anabaena cylindrica* (Publication 4). This Cyanobacterium has a specific requirement for sodium and it seemed likely that the function of sodium in *Anabaena* might be similar to its function in *Atriplex.* The alga has the advantages over higher plants for metabolic studies in having short generation time, and being easy to sample and manipulate in metabolic experiments. Our finding of greatly increased nitrate reductase activity in sodium-deficient *Anabaena* could not be shown in sodium-deficient *Atriplex* suggesting that the organisms required sodium for different functions.

Following the discovery of the correlation between the possession of the C_4 pathway and the requirement for sodium (Publication 6) and the link between sodium requirement and the CAM mechanism (Publication 7) , it appeared likely that sodium might play a role within the C_4 dicarboxylic system common to C_4 and CAM plants. It seemed possible that further information on the role of sodium in C_4 plants plants could be obtained by studying the effects of sodium on C_3/C_4 species hybrids. Although attempts to breed the hybrids were successful (Publication 24), the small

numbers of F_1 and F_2 individuals obtained made experimental work on interspecific hybrids impractical.

Section 5: The effect of CO_2 and O_2 concentrations on the growth of sodium-deficient plants.

Effect of carbon dioxide concentration on growth

The discovery of the requirement for sodium by plants having C_4 but not C_3 photosynthesis suggested that sodium was required for the operation of the C_4 appendage in transporting CO_2 to the bundle sheath cells where it is reduced to carbohydrate. In support of this, we found that the signs of sodium-deficiency were alleviated in plants grown in atmospheres with elevated CO_2 concentrations. In experiments in which the C_4 plant, *Amaranthus tricolor*, was grown under conditions of low concentrations of CO_2 (15 µL CO_2 L^{-1}), signs of deficiency were accentuated. The sodium-deficient plants resembled the C_3 species, *Atriplex hastata* and tomato, in their response to CO_2 treatments. Growth and chlorophyll concentrations increased in both sodium-deficient C_4 and C_3 plants when they were grown in conditions of increased CO_2 concentrations. It is suggested that in sodium-deficiency, CO_2 transport to the bundle sheath cells is decreased, thus limiting the rate of photosynthesis. When the atmospheric concentration of CO_2 in which the plants are grown is elevated to about 1,500 µL CO_2 L^{-1}, the CO_2 enters the bundle sheath cells by diffusion (Publications 10 and 13). This is shown schematically in Fig. 2a, 2b, 2c.

Increasing ambient CO_2 concentration to 6,000 µL CO_2 L^{-1} alleviated but did not

completely overcome the symptoms of sodium-deficiency in plants of *Amaranthus tricolor*. Thus, the chlorophyll *a/b* ratios of sodium-deficient plants which were significantly lower than those of sodium-sufficient plants, were not significantly affected by ambient CO_2 concentration, indicating that increased CO_2 concentration alleviated the signs of sodium-deficiency by by-passing the sodium-requiring CO_2 transporting process (Publications 14 and 15).

Effects of oxygen concentration on growth

The yield of sodium-deficient C_4 plants decreased significantly with increased oxygen concentrations, whereas the control plants showed no response (Publication 18). These data suggest that sodium-deficient plants are more sensitive to oxygen concentrations than control plants. The increased ambient oxygen concentration may lead to an increased concentration of oxygen within the bundle sheath. In sodium-deficient plants where the C_4 cycle may be impaired, the CO_2 concentration would be reduced and the consequent increase in the O_2/CO_2 ratio in the bundle sheath could lead to increased activity of the photosynthetic carbon oxidation (PCO) cycle. The observed significant reduction in yield in sodium-deficient plants with increased ambient oxygen concentration supports this hypothesis. This is shown schematically Fig. 3a, b, c, d.

Fig. 2. Scheme illustrating the effect of different ambient CO_2 concentration on sodium-deficient and control plants.

a) Control plant grown in CO_2 concentration equal to 15 or 30 $\mu L\ CO_2\ L^{-1}$.

b) Sodium-deficient plant grown in atmospheric CO_2 concentration.

c) Sodium-deficient plant grown in elevated ambient CO_2 concentrations.

Broken lines indicate low flow rate.

from Grof (1988)

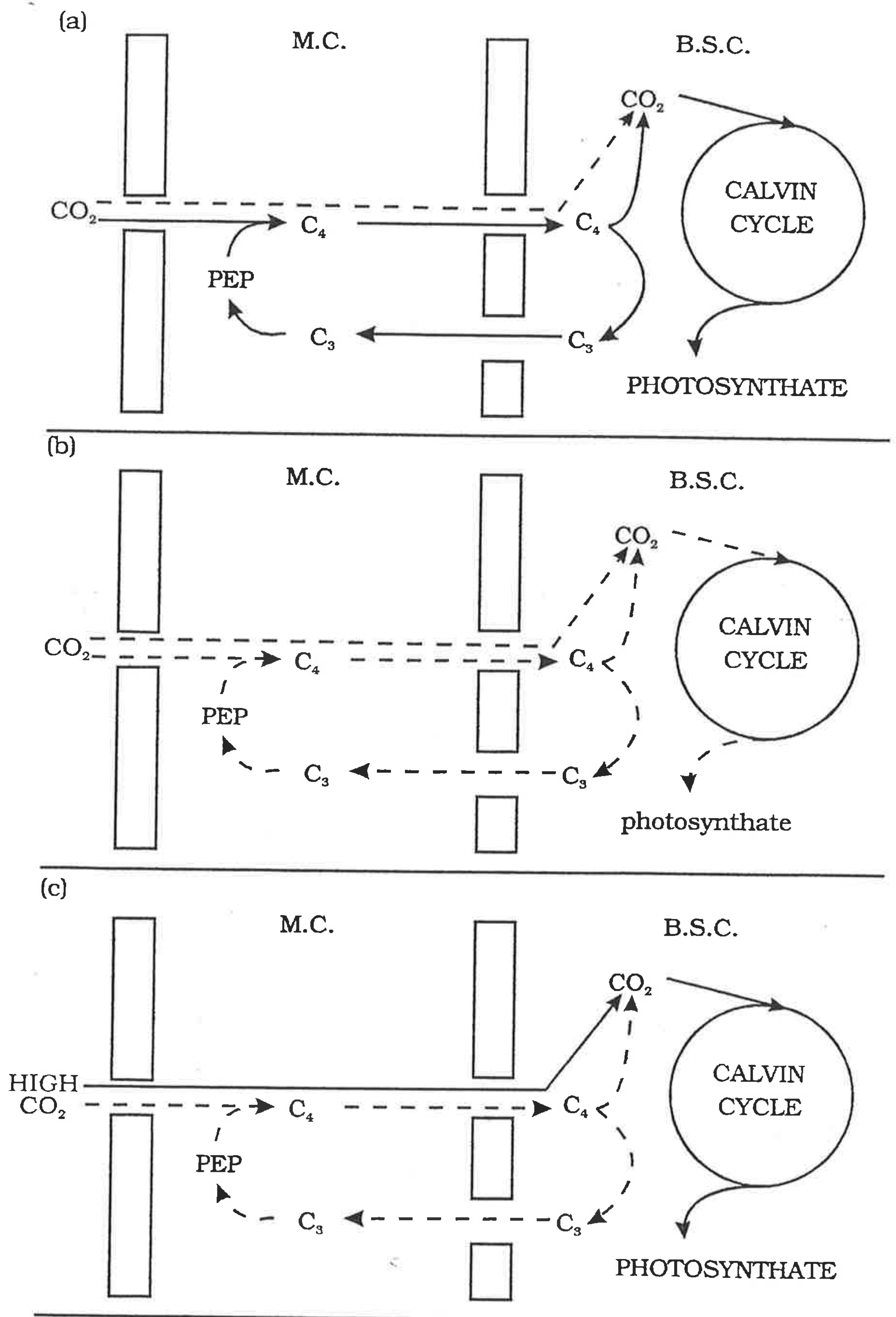

(a)
M.C.
B.S.C.
CO2
CO2
C4
C4
PEP
C3
C3
CALVIN CYCLE
PHOTOSYNTHATE
(b)
M.C.
B.S.C.
CO2
CO2
C4
C4
PEP
C3
C3
CALVIN CYCLE
photosynthate
(c)
M.C.
B.S.C.
CO2
HIGH CO2
C4
C4
PEP
C3
C3
CALVIN CYCLE
PHOTOSYNTHATE

Fig. 3. Scheme illustrating the effect of different O_2 concentrations on sodium-deficient and control plants.

a) Control plants grown in 21% O_2.

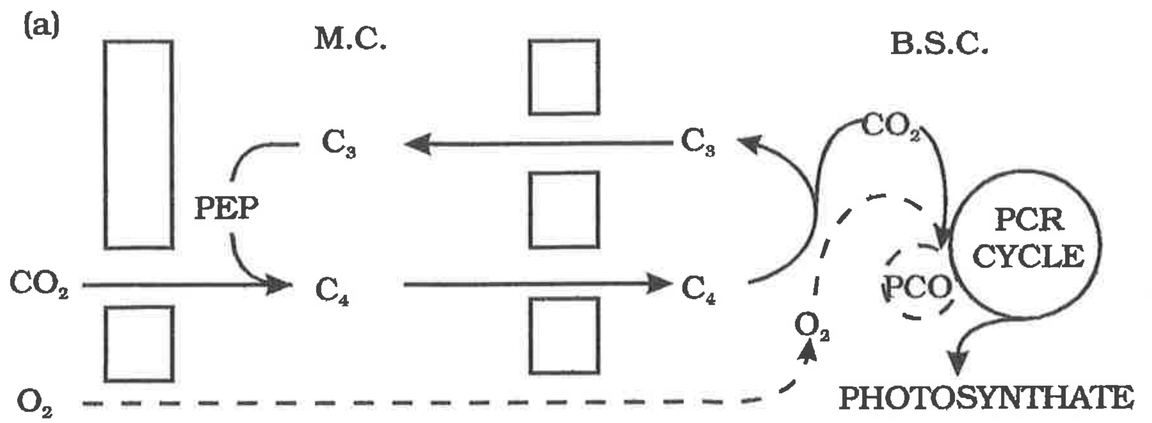
b) Sodium-deficient plants grown in 21% O_2.

c) Control plants grown in 42% O_2.

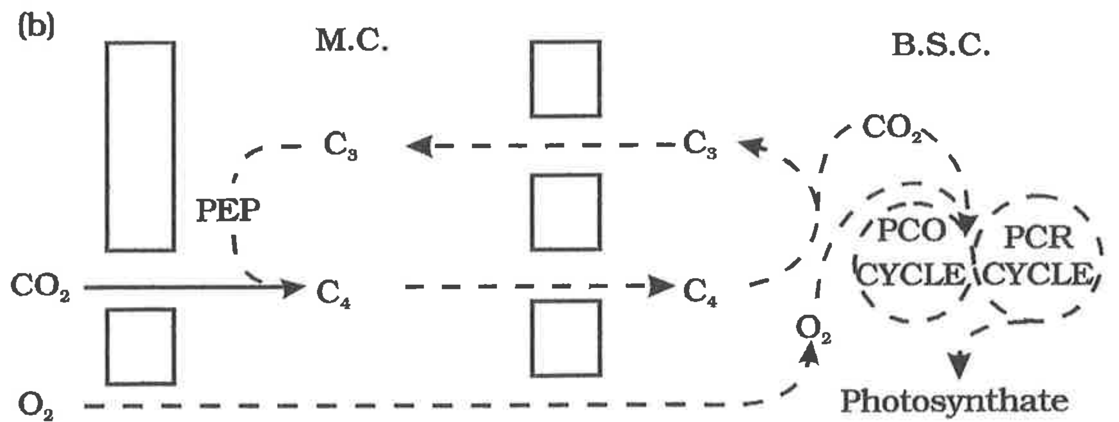
d) Sodium-deficient plants grown in 42% O_2.

Broken lines indicate low flow rate.

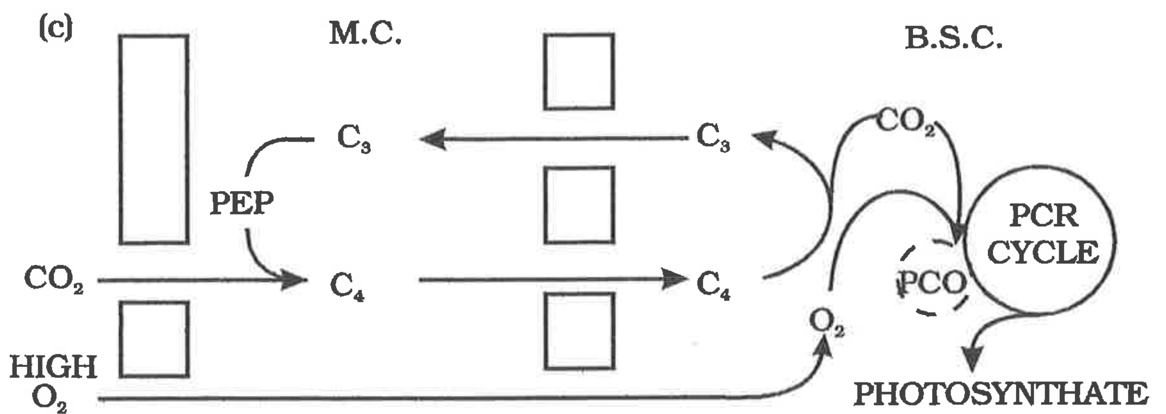
from Grof (1988)



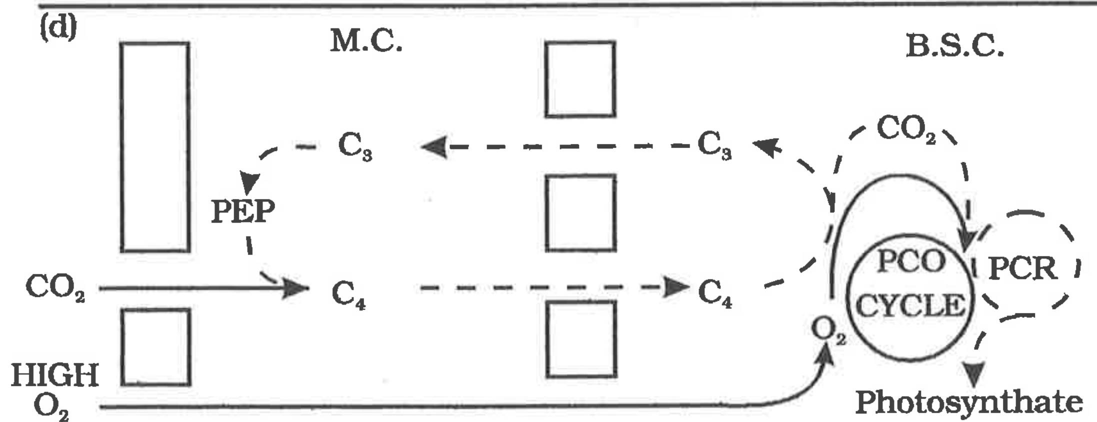
(a)
M.C.
B.S.C.
C3
C3
CO2
PEP
PCR
CYCLE
CO2
C4
C4
PCO
O2
O2
PHOTOSYNTHATE



(b)
M.C.
B.S.C.
C3
C3
CO2
PEP
PCO
CYCLE
PCR
CYCLE
CO2
C4
C4
O2
O2
Photosynthate



(c)
M.C.
B.S.C.
C3
C3
CO2
PEP
PCR
CYCLE
CO2
C4
C4
PCO
O2
HIGH
O2
PHOTOSYNTHATE



(d)
M.C.
B.S.C.
C3
C3
CO2
PEP
CO2
C4
C4
PCO
CYCLE
PCR
O2
HIGH
O2
Photosynthate

The finding of generally increased levels of amino acid intermediates of the photorespiratory pathway, glycine and serine, in sodium-deficient plants and the accumulation of ^{14}C label in these compounds reported by Boag (1981) also supports the hypothesis outlined above An alternative explanation requires that the C_4 pathway in both sodium-deficient and control plants functions at a similar rate. Under normal conditions, the concentration of the intermediate pools of CO_2 plus HCO_3^- range from 0.6 mM to 2 mM in the bundle sheath cells of C_4 plants (Hatch 1976; Hatch and Osmond 1976) creating a concentration gradient between bundle sheath and mesophyll cells. The reduction in yield observed in sodium-deficient plants could be attributed to a possible increased leakiness of the bundle sheath to both CO_2 and O_2 thus negating the CO_2 concentrating effect of the C_4 appendage. If this were the case, the CO_2 and O_2 concentrations would be predominantly governed by the ambient gas concentration. However, Boag (1981) found that the quantum yield in both sodium-deficient and control plants of *Amaranthus edulis* and *Digitaria sanguinalis* were similar. This would not be so if the bundle sheath were leakier in sodium-deficient C_4 plants, as additional energy would be required to carry out the CO_2 concentrating process in the bundle sheath cells.

Boag (1981) found the long-term exposure to supra-atmospheric O_2 concentrations led to a decrease in the rate of CO_2 assimilation in both sodium-deficient and control plants of *Chloris barbata*. Other work indicates that the response of plants to supra-atmospheric O_2 concentration and the effect on C_4 photosynthesis depends upon the species and the length of exposure (Forrester *et al.* 1966a; 1966b; Gale

and Tako 1976; Ku and Edwards 1980). Boag (1981) examined CO_2 assimilation rates in plants of *Echinochloa crus-galli, Chloris barbata,* and *Eleusine indica.* He observed no significant change in photosynthesis in either sodium-deficient or control plants when they were exposed to 2%, 21% or 50% O_2. However, the exposure period was only 45 minutes. Unlike C_3 plants, which may equilibrate rapidly, C_4 plants may take hours to reach steady state photosynthesis (Gale and Tako 1976; Ku and Edwards 1980). The conclusion drawn by by Boag (1981) that C_4 pathway metabolism effectively suppresses photorespiration in sodium-deficient plants may only apply in short-term observations.

Section 6: The requirement for sodium for the conversion of pyruvate to phosho*enol*pyruvate (PEP)

A marked accumulation of alanine was observed in sodium-deficient C_4 plants (Publication 12). This suggested that alanine which is in equilibrium with pyruvate, builds up as a result of the block in the conversion of pyruvate to phospho*enol*pyruvate (PEP) in mesophyll cells of these plants. This is schematically shown in Fig. 4. The pool sizes of the other key C_4 intermediates provided further evidence for this block (Publications 16 and 21).

Consistently lower chlorophyll *a/b* ratios were found in the leaves of sodium-deficient compared to normal C_4 plants (Publication 14). Previously, it had been observed that the chlorophyll *a/b* ratio in the C_4 plants, *Kochia childsii* and *Chloris barbata*, was unaffected by sodium nutrition (Publications 8, and 11). This apparent inconsistency was later found to be due to differences in the techniques used in the chlorophyll determination (Publication 14).

Fig. 4. Proposed scheme to illustrate possible location of lesion caused by sodium-deficiency in C_4 plants.

a) Control plant.

b) Sodium-deficient plant.

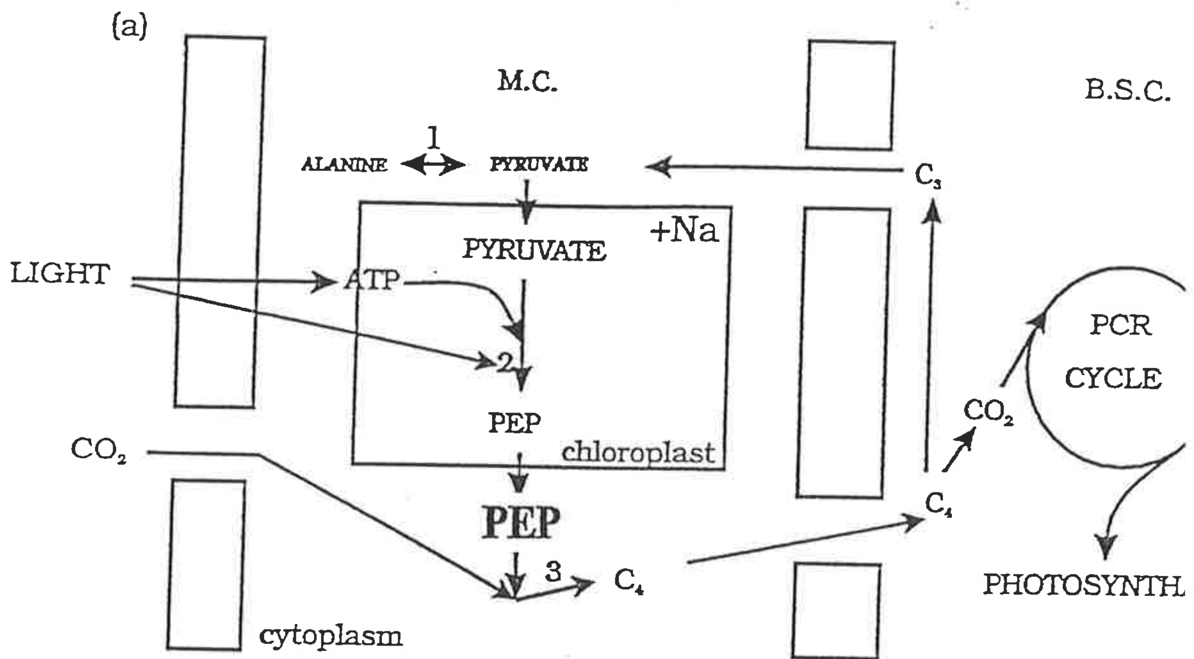
1. Alanine aminotransferase.

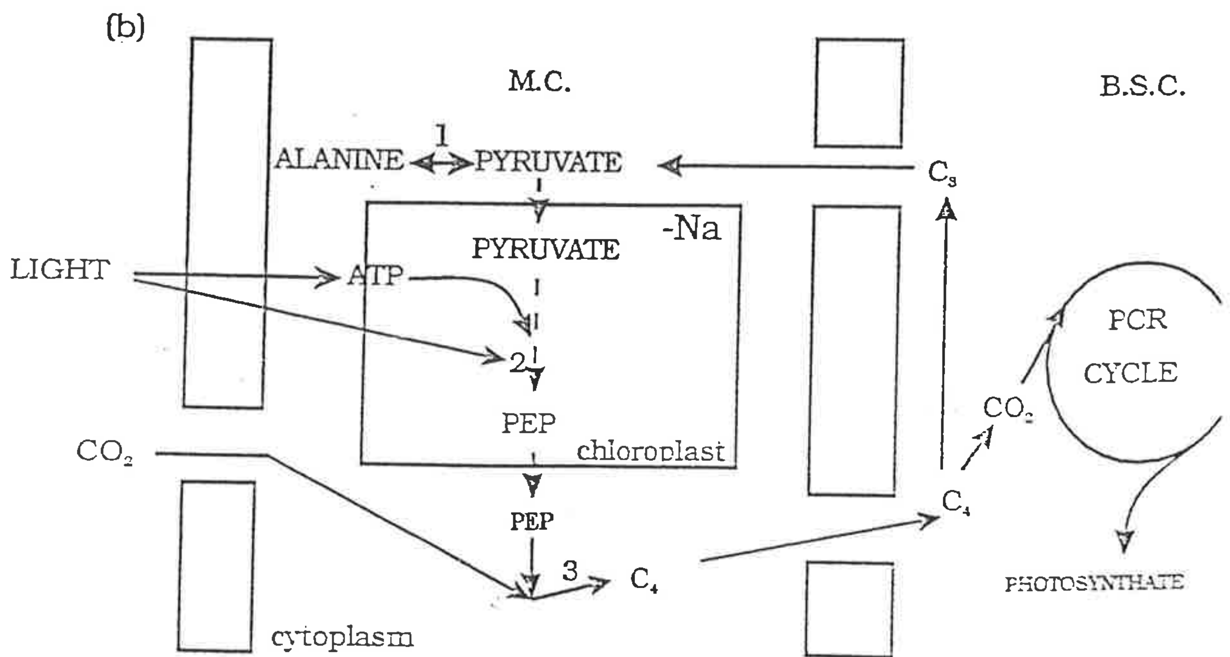
2. Pyruvate orthophosphate dikinase.

3. Phospho*enol*pyruvate carboxylase.

Broken lines indicate low flow rate.

from Grof (1988)


(a)
M.C.
B.S.C.
1
ALANINE
PYRUVATE
C_3
PYRUVATE
+Na
LIGHT
ATP
2
PCR
CYCLE
CO_2
PEP
chloroplast
CO_2
PEP
C_4
3
C_4
PHOTOSYNTH
cytoplasm


(b)
M.C.
B.S.C.
1
ALANINE
PYRUVATE
C_3
PYRUVATE
-Na
LIGHT
ATP
2
PCR
CYCLE
CO_2
PEP
chloroplast
CO_2
PEP
C_4
3
C_4
PHOTOSYNTHATE
cytoplasm

The lower chlorophyll *a/b* ratio was shown to be localised in the mesophyll chloroplasts (Publication 22). This suggested far reaching implications in terms of energy production in the mesophyll chloroplast. The lower chlorophyll *a/b* ratio suggests less cyclic compared to non-cyclic electron flow and a potentially lower ratio of ATP/NADPH production in sodium-deficient compared to normal plants. This decrease in production of ATP could restrict the conversion of pyruvate to PEP as this is an energy requiring reaction.

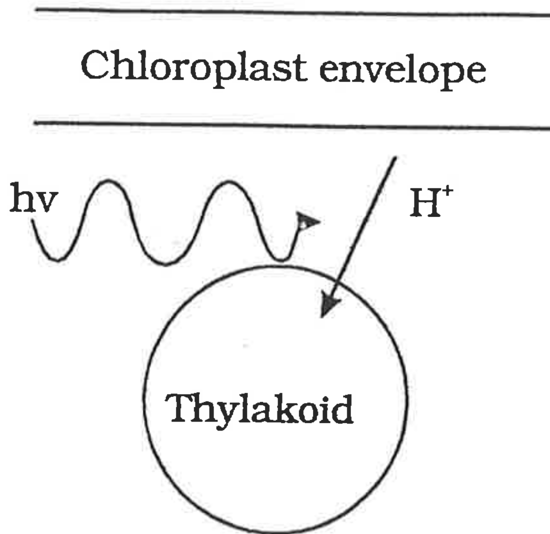
Section 7: The involvement of sodium in the regeneration of phospho*enol*pyruvate from pyruvate in the mesophyll chloroplast

The major steps in the conversion of pyruvate to PEP involve the transport of pyruvate into the mesophyll chloroplast, its enzymatic conversion to PEP and the provision of energy required for its transport and/or conversion. No effect of sodium on the *in vitro* activity of pyruvate orthophosphate dikinase, the enzyme that catalyses the conversion, has been observed (Boag 1981; Dorney 1985). It is still possible that sodium could affect the *in vivo* activity of the enzyme but there is no evidence for this. There is convincing evidence that the transport of pyruvate into the chloroplast is sodium-dependent (Ohnishi and Kanai 1987; Ohnishi *et al,* 1990). The exceptions to this are members of the Tribe, Andropogoneae, in which the transport mechanism may be proton- rather than sodium-dependent (Aoki *et al,* 1992). This is shown schematically in Fig. 5. Sodium may be needed for the maintenance of the energy supplying system required to drive the reaction in which PEP is produced

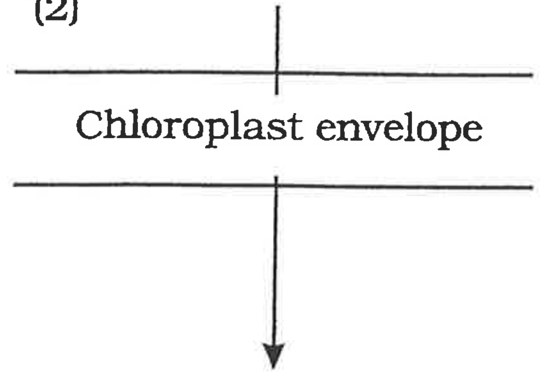
Fig. 5. The possible involvement of sodium in the transport of pyruvate into the mesophyll chloroplast envelope as proposed by Ohnishi and Kanai (1987).

from Albertson (1988)

(1)

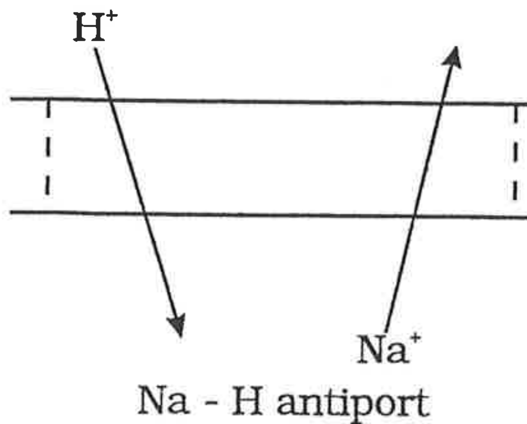


Light drives protons from the stroma into the Thylakoid lumen.



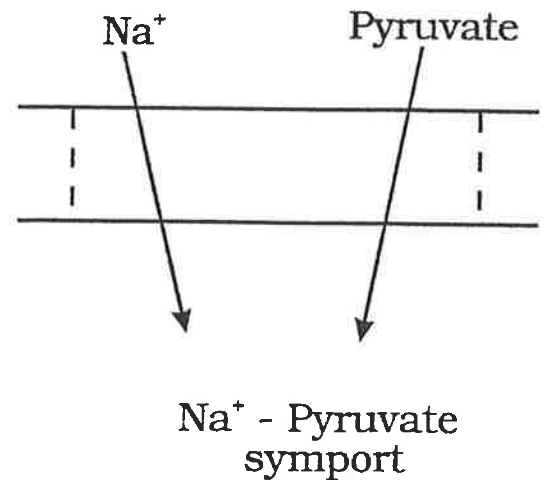
As a result, the stroma becomes more alkaline; a proton gradient is established across the envelope.

(3)



Through an antiport protons enter exchanging for sodium ions.

(4)



Sodium ions now re-ente accompanied by pyruvat ions.

from pyruvate and/or the active transport of pyruvate into the chloroplast. If sodium is deficicient there is evidence of damage to this system such as decreased chlorophyll *a/b* ratios (Publications 14 and 22), decreased fluorescence ratios (Publications 17 and 20) and decreased Photosystem II activity (Publication 22). There are also changes to the ultrastructure, particularly manifested by decreased thylakoid stacking in the mesophyll but not the bundle sheath chloroplasts (Publication 19). Sodium may be essential to maintain the structural and functional integrity of the thylakoids of mesophyll chloroplasts. It is difficult to determine whether these changes are a direct consequence of sodium-deficiency or are a result of damage to the thylakoid system from the excess energy normally consumed in the conversion of pyruvate to PEP.

Section 8: The role of sodium in the nutrition of Cyanobacteria.

Sodium appears to affect assimilation of nitrogen and carbon in Cyanobacteria. . Nitrate reductase activity has been shown to markedly increase in sodium-deficient cells of *Anabaena cylindrica* (Publication 4; Cole 1983) resulting in more rapid nitrate utilisation and build up of nitrite in the cells and in the medium. Sodium appears to have a regulating effect on this enzyme. Decreased rates of acetylene reduction by cells grown under sodium-deficient conditions have been observed by Ward and Wetzel (1975) and Smith (1977). $^{15}N_2$ incorporation into cell protein was found to be reduced in sodium-deficient cells (Publication 4). These data suggest that sodium is required for reduction of N_2 to NH_3.

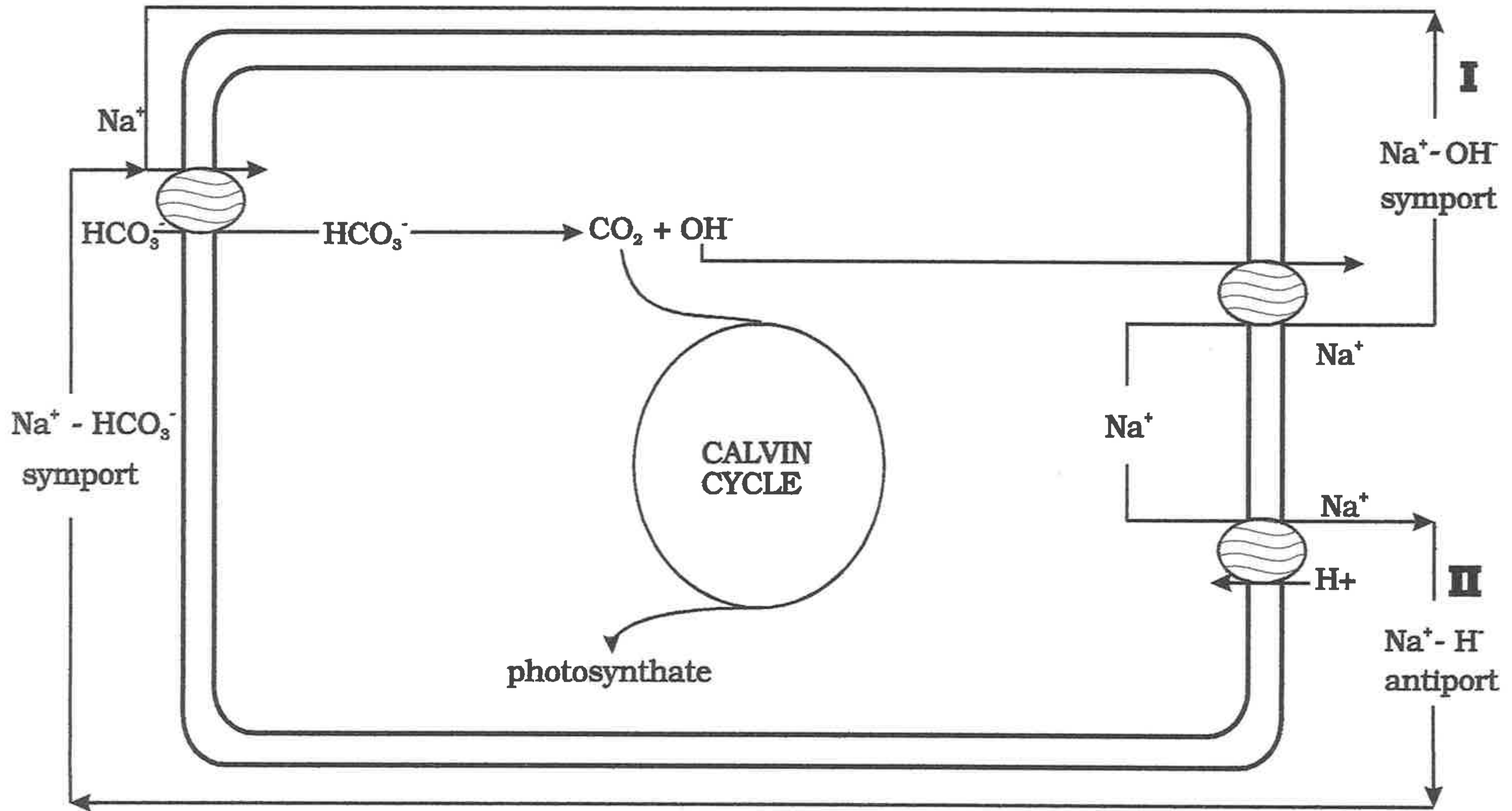
Carbon assimilation measured as ^{14}C was found to be 65% more rapid in cells of *Anabaena cylindrica* supplied with 2.2 mM (50.6 μg mL^{-1}) Na as compared to

those grown in low sodium concentrations (0.22 mM; 5.06 μg mL^{-1}). The excretion of carbon has also been found to increase in low-sodium cultures (Ward and Wetzel 1975; Smith 1977).

Sodium-deficiency decreases the efficiency of carbon utilisation. The photosynthetic O_2 evolution of *Anabaena variabilis* cells in high pH solutions (> 9.0) increased markedly upon the addition of NaCl. Other chloride salts, viz KCl and $MgCl_2$ did not increase O_2 evolution, whereas Na_2SO_4 produced the same response as NaCl (Reinhold *et al.* 1984; Faithful 1985). These authors concluded that sodium strongly promoted HCO_3^- transport in *Anabaena variabilis* and suggested that the transport is a Na^+/HCO_3^- symport or alternatively a Na^+/H^+ antiport (or Na^+/OH^- symport). The efflux of OH^- ions would result from exchange with in-coming HCO_3^- ions and could be limited by the Na^+ regulated antiport (Reinhold et al. 1984). This is shown schematically in Fig. 6.

Fig. 6. Scheme showing the possible pathways in which sodium is involved in the transport of HCO_3^- in *Anabaena cylindrica* according to Reinhold *et al.* (1984). HCO_3^- ions are shown to accompany Na^+ ions entering the cell via a symport. The Na^+ ions are circulated from within the cell. They leave the cell accompanied by OH^- ions through a symport or via an antiport in exchange for H^+ ions.

from Albertson (1988)



Na+
HCO3-
HCO3-
CO2 + OH-
CALVIN
CYCLE
photosynthate
Na+ - HCO3-
symport
I
Na+- OH-
symport
Na+
Na+
Na+
H+
II
Na+- H-
antiport

Section 9: Overview

The publications submitted in this thesis provide clear evidence for sodium being an essential micronutrient element for *Atriplex vesicaria* and other Australian *Atriplex* species. A strong correlation between the requirement for sodium and the possession of the C_4 pathway has been established although it is possible that species of the Tribes Andropogoneae and Arundinelleae may not have a sodium requirement even though they are C_4 NADP-ME plants.

Sodium has been shown to be involved in the transport of CO_2 to the bundle sheath cells where it is reduced to carbohydrates by the photosynthetic carbon reduction cycle. The sodium-deficiency symptoms of C_4 plants were intensified and their growth reduced when grown under atmospheres of low CO_2 concentration. On the other hand, in elevated concentrations of CO_2, growth was increased and the symptoms of sodium-deficiency were alleviated. It is postulated that under high CO_2 conditions, the CO_2 reaches the bundle sheath cells because of increased rates of diffusion thus by-passing the C_4 system (Publications 13 and 15).

It now appears that under sodium-deficiency, the growth of plants is limited by a decrease in the rate of supply of CO_2 to the bundle sheath cells. Of the factors that may limit the supply of CO_2 to the bundle sheath cells, stomatal conductance is unlikely to be of prime importance. Boag (1981), working with species representing the three types of C_4 plants (Guterriez *et al.* 1974; Hatch *et al.* 1975) showed stomatal physiology and function to be unaffected by sodium

nutrition. He concluded that the internal concentration of CO_2 would be unlikely to limit photosynthesis at saturating irradiances. It also appeared possible that photosynthesis in sodium-deficient plants might be limited because of reduced activities of carbonic anhydrase in their leaves. This enzyme catalyses the interconversion of CO_2 and HCO_3^-, the latter being the substrate of PEP carboxylase in C_4 and CAM plants (Hatch and Osmond 1976). On the basis of observations on representatives of each of the types of C_4 plants (Guterriez *et al.* 1974; Hatch *et al.* 1975), Hatch and Burnell (1990) concluded that the activity of the enzyme was only just sufficient to prevent the rate of conversion of CO_2 to HCO_3^- from limiting C_4 photosynthesis. Carbonic anhydrase is apparently restricted to the mesophyll cells of C_4 plants with the bundle sheath containing little or none of the enzyme (Burnell and Hatch 1988). However, carbonic anhydrase was found to be about twice as active in sodium-deficient as in control plants when expressed on fresh weight or protein bases and three times as active on a chlorophyll basis (Publication 25). It appears unlikely, therefore, that the activity of this enzyme is a limiting factor in sodium-deficient plants. These findings are consistent with the hypothesis of Nable and Brownell (1984) [Publication 12] that the regeneration of the primary CO_2 acceptor, PEP, in the mesophyll chloroplasts limits photosynthesis in sodium-deficient C_4 plants (Fig. 5). High concentrations of alanine and pyruvate and low concentrations of PEP, malate, and aspartate in the leaves of sodium-deficient compared to normal plants provide evidence for this block in the conversion of pyruvate to PEP (Publications 12, 16, 21).

Further evidence for this block has been obtained in experiments using illuminated intact mesophyll chloroplasts of *Amaranthus tricolor* (Murata *et al.* 1992). It was shown that the rate of conversion of pyruvate to PEP was greater when sodium was added to the chloroplasts. Recently, we have found that the rate of PEP regeneration from pyruvate is greater in intact chloroplasts isolated from sodium-fed than those isolated from sodium-deficient plants of *Kochia childsii*. In addition, if these chloroplasts were washed several times in sodium-free resuspension medium, markedly increased rates of PEP formation from pyruvate were obtained on the addition of sodium salts to the medium (Fig. 7). It is concluded that the chloroplasts from sodium-deficient plants may have certain defects resulting in poorer rates of PEP regeneration. The mechanism by which sodium restores the rates of PEP regeneration when added to the isolation medium during the chloroplast preparation is not clear. Sodium ions may preserve the functional integrity of the chloroplasts and/or they may be involved in the transport of pyruvate into the chloroplasts.

From our research, we have established that sodium is involved in the process in which pyruvate is converted to PEP in the mesophyll chloroplast of C_4 plants. This finding has been confirmed by workers in other laboratories. We now need to determine the specific action of sodium in this process. The major steps in the process in which sodium may be involved are:-

1) The transport of pyruvate into the mesophyll chloroplast: There is evidence for the transport of pyruvate into the mesophyll chloroplast being sodium-dependent except in members of Andropogoneae and

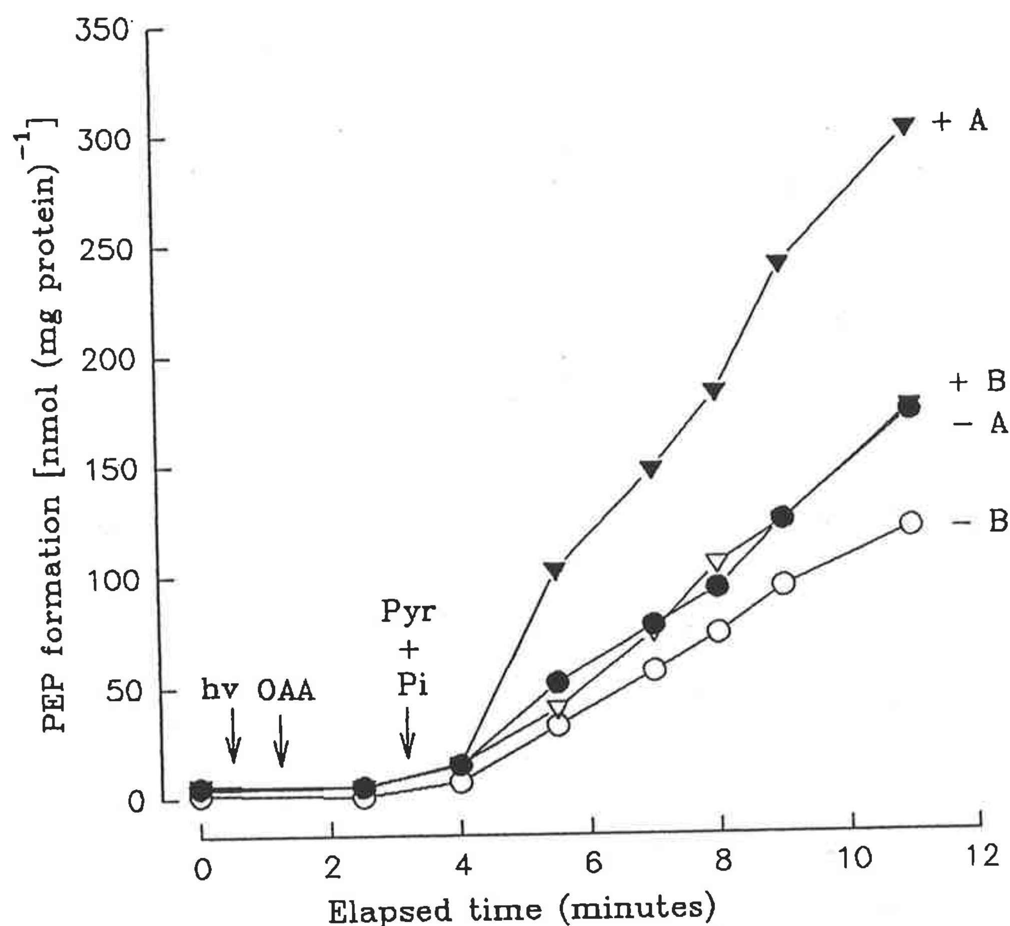


Fig. 7. Phospho*enol*pyruvate formation by mesophyll chloroplasts from sodium-deficient and normal plants of *Kochia childsii*. The following treatments were applied at times indicated in the graph:-

hv	Light 'on' at photon irradiance of approximately 400 µmol m^{-2} s^{-1}
OAA	1.0 mM oxaloacetate added
Pyr + Pi	20 mM pyruvate plus 8 mM Pi added
- / +	Plants grown in 'sodium-free' conditions (-) or supplied with sodium (+)
B/A	Mesophyll chloroplasts resuspended in 'sodium-free' (B) or sodium-containing (A) medium

P.F. Brownell and L.M. Bielig, Unpublished work

Arundinelleae in which the transport may be proton- rather than sodium-dependent (Aoki *et al.* 1992).

2) The enzymatic conversion of pyruvate to PEP: There is no evidence of sodium nutrition affecting the *in vitro* activity of pyruvate orthophosphate dikinase, the enzyme catalysing the conversion of pyruvate to PEP (Boag 1981; Dorney 1985). It is still possible that sodium could affect the *in vivo* activity of the enzyme but this has not been investigated.

3) The maintenance of the integrity of the light harvesting system as a source of energy for the conversion and/or transport of pyruvate into the chloroplasts: In our work, we have several lines of evidence (Publications 14, 17, 19, 20, 22 [p.27]) indicating damage to the light harvesting system in mesophyll cells from sodium-deficient plants. Furthermore, the damage was rapidly repaired upon the addition of sodium to the plants. It is difficult to determine whether these phenomena are a direct consequence of sodium-deficiency or are the possible result of damage to the system because of excess energy which would normally have been utilised in the conversion of pyruvate to PEP (Publication 23).

Aoki *et al.* (1992) reported that members of Andropogoneae and Arundinelleae, including sugar cane, sorghum and maize, utilise protons rather than sodium ions for the transport of pyruvate into the mesophyll chloroplast. This finding suggests that there may be rare exceptions to the general requirement for sodium

by C_4 plants. We are currently testing members of these tribes for their sodium requirement. If sodium is not required by species with proton-activated transport, it will suggest that the sole function of sodium is to activate pyruvate translocators in the species for which sodium is essential. On the other hand, if sodium is required by species utilising proton transport, it may be concluded that sodium is involved in other functions than pyruvate transport.

It is of particular interest that Cyanobacteria, in addition to most C_4 plants, utilise Na^+ ions for metabolite transport. At present, only one Na^+-activated transport system has been defined in the C_4 mesophyll chloroplast whereas in Cyanobacteria, several Na^+-activated systems for the transport of $H_2CO_3^-$, CO_2, Cl, and P have been described (Valiente and Avendana 1993). As the mesophyll chloroplast of C_4 plants and Cyanobacteria have prokaryotic origins in common, it is possible that further Na^+-activated transport systems might be discovered in the mesophyll chloroplasts of C_4 plants.

Aoki *et al.*(1992) briefly reviewed the specificity of Na^+-activated translocators pointing out that the melibiose translocator of *Escherichia coli* utilises both Na^+ ions and protons and that mutagenesis studies show that a single substitution of one amino residue may change cation specificity. Similarly, they postulate that the pyruvate translocator of the C_4 mesophyll chloroplast may also have changed its cation specificity through a minor modification during evolution of the Andropogoneae and Arundinelleae species from ancestral species.

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Sodium as an Essential Micronutrient Element for *Atriplex vesicaria*, Heward

SODIUM has been recorded as an essential nutrient element only for the blue-green alga *Anabaena cylindrica* Lemm.[1]. It has, however, been shown that increase in dry weight occurs following application of sodium salts to various higher plants growing in low-potassium culture solution and also to members of the Chenopodiaceae growing in solutions containing adequate potassium; views have been expressed that sodium may partially replace the function of potassium or may prevent toxic accumulations of other elements[2]. The object of this communication is to record sodium as an essential micronutrient element for the bladder saltbush (*Atriplex vesicaria*, Heward), to describe the visual deficiency symptoms when plants were grown in solutions carefully freed from sodium and the recovery of such deficient plants on addition of sodium salts.

This species occupies large areas of arid Australia, is an important fodder plant and was chosen for investigation because it accumulates large quantities of sodium chloride in its leaves[3].

Plants of saltbush were grown in a basal culture solution containing the following macronutrient ions (in millimoles per litre): K^+, 6; Ca^{++}, 4; NO_3', 13; NH_4^+, 2; H_2PO_4', 1; HPO_4'', 1; SO_4'', 1; Mg^{++}, 1; and micronutrient elements (in micromoles per litre): iron, 90; boron, 46; manganese, 9·1; zinc, 0·76; copper, 0·31; molybdenum, 0·10; chlorine, 350. Nutrient salts were recrystallized many times, their sodium content being determined each time by flame photometry; salts of the nutrient culture solution contributed less than 0·075 p.p.m. sodium to the nutrient solution. Water, thrice distilled from tinned metal stills, stored in polythene containers, contained 0·0005–0·0008 p.p.m. sodium.

In a preliminary experiment in an ordinary glasshouse, plants growing in the basal culture solution showed significant increases in dry weight when sodium salts (but not when additional potassium salts in equivalent amounts) were supplied at levels of 0·35, 1·75 and 7·0 m.equiv. sodium/litre. When the plants were relatively large and had acquired tertiary branches, the leaves of all plants not receiving sodium appeared paler green than those of plants with added sodium. Analysis of plant organs and culture solutions at the end of the experiment

Table 1. EFFECT OF VARIOUS TREATMENTS OF SODIUM OR POTASSIUM SALTS ON DRY-WEIGHT PRODUCTION OF SALTBUSH PLANTS

Treatment		Mean dry weight per vessel
I	No Na_2SO_4	0·056 gm.
II	Na_2SO_4, 0·02 m.equiv./litre	0·340 ,,
III	,, 0·10 ,, ,,	0·513 ,,
IV	,, 0·60 ,, ,,	0·493 ,,
V	K_2SO_4 0·60 ,, ,,	0·066 ,,

Statistical treatment: II, III, IV > I, V at 0·1 per cent level of significance; III > II at 5 per cent level of significance; III and IV indistinguishable; I and V indistinguishable.

showed an increase of 300 μequiv. sodium above the amount supplied in the culture solution and seeds. This suggested that plants had received sodium, presumably as cyclic salt, from the atmosphere.

To preclude this possibility, a temperature-controlled cabinet was constructed with walls of clear polyvinylchloride sheeting and supplied continuously with filtered air both to culture vessels and to the cabinet itself, which was maintained at a slightly positive pressure to prevent entry of dust.

Bracteoles were removed from seeds, which were then washed in distilled water and germinated on waxed gauze over distilled water; after emergence of the radicle, the distilled water was replaced by basal culture solution of one-fifth full concentration.

Fig. 1

Table 2. DRY-WEIGHT CHANGES AFTER RECOVERY OF SODIUM-DEFICIENT PLANTS FOLLOWING APPLICATION OF SODIUM SULPHATE TREATMENT

Treatment	Mean dry weight per vessel
I No Na_2SO_4	0·149 gm.
II 0·10 m.equiv./litre Na_2SO_4 applied on day 31	0·728 ,,
III 0·10 m.equiv./litre Na_2SO_4 applied on day 16	1·656 ,,

Statistical treatment: II > I at 1 per cent level of significance; III > I at 0·1 per cent level of significance; III > II at 5 per cent level of significance.

When 11 days old, the seedlings were transferred to polythene containers, four per container, with 'Perspex' tops and placed in the cabinet. They were continuously aerated through polyvinylchloride tubing dipping into the culture solutions. On the fifteenth day, differential treatments were applied. When harvested on the forty-eighth day, the results shown in Table 1 were obtained.

On the twenty-fifth day, plants which had not received sodium sulphate could be distinguished from those which had by their yellow colour and fewer leaves of smaller area. Necrotic areas, white in colour, appeared along tips and margins of cotyledons and older leaves on the thirtieth day (Fig. 1) and plants began to die by the thirty-fourth day.

In another experiment, plants were transferred on the fourteenth day after germination to culture vessels containing the basal culture solution, without added sodium. On the sixteenth day sodium sulphate (0·10 m.equiv./litre) was added to one set of culture vessels and seven days later the plants growing in these cultures could be distinguished from the controls by their darker green colour. Symptoms similar to those described above again appeared in cultures which had not received sodium, and by the thirty-first day, when a second set of culture vessels received a treatment of 0·10 m.equiv./litre of sodium sulphate, symptoms were severe. Four days after receiving this delayed sodium treatment, plants showed signs of recovery by a progressive change of colour in older leaves (and in some cases cotyledons) from yellow to green; greening commenced at tips and around the midribs, and gradually spread over the lamina. Plants growing in the set of cultures which received no sodium treatment throughout the experiment became progressively more chlorotic, making little further growth. On the other hand, marked growth occurred in both sets of cultures which received additional sodium. When harvested on the forty-ninth day, the results shown in Table 2 were obtained.

Leaf material from this experiment was analysed

for sodium. On a dry-weight basis, leaves of plants from 'no sodium sulphate' cultures contained 350 p.p.m. sodium, whereas leaves from plants which received 0·10 m.equiv./litre sodium sulphate in their culture solutions contained 9,100 p.p.m. sodium.

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A Simple Medium for the Demonstration of Chlamydospores of *Candida albicans*

WHILE investigating the formation of chlamydospores by different species of the fungus *Candida*, it was observed that human serum as a medium gave good results. Cultures of *Candida albicans* 10, *Candida tropicales* 10, *Candida krussei* 10, and *Candida stellatoidea* 10, *Candida guillarmondi* 1, and *Candida pseudotropicales* 1, were examined by the following technique: 24-hr. growth in Sabouraud's dextrose agar medium was emulsified with 1 c.c. of sterile normal saline; then 1 c.c. of this emulsion was added to a test-tube containing 4 drops of normal human serum, and the tube was incubated at 37° C. in a water-bath. After a lapse of 24, 48 and 72 hr., cover-slip preparations were examined under the microscope. In each instance, where cultural and biochemical tests for a strain conformed to those for *Candida albicans*, chlamydospores were produced after a lapse of 24 hr. Chlamydospores, however, did not develop with other types. Thus the above findings show that this method is very simple, less expensive and less time-consuming than routine methods.

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Growth of a Nematode in Tomato Plants grown on Sodium-deficient Water Cultures

HOST plants deficient in nitrogen, phosphorus, potassium, magnesium, sulphur and iron will support a population of the root-knot nematode *Meloidogyne javanica*[1], whereas plants on full nutrient and heavily infected with *M. incognita acrita* do not show deficiencies of these elements[2]. It has been reported[3], however, that tomatoes infected with *M. incognita acrita* show less sodium in their tissues than do uninfected plants.

It may be that members of the genus *Meloidogyne* require large amounts of sodium during their development, and the following experiment was undertaken to test the effect of 'sodium deficiency' on the growth and reproduction of *M. javanica* grown in tomatoes. The terms 'sodium deficient' and 'sodium free' used here refer to plants grown in water cultures in which the presence of sodium has been reduced to a minimum.

All seeds used came from a single susceptible tomato plant of the variety Pan America. They were washed six times in water distilled from a silica still which was changed hourly. This water, which was used throughout the experiment, contained 0·0002 p.p.m. sodium.

Nutrient salts were re-crystallized as many as six times, and their sodium content estimated before use. The seeds were germinated on nylon mesh over distilled water. When the radicles were about 1 cm. in length, a culture solution of half concentration containing 0·0042 p.p.m. sodium was substituted for the distilled water. The concentrations of salts used and the methods of growth in polythene containers have been described[4].

Sodium was estimated, using a 'Si-Ro-Spec' atomic absorption instrument[5], on a dry-weight basis. The methods used in infecting the plants and in measuring the growth of the nematodes have been described[1], except that the agar was dialysed against distilled water as a 5 per cent gel for five days with six changes before it was used. A 0·5 per cent solution of the dialysed agar was used as the infecting medium.

Infective larvæ of *M. javanica* were washed several times over a sintered glass filter of pore diameter 5–15μ before being placed in the agar. The plants were exposed to infection for a period of 48 hr. and were then removed. The roots were washed in distilled water before being returned to the nutrients.

The plants were grown in a cooled glass-house from which dust contamination was reduced by a positive pressure of filtered air. A diurnal temperature variation between 60° and 110° F. was maintained during the experiment.

Plants grown in the presence of sodium were grown in culture solutions identical with those without sodium except that they had 0·1 m.equiv./litre of sodium sulphate added. The roots and leaves of these plants were hydrolysed separately in silica Kjeldahl tubes using a mixture consisting of 5 ml. concentrated nitric acid, 1 ml. concentrated sulphuric acid and 1 ml. concentrated perchloric acid, all these chemicals being A.R. grade. Blanks consisting of these chemicals were included in all hydrolyses and sodium estimations.

Throughout the experiment the growth of plants in all treatments was similar. Plants were harvested at 7, 14, 21 and 28 days, and their dry weights were similar, whether or not they were infected or whether or not they had sodium. At the final harvest, average weights of twelve plants from each of the two treatments were approximately the same (6·4 gm.).

The amounts of sodium in the roots and leaves of these plants showed marked differences. Tomato plants grown on a 'sodium free' culture solution showed uniform amounts of sodium which ranged from 20 to 50 p.p.m. throughout the entire experiment for both roots and leaves; the roots always contained sodium in a slightly higher concentration than the leaves.

Plants grown with sodium showed a steady increase in uptake of this element in the roots throughout the entire experiment, ranging from 120 p.p.m. at the first (seventh day) harvest to 1,410 p.p.m. at the final (twenty-eighth day) harvest. The leaves of these plants, however, did not show an increase in sodium concentration throughout the experiment.

These results suggest that sodium accumulates in the roots of tomato plants when the amounts mentioned above of sodium are supplied. Infection was not consistent using agar, and although great care was taken to make this procedure as uniform as possible, in several instances plants remained uninfected.

Infected 'sodium-free' plants were harvested at 7, 14 and 28 days, and galled roots were cut away from the plant and fixed in buffered 1 per cent osmium tetroxide at pH 7·2. They were washed in several changes of distilled water and at each harvest fifty nematodes were dissected at random and their cross-sectional area measured as previously described[1].

Growth of these nematodes on tomatoes containing 20–50 p.p.m. sodium was quite normal. They moulted, produced egg sacs, eggs and viable larvæ which were capable of re-infecting tomato seedlings,

and it appears that the amount of sodium removed from 'sodium-deficient' tomato plants by light infections (several hundred per plant) of these parasites is negligible, as at the final harvest infected 'sodium-free' plants gave a reading of 40 p.p.m. sodium compared with 35 p.p.m. sodium for uninfected 'sodium-free' plants. From these observations it is clear that *Meloidogyne javanica* does not require a large amount of sodium during its development.

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Mechanism of Trail-laying in Two Species of Termites

DURING recent investigations on the behaviour of the primitive termite *Zootermopsis nevadensis* (Hagen), evidence for trail-laying was obtained when it was noted that nymphs accurately followed the path taken by one nymph over a clear unobstructed surface in an artificial nest, even when the direction of the light and the position of the nest were altered. This, to my knowledge, is the first mention of trail-laying in a lower termite.

Subsequent experiments using squashes and petroleum ether extracts of various parts of the body and organs of individuals of *Z. nevadensis* and *Nasutitermes cornigera* Motschulsky have shown that in these two genera, belonging to two widely separated families (Hodotermitidae and Termitidae), trails are produced by a substance secreted by a gland in the ventral part of the fifth segment of the abdomen. Grassé and Noirot[1], however, from work on *Macrotermes bellicosus* (Smeathman), *Macrotermes natalensis* (Haviland) and *Odontotermes magdalenae* (Grassé and Noirot) believe that in these species trails are marked by fæcal droppings or pellets of earth. In *Z. nevadensis* and *N. cornigera* no trails made with squashes or petroleum ether extracts of the gut, contents of alimentary canal or rectum and drawn on a white card were followed, whereas similar trails drawn with extracts and squashes of the gland produced clear-cut trail-following. *Nasutitermes* does appear to follow a visible trail of fæcal droppings, however, but this is thought to be a consequent phenomenon and not the initial trail marker. When a scarcely visible, by-pass trail, made from the cleaned integument containing the gland, was drawn in competition with a natural trail that had reached the stage where it was quite visibly marked by fæcal droppings, both the workers and soldiers of *Nasutitermes* followed the by-pass in preference to the old trail.

The secretion from the gland has been shown to act as a trail releaser as well as marking the trail.

The trail-laying gland in *Zootermopsis* and *Nasutitermes* appears homologous to that described very briefly in *Kalotermes flavicollis* (Fabr.) by Grassi and Sandias[2], and by Montalenti[3], and in *Rhinotermes nasutus* (Perty) by Holmgren[4]. Grassé[5] has stated that the function of the gland in these species is unknown. In *Zootermopsis* the gland can empty through numerous small pores in the cuticle into a crude external reservoir formed by the almost complete overlapping of the fifth sternite by the preceding one. The nymphs of *Zootermopsis* are often seen dragging their abdomens along the ground when moving, thus bringing segment 5 in contact with the substrate, while the segments at the extremity of the abdomen are clear of the ground. The substance from the gland can thus quite easily escape from the reservoir on to the surface on which the insect is crawling.

All the observations and experiments on *Zootermopsis* were made on laboratory colonies which had been conditioned to live in constant illumination provided by a 'Daylight' fluorescent lamp.

A full account of this work and a description of the gland in *Zootermopsis* and *Nasutitermes* will be published elsewhere.

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CYTOLOGY

Auto-Alloploid Nature of *Pennisetum squamulatum* Fresen

CYTOLOGICAL observations carried out in this laboratory on *Pennisetum squamulatum*, an East African[1] grass species, and on its F_1 hybrid[2] with pearl millet (*Pennisetum typhoides* Stapf and Hubb.) have suggested that *P. squamulatum* is an auto-allopolyploid.

Raman, Chandrasekharan and Krishnaswamy reported that the species possessed $2n = 54$ chromosomes and that, during meiosis in the pollen-mother cells, bivalents and quadrivalents (1 per cell) were formed. We re-examined meiosis in the pollen mother cells of this species and observed the types of chromosome association which suggested the polyploid nature of this species (Table 1).

Table 1

	Quadrivalents	Trivalents	Bivalents	Univalents
Average frequency per cell	3·14	1·06	17·90	1·91
Range	1–8	0–2	11–23	0–4

Total cells observed: 3

During 1958 the cross, *P. typhoides* × *P. squamulatum*, was attempted on an extensive scale. For this pollen from a single plant of *P. squamulatum* was dusted on a large number of ears of *P. typhoides* without emasculation. In 1959, 11 plants were raised in the field from the seed set on the male parent and about 600 plants from the seed set on the ears of *P. typhoides* used for crossing. Seven out of these 11 plants of *P. squamulatum* were examined cytologically, and all of them were found to posses

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THE PLANT ECOLOGY OF THE MOUNT LOFTY RANGES, SOUTH AUSTRALIA
2. THE DISTRIBUTION OF EUCALYPTUS ELAEOPHORA

By R. L. Specht, P. F. Brownell and P. N. Hewitt

THE PLANT ECOLOGY OF THE MOUNT LOFTY RANGES, SOUTH AUSTRALIA

2. THE DISTRIBUTION OF *EUCALYPTUS ELAEOPHORA*

by R. L. Specht,[1] P. F. Brownell[2] and P. N. Hewitt[3]

(Read 10 August 1961)

SUMMARY

Eucalyptus elaeophora has a disjunct distribution pattern in south-eastern Australia, extending in a discontinuous arc in the highlands from northern New South Wales, through Victoria, into the Mount Lofty Ranges of South Australia and northward into the Flinders Ranges. The distribution of the species is examined in the Mount Lofty Ranges and discussed in relation to climatic and edaphic factors.

In South Australia the species is found in the dry sclerophyll forest formation on very infertile soils developed from a wide variety of rocks — residual Tertiary laterites, Aldgate sandstone, Stonyfell Quartzite, Archaean schists and gneisses and even phyllites where the soil is highly leached. The climate of the area is of Mediterranean type with cool (mean July temperature 45° F.), wet winters alternating with hot (mean January temperature 65° F.), dry summers. Within this area *E. elaeophora* is widespread between the 27 and 36 inch isohyets. Small pockets are found in rainfall areas as low as 23 inches per annum, where infertile soil is found with a high water-retaining capacity. The species in South Australia appears to have reached its maximum southward distribution in the Mount Lofty Ranges.

The ecological limits, thus defined for the Mount Lofty Ranges, are compared with those found for other areas within Australia. As the species extends from a region of winter rainfall to one where summer rain predominates, corrections had to be made. In all areas, foliage growth is greatest during summer; soil moisture stored from winter rains is utilized during this growth period; some 25 p.c. of the winter rainfall is lost by evapotranspiration before growth occurs. After this correction, the predominantly winter rainfall range (27 to 36 inches per annum) of the Mount Lofty Ranges is equivalent to that in the Monaro Region of New South Wales (19 to 26 inches per annum) where summer rainfall predominates.

If these limits are correct, the species is unlikely to occur in wetter areas (as high as 48 inches per annum) as recorded in the Dandenong Ranges, Victoria; this observation could be due to the difficulty of distinguishing *E. elaeophora* from *E. goniocalyx*, with which it hybridises.

The distributions of the other eucalypts in the area are compared with those found to the south of the Torrens Gorge.

The disjunct distribution of *E. baxteri* — Black Hill, Mount Gawler, Tanunda Creek — is of interest.

INTRODUCTION

In the first paper in this series on the plant ecology of the Mount Lofty Ranges, Specht and Perry (1948) discussed the distribution of *Eucalyptus* species between the Torrens Gorge and Port Noarlunga. In this area, nine species were found in extensive and complex patterns of distribution which enabled the authors to prescribe on the basis of soil nutrients and climate, geo-

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graphical limits for each species. As *Eucalyptus elaeophora* F. Muell. appeared in only the most northerly portion of the area mapped, and was known to be common farther north, a more comprehensive study of this species was deferred. This paper presents the results of this later investigation.

Adamson and Osborn (1924) gave an excellent introductory account of the distribution of *E. elaeophora.* They indicated (1) that the southern boundary of the species was just south of the Torrens Gorge; (2) that the forests occurred on rugged hills with shallow, stony soils formed from metamorphosed Archaean (then considered to be Pre-Cambrian) rocks, notably hard schists, crystalline quartzites and gneisses; and (3) that less water was available in forests of *E. elaeophora* than in the *E. obliqua* forests to the south. Winter mists, common in the Ranges to the south of the *E. elaeophora* zone, would induce wetter conditions favouring *E. obliqua* even in areas of similar rainfall, viz. 30 to 35 inches per annum where *E. elaeophora* was found.

Wood (1930) reiterated the above conclusions and later (1937) in his book, "The Vegetation of South Australia", added a little further information on its distribution – "from about Mount Crawford southwards to the Torrens Gorge", the general distribution of the Archaeozoic rocks – and noted that the soil formed from these rocks is shallow, somewhat podsolized and that, in some areas, lateritic residuals may be found.

Specht and Perry (1948), as mentioned above, noted the presence of *E. elaeophora* south of the Torrens Gorge, and indicated that it was found in podsolic soils developed over the "Stonyfell quartzite" on Black Hill and on soils, erroneously termed ferrimorphic soils, developed over Archaean schists and gneisses.

In a recent soil survey, Jackson (1957) made some general remarks on the vegetation. He noted that both *E. obliqua* and *E. elaeophora* were the most common trees in the sclerophyll forests he examined (see area C, Fig. 1). The two species were co-dominant in scrubs north of Millbrook Reservoir, on the Mount Gawler, Millbrook, Kersbrook, Mount Gould and Horse Gully soil associations. To the east of the longitude of Birdwood, on the Birdwood soil association, *E. elaeophora* was co-dominant with *E. fasciculosa* while *E. obliqua* was sole dominant in sclerophyll forests on the Lobethal and Kangaroo Creek soil associations on the south boundary of the Hundred of Talunga.

This new evidence of Jackson together with the detailed maps presented in this paper enables a much broader picture of the distribution of *E. elaeophora* in the Mount Lofty Ranges to emerge than Adamson and Osborn could possibly have seen in their reconnaissance survey almost forty years ago.

The main area selected for study extended from the Torrens Gorge in the south to Mount Gawler in the north, from Golden Grove in the west to Mount Gould in the east (see area A, Fig. 1). This was chosen, because it was a logical extension of Specht and Perry's original survey, and also because it showed an excellent sequence of rocks of Archaeozoic and Pre-Cambrian age (Torrensian series) varying greatly in mineral composition (Sprigg *et al.*, 1951). As well, deep Eocene sands are found on the western boundary of the area below the Eden scarp in the Anstey Hill region.

A smaller area, the Barossa Goldfields, mapped by one of us (P.N.H.) was chosen because it illustrates the most northerly limits of *E. elaeophora* in the Mount Lofty Ranges, an area where the rocks mentioned above are largely masked by Tertiary laterites and deep sands (Campana and Whittle, 1953).

A mapping procedure similar to that used by Specht and Perry (1948) was used in this survey; namely, the distribution of the Eucalyptus species on every ridge and valley was projected onto contour maps (Military Ordnance maps)

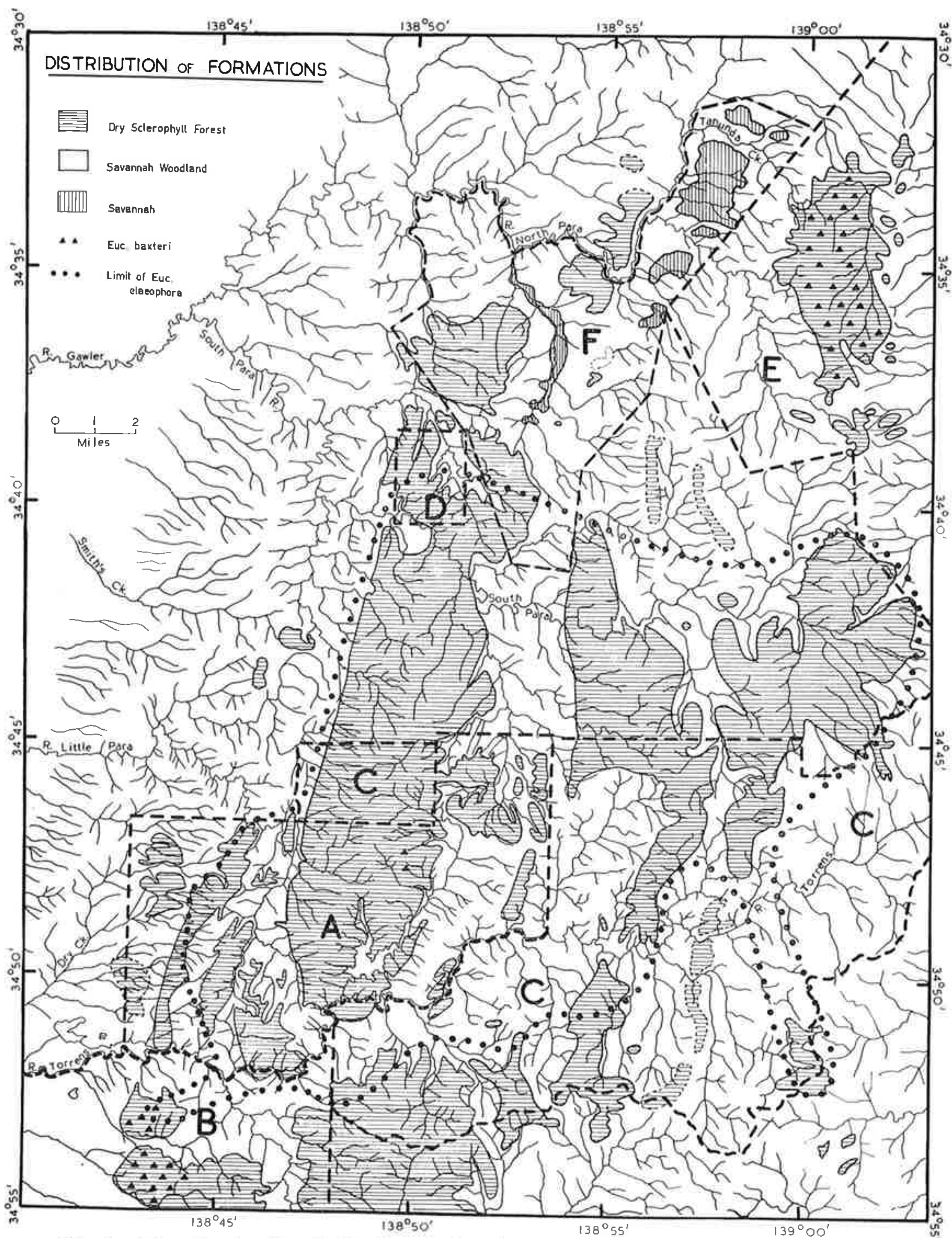

Fig. 1. Map showing the distribution of plant formations, savannah woodland, dry sclerophyll forest and savannah, between the Torrens Gorge and the Barossa Valley. The distribution of both *E. elaeophora* and *E. baxteri* within the dry sclerophyll forest is indicated. Detailed vegetation maps of Areas A and D are included in this paper (see Figs. 3 and 4); Area B is discussed by Specht and Perry (1948). Soil surveys of Jackson (1957) and Northcote and deMooy (1957) and Northcote (1959) cover the areas indicated by Areas C, E and F respectively.

TABLE 1.

Distribution of dry sclerophyll forest and savannah woodland formations in relation to geology and soils.

	Dry Sclerophyll Forest Formation			Savannah Woodland Formation		
	Parent Material	Soil Mapping Unit	Reference	Parent Material	Soil Mapping Unit	Reference
Recent to Tertiary	*Alluvial Deposits*	Nuriootpa family RS2 (unnamed soil group) Moppa-Vine Vale family Nuriootpa family RS3 (unnamed soil group)	Northcote (1959) Northcote (1959) Northcote (1959)	*Alluvial Deposits*	Para family Kroemer family Bilyara series 4 Bethany, Kabininge families Torrens, Gumeracha Associations	Northcote (1959) Northcote (1959) Northcote (1959) Northcote (1959) Jackson (1957)
	Eocene lacustrine sands	Birdwood B. Association Deep silicous sands of strongly acid reaction* Blewitt Springs Sand	Jackson (1957) Specht & Perry (1948) Rix & Hutton (1953)	*Lacustrine sediments* *Eocene marls*	Kabininge family Paradise Association	Northcote (1959) Aitchison & Sprigg (1954)
	Tertiary laterites	Birdwood C. Association Residual lateritic podsols** Incrassata family, Unnamed soil series 1 Unnamed soil group SC1 Barossa	Jackson (1957) Specht & Perry (1948) Northcote (1959) Northcote (1959) Northcote (1959)			
Early Palaeozoic	*Mt. Kitchener and Tanunda Creek Granites*	Ridge Vale — Potts Spring — Yamba Soil combination	Northcote and De Mooy (1957)	*Kanmantoo schists*	Mount Pleasant, McVittie Hill Associations	Jackson (1957)

Precambrian	*Aldgate sandstone*	Horse Gully Association. Podsols and skeletal quartzites.	Jackson (1957) Specht & Perry (1948)	*Castambul, Montacute and Beaumont dolomites*	Terra Rossa	Specht & Perry (1948)
	Stonyfell quartzite	Mt. Gould Association Podsols and skeletal quartzites Birdwood A Association The Peak Soil Combination	Jackson (1957) Specht & Perry (1948) Jackson (1957) Northcote (1957)	*Upper and Lower Phyllites* (Rainfall<35 in. p.a.)	Forreston and Cudlee Creek Assns. Trial Hill Soil Combination Grey brown podsols (with high nutrient status)	Jackson (1957) Northcote (1957) Specht & Perry (1948)
	Upper and Lower phyllites (rainfall>35 in. p.a.)	Lobethal, Kangaroo Creek. Grey brown podsols (with low nutrient status)	Jackson (1957) Specht & Perry (1948)	*Glen Osmond Slates*	Grey brown podsols (with high nutrient status)	Specht & Perry (1948)
Proterozoic	*Torrensian series. Jointed quartzites and associates*	Unnamed soil group Sk1 Unnamed soil series YP1	Northcote (1959) Northcote (1959)	*Torrensian series. Phyllitic slates and associates*	Wilsford family	Northcote (1959)
Archaean	*Schists and gneisses*	Mt. Gawler, Millbrook Associations Ferrimorphic soil†	Jackson (1957) Specht & Perry (1948)	*Granulites (formerly "Houghton diorite")*	Inglewood Association	Jackson (1957)

* These were subsequently examined in the Blewitt Springs area by Rix and Hutton (1953). They are allied to the Golden Grove-Tea Tree Gully sands. Sandy soils in the Barossa Goldfields are solodised solonetz soils similar to those described by Northcote *et al.* (1954)

** Jackson (1957) combines three distinct soil associations in his Birdwood Association.

† Now known to be an error.

after a detailed ground survey. As the density of the species has been greatly altered by fire, woodcutting and clearing, no attempt was made to indicate the relative dominance of the various species; only the presence of a species in any area was delineated.

A reconnaissance soil survey of the areas studied was made, but as Jackson's soil survey (1957) overlaps half of our area, it is redundant to publish our map. Divergent interpretations and notes on the area outside his survey will be noted in the text.

DISTRIBUTION OF PLANT FORMATIONS

In order to see the distribution of *E. elaeophora* in the Mount Lofty Ranges in perspective the distribution of the two major plant formations, dry sclerophyll forest and savannah woodland, was mapped throughout the area concerned by means of aerial photographs and ground reconnaissance.

In Fig. 1 the limits of the dry sclerophyll forests are shown from the Barossa Valley (Tanunda) in the north to Lobethal in the south, from the edge of the Ranges in the west to Mount Pleasant in the east. The formation does not appear to extend into areas of the Mount Lofty Ranges to the east of longitude 139°03′ E.

Adamson and Osborn (1924) noted trees 60 to 70 feet in height, but usually the eucalypts were somewhat smaller. In the drier limit (20 to 25 inches per annum) the eucalypts, usually *E. fasciculosa*, are stunted, twisted and scattered; the formation is a tall sclerophyll shrub woodland (Wood and Williams, 1960). This is particularly so on the deep Tertiary sands between Golden Grove, Tea Tree Gully and Highbury (Sprigg *et al.*, 1951), and again on the sands extending from the Barossa Goldfields through Sandy Creek and along the eastern edge of the Barossa Valley (Campana and Whittle, 1953). Here the soils show characteristics, such as an increase in sodium in the clay complex, typical of solods and solodized solonetz soils (Northcote *et al.*, 1954). Associated with these soils, it is not surprising to find certain mallee species (*E. incrassata* – Section 46, Hundred of Moorooroo; *E. gracilis* – Barossa Goldfields) appearing together with *Melaleuca uncinata* and *Baeckea behrii* amongst the usual assemblage of undershrubs. These stands approach a sclerophyll mallee subformation (Wood and Williams, 1960). This subformation formerly extended some distance to the north of Fig. 1, but is now largely cleared for vineyards.

In general, the dry sclerophyll forest formation or any of its variants is restricted in its distribution. The savannah woodland formation is much more widespread. In addition to the two major plant formations, dry sclerophyll forest and savannah woodland, there are small areas of savannah associated with the following soils; Wiesenboden-Lyndoch, grey and brown soils of heavy texture – Tanunda and grey and brown soils of heavy texture – Altona (see Table 1, Northcote, 1959). These are indicated in Fig. 1.

TOPOGRAPHY AND GEOLOGY

Aspects of the physiography and geology of the area over which *Eucalyptus elaeophora* is distributed (Fig. 1) have been examined by many geologists over the last eighty years. Earlier work by Scoular (1879, 1880, 1882), Brown and Woodwood (1885), Howchin (1906, 1915, 1926), Benson (1909), Hossfeld (1925, 1935), England (1935), and Alderman (1938, 1942) provided a sound basis for the more recent and comprehensive studies of Sprigg (1945, 1946), Spry (1951), Sprigg *et al.* (1951) and Campana and Whittle (1953, 1955). Most of the geological strata of the area are of Archaean or Pre-Cambrian (Torren-

sian Series) age. The Archaean rocks, various schists and gneisses, form the core of the ranges and are often masked by deep beds of Pre-Cambrian sandstones, grits, quartzites, limestone and phyllites. In some areas, especially east of the Williamstown-Lyndoch fault line, regional metamorphism has altered these rocks to epidote quartzites, saccharoidal quartzites, marbles, sericite schists, knotted schists, etc.

During the early Palaeozoic these beds were extensively folded, viz. Humbug Scrub and Lookout Tower (Warren Reservoir) anticlines; Gould Creek, Williamstown (along Williamstown-Lyndoch road) and Mount Crawford-Barossa Range synclines. Subsequently they were extensively peneplained until little relief was apparent other than the monadnock structures of Mounts Lofty, Barker, Torrens, Gawler, Crawford, Pewsey Vale Peak and Kitchener. Meandering streams such as the Onkaparinga, Torrens, North Para and South Para rivers drained the landscape.

Early in the Tertiary (Eocene) considerable depths of sands, clay and ferruginous gravel were deposited in the beds of lakes and streams on the low-lying countryside. These non-marine sediments, as well as the older rocks, were subsequently subjected to widespread lateritisation which formed ironstone crusts resistant to erosion.

Tertiary (Miocene-Pleistocene) tectonic movements rejuvenated the relief by a general uplift of the hills of the area. Differential block-faulting, related to the Early Palaeozoic folding, has produced the present orography. In the area under discussion, the scarps produced by the Para, Eden and Kitchener Faults dominate the landscape. Considerable erosion by the antecedent streams (Onkaparinga, Torrens and Para Rivers) and many later ones have dissected the fault blocks so formed into the present topography. The rate of dissection is influenced by the nature of the rock; rocks such as quartzites, sandstones and grits resist erosion to produce rugged topography which contrasts with the rounded ridges with gentle slopes produced from more easily weathered rocks.

The lateritic duricrust has also resisted erosion on the plateau surface of some fault-blocks, e.g. around Paracombe, Humbug Scrub and even in small areas on the slopes of the former monadnock, Mount Gawler, but, in general, the laterite has been completely dissected. A general picture of the topography can be gained from Fig. 2 on which contours for the 500 feet levels are indicated. The former monadnock, Mount Kitchener (1,965 feet), Mount Pewsey Vale Peak (2,064 feet), Mount Crawford (1,844 feet), Mount Gawler (1,779 feet), Mount Lofty (2,384 feet) and Mount Torrens (1,913 feet) dominate the landscape.

Considerable areas of the Eocene lacustrine deposits are now seen from the Barossa Valley south-westwards into the Barossa Goldfields district. South of this district they are found in small, dissected areas formerly continuous with the Golden Grove-Paradise area where considerable deposits are still obvious. At Paradise they disappear beneath the Upper Eocene marine strata. A similar area may be seen in the old lake basin east of Mount Crawford.

Elsewhere the underlying Pre-Cambrian and Archaean rocks have been exposed. The Archaean is prominent in the anticline running from the Torrens Gorge south of Mount Gawler through the Humbug Scrub area as far north as the Barossa Goldfields. Areas of Archaean rock near Inglewood and Kersbrook are apparently "granulites derived from lime-magnesia rich sediments by high-grade regional metamorphism combined with potash and soda metasomatism" (Spry, 1951). These areas were formerly called "Houghton diorite" by Benson (1909).

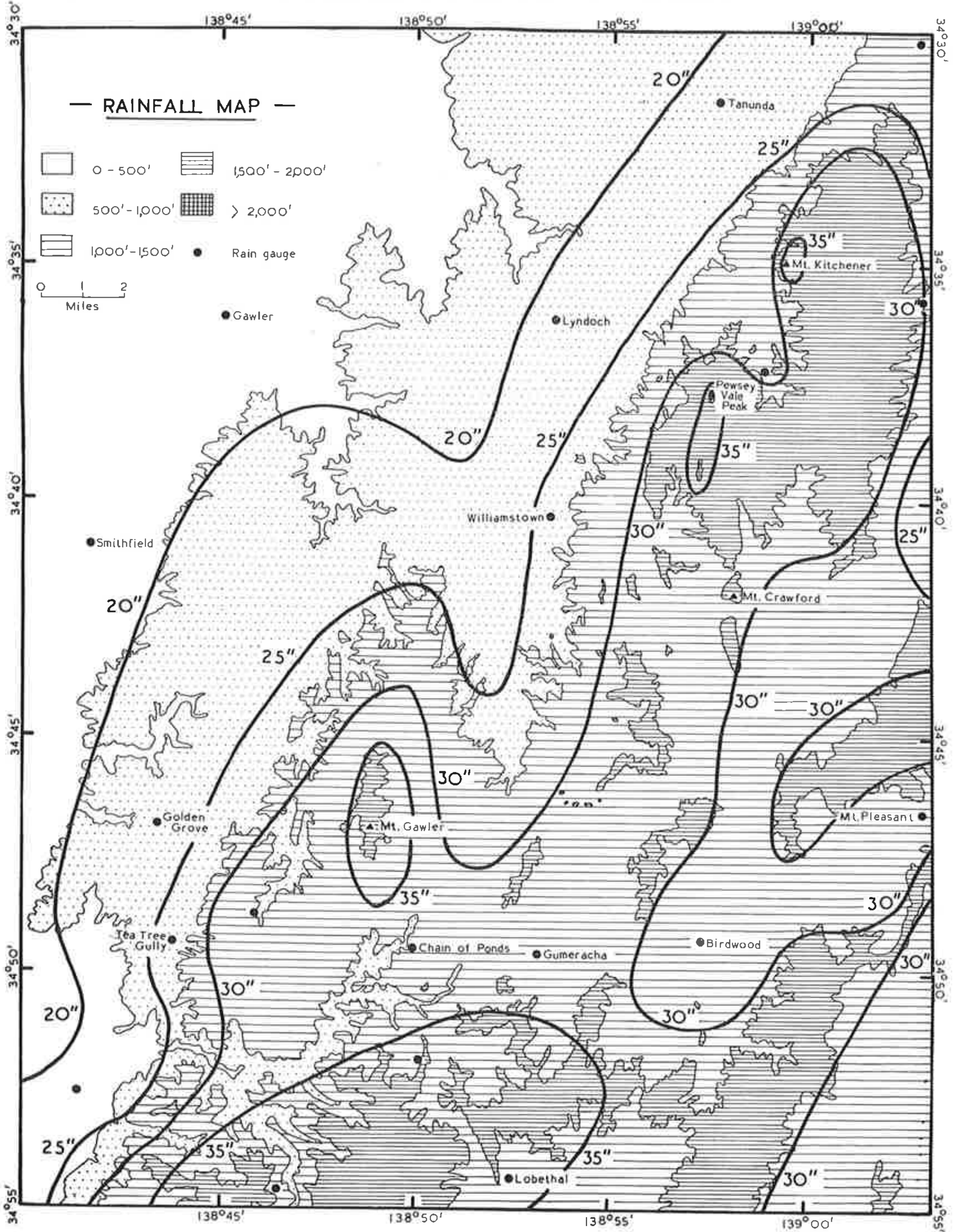

Fig. 2. Rainfall-altitude map of the area shown in Fig. 1.

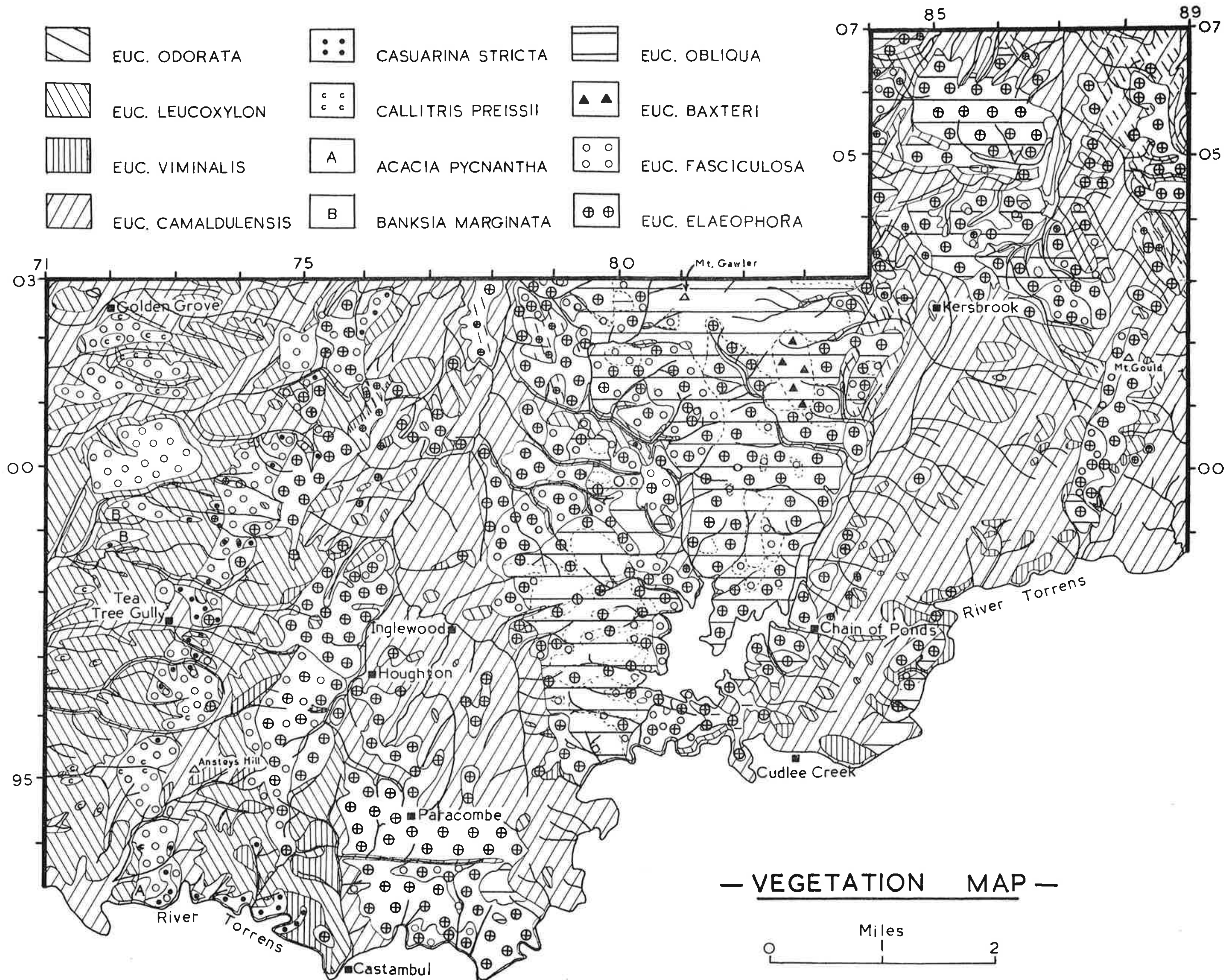

Fig. 3. Detailed vegetation map of area A shown in Fig. 1. Grid references on the Adelaide military ordnance map are indicated.

The basal beds of the Pre-Cambrian rocks (Aldgate sandstone) outcrop both on the east and the west of these Archaean rocks. The same basal rocks, though highly metamorphosed, also outcrop along the anticlinal ridge just west of the Warren Reservoir (the Lookout Tower Anticline).

Considerable depths of slates and phyllites interspersed with many bands of quartzite and some shallow beds of limestone or dolomite are prominent in the younger Pre-Cambrian outcrops. Ecologically the narrow outcrops of the Castambul, Montacute and Beaumont dolomites are of little significance. However, the many quartzitic beds, especially the thick Stonyfell quartzite, play a large part in the distribution of the vegetation. North of the Torrens Gorge and west of the Archaean rocks, quartzite outcrops along the Eden scarp in quite considerable areas; north of the latitude of Golden Grove, however, the outcrops are reduced to narrow bands usually too thin to be of much ecological significance. To the east of the Archaean core, three major quartzitic outcrops are found running roughly north and south – one ridge through the Mount Gould area and two ridges from Mount Crawford forest southward. One of the latter runs towards Lobethal and the other towards Mount Torrens. Similar ridges, though much metamorphosed, may be seen to the north of the Mount Crawford Forest where they form the backbones of the Mount Crawford-Barossa Range area.

Palaeozoic schists and schistose quartzites of the Kanmantoo Series are prominent on the eastern edge of the area. Granitic rocks have intruded through them in the Mount Kitchener and Tanunda Creek area.

From the broad ecological viewpoint, there are two major geological groups. One group of rocks supports a dry sclerophyll forest and/or woodland formation, viz.:

Caenozoic	Tertiary laterites.
	Eocene lacustrine sands.
Early Palaeozoic ...	Mount Kitchener and Tanunda Creek granites.
Pre-Cambrian	Aldgate sandstone.
(Torrensian Series) ...	Stonyfell quartzites.
	Other quartzitic beds if not greatly intermixed with slates or phyllites.
Archaean	Schists, gneisses and augen-gneisses.

The other group of rocks supports a savannah woodland formation, viz.:

Caenozoic	Recent alluvial floodplains.
	Eocene marls (near Paradise).
Early Palaeozoic ...	Kanmantoo schists and schistose quartzites.
Pre-Cambrian	Castambul, Montacute and Beaumont dolomites.
(Torrensian Series) ...	Lower and Upper phyllites with their minor quartzitic bands.
	Glen Osmond slates.
Archaean	Granulites (formerly called "Houghton diorite").

SOILS

The diverse geological formations mentioned above have had a marked influence on the soils to be found in the area. Since Specht and Perry published their ecological survey in 1948, a number of soil surveys has been made in the Mount Lofty Ranges, viz. Rix and Hutton (1953), Aitchison and Sprigg (1954), Northcote *et al.* (1954), Clarke (1957), Jackson (1957), Northcote and de Mooy (1957), and Northcote (1959). Table 1 summarises the soil-geological relation-

TABLE 2.

Chemical analyses of soil samples from dry sclerophyll forest and savannah woodland formations in the Mount Lofty Ranges.

Chemical Property	Soil Depth (inches)	No. of samples	*Savannah Woodland Formation.* Mean and Standard Error of mean	No. of samples	*Dry Sclerophyll Formation* Mean and Standard Error of mean	**Difference Significant at
pH	0-12	50	6·47 ±·112	26	5·70 ±·067	$P<\cdot01$
Nitrogen (% N.)	0-12	29	0·127±·0123	18	0·074±·0104	$P<\cdot01$
Phosphorus (% P.)	0-12	24	0·026±·0041	19	0·007±·00092	$P<\cdot01$
Potassium (% K.)	0- 3	21	0·29 ±·043	8	0·15 ±·068	N.S.†
Total Soluble salts %	0-12	40	0·022±·0038	17	0·016±·0027	N.S.†
Chloride as NaCl %	0-12	40	0·008±·0021	17	0·006±·0017	N.S.†
Exchangeable cations						
i) Exchangeable Ca*	0- 3	37	7·42 ±·118	12	3·08 ±·318	$P<\cdot01$
ii) Exchangeable Mg*	0- 3	37	1·89 ±·171	12	1·38 ±·231	N.S.†
iii) Exchangeable K*	0- 3	37	0·68 ±·089	12	0·28 ±·038	$P<\cdot01$
iv) Exchangeable Na*	0- 3	37	0·25 ±·054	12	0·15 ±·026	N.S.†

* M-equiv./100 g. soil.

** Analyses were carried out by t-test where variances were equal; where they were unequal, the Sukhatme test was used.

† N.S.=not significant at $P=0\cdot05$

ships found by these authors and compares them with the broad classification used by Specht and Perry. It is obvious that the two plant formations of the area are found on a wide variety of soil associations. In 1948, Specht and Perry analysed some 23 soil profiles, usually only subdivided into A and B horizons, for texture, hydrogen ion concentrations, phosphorus and nitrogen. These preliminary analyses indicated that nutrient status of the soil controlled the distribution of the two plant formations found in the area. The dry sclerophyll forest was found on those soils of low nutrient status (as indicated by phosphorus and nitrogen levels); the savannah woodland on soils with higher fertility.

Detailed soil surveys of subsequent workers have been accompanied by comprehensive mechanical and chemical analyses of typical profiles by the Soil Chemistry Section of the Soils Division of C.S.I.R.O. It is possible now to contrast the fertility of soils of these two formations with much greater precision. As most of Specht and Perry's analyses were made on the surface twelve inches of soil, comparable values were calculated from the more recent data. The levels thus calculated though lower than those of shallower layers of soil, showed the same order of difference as that observed in the surface two or three inches. Some analyses (namely, exchangeable cations, percentage potassium) had been made on only the surface two or three inches of soil. The data, thus computed, are presented in Table 2.

Values for hydrogen ion concentration, nitrogen, phosphorus, exchangeable calcium and potassium for soils of the savannah woodland formation are significantly higher than those of the dry sclerophyll forests. Other analyses (total potassium, total soluble salts, chlorides, exchangeable magnesium and sodium), although they appear lower in soils of the dry sclerophyll forest formation, are not significantly different. In effect, the hypothesis of Specht and Perry (1948) that the savannah woodland is found on soils of higher fertility than that of the dry sclerophyll forest is confirmed.

CLIMATE

Recently Coote and Cornish (1958) made a detailed statistical analysis of the rainfall of the Mount Lofty Ranges. A close correlation between rainfall, altitude, latitude and longitude was shown. From this they were able to construct maps of the mean monthly rainfall of the Ranges.

Unfortunately they did not compute mean annual isohyets. Using their monthly regression equations (see Martin, 1960), mean annual rainfall was calculated for every 5-second latitude and longitude intersection of the area. These figures, plus the infrequent rain-gauge records, were used to construct the annual rainfall-topography map of Fig. 2. The high peaks of Mt. Kitchener, Mt. Pewsey Vale, Mt. Gawler and the country to the south of Millbrook Reservoir induce the highest rainfall (35 to 40 inches per annum) of the area. Much lower annual rainfall (less than 20 inches per annum) occurs on the lowlands to the west and north as well as in the rain shadow towards the east.

As indicated in the first paper in this series (Specht and Perry, 1948), this rainfall has a well-marked winter maximum, coinciding with low temperatures (mean July temperature is 45° F.), alternating with a summer minimum when temperatures are high (mean January temperature is 65° F.). This is a typical Mediterranean climate.

Distribution of the two plant formations, dry sclerophyll forest and savannah woodland, is little influenced by climate in this area (compare Figs. 1 and 2). The Castambul-Lobethal area may be an exception where the higher rainfall (greater than 35 inches per annum) may have markedly influenced the fertility

VEGETATION MAP — BAROSSA GOLDFIELDS

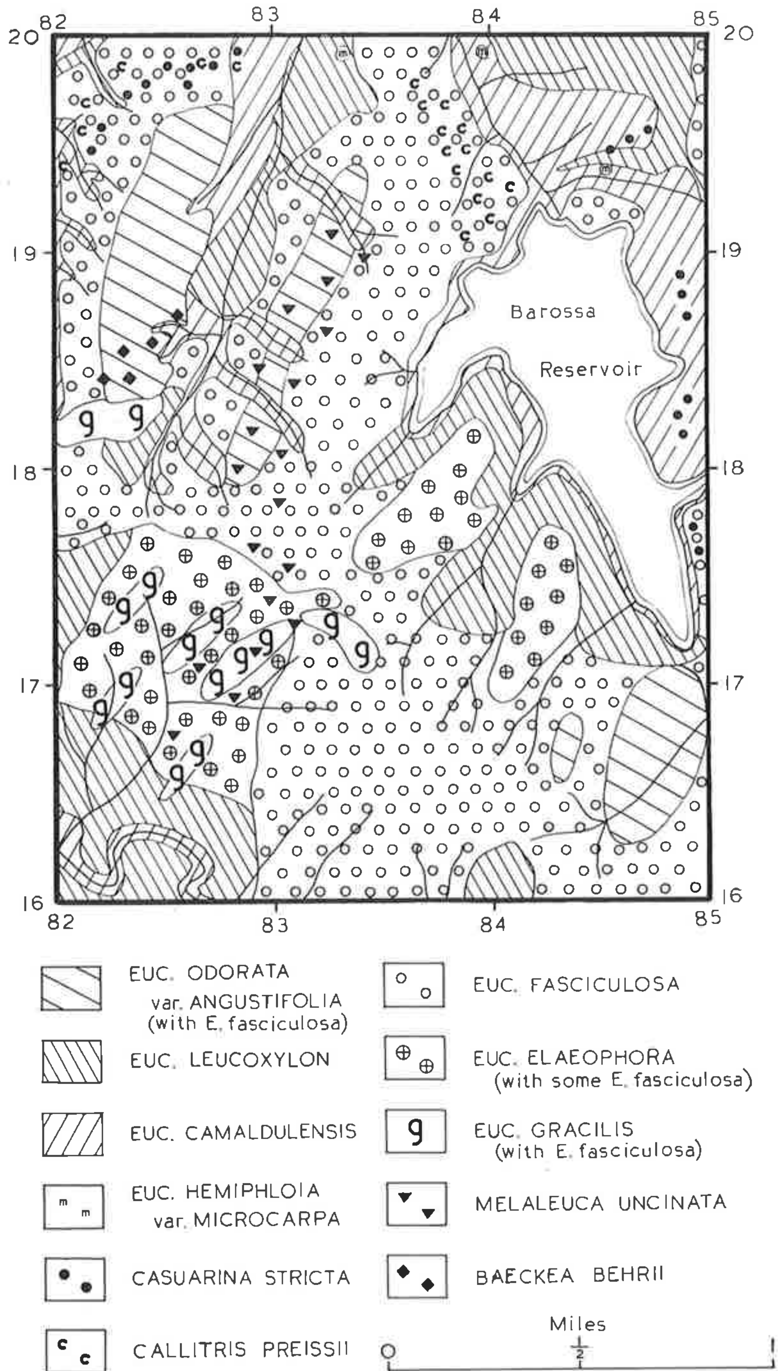

Fig. 4. Detailed vegetation map of Barossa Goldfields, Area D of Fig. 1. Grid references on the Gawler military ordnance map are indicated.

of soils such as Lobethal and Kangaroo Creek associations in favour of the dry sclerophyll forest (see Specht and Perry, 1948, p. 103).

However, distribution of individual species within each plant formation is markedly influenced by climate, especially microclimate, as will be shown below.

DISTRIBUTION OF *EUCALYPTUS ELAEOPHORA*

(1) *In the Mount Lofty Ranges*

The distribution of *Eucalyptus elaeophora* in the Mt. Lofty Ranges is indicated in Fig. 1. The species does extend a short distance outside this boundary, but always as an insignificant part of the community and then often represented by trees which suggest that they are hybrids of *E. elaeophora* and *E. viminalis* (probably not even pure *E. viminalis* but hybrids between it and *E. aromaphloia* (Pryor, 1955b) – formerly considered as *E. viminalis* var. *huberiana* by Burbidge, 1947). Such limited areas of these hybrid forms may be seen on the Tertiary laterites just west of Tungkillo (lat. 34°52′S., long. 139°00′E.), on the narrow quartzite outcrop running north from Mt. Torrens to the River Torrens, and on the narrow, metamorphosed quartzite ridges just north of Mount Crawford. This information is contrary to that of Jackson (1957, p. 13), who stated that *E. elaeophora* was co-dominant with *E. fasciculosa* to the east of the longitude of Birdwood. *E. elaeophora* is really quite rare in this area.

In all areas, *E. elaeophora* is found only in the dry sclerophyll forest formation. Within that formation, it appears to be much more widespread than originally thought (Adamson and Osborn, 1924; Wood, 1937), being found on Tertiary laterites, Aldgate sandstone, Stonyfell quartzites as well as the Archaean schists and gneisses. Small pockets of the species also extend onto the phyllites of low nutrient status (presumably leached by the rainfall greater than 35 inches per annum), though these pockets are rare. In fact, the species may be found on all rocks supporting a dry sclerophyll forest mentioned in Table 2, except the deep Eocene lacustrine sands and the Mount Kitchener-Tanunda Creek granites. The deep sands are found on the drier limit of the species thus precluding its establishment. The Tanunda Creek Granites appear to be the most infertile soils of the area, possibly too infertile to support *E. elaeophora*, only gnarled *E. baxteri* and *Xanthorrhoea semiplana* growing there.

It is clear from Fig. 3 that the distribution of *E. elaeophora* overlaps that of *E. obliqua*, *E. fasciculosa*, and even the small area of *E. baxteri* on the western slopes of Mount Gawler. Because of this overlap, the mean soil data shown in Table 2 for the dry sclerophyll forest, were almost identical with those for the component eucalypt species.*

The rainfall limits of *E. elaeophora* are well defined on the map. On the drier limit, the species is first found in isolated pockets amongst *E. fasciculosa* on the skeletal quartzites on top of the Eden scarp (Anstey's Hill-Golden Grove). The species seems to occur in the wetter habitats of gullies and southerly slopes. This area lies approximately along the 27-28 inch annual isohyet. Not far to the east of the escarpment the soil profile becomes well developed on quartzites allied to those on the scarp. Here *E. elaeophora* is widespread, sometimes co-dominant with *E. fasciculosa*, sometimes in almost pure stands.

Farther east, *E. elaeophora* extends into the wetter areas of the Archaeozoic rocks (with or without lateritic remnants). In the Paracombe area it again

* The limited soil data for *E. baxteri* were the only exceptions; percentage nitrogen (0·047), percentage phosphorus (0·004), exchangeable calcium (2·1 milli-equivalents p.c.) and exchangeable magnesium (0·5 milli-equivalents p.c.) were half to two-thirds that of the mean for the formation. *E. elaeophora* was absent from all these soils.

forms almost pure stands, mixed occasionally with trees of *E. fasciculosa*. However, in the wettest part of the area around the massif of Mount Gawler, the species is greatly admixed with *E. fasciculosa* and especially with *E. obliqua*. On these Archaeozoic rocks, *E. fasciculosa* is mainly found with *E. elaeophora* on shallow soils usually with a northerly aspect. On deeper soils *E. obliqua* is found co-dominant with *E. elaeophora* and forms almost pure stands in the wettest sites on the east side of Mount Gawler and on the ridge to the west of Millbrook Reservoir. It is apparent then that the upper rainfall limit of *E. elaeophora* in the area is 35 to 36 inches per annum.

The lower rainfall limit of 27-28 inches per annum shown on Fig. 3 may be a little high. The species is present in small areas of deep infertile soil on the

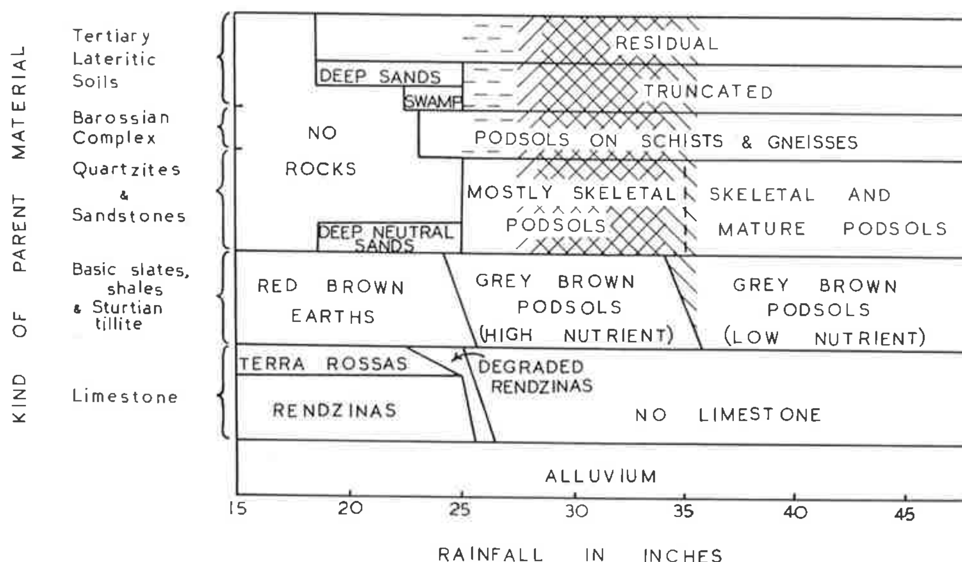

Fig. 5. Diagram illustrating the distribution of *Eucalyptus elaeophora* in relation to soils and rainfall. The figure is altered slightly from that given by Specht and Perry (1948); the schists and gneisses of the Barossian Complex are found in rainfall areas as low as 23 inches per annum. Hatching from right to left (downwards) indicates presence on south-facing aspects only. Cross hatching indicates presence on both aspects. Broken horizontal lines indicate presence only on soils of high water-retaining capacity.

Barossa Goldfields (Fig. 4), where, although meteorological records are scanty, the rainfall may be as low as 23 inches per annum. Here, however, the soil probably has better water-relationships than those found in areas of similar rainfall on Fig. 3, as it is a deep soil with a clay-loam, A horizon.

It appears then that *E. elaeophora* can grow on a wide range of infertile soils which characteristically support a dry sclerophyll forest. Exceedingly low fertility may exclude the species for it is not found on deep sands or on soils developed from Tanunda Creek granites where *E. baxteri* or *E. fasciculosa* survive. In general, the species extends from approximately 27 to 36 inches of rainfall per annum, although it may be found in drier areas (as low as 23 inches per annum) in moist pockets. This distribution is summarized in Fig. 5.

These climatic and edaphic limits give some clues which may explain why *E. elaeophora* is not found farther to the south. Much of the Ranges

TABLE 3.

Environmental factors controlling the distribution of *Eucalyptus elaeophora* in various parts of Australia.

State	Locality	Topography	Rainfall Limits	Soil	Reference	Mean monthly temperature (July and January)
S. Aust.	Hills surrounding Wilpena Pound (3 peaks on northern side including St. Mary's Peak)	Highlands: From 2,500 feet to the summits.	Approx. 26 inches p.a.	In rock clefts (Pound quartzites).	Brooker (personal communication.)	50-78°F*
	Elders Range (Flinders Range)	Highlands:—towards the summit.	Approx. 26 inches p.a.	In rock clefts (Pound quartzites).	Boomsma (1960)	50-78°F*
	Southern Flinders Ranges	Highlands (1,500 ft. to the summits)	Greater than 26 inches p.a.	Skeletal soil, podsols from quartzite.	Boomsma (1946)	48-73°F**
	Western Clare Hills	Highlands (1,500 feet)	27-30 inches p.a.	Skeletal soil, podsols (sandy loam over clay subsoil).	Boomsma (1949)	47-70°F†
	Mount Lofty Ranges	Highlands (800-1,800 ft.)	27-36 inches p.a. (may be lower on favourable soils).	Podsolic soils, laterites, etc.	Specht *et al.* this paper.	45-65°F††
Vic.	Dandenong Ranges	Ridges and steep slopes of Lilydale and Beaconsfield Hills. North and west aspects of Dandenong Range (300-1,700 feet).	30**-48 inches p.a.	Skeletal soil, podsols (silty loam and sandy loam). krasnozem.	Clifford (1953)	45-65°F
	S. Riverina	Highlands — south and east slopes (1,500-2,000 feet).	25-27 inches p.a.	Shallow soil with little profile development.	Moore (1953)	45-70°F
	Monaro Region	Highlands (1,500 - 3,000 feet).	19-26 inches p.a.	Well drained podsols and lithosols	Pryor (1938) Costin (1954)	40-70°F
All States	General	500-2,000 (or 3,000 feet).	18-20 inches p.a.	Rocky sites, poor slate and granite soils.	Rodger (1953)	45-70°F

Recorded temperatures at * Angorichina ** Georgetown † Clare, will be higher than at actual sites of *E. elaeophora* in the highlands; those of Mt. Crawford†† are characteristic for the centre of the sand in the Mt. Lofty Ranges. ††This is the minimum rainfall of the area surveyed; *E. elaeophora* extends into drier habitats throughout Victoria.

to the south, centred around the Mount Lofty massif, have a rainfall greater than 35 inches per annum (Specht and Perry, 1948, p. 93). The only areas which support the dry sclerophyll forest formation to the west of the isohyet are (1) Black Hill, (2) Rocky Hill, Morialta, (3) Stonyfell Ridge, until the Eden-Moana Fault Block is reached at Belair (Specht and Perry, 1948, p. 115). *E. elaeophora* is found in small pockets on Black Hill, not on the drier side where the soil is predominantly steep, skeletal quartzite, and thus a drier habitat than is at first apparent, but on the mature podsolic soils developed on the eastern side of the hill. Such mature podsols are rare on the west side of

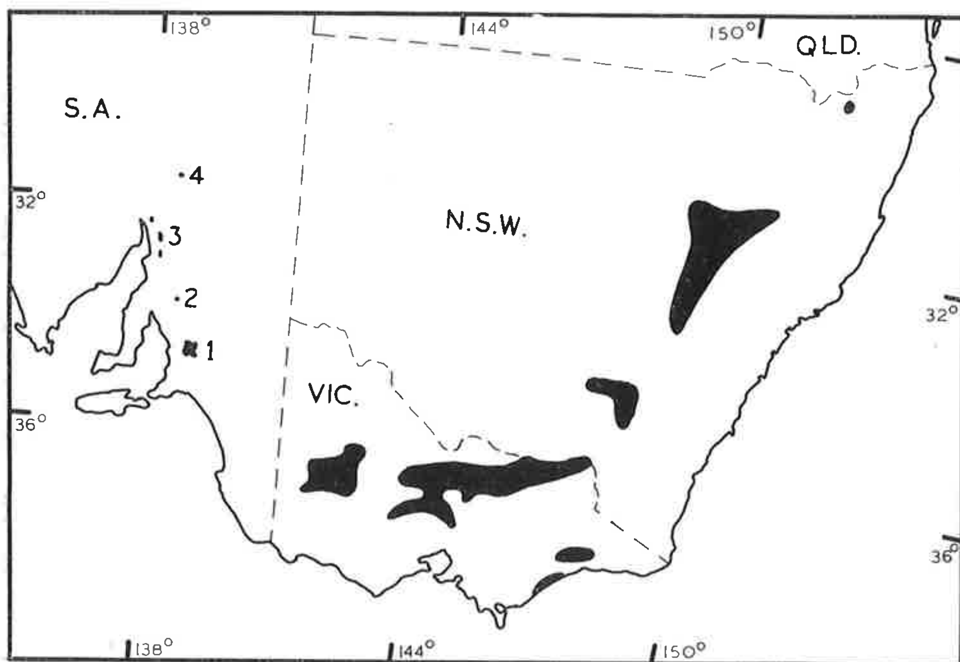

Fig. 6. Distribution of *Eucalyptus elaeophora* throughout Australia. The areas 1 to 4 in South Australia are discussed by Specht *et al.* (this paper), Boomsma (1949), Boomsma (1946), and Boomsma (1960), respectively. The distribution in the eastern States is mainly after Clifford (1953).

Rocky Hill and Stonyfell Ridge where the rainfall is less than 35 inches per annum. In addition to the effect of distance on the availability of seed, it is unlikely that these sites are suitable habitats for germination and establishment of *E. elaeophora*.

Suitable soils are even less common on the eastern sides of the Ranges in areas where the rainfall falls below 35 inches per annum (Fig. 2). Small ridges like Mount Charles and Mount Barker are found, but they are so isolated from *E. elaeophora* stands that the species has not yet established itself. To the north of its present distribution in the Mount Lofty Ranges, both the soils (solonized sands in the Barossa Valley and very infertile soils on Tanunda Creek granites) and the climate appear to be so unsuitable as to preclude further spread of the species.

It appears then that this relic species has effectively occupied most suitable sites within the area and is prevented from expanding both to the north and the south by unfavourable climate and soils.

(2) *In Other Localities in Australia*

Fig. 6 indicates the scattered distribution of *E. elaeophora* throughout Australia. It is found in disjunct areas extending in an arc from the highlands near Queensland, through New South Wales, Victoria to the Mount Lofty Ranges and then up into the Flinders Ranges. The species is thus one of the many examples which indicate that major climatic changes have occurred in Australia in the not-so-distant past (Crocker and Wood, 1947).

Some environmental factors which may control the distribution of the species in these disjunct areas are summarized in Table 3. In all areas noted in this table, *E. elaeophora* is found in either a dry sclerophyll forest formation or in closely allied communities. It is usually found on ridges, always on infertile podsolic or related soils, the depth of which depends largely on the rainfall, e.g. deeper soils on the drier sites; shallow, skeletal, or exposed soils on the wetter sites. In all cases, the soils are well-drained.

As Rodger (1953) indicated, *E. elaeophora* is found in a wide range of climates from Mediterranean type with winter rainfall (South Australia), through a climate where rainfall is uniform throughout the year (Victoria-southern New South Wales), to a climate where summer rainfall tends to predominate (northern New South Wales).

In many areas, the highest rainfall where *E. elaeophora* occurs is far below its upper limit, e.g. in Flinders Ranges, Clare Hills, and S.E. Riverina. However, in the Mount Lofty Ranges, and the Monaro Region, the upper rainfall limit is certainly reached. It can be seen in Table 3 that under conditions of winter rainfall the *E. elaeophora* generally occurs in areas receiving a higher mean annual rainfall than it does under conditions of uniform or summer rainfall incidence, e.g. the limits of mean annual rainfall for the distribution of *E. elaeophora* in the Mount Lofty Ranges (winter rainfall incidence) and in the Monaro Region (uniform rainfall incidence) are compared below:

	Mean Annual Rainfall (in.)		Ratio Mean Annual Rainfall
	Mount Lofty	Monaro Region	Monaro Region/Mt. Lofty
Lower Limit	27	19	0·73
Upper Limit ...	36	26	0·72

A possible exception to the above is the reported occurrence by Clifford (1953) of *E. elaeophora* in areas in the Dandenong Ranges, Victoria, with uniform rainfall incidence receiving up to 48 inches mean annual rainfall. However, *E. elaeophora* hybridises freely with *E. goniocalyx*, a species common in wetter areas, thus making it difficult to determine the limits of the two species (Clifford and Binet, 1954). The upper limit of the mean annual rainfall given by Clifford (1953) may be too high.

The mean annual rainfall probably gives a poor indication of the water available at any particular time of the year for the maintenance of stands of *E. elaeophora*. Eucalypts generally make most active foliage growth in the summer (Specht and Rayson, 1957; Burbidge, 1960) and losses of moisture due to transpiration are highest at this period (Martin, 1960). It would, therefore, be expected that the amounts of water available (as rain and stored soil moisture) would be more critical at this time of year than at any other for the maintenance of stands of *E. elaeophora*. Thus, the amounts of available water at each month in localities where *E. elaeophora* occurs under conditions of different

rainfall incidence have been calculated by the method used by Martin (1960) and are shown in Fig. 7.

It has been necessary to use the relationship established by Martin (1960) between amount of available water and the Index of Evapotranspiration in communities of *E. elaeophora* in the Mount Lofty Ranges throughout all calculations, as, due to lack of data, the relationship has not been determined in other areas. In all these calculations it has been assumed that up to four inches of water can be stored within the root zone without loss by drainage.

It can be seen in Fig. 7 that at Queanbeyan or at Tarcutta (uniform rainfall incidence) the amounts of available water during the summer months (viz. January, February and March) are about twice as great as at Mount Crawford or at Clare (winter rainfall incidence), even though the mean annual rainfall at the sites of *E. elaeophora* at Mount Crawford (28 inches) or at Clare (27-30 inches)* is greater than at Queanbeyan (22 inches) or at Tarcutta (26 inches).

In winter rainfall areas with a mean annual rainfall less than Mount Crawford, it is conceivable that the amount of available water during the summer months may fall too low to support growth of *E. elaeophora*. Summer rainfall, however, enables this species to grow in areas of even lower annual precipitation.

DISTRIBUTION OF OTHER EUCALYPTS

All the other eucalypts found north of the Torrens Gorge have distribution patterns almost identical with those indicated by Specht and Perry (1948) for the area to the south.

A. *Species of the Savannah Woodland*

1. *Eucalyptus odorata* is found in only the north-west corner of Fig. 3 (Section 2165, Hd. Yatala, with a few isolated trees also in Sections 2180 and 5575). However, in the drier habitats to the west of the area, it is the dominant tree in the savannah woodland formation.

It is common along the uncleared areas of the Para scarp (e.g. Sections 2102-5, 2108, 2110, 2114-6, 2144, 2152 and 2284, Hd. Yatala, as well as areas to the north), and in small pockets on the Para Fault Block (Sections 2148, 2167 and 1560, Hd. Yatala), which have escaped agricultural development. Much of this area lies within the 20 to 25-inch isohyets on reasonably fertile soils. In the areas under survey, the species rarely extends into sites with rainfall as high as 30 inches per annum, as was noted by Specht and Perry further to the south, e.g. into the Belair National Park. The small stands in Sections 2180 and 5575, as well as clumps of hybrids (*E. odorata* x *E. leucoxylon*, similar to those recorded by Pryor, 1955a, in the vicinity of Burnside) in Sections 5461 and 5476, Hd. Yatala, were the only trees observed in the higher rainfall zone (25-30 inches per annum). This was not surprising as most of the fertile soils of this zone were deep, alluvial soils probably with a high water-retaining capacity, quite unlike the shallow, almost skeletal, soils of National Park.

2. *Eucalyptus leucoxylon* is a common tree in the savannah woodland formation between the 25 to 30 inch isohyets (Figs. 3 and 4). Here the species

* The mean annual rainfall estimated by Boomsma (1949) for the actual sites of *E. elaeophora* in the Western Clare Hills, given above, is greater than that recorded at Clare, from where the mean monthly data were obtained for the calculation of the available water at each month. The difference between the means of annual rainfall at Clare and at the actual sites of *E. elaeophora* can be attributed to differences in their altitudes. This topographic effect would be much more significant in the winter months when most of the rain is received in this district, and the effect in summer months would be almost negligible. The amounts of available water at each month during the summer would, therefore, be expected to be approximately the same at Clare as at the actual sites of *E. elaeophora*.

forms pure stands over the rolling countryside of the Para Fault Block and again on the broad, fertile ridges of the Eden Fault Block. The deeper soils of these broad ridges, as mentioned above, have a high water-retaining capacity, thus excluding *E. odorata* in favour of *E. leucoxylon*. This is markedly in

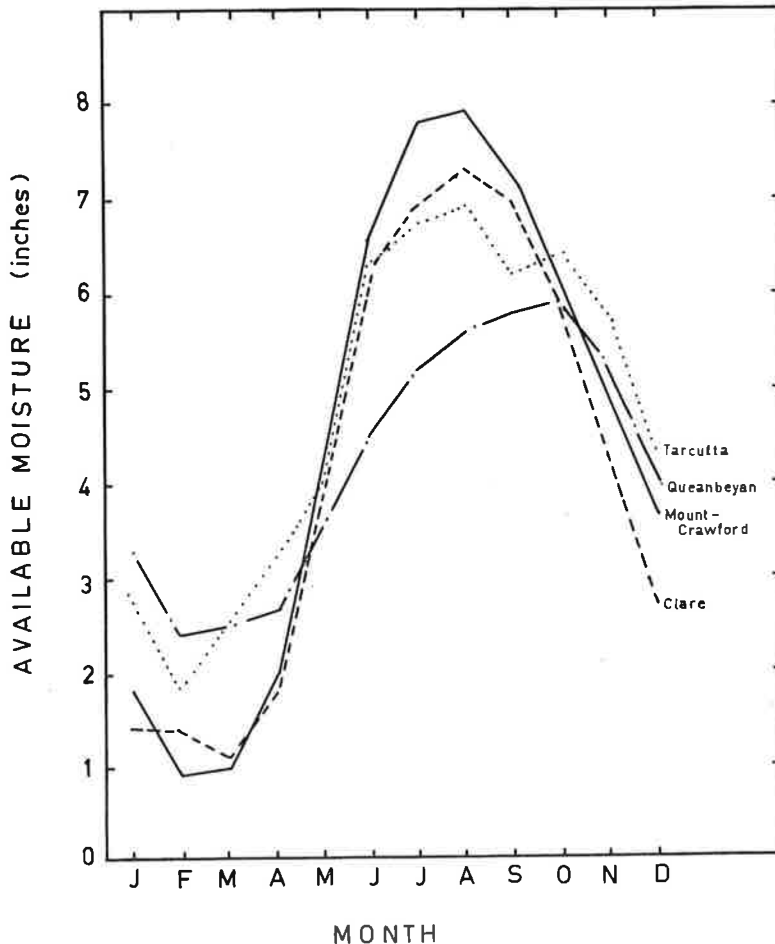

Fig. 7. The amounts of available moisture (rainfall plus stored moisture) in localities where *E. elaeophora* occurs. The amounts of available moisture for each month were calculated by the method used by Martin (1960). The relationship between the amount of available water and Index of Evapotranspiration in communities of *E. elaeophora* in the Mount Lofty Ranges established by Martin (1960) was used in all calculations, owing to lack of data from other areas. It was assumed that up to four inches of water could be stored within the root zone without loss by drainage.

contrast to the sharply dissected topography further south where shallow soils and aspect play a large part in the distribution of the two species in this climatic zone (Specht and Perry, 1948).

In areas with rainfall greater than 30 inches per annum, the species is restricted to exposed ridge tops, the rest of the savannah woodland is dominated by *E. camaldulensis*.

The species is also found in the more fertile valleys of the Archaeozoic complex, especially around Mount Gawler, where the vegetation is typically a dry sclerophyll forest formation. This distribution is identical with the stands of *E. leucoxylon* tonguing into the dry sclerophyll forest at Blewitt Springs to the south (Specht and Perry, 1948).

3. *Eucalyptus viminalis* is almost invariably found on southern aspects within the 30 to 36 inch isohyets, in habitats identical with those in which it is found south of the Torrens Gorge. Its distribution north of the Torrens Gorge is markedly restricted. *E. camaldulensis* appears to occupy all suitable habitats.

The hybrid, *E. viminalis* x *E. aromaphloia* (Pryor, 1955b), formerly referred to as *E. viminalis* var. *huberiana*, is found on the quartzitic ridges running north from Mount Torrens and again north of Mount Crawford. These hybrids are in communities allied to a dry sclerophyll forest and appear to replace *E. elaeophora*, rare trees of which may be found among the hybrids.

4. *Eucalyptus camaldulensis* confined to stream-beds below 27 inches per annum spreads out over both north and south aspects of most hills with rainfall between 27 to 35 inches per annum. As the headwaters of the Torrens and Para Rivers lie within this rainfall belt, much of the savannah woodland of the area is dominated by *E. camaldulensis*, with only small pockets of *E. leucoxylon* and *E. viminalis.*

The environmental range is identical with that indicated by Specht and Perry (1948).

5. *Eucalyptus hemiphloia* var. *microcarpa*. Three specimens of this tree were found to the north of the Barossa Reservoir (Fig. 4).

6. *Callitris preissii*. Numerous small stands of this species occur on the shallow Eocene lacustrine sands in the Golden Grove and Barossa Goldfield areas (Figs. 3 and 4). In both areas the annual rainfall is about 25 inches.

B. *Species of the Dry Sclerophyll Forest*

1. *Eucalyptus fasciculosa* occurs in almost pure stands in the dry sclerophyll forest formation on the infertile Golden Grove lacustrine sands, the quartzitic soil of the Eden (Ansteys Hill) scarp, and the lateritic soils of the Barossa Goldfields. Above a rainfall of 30 inches per annum, the species is intermixed with *E. elaeophora* and *E. obliqua*. In the wettest locality east of Mount Gawler (approximately 36 inches per annum) the species is only a minor component in the exposed habitats of the *E. obliqua* forest.

This distribution is similar to that observed by Specht and Perry (1948) to the south of the Torrens Gorge.

2. *Eucalyptus baxteri* was found by Specht and Perry (1948) to occupy only the most impoverished soils where the rainfall was above 30 inches per annum. Such habitats are rare north of the Torrens Gorge. One small area of lacustrine sandstone is found on the eastern slopes of Mount Gawler; the Tanunda Creek granites are also very low in nutrients. *E. baxteri* is found on both these sites (Fig. 1), and, as yet, has not been recorded anywhere else in the dry sclerophyll forest of this area.

The disjunct distribution of this species is of note: Black Hill, Mount Gawler, Tanunda Creek.

3. *Eucalyptus obliqua*, noted by Specht and Perry (1948) as being present only above 33 inch isohyet (widespread above 35 inches per annum) on infertile soils, occupies similar habitats on the Archaeozoic rocks from the River Torrens south of Millbrook Reservoir to Mount Gawler. On the ridge to the west of Millbrook Reservoir and on the eastern slopes of Mount Gawler the species is almost the sole tree of the dry sclerophyll forest. Elsewhere, below 35 inches per annum, it is co-dominant with *E. elaeophora* and *E. fasciculosa*.

C. *Species of the Sclerophyll Shrub Woodland (Mallee)*

1. *Eucalytus odorata* var. *angustifolia* was recorded by Specht and Perry (1948) from two small areas on sandy, detrital soils at the foot of Black Hill. The same "whipstick mallee" is found in several stands in the Barossa Goldfields (Fig. 4), on sandy soils and almost identical rainfall (25 inches per annum).

2. *Eucalyptus gracilis.* This species, together with *Melaleuca uncinata*, is found on the solodic sands of Eocene lacustrine origin in the Barossa Goldfields (Fig. 4).

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Publication 2

Sodium as an Essential Micronutrient Element for a Higher Plant (Atriplex vesicaria)[1]

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Introduction

This paper presents evidence that sodium is essential for the growth of *Atriplex vesicaria* Heward ex Benth. (Bladder salt bush). Prior to a preliminary report of this work (4), Allen and Arnon (1) had shown sodium to be essential only for the blue-green alga *Anabaena cylindrica*. The optimum growth of the alga was obtained in media containing 218 μeq/liter (5 ppm) or higher of sodium. The requirement for sodium was specific; lithium, potassium, rubidium, and caesium did not substitute for sodium.

The effects of sodium on higher plants have not been clear. In many observations the dry weight production of plants growing in the field or in soil culture has increased following the application of salts of sodium. The literature dealing with such observations has been reviewed by Harmer and Benne (5), Lehr (9,10) and Wybenga (17). The results of these investigations although of possible economic significance give little information on the actual role of sodium in plant nutrition. Such increases in yield could have been due to the effects of the salt in either modifying the soil, or in increasing the uptake of the other ions or to the anion associated with the sodium being involved in the nutrition of the plant.

An increase in dry weight occurs following the application of salts of sodium to various higher plants growing in low potassium culture solutions (13). This would suggest that sodium partially replaces potassium in some species. There are also suggestions in the literature that the dry weight of some plants, mostly members of *Chenopodiaceae*, increases in solutions containing adequate potassium following the application of sodium (6,8,14,16). This latter evidence suggests that sodium may have an independent role as a nutrient element.

The possibility existed that higher plants might require sodium, but in such small amounts that these needs were always satisfied where plants grow under natural conditions. If so, evidence that sodium is essential for higher plants would be obtained by studying their growth only under conditions in which sodium was rigorously excluded. Such conditions, due to the ubiquity of sodium, are difficult to achieve, and the possible sources of sodium, the seed, nutrient salts, water, culture vessels, and the atmosphere, were critically examined.

This paper describes techniques used to reduce the sodium contamination from these sources and a series of experiments in which sodium was shown to be essential for *Atriplex vesicaria*. This species occupies large areas of arid Australia, and was chosen for investigation as it accumulates large quantities of sodium in its leaves (15).

Materials and Methods

Determination of Sodium. Sodium was determined in distilled water by carefully evaporating it down to one-thousandth of its original volume in a silica beaker. A filter flame photometer was used in these determinations.

When estimating sodium with a filter flame photometer in solutions of nutrient salts, particularly in those of calcium and potassium, the relative errors due to interference by other ions increased as the salts were progressively purified. Interference was also experienced to a less extent, in the estimation of sodium in solutions containing iron, boron and phosphate. Estimations of sodium with an atomic absorption instrument (3) were virtually free of interference but when estimating sodium in highly concentrated solutions of nutrient salts and digests of plant material, the response by the instrument to sodium was reduced. Under these conditions, it was necessary to prepare calibration curves showing the responses of the atomic absorption instrument to known concentrations of sodium in solutions similar to those in which sodium was to be estimated.

Sodium was estimated in plant material by the following method. From finely ground plant material 2 representative fractions were taken of less than 0.5 g. These were dried at 95°, until they had reached constant weight, and then placed in a desiccator. When at room temperature, they were reweighed and placed in quartz Kjeldahl tubes of approximately 15 ml capacity and digested with 1 ml H_2SO_4 (S.G. 1.86), 1 ml $HCLO_4$ (S.G. 1.70), and 5 ml HNO_3 (S.G. 1.42) (which had been redistilled in silica). The digestion was carried out in accordance with the method described by Piper (11). When the digestion was complete, the digest was made up to a suitable volume with distilled water, and the concentration of

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sodium determined with the atomic absorption instrument. Digests from material containing only very low concentrations of sodium were made up to small volumes so that sodium would be at a concentration high enough to be determined with the atomic absorption instrument. It was found that the response of the instrument to known concentrations of sodium in these highly concentrated solutions was less than in water. The depression in the response to sodium in the solution was found to be attributable to the amounts of sulfuric acid used in the digestion of the plant material and calibration curves were prepared using similar amounts of sulfuric acid.

Purification of Water. Rainwater containing approximately 87 μeq/liter (2 ppm) of sodium was passed through a commercial deionizer consisting of columns of cation and anion exchange resins arranged in series. Treated water contained 3.5 μeq/liter (0.08 ppm) of sodium. This water after distillation in a silica still contained less than 0.0087 μeq/liter (0.0002 ppm) of sodium and was stored in stoppered polythene containers.

Purification of Air. In a preliminary experiment carried out in a glasshouse of conventional design, analysis of plant organs and culture solutions at the end of the experiment showed an increase of about 294 μeq of sodium above the amounts supplied in the culture solution and seeds (table I). This suggested that cultures had received sodium possibly as cyclic salt from the atmosphere. This possibility was tested by placing 4 filter papers horizontally in different sites within the glasshouse, and determining the increase in sodium of each paper at the end of each week of exposure when the papers were replaced by another set. The mean amount of sodium collected per cm^2 per week during the whole period was 0.02 μeq. Subsequent experiments were conducted in a small greenhouse designed to prevent contamination of plants and their cultures by sodium from the atmosphere. A slightly positive pressure was maintained within the greenhouse by a compressor which supplied air continuously to both the cultures for aeration and to the greenhouse itself, through a series of Whatman No. 1 filter papers and washed absorbent cotton wool contained in metal cylinders.

The amount of sodium known to have been present in the culture solution, water and seeds of cultures to which no sodium had been intentionally added was approximately the amount of sodium recovered in the culture solution and plant organs at the end of an experiment in this greenhouse (table I). No increase in the amount of sodium could be detected after the cultures had remained in the experimental greenhouse for 49 days.

All air entering the compressor was drawn through Whatman No. 1 filter papers which were changed at 24 hourly intervals and the sodium they had trapped determined. The amounts of sodium trapped per day (from about 10^5 liters of air) rose and fell periodically (see fig 1). It was found that the amounts were greatest when strong winds blew from the west (the seaward side). Under these conditions opening of the cabinet and manipulation of cultures was avoided.

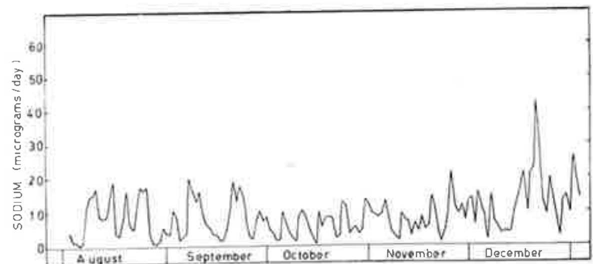

FIG. 1 Amounts of sodium trapped per day from air drawn through a Whatman No. 1 filter paper; about 10^5 liters of air passed through the filter per day.

Table I. *Sodium Supplied; Sodium Recovered*

Conditions of experiment	Amount supplied (μeq)				Amount recovered (μeq)				
	Seeds	Culture solution	Water†	Total	Leaves	Stems and petioles	Roots	Remaining in culture Solution	Total
In conventional glasshouse*	3 (10 Seeds)	43***	80	126	153	33	117	117***	420
In pressurized cabinet**	1.07 (4 Seeds)	5***	0.04	6.11	1.52	1.64	1.66	1.13***	5.95

* Ten plants of *Atriplex vesicaria* were grown in 4.5 liters of basal culture solution to which no sodium had been intentionally added. Experiment was of 93 days duration.

** Four plants of *Atriplex vesicaria* were grown in 2 liters of basal culture solution to which no sodium had been intentionally added. The experiment was of 48 days duration.

*** These data were obtained using emission flame photometry. Due to positive interference from ions in the culture solution, these values are generally higher than they should be.

† Water used in the experiment in the glasshouse contained 4 μeq Na/liter and water used in the pressurized cabinet contained 0.010 μeg Na/liter. Amounts of sodium were calculated on the total volume of water supplied to the culture during the experiment.

Preparation of Nutrient Salts Containing only Minute Amounts of Sodium. The following procedures were used in the preparation of nutrient salts containing only the smallest contaminations of sodium. In table II, concentrations of sodium in solution of purified salts and untreated salts are compared.

Calcium Nitrate: About 700 g calcium salicylate were prepared by slowly adding 500 g salicylic acid and 200 g calcium carbonate simultaneously, with rapid stirring to 2 liters of distilled water at approximately 95°. The resulting solution was heated with rapid stirring until the evolution of CO_2 ceased, and was then filtered, while still hot, through Whatman No. 1 filter paper at a reduced pressure. The filtrate, run into 800 ml silica evaporating dishes, was placed in a refrigerator overnight. The calcium salicylate was recrystallized a further 8 times. The crystals obtained were transferred to a platinum vessel and heated strongly over a bunsen burner. After a short time, the calcium salicylate burst into flames, leaving black ashes. These were then placed in a muffle furnace and heated for about 5 hours at 500°. When the contents of the vessel had been converted to a white powder of calcium oxide free from particles of carbon, the vessel was placed in a desiccator and cooled to room temperature. This was quickly weighed and an equivalent amount of HNO_3 (which had been redistilled in silica) was slowly added to the oxide to form a solution of calcium nitrate. A slight excess of calcium oxide was added to ensure complete neutralization of the acid. The concentration of calcium of this solution was compared with that of a standard solution of calcium nitrate by flame photometer. The solution, diluted to give a concentration 400 times that required in the full culture solution was stored in a stoppered polythene bottle.

Potassium Nitrate: About 100 to 400 g of KNO_3 to be purified was dissolved in twice distilled water in a silica vessel to give an almost saturated solution at 90°. After filtering the solution through Whatman No. 1 filter paper held in a polythene funnel into a silica evaporating dish, the solution was cooled to room temperature and placed in a refrigerator overnight. The crystals formed were washed quickly with chilled distilled water, after the supernatant fluid had been discarded. The recrystallization process was repeated up to 12 times without further filtration and the resulting crystals were dried in an oven at 60° and placed in a desiccator.

Potassium Dihydrogen Phosphate: KH_2PO_4 was recrystallized 6 times by the procedure described for the recyrstallizations of KNO_3.

Diammonium Phosphate: About 80 ml of phosphorus oxychloride (b.p. 105°) was redistilled (in silica) and collected in a platinum vessel. The amount of phosphorus oxychloride equal to a third of its molecular weight (51.13 g) was weighed out and an equivalent amount of twice distilled water (in silica) was cautiously added with a silica pipette. The resulting solution was then boiled with slight excess of water until no further HCl was evolved. The normality of the phosphoric acid produced was found to be 3.77 by carrying out a potentiometric titration of an aliquot against a standardized solution of NaOH. By the addition of 31.8 ml of the 3.77 N phosphoric acid to 6 ml of 6.67 N ammonium hydroxide (redistilled in silica). 10.55 g of diammonium phosphate was formed. When this was made up to 100 ml it had a concentration 400 times as great as that in the full concentration culture solution and had a pH of 8.10.

Magnesium Sulfate and Manganese Sulfate: $MgSO_4$ was recrystallized 6 times and $MnSO_4$ 5 times by the procedure described for KNO_3.

Boric Acid: A saturated solution of boric acid in ethanol (redistilled in silica) was made up in the boiling flask of a silica still. The boron was vola-

Table II. *Sodium Contributed to the Culture Solution as Impurities of Component Salts before and after Purification*

Salt	Conc. of salt in culture solution (μM)	Sodium contributed to culture solution by component salts (μeq/2-l culture)	
		Untreated analytical reagent salts	Prepared salts
Calcium nitrate	4,000	4.35	0.0174
Potassium nitrate	5,000	0.52	0.0109
Potassium dihydrogen phosphate	1,000	2.18	0.0174
Diammonium phosphate	1,000	0.52	0.0347
Magnesium sulfate	1,000	0.26	0.00174
Boric acid	46	0.0026	0.00087
Manganese sulfate	9.1	0.0252	0.01320
Copper sulfate	0.31	0.00026	0.00026
Zinc sulfate	0.76	0.0065	0.000435
Ammonium molybdate	0.10	0.00022	0.000218
Ferric ammonium ethylene tetra acetate	90	1.39	0.0347
Ammonium chloride	350	0.00569	0.00565
Total sodium in culture solution due to sodium impurities of all component salts		9.26	0.137

tilized as the ethyl ester of boron and the distillate collected in a platinum vessel. After slowly drying the distillate in a water bath, boric acid remained. This was placed in a desiccator until its weight was constant, then made up into a stock solution 10,000 times as concentrated as it was in the final solution. The concentration of sodium was reduced from 435 meq/Kg (10,000 ppm) in boric acid (to which sodium had been previously added) to less than 218 μeq/Kg (5 ppm) in boric acid purified in this way.

Copper Sulfate, Zinc Sulfate, and Ammonium Molybdate: Solutions of copper sulfate, zinc sulfate, and ammonium molybdate were made up from A. R. grade salts 20.000 times as concentrated as they were required in the final culture solution without purification as the amounts of sodium they contributed to the culture solutions were extremely small.

Iron: Iron was supplied to cultures in a single addition of ferric ammonium ethylene diamine tetra acetate. This was prepared by a method similar to that of Jacobson (7) for the preparation of ferric potassium ethylene diamine tetra acetate except that ammonium hydroxide was used in the place of potassium hydroxide in an equivalent amount. KOH, which contained much sodium as an impurity would have been difficult to purify, whereas the ammonium hydroxide redistilled in silica contained an amount of sodium too small to be detected. Other compounds used in the preparation of ferric ammonium ethylene diamine tetra acetate were purified by the following methods. Ferrous sulfate was recrystallized 6 times from solutions acidified with small quantities of H_2SO_4, and the resulting crystals were dried in an oven at 50°. Ethylene diamine tetra acetic acid was dissolved in 2 N ammonium hydroxide (redistilled in silica) and then precipitated by the addition of 2 N HCl (redistilled in silica). This procedure was repeated 4 times and the resulting precipitate was washed in several changes of distilled water and dried in an oven at 50°.

Ammonium Chloride: NH_4Cl was formed by the addition of ammonium hydroxide to an equivalent amount of HCl (both redistilled in silica). The resulting solution was concentrated by boiling, cooled to room temperature, and placed in a refrigerator overnight. The ammonium chloride crystals formed were dried in a desiccator to constant weight and made up in a stock solution 20,000 times the concentration required in the full concentration culture solution.

Culture Apparatus. Seeds were germinated on nylon gauze sewn onto a circle of polythene tubing to form a flat disc which was supported by polystyrene legs in a circular polythene vessel. Water or culture solutions in this vessel were aerated through a fine bore silica tube.

Culture vessels of 2-liter capacity were made from half-gallon polythene containers by cutting off their tops.

The vessels had covers of black or grey Perspex which held 4 evenly spaced plants, secured by white terylene (equivalent to Dacron) fibres washed in many changes of silica distilled water, clamped between split corks made from polythene tubing. Cultures were aerated continuously, with air filtered through cotton-wool and bubbled through frequently changed distilled water and filter papers, by means of centrally placed silica tubes dipping to the bottom of culture vessels.

Paper, black on 1 side and white on the other, was wrapped around each culture vessel to exclude light from the culture solution and roots of the plants.

Samples of all materials associated with the cultures were boiled in small amounts of concentrated HNO_3 (redistilled in silica); the amounts of sodium extracted by this drastic treatment were small in all cases.

Composition of Culture Solution. The composition of the basal culture solution, expressed in μmoles/liter was as follows: KNO_3, 5,000; $Ca(NO_3)_2$, 4,000; $MgSO_4$, 1,000; $(NH_4)_2$ HPO_4, 1,000; KH_2PO_4, 1,000; H_3BO_3, 46; $MnSO_4 \cdot 7H_2O$, 9.1; $CuSO_4 \cdot 5H_2O$, 0.31; $ZnSO_4 \cdot 7H_2O$, 0.76; $(NH_4)_6$ Mo_7 $O_{24} \cdot 4H_2O$, 0.1; NH_4Cl, 350. Iron was supplied as the ferric ammonium ethylene diamine tetra acetate (see above) at 90 μmoles/liter in the basal culture solution.

Procedure. Seeds of *Atriplex vesicaria* were removed from their bracteoles and washed in many changes of distilled water until the amount of sodium in the wash water could not be detected with the flame photometer (adjusted to its maximum sensitivity); the seed then contained a mean amount of 0.27 μeq of sodium per seed. The washed seeds were germinated on distilled water in the seedling culture. After the emergence of the radicle, the distilled water was replaced by the basal culture solution of one-fifth full concentration. When 11 to 14 days old, the seedlings which had acquired cotyledons and apical buds were selected for uniformity and transferred to the polythene culture vessels containing 2 liters of culture solution. Different treatments were applied at this stage.

At harvests, the tops of plants were removed by severing the hypocotyl at the level of the top of the cork. The tops were rapidly separated into leaf-blade and stem and petiole fractions, which were weighed immediately to obtain their fresh weight. The roots were removed from the culture vessels, and, after they had been dried between cleansing tissues, they were dried on aluminum foil trays (previously washed in distilled water and dried) placed in a well-ventilated oven at 95°. After 36 hours the various fractions were cooled in a desiccator to room temperature and their dry weights obtained.

Results

Experiment A. Effect of Small Graduated Amounts of Sodium on Growth of Atriplex vesicaria.

Table III. *Yields of Atriplex vesicaria following the Application of the Sulfates of Sodium and Potassium*

All values are the means of yields from 4 2-liter cultures of 4 plants each. The statistical treatment of total dry weight data was as follows: II > I at 1 % level of significance; III > I at 0.1 % level of significance; III > II at 5 % level of significance.

Treatment	Fr wt/vessel (g)				Dry wt/vessel (g)			
	Leaf-blades	Stems and petioles	Roots	Total	Leaf-blades	Stems and petioles	Roots	Total
I. No addition	0.301	0.021	0.221	0.543	0.0324	0.0042	0.0153	0.0519
II. 0.02 meq/liter Na_2SO_4	2.101	0.144	1.441	3.686	0.2354	0.0212	0.0913	0.3479
III. 0.10 meq/liter Na_2SO_4	2.926	0.224	2.148	5.298	0.3468	0.0324	0.1357	0.5149
IV. 0.60 meq/liter Na_2SO_4	2.940	0.228	2.940	6.108	0.3475	0.0355	0.1286	0.5116
V. 0.60 meq/liter K_2SO_4	0.436	0.019	0.326	0.781	0.0433	0.0031	0.0206	0.0670

Different treatments were applied to the cultures in each of 4 blocks (table III).

The concentration of sodium in the full concentration of culture solution due to the sodium contributed by potassium sulfate was reduced from 7.1 to 0.039 μeq/liter by recrystallizing the potassium sulfate 5 times. The cultures within each block were placed in random positions at the beginning of each experiment.

By the twenty-fifth day after germination, plants which had not received sodium sulfate could be distinguished from those which had, by their yellow color and fewer leaves each of smaller area. White necrotic areas appeared along tips and margins of the cotyledons and older leaves on the thirtieth day. Some plants died by the thirty-fourth day. A plant which showed symptoms just described and another which had died, were examined by plant pathologists at the Waite Agricultural Research Institute for the presence of pathogenic organisms. None were found in these plants. When harvested on the forty-eighth day, plants which had received sodium sulfate appeared markedly different from those which had not, having many more leaves of darker green colour which showed no necrosis (fig 2).

The difference between the root systems of plants grown with and without the addition of sodium sulfate was observable at a very early stage (fig 3).

From the results shown in table III obtained when plants were harvested on the forty-eighth day, the yield is seen to have increased asymptotically with increasing sodium sulfate. As plants which received 0.60 meq/liter of potassium sulfate, a concentration equivalent to the highest concentration of sodium sulfate treatment in their cultures, could not be distinguished from the plants grown in the "no addition" cultures, it was evident that the increase in yield with increasing sodium sulfate was not due to the sulfate but to the sodium of the sodium sulfate treatment. This also showed that the part played by sodium in the nutrition of *Atriplex vesicaria* could not be performed by additional potassium when supplied in an amount equivalent to the highest sodium sulfate treatment. The lowest sodium sulfate treatment for maximum dry weight production was about 0.2 meq/2-liter culture and the leaf material contained about 80 μeq/g (dry basis). Although these data would be expected to vary marked-

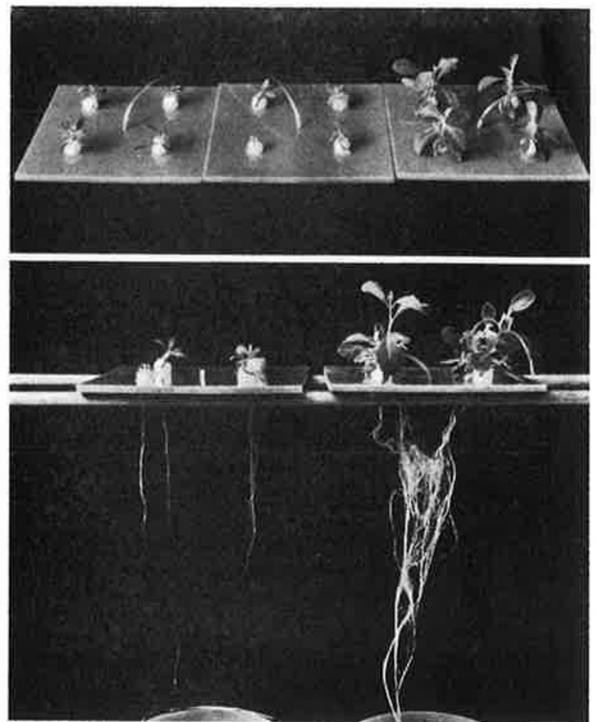

FIG. 2 Comparison between the growth of tops of plants of *Atriplex vesicaria* growing in the basal culture solution with the addition of 0.60 meq/liter potassium sulfate (left), with no addition (center) and with 0.02 meq/liter sodium sulfate (right). The plants had a height of approximately 2.5 cm (left), 2.5 cm (center) and 5.1 cm (right). Photograph was taken on the forty-eighth day.

FIG. 3. Comparison between the top and root growth of plants grown in the basal culture solution which received no addition (left), and 0.60 meq/liter sodium sulfate (right). The heights of the tops of the plants were approximately 2.5 cm (left) and 6.4 cm (right). The photograph was taken on the forty-eighth day.

ly according to the conditions of the experiment, the sodium requirements by *Atriplex vesicaria* were high in comparison with the requirements of micronutrients by plants of other species (17).

Recovery of Sodium Deficient Plants of Atriplex vesicaria Following the Application of Sodium. Fourteen days after germination, plants selected for uniformity were transferred from seedling cultures to culture vessels containing the basal culture solution without added sodium. On the sixteenth day sodium sulfate (0.10 meq/liter) was added to 1 set of culture vessels and 7 days later plants growing in these cultures could be distinguished from the controls by their darker green color. Symptons similar to those obtained in the previously described experiment again appeared in cultures which had not received sodium, and by the thirty-first day, when a second set of deficient cultures received a treatment of 0.10 meq/liter of sodium sulfate, symtoms were severe.

Four days after receiving this delayed sodium treatment, plants showed signs of recovery by a progressive change of color in older leaves (and in some cases cotyledons) from yellow to green; greening commenced at tips and around midribs, and gradually spread over the laminae.

Plants growing in the set of cultures which received no sodium treatment throughout the experiment became progressively more chlorotic, making little further growth. On the other hand, marked growth occurred in both sets of cultures which received added sodium. When harvested on the forty-ninth day, the results in table IV were obtained.

The complete recovery of plants growing in cultures which received a small addition of sodium sulfate (even though they were adequately supplied with sulfate), is convincing evidence for sodium being an essential nutrient element for *Atriplex vesicaria*.

Experiment B. Effects of Lithium, Sodium, Potassium or Rubidium on Sodium-Deficient Plants of Atriplex vesicaria. On the fifteenth day after germination, seedlings selected for uniformity were transferred from seedling cultures to culture ves-

Table IV. *Fresh and Dry Weight Changes after Recovery of Sodium Deficient Plants of Atriplex vesicaria following the Application of Sodium Sulfate Treatments*

All values are the means of yields of 2 vessels of 4 plants each. The statistical treatment of total dry weight data was as follows: III > I at 1 % level of significance; II > I at 0.1 % level of significance; II > III at 5 % level of significance.

Treatment	Fr wt/vessel (g) Leaf-blades	Fr wt/vessel (g) Stems and petioles	Fr wt/vessel (g) Roots	Fr wt/vessel (g) Total	Dry wt/vessel (g) Leaf-blades	Dry wt/vessel (g) Stems and petioles	Dry wt/vessel (g) Roots	Dry wt/vessel (g) Total
I. No sodium sulfate	0.877	0.078	0.671	1.626	0.0894	0.0124	0.0481	0.1499
II. 0.10 meq/liter Na_2SO_4 applied on day 16	8.139	1.007	6.923	16.069	1.0236	0.1619	0.4810	1.6665
III. 0.10 meq/liter Na_2SO_4 applied on day 31	3.713	0.334	2.800	6.847	0.4713	0.0544	0.2133	0.7390

Table V. *Effects of Equivalent Amounts of the Sulfates of Lithium, Sodium, Potassium or Rubidium when Applied to Cultures of Sodium-deficient Plants of Atriplex vesicaria*

Plants were harvested on the forty-fourth day. The statistical treatment of total dry weight data was as follows: III > I, II, IV, V at 0.1 % level of significance; I, II, IV, V, indistinguishable.

Treatment	Sodium/2–1 culture (µeq) As impurity of basal culture solution	Sodium/2–1 culture (µeq) Due to treatment salts	Sodium/2–1 culture (µeq) Total	Yield (Each value is the mean of 4 replications) Mean dry wt/culture (g) Leaf-blades	Stems and petioles	Roots	Total
I. Control	0.14	(No addition)	0.14	0.179	0.030	0.061	0.270
II. Li_2SO_4 0.10 meq/liter	0.14	0.052 (Impurity)	0.192	0.163	0.027	0.051	0.241
III. Na_2SO_4 0.10 meq/liter	0.14	200 (Treatment)	200.14	0.761	0.183	0.288	1.232
IV. K_2SO_4 0.10 meq/liter	0.14	0.017 (Impurity)	0.157	0.169	0.024	0.049	0.242
V. Rb_2SO_4 0.10 meq/liter	0.14	0.069 (Impurity)	0.209	0.220	0.045	0.071	0.336

sels which were placed in the pressurized greenhouse. On the twenty-second day, when symptoms of sodium deficiency were clearly recognizable, 4 cultures each containing 4 plants were harvested. The mean dry weight per culture was 0.0187 ∓ 0.0012 g. On the same day, the differential treatments shown in table V were applied to the cultures of each of 4 blocks. The concentration of sodium in the culture solution due to the sodium associated with the treatment application is also given and was not greater than 0.035 μeq/liter (0.0008 ppm) in any of the treatments other than that of sodium sulfate. On the twenty-seventh day, plants which had received the sodium sulfate treatment showed signs of recovery. Plants growing in cultures which received no sodium treatments became progressively more chlorotic, making little further growth. On the other hand, marked growth occurred in the set of cultures which had received sodium.

Plants in untreated, and in lithium, potassium and rubidium sulfate treated cultures were indistinguishable. By the thirty-third day, some plants had died in the cultures which had not received sodium. The mean dry weights per culture of 4 plants obtained for each treatment on the forty-fourth day are shown in table V.

At the final harvest, the plants in cultures which received sodium sulfate had made about 4 times as much growth as the plants in the cultures which had received the other treatments. The results of this experiment show clearly that the essential function of sodium in the nutrition of *Atriplex vesicaria* cannot be performed by equivalent amounts of lithium, potassium, or rubidium. This finding is similar to that of Allen and Arnon (1) who showed that the blue-green alga, *Anabaena cylindrica* Lemm, has a specific requirement for sodium in its nutrition which is not satisfied by supplying any of the other group 1 elements in the same concentrations.

Uptake and Distribution of Sodium and Potassium in Plants of Atriplex vesicaria. The effects of graduated amounts of sodium sulfate on dry weight production and on the concentrations of sodium and potassium in leaves, stems and roots of *Atriplex vesicaria* are shown in table VI. Seedlings were transferred to cultures 9 days after germination, the different treatments applied on the tenth day, and the plants harvested on the fifty-first day.

The dry weight production increased asymptotically with increasing applications of sodium sulfate. The concentrations of sodium increased strikingly in all fractions, especially in the leaves where the increase was more than 100-fold when 0.60 meq/liter of Na_2SO_4 was supplied.

The concentrations of potassium in leaves, stems, and roots increased when 0.01 meq/liter of Na_2SO_4 was supplied, but decreased when the amounts of sodium sulfate were further increased to 0.60 meq/liter.

Discussion

Experiments have been described in which sodium was shown to be an essential nutrient element for *Atriplex vesicaria* according to the criteria of Arnon and Stout (2). Plants, protected from atmospheric contamination of sodium, grown in culture solutions containing only small amounts of sodium showed characteristic deficiency symptoms by the yellowing of their leaves and development of white necrotic patches on their tips and margins. Plants developed few or no secondary shoots and in some cases died at an early stage; no pathogenic organisms could be found in their tissues. Thus, the first of the criteria of Arnon and Stout (2), viz., "a deficiency of it makes it impossible for the plant to complete its life cycle," was satisfied.

In the second experiment described, sodium-deficient plants recovered after an application of a small amount of sodium which had been delayed until the onset of severe symptoms; recovery took about a week.

Table VI. *Effects of Treatments of Sodium Sulfate on Dry Weight Production and Concentrations of Sodium and Potassium in Leaves, Stems and Roots of Atriplex vesicaria*

The statistical treatment of total dry weight data was as follows: VI, V, IV, III, II > I at 0.1 % level of significance; VI > V at 5 % level of significance; II, III; III, IV; IV, V indistinguishable.

Treatment	Dry wt (g) All values are the mean of 5 cultures of 4 plants each				Conc. of sodium and potassium (meq/Kg) All values are the means of duplicate samples taken from 5 replicated cultures of each treatment					
					Sodium			Potassium		
	Leaves	Stems	Roots	Total	Leaves	Stems	Roots	Leaves	Stems	Roots
I. No sodium sulfate	0.0560	0.008	0.022	0.086	10.0	7.1	2.6	2,834	1,913	1,547
II. 0.01 meq/liter Na_2SO_4	0.257	0.043	0.098	0.398	47.8	6.5	6.5	4,450	2,583	1,442
III. 0.02 meq/liter Na_2SO_4	0.377	0.066	0.138	0.581	78.3	11.7	7.0	2,504	2,197	1,563
IV. 0.06 meq/liter Na_2SO_4	0.461	0.088	0.173	0.722	213.0	20.2	11.7	2,476	2,169	1,540
V. 0.10 meq/liter Na_2SO_4	0.489	0.089	0.193	0.771	295.7	51.0	29.1	2,225	2,205	1,683
VI. 0.60 meq/liter Na_2SO_4	0.685	0.149	0.267	1.101	1,129	338.7	257.8	1,688	1,934	1,445

The results of the third experiment showed that of the group 1 elements, lithium, sodium, potassium or rubidium only sodium brought about the recovery of sodium-deficient plants of *Atriplex vesicaria*. The plants receiving no sodium additions to their cultures could not be distinguished from those receiving the treatments of lithium, potassium or rubidium. It appeared, therefore, that the second of the criteria, viz., "such deficiency is specific to the element in question and can only be prevented by supplying this element," has almost certainly been satisfied.

The fulfillment of the third of the criteria (2), viz., "the element is directly involved in the nutrition of the plant quite apart from its possible effects in correcting some unfavorable microbiological or chemical condition of the soil medium," is difficult to achieve. However, plants in these experiments were grown in solution culture so that the sodium supplied in the treatments was more likely to have exerted its effects directly in the nutrition of the plant than if more complicated media had been used. Even so the possibility still exists that the sodium corrected an unfavorable chemical or microbiological condition of the culture solution, and this possibility cannot be dismissed until a specific essential role of sodium in the metabolism of the plant has been demonstrated. These experiments were of short duration so that the possible complicating effects due to the depletion of nutrients in the culture solution were avoided, and the risk of the heavy infection of the cultures by organisms such as algae, fungi and bacteria were minimized.

The potential sources of sodium to the plant are the culture solution, water, culture vessels, air in greenhouse and seed, which all require further critical examination if very low sodium conditions are to be achieved in cultures.

The salts of the culture solution contribute less than 0.07 μeq/liter (0 0016 ppm) of sodium to the solution. This amount is calculated from the sum of the impurities of sodium remaining in the concentrated solutions of the individual salts of the culture solutions. These were estimated with an atomic absorption instrument, which was working in some cases at the limits of its sensitivity. To make further progress in the purification of certain salts a more sensitive instrument would be needed to determine the efficiency of the procedures used.

The culture vessels appeared satisfactory as the amounts of sodium they contributed to the culture solution could not be detected, nor could any increase in the sodium in the water they contained be detected after open culture vessels had remained in the pressurized greenhouse for a fortnight. However, the concentration of sodium in water in a culture vessel after a fortnight of continuous aeration even when covered, increased by 0.4 μeq/liter (0.01 ppm). At this rate of contamination, the amount of sodium in the most highly purified solution used in this study would be increased many times in even a short experiment. However, in subsequent experiments the air used for aeration was effectively freed from sodium by passing it through distilled water contained in a train of plastic vessels.

Water from the silica still contained less than 0.0087 μeq/liter (0.0002 ppm) of sodium; this is low compared with the concentration of sodium in the culture solution and would not be an important source of sodium except in an experiment in which a large amount of water was used. The amount of sodium contributed to a culture by the seeds of *Atriplex vesicaria* was approximately 0.27 μeq per seed after washing in several changes of distilled water.

Summary

Methods are described by which plants were grown under conditions from which sodium was carefully minimized. Culture solutions prepared from purified salts contained less than 0.07 μeq/liter (0.0016 ppm) of sodium as an impurity and water contained less than 0.0087 μeq/liter (0.0002 ppm) of sodium.

Cultures were protected from atmospheric contamination by sodium in a small greenhouse maintained at a slightly positive pressure by a continuous supply of filtered air. Under these conditions it was not possible to detect any increase in the amount of sodium in a culture or its plants over the period of an experiment.

Characteristic deficiency symptoms developed on about the twentieth day by plants of *Atriplex vesicaria* Heward ex Benth. (Bladder salt bush) which had not received an application of sodium to their cultures. Leaves became chlorotic and developed necrotic patches at their tips and along their margins after which little further growth was made. By about the thirty-fifth day some plants died. Plants receiving 0.02 meq/liter (0.46 ppm) Na_2SO_4 made favorable growth and when harvested on the forty-eighth day had approximately 10 times the dry weight of plants which had not received sodium. Plants which had developed severe symptoms of sodium deficiency recovered within about 7 days of receiving an application of sodium to their culture solutions.

Only sodium of the group 1 elements effected the recovery of sodium-deficient plants of *Atriplex vesicaria*. Plants receiving equivalent amounts of lithium, potassium or rubidium in their cultures could not be distinguished from those to which no addition of sodium had been made.

It is concluded that small amounts of sodium are essential for the growth and development of *Atriplex vesicaria*.

Acknowledgments

The author is grateful to the late Professor J. G. Wood for the encouragement and advice he gave throughout this investigation.

Dr. R. L. Specht and Miss Margaret E. Jackman gave constructive criticism and practical assistance; Dr. A. Walsh and Mr. G. F. Box of the Division of Chemical Physics, C.S.I.R.O. especially designed and constructed an atomic absorption apparatus which made possible precise determinations of sodium in solutions containing high concentrations of other ions. Mr. A. F. Jenkinson carried out statistical analyses and figures 2 and 3 were prepared by Mr. K. P. Phillips of the Waite Agricultural Research Institute. Grateful acknowledgment is given for this help.

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Changes During Recovery from Sodium Deficiency in Atriplex

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Summary. Although the concentration of sodium in leaves of *Atriplex* plants increased rapidly after receiving sodium, no growth response was detectable for about 6 days. It was found that respiration rate increased to its maximum within 3 days. Chlorophyll content also increased from an early stage, whereas the concentrations of sugars and starch did not increase, and ratios of soluble to total nitrogen did not decrease until later.

The respiratory response appears to be specific to sodium as different salts of sodium caused similar responses, and no other univalent cation substituted for sodium. In addition, both growth response and respiration rate tended towards their maxima with the same concentration of applied sodium. The rate of anaerobic CO_2 production increased when sodium was fed to leaves, suggesting that the effect of sodium is in the glycolytic sequence.

The first decisive evidence that sodium is an essential element for plant growth was obtained by Allen and Arnon (1), who showed that small amounts of sodium are required by the blue-green alga, *Anabaena cylindrica*. Subsequently, it was found that sodium is essential for the angiosperm, *Atriplex vesicaria* Heward ex Benth (4, 5), and more recently, a similar requirement has been shown in certain other species of *Atriplex* (unpublished experiments).

Preliminary experiments indicated that there is a delay of about 6 days following the supply of sodium to sodium-deficient *Atriplex* plants before a significant increase in dry weight occurs. A metabolic response to sodium detected early in this period may represent a primary step in recovery, and hence contribute to an understanding of the function of sodium in the nutrition of *Atriplex*. Therefore, a study was made of the changes in leaf respiration rate, chlorophyll content, soluble and insoluble nitrogen fractions, and sugar and starch contents, following the addition of sodium to sodium-deficient plants.

Materials and Methods

Plant Culture. The species were *Atriplex nummularia* Lindl, and *A. inflata* F.v.M., and seeds were obtained from Griffith, New South Wales, and Renmark, South Australia respectively. *A. nummularia* was used except where otherwise indicated. The procedures for germination and growth of plants under conditions of low sodium have been described previously (4). The capacity of culture vessels was 2 liters. The age of plants in all experiments ranged from 34 days in summer to 46 days in winter. Except in 1 experiment in which a short-term response was sought, sodium was fed by the addition of sodium sulfate to the culture solution in amounts varying between 0.1 and 0.6 meq/liter. The concentration used is included with the appropriate experimental results.

Sodium Content. Sodium determinations in plant material were carried out on boiling water extracts (2) using an atomic absorption instrument (3).

Leaf Respiration. Respiration rate of leaf tissues was determined manometrically by measuring O_2 uptake of intact leaves (0.2-1 g fr wt) placed on moist, convoluted filter paper in the dark at 27°. Changes in rate of uptake over a period of 1 to 2 hours were small (fig 4).

For later experiments (see below) alternative methods of introducing sodium to the tissue were tested, the aim being to supply sodium to all cells in the tissue as rapidly as possible. The O_2 uptake of leaf disks (7 mm in diameter) of sodium-fed plants (0.6 meq/liter sodium sulfate 20 days previously) was measured under the following conditions: A) disks placed on moist, convoluted filter paper, 590 μl/g fresh weight per hour; B) vacuum infiltrated with water and placed on moist convoluted filter paper, 265 μl/g fresh weight per hour; C) submerged in water, 360 μl/g fresh weight per hour; and D) vacuum infiltrated and submerged, 282 μl/g fresh weight per hour. Thus, submerging or vacuum infiltrating the tissues markedly reduced the rate of O_2 uptake compared with that of the untreated tissue. This inhibitory effect of water on O_2 uptake is similar to that found by Yocum and Hackett (14) work-

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ing with aroid spadix, and Ohmura and Howell (12) working with corn, bean and barley tissues. In addition the rate of O_2 uptake by submerged tissues could be increased to that of the untreated tissues by replacing air in the flask with O_2, although the rates of O_2 uptake by untreated tissues in air and O_2 were similar. Even though this limitation to gas exchange can apparently be overcome, an increase in O_2 tension could conceivably induce other complicating effects. For this reason the sodium was introduced through cut stems. The cut ends of sodium deficient shoots were placed in sodium sulfate solution (2.2 meq/liter) for a period (1 and one-half hours) determined by observing the progress of a dye (eosin) into a similar shoot under the same conditions for transpiration. At the same time control shoots were placed in distilled water. Immediately following this treatment, the leaves were cut from the stems and the rate of O_2 uptake measured.

A similar method was used to feed sucrose, but in this experiment cut petioles of detached leaves were immersed in 0.1 M sucrose for 1 and one-half hours before O_2 uptake was measured. Sodium was added to the culture solution 43 hours before harvesting.

Anaerobiosis. CO_2 evolution by leaves was measured for at least 1 hour in Warburg vessels containing O_2-free nitrogen. After the anaerobic measurements, the leaves were returned to air, and the rate of O_2 uptake determined.

Chlorophyll. Samples of leaf material (0.3–6 g fr wt) were ground in cold 80 % (v/v) acetone and filtered. The OD of the solutions were measured at 645 and 663 mμ using a Shimadzu Type QR-50 spectrophotometer after adjusting the volume to give readings between 0.1 and 0.6. The concentrations of chlorophylls a and b and total chlorophyll were then calculated from the extinction coefficients reported by MacKinney (11).

Soluble and Insoluble Nitrogen Fractions. Fresh leaf material (0.5–3 g) was ground in ice cold 80 % (v/v) ethanol, centrifuged at 1350 × g for 4 minutes, and the nitrogen contents of both supernatant fraction and residue determined by the method of McKenzie and Wallace (10).

Sugars and Starch. Harvests were made at noon. Sugars were extracted from leaves (0.5–2 g fr wt) with boiling 80 % (v/v) ethanol, the starch solubilized from the residue with dilute perchloric acid, and both were then estimated using the anthrone method (9).

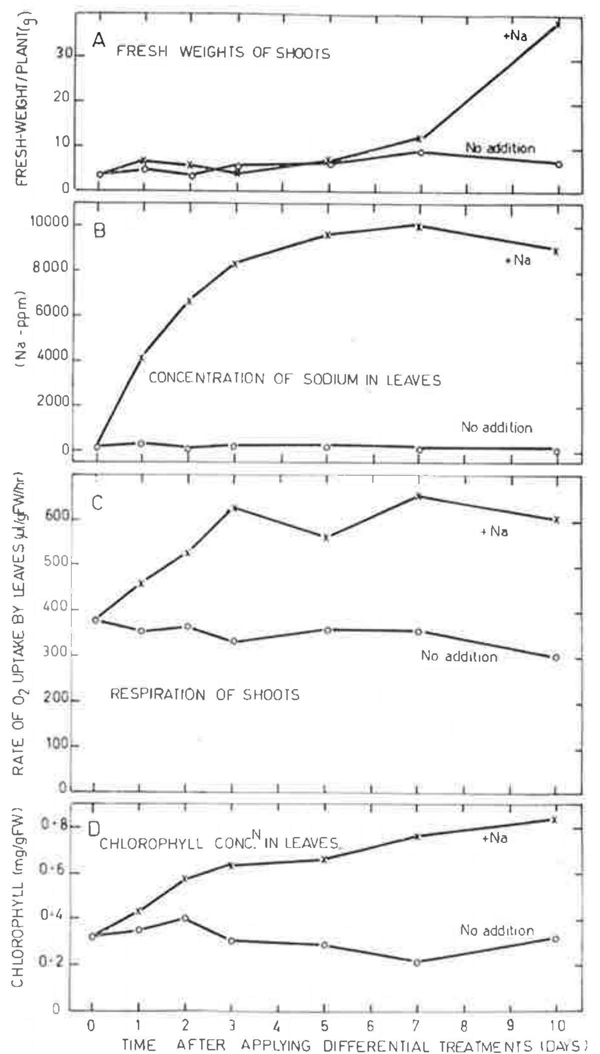

FIG. 1. Changes in fresh weight of shoots (A), sodium concentration (B), rate of O_2 uptake by leaves (C), and chlorophyll concentration (D) following the addition of sodium sulfate (0.6 meq/liter) to 32-day-old sodium-deficient plants. All harvests were made at 9 AM. Each point is derived from 2 cultures of 8 plants, of which 6 plants were used for chlorophyll determinations, and the remainder for measurements of sodium concentration and respiration rate. Total fresh weights of shoots of all 16 plants were recorded.

Results and Discussion

Changes During Recovery from Sodium Deficiency. The sequence of changes that follow the feeding of sodium to sodium-deficient *Atriplex* plants is shown in figure 1. For at least 5 days, growth of plants which received sodium (expressed as fr wt of shoots) was similar to that of plants to which no sodium was added (fig 1 A). After 7 days however, the growth of sodium-fed plants began to increase rapidly compared with that of the untreated controls. This delay in growth response occurred despite a rapid uptake of sodium into the shoots from the time of application (fig 1 B). By the fifth day the plants had taken up most of the sodium supplied in the culture solution, and the concentration in the leaves thus remained steady until after the seventh day when it showed a slight decrease presumably due to the onset of rapid growth.

The respiration rate per unit fresh weight of shoots (fig 1 C) increased rapidly (for about 3 days)

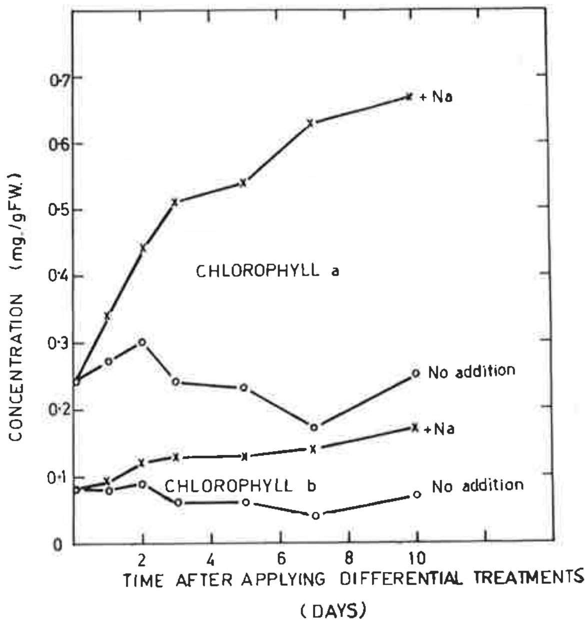

FIG. 2. Changes in concentrations of chlorophylls a and b following the addition of sodium sulfate to sodium-deficient plants. Data obtained from the same experiment as figure 1.

after receiving sodium, reaching a rate about twice that of the sodium-deficient plants. Thereafter there was little further change in rate.

The concentration of chlorophyll in the leaves increased rapidly to almost double the initial concentration by the third day after the addition of sodium (fig 1 D), and continued to increase throughout the 10 days of the experiment. During this period the chlorophyll a concentration in sodium-fed plants increased to a value nearly 3 times the initial value, while chlorophyll b concentration doubled (fig 2). These results were obtained during the summer, and the increase in chlorophyll concentration was much more rapid than in the winter months when growth was slow.

From this experiment it appears that even though fresh weight responses do not occur until some time after the addition of sodium to sodium-deficient *Atriplex* plants, both respiration rate and chlorophyll concentration increase at an early stage.

Marked differences in ratios of 80 % ethanol-soluble to total nitrogen were apparent between sodium-fed and deficient shoots by 20 days after supplying sodium but no difference was detectable 2 days after the addition of sodium (table I), although by this time there was a definite respiratory increase. Hence, the apparent long-term effect of sodium on nitrogen fractions is likely to be indirect, and only one of many changes expected to take place in the later stages of recovery.

The concentrations of both sugars and starch in leaves of sodium-deficient plants were low (fig 3). During recovery, however, these levels gradually rose to several times those found in deficient plants.

Further Respiration Experiments. Since it was one of the first detected, the respiratory response was

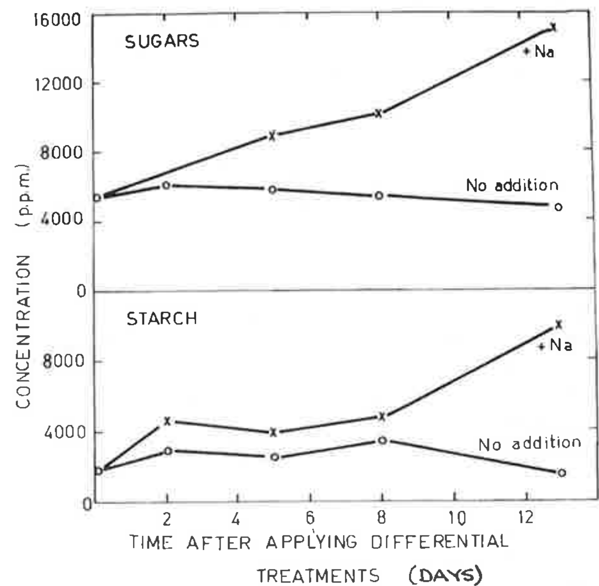

FIG. 3. Changes in concentrations of sugars and starch following the addition of sodium sulfate (0.6 meq/liter) to 45-day-old sodium-deficient plants. Points are the means of determinations on 6 samples each containing the leaves of 2 plants.

Table I. *Concentrations of Soluble and Insoluble Nitrogen in Leaves of* Atriplex

	*Atriplex nummularia** 2 days after receiving sodium				*Atriplex inflata*** 20 days after receiving sodium			
	Fr wt per plant (g)	Nitrogen content (% fr wt)		Soluble N / Total N	Fr wt per plant (g)	Nitrogen content (% fr wt)		Soluble N / Total N
		Ethanol extract	Residue			Ethanol extract	Residue	
No sodium added	1.33	0.29	0.42	0.41	0.11	0.32	0.39	0.45
Sodium added***	1.76	0.25	0.39	0.39	0.50	0.17	0.46	0.27

* Data are the means of determinations on 5 individual plants.

** Data are the means of determinations on 4 samples of 2 plants when no sodium was added and 1 plant when sodium was fed.

*** 0.60 and 0.10 meq/liter sodium sulfate to *Atriplex nummularia* and *Atriplex inflata*, respectively.

studied more fully by examining the effects of different salts of sodium, other univalent cations, varying concentrations of sodium, feeding sodium to cut shoots, feeding sucrose, and the effect of sodium on rate of anaerobic CO_2 production.

Whether supplied as chloride or as sulfate, equivalent amounts of sodium brought about similar increases in rate of O_2 uptake (fig 4). It appeared, therefore that these increases depended on the sodium ion, and were not due to excess cation absorption (6, 7), as, with the latter phenomenon, different rates of O_2 uptake could be expected as a result of the different mobilities of chloride and sulfate ions.

The rates of O_2 uptake by leaves of sodium-deficient plants 48 hours after receiving (in the culture solutions) sulfates of the univalent cations (0.10 meq/liter) were as follows: lithium, 368 µl/g fresh weight per hour; sodium, 640 µl/g fresh weight per hour; potassium, 364 µl/g fresh weight per hour; rubidium, 354 µl/g fresh weight per hour; and caesium, 345 µl/g fresh weight per hour. There was no difference from the control (370 µl/g fr wt per hr) except for the marked increase in the case of sodium. It seems improbable that this is salt-stimulated or anionic respiration (8, 13) for 2 reasons; first, the salt-stimulated response generally occurs only in tissues initially containing low concentrations of ions, whereas the concentrations of ions in leaf tissues of *Atriplex* are high in comparison with the low concentrations

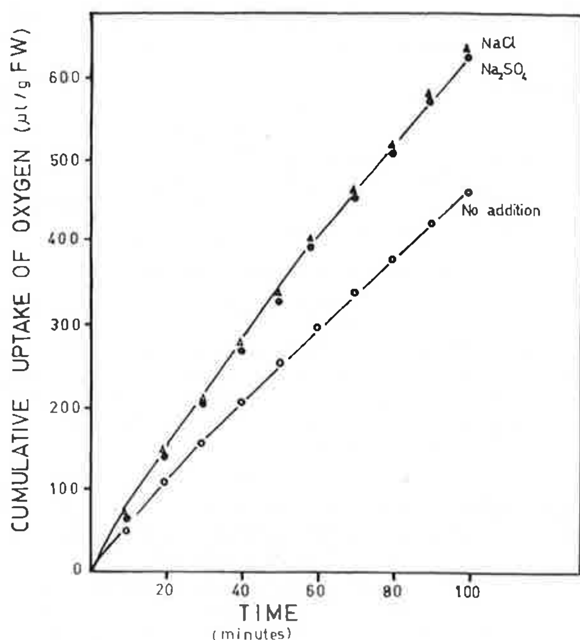

FIG. 4. Effect of adding sodium as either chloride or sulfate on the rate of O_2 uptake by sodium-deficient plants. Sodium chloride or sodium sulfate (0.22 meq/liter) was added to the culture solution 48 hours before harvesting. Points are the mean rates of O_2 uptake of 4 Warburg flasks each containing the leaves of 2 plants.

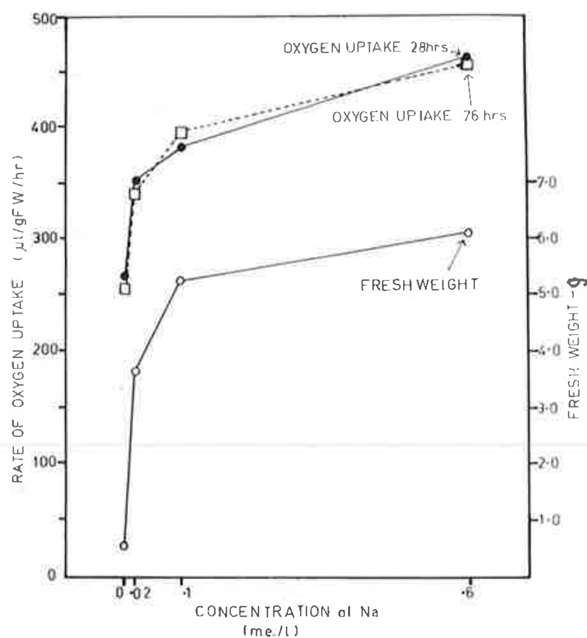

FIG. 5. Effect of concentration of sodium in culture solution on fresh weight and rate of O_2 uptake. Fresh weight data are means of 5 cultures of 4 plants each, sodium being supplied 16 days after germination and the plants harvested 32 days later. The rates of O_2 uptake were determined 28 and 76 hours after supplying sodium to 46-day-old plants. Points are the mean rates of uptake of 3 Warburg flasks each containing the leaves of 3 plants.

of sodium required to elicit a response; second, salt-stimulated respiration may be brought about by various ions, unlike the respiratory response in *Atriplex* where there is apparently a specific requirement for sodium.

Figure 5 shows that both respiration rate (O_2 uptake) and growth (fr wt) tend towards a maximum value with similar concentrations of sodium in the culture solution.

From the results of this work, it appears that lack of sodium limits the rate of respiration of *Atriplex* leaf tissues. If this leads to a reduction in phosphorylation, growth could conceivably be restricted by the decreased amounts of high energy phosphate compounds available for cellular work and syntheses.

When sodium was introduced to the tissue through cut stems, the rate of O_2 uptake by the leaves was 20 % greater than that of the untreated leaves (table II) and analyses showed that there had been an increase in the sodium concentration. This respiration response, only 1 and one-half hours after the application of sodium, was detected in a considerably shorter time than in other experiments when sodium was added to the culture solution. It is difficult to determine precisely the time required for this response to sodium, first because it is not known when sodium arrives at the actual site of action, and second because

Table II. *Effect of Feeding Sodium to Cut Shoots on Rate of O_2 Uptake and Sodium Concentration of Leaves*

	O_2 uptake** (μl per g fr wt per hr)	Sodium Conc*** (meq Na per kg dry wt)
No sodium added	211	12.1
Added sodium*	256	18.5

* Cut shoots were placed in sodium sulfate (2.2 meq/liter) for 1 and one-half hours in the light.
** Values are the mean rates of uptake of 6 Warburg flasks each containing the leaves of 2 to 3 plants.
*** Values are the means of 2 determinations on the leaves from 6 Warburg flasks.

it would not be expected to reach all cells in a tissue at the same time.

Since the concentrations of sugars and starch were low in leaves of sodium-deficient plants (fig 3), it seemed possible that lack of respiratory substrates could limit their respiration rate. Hence, cut leaves were supplied with sucrose and their respiration rates measured. The results (table III) showed that addition of sucrose increased the respiration rates of leaves from both sodium-deficient and sodium-fed plants suggesting that the small amounts of endogenous substrate were limiting the rate of respiration.

Table III. *Effect of Feeding Sucrose to Cut Leaves on Rate of O_2 Uptake*

Data are the mean rates of O_2 uptake of 3 Warburg flasks each containing the leaves of 2 to 3 plants.

	O_2 uptake (μl/g fr wt/hr) Without sucrose	With sucrose**
No sodium added	244	322
Added sodium*	302	400

* 0.6 meq/liter sodium sulfate added to culture solution 43 hours before harvesting.
** Cut petioles were immersed in 0.1 M sucrose for 1 and one-half hours in the light.

The supply of sodium to the culture solution 43 hours before harvesting also caused an increase in the respiration rate of leaves which received no additional sucrose. This could have been due to either a direct effect of sodium on the respiratory system or alternatively to an indirect effect, for example by increasing the amount of substrate derived from photosynthesis during the period after addition of sodium. When sucrose was fed in an attempt to remove the substrate limitation, there was still a response to sodium, suggesting that its effect is a direct one on some part of the respiration process. If however, the substrate limitation was not entirely removed by the exogenous sucrose, the apparent effect of sodium on respiration rate could be through its effect on photosynthesis. In all experiments, sodium was supplied in the light when conditions were favourable for high transpiration.

In an initial attempt to locate the part of the respiratory system requiring sodium, rates of CO_2 output of sodium-deficient and sodium-fed plants were compared. The results obtained (table IV) showed that the addition of sodium to sodium-deficient plants stimulated the rate of anaerobic CO_2 production and

Table IV. *Effect of Sodium on Rate of Anaerobic CO_2 Production of Sodium-deficient* Atriplex nummularia *Leaves*

Data are the mean rates of gas exchange of 16 Warburg flasks each containing the leaves of 2 to 3 plants.

	Anaerobic phase CO_2 Output	Aerobic phase O_2 Uptake
No sodium added	85	316
Added sodium*	105	426

* 0.1 meq/liter sodium sulfate was supplied to the culture solution 24 hours before harvesting.

that the rate of O_2 uptake when leaves were returned to air was unaffected by the anaerobic treatment. Hence, the increase in rate of CO_2 production under anaerobic conditions, suggests that sodium acts during the glycolytic stages of respiration. However, the possibility of an earlier effect of sodium on some other system leading to this respiratory response should not be overlooked.

Acknowledgment

We thank Miss Helen Cameron for her assistance in the respiration determinations and for the preparation of figures and Mr. Spencer Knowles for his help in the analysis of starch and sugars.

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Some Effects of Sodium on Nitrate Assimilation and N_2 Fixation in Anabaena Cylindrica

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Summary. Anabaena cylindrica grown with nitrate required higher levels of sodium (0.4 meq/l NaCl) to prevent chlorosis than when grown without combined nitrogen (0.004 meq/l NaCl). Nitrite accumulated in sodium-deficient cultures containing nitrate. Amounts of nitrite similar to those found in deficient cultures when added to normal cultures resulted in a chlorosis of the cells. Thus loss of chlorophyll was caused by nitrite toxicity.

A deficiency of sodium resulted in an increased incorporation of $^{15}NO_3$, $^{15}NO_2$, $^{15}NH_3$ or ^{14}C glutamate into protein compared with normal cells. The enzyme nitrate reductase was markedly increased in cells grown without sodium.

Evidence from chloramphenicol treatment of the cells suggests that sodium may exert its control of nitrate reductase through a protein factor(s).

By contrast, N_2 fixation was reduced in sodium deficient cells. Since the incorporation of ammonia or glutamate into protein was increased under these conditions, it is likely that the element is required for the conversion of N_2 gas into ammonia. Various nitrogenous compounds including ammonium chloride, amides and amino acids at low concentrations (0.1 mM) greatly reduced the nitrite accumulation in sodium-deficient cultures.

The first decisive evidence that sodium is an essential element for plants was obtained by Allen and Arnon (2) who showed that small amounts of the element are specifically required for the growth of the blue-green alga. *Anabaena cylindrica.* Subsequently, Brownwell and Wood (7) found that sodium is essential for the angiosperm, *Atriplex vesicaria* and more recently certain other species have been shown to require sodium (5). Significant yield responses to small amounts of sodium have been obtained with barley (5), tomatoes (19) and *Halogeton glomeratus* (18).

Little is known of the role of sodium in plant metabolism. The respiration of leaves of sodium-deficient *Atriplex nummularia* is markedly depressed and is restored within a few hours of adding sodium to the deficient culture solution whereas the leaf symptoms are not alleviated until the fifth day. The output of CO_2 under anaerobic conditions was restored when sodium was fed to deficient plants (6).

Some effects of sodium on the nitrogen metabolism of *Anabaena cylindrica* are described in this paper.

Methods

A culture of *Anabaena cylindrica* was kindly supplied by Professor G. E. Fogg (Department of Botany, Westfield College, London).

The alga was grown in liquid culture similar to that described by Allen and Arnon (2). Two culture solutions were used, one containing combined nitrogen as potassium nitrate and the other without combined nitrogen since the potassium nitrate was replaced by an equivalent amount of potassium chloride. The compositions of culture solutions were as follows (mM): $MgSO_4$, 1: KH_2PO_4/K_2HPO_4 (pH 7.2), 2: $Ca(NO_3)_2$, 0.5: and KCl, 10 (in the nitrogen-free medium), or KNO_3, 10 (in the nitrate-containing medium). Micronutrients were supplied (μg/ml) as follows: Fe (as the EDTA complex 5, Mn (as $MnSO_4 \cdot 4H_2O$) 0.5, Mo (as $(NH_4)_6 Mo_7 O_{24} \cdot 4H_2O$), 0.1, Zn (as $ZnSO_4 \cdot 4H_2O$) 0.05, Cu (as $CuSO_4 \cdot 5H_2O$) 0.02, B (as H_3BO_3) 0.050, Co (as Co $(NO_3)_2$, 0.01.

All cultures were dispensed in water distilled from silica and sodium was removed from the salts by methods described by Brownell (4). The purified culture solution then contained < 0.17 μeq/l Na.

Culture solutions without phosphate and the phosphate solution alone were autoclaved separately in silicone-coated pyrex vessels. The phosphate was added asceptically after cooling to room temperature. To obtain extreme deficiency symptoms in cultures not containing combined nitrogen, it was necessary to subculture the inoculum in polythene or polystyrene vessels to avoid sodium contamination from glass vessels. In addition it was

essential to aerate through a silica rather than a glass tube. During the subculturing stage, solutions were irradiated with an ultraviolet probe unit to kill bacteria and stock culture solutions were kept in the dark at 4° to prevent the growth of other algae. Routine microscopic examination of the solutions and inocula were carried out at each stage to check the absence of contaminants.

Cultures containing nitrate were maintained in Roux bottles at approximately 25° and illuminated by warm-white fluorescent lamps at an intensity of about 600 ft-c. They were shaken continuously on a reciprocator (80 strokes/min) (3). Cultures without combined nitrogen were grown in plastic containers (500 ml capacity) and aerated with filtered air.

The cultures were well dispersed in a teflon/glass homogenizer. Subsamples were then centrifuged at 4° for 15 minutes at 2000 × g and then washed with 0.05 M phosphate buffer (pH 7.5). When cells were grown in nitrate nitrogen, washing was continued until no trace of nitrite could be detected. Dry-weights were determined on cells collected by centrifuging for 10 minutes at 2000 × g, washed in distilled water and dried to constant weight at 105°.

Nitrite was determined by a modification of the Griess-Ilosvay colorimetric method (12).

Chlorophyll was assayed in methanol extracts (8). Phycocyanins were extracted with water from cells autolysed by alternate freezing and thawing and relative concentrations measured at 620 mμ (9).

Nitrate reductase activity was determined in crude extracts of cells washed with 0.05 M phosphate buffer (pH 7.5) to remove nitrite. Cells suspended in the cold buffer were disrupted by passing them through a French Press at 7000 to 12,000 pound pressure/square inch at 4° or by ultrasonic disruption in an M.S.E. Ultrasonic disintegrator (20 kilocycles per second) for 5 minutes (as specified in the tables). The extract was centrifuged at 2000 × g for 10 minutes to remove cell wall material and other debris. The supernatant fraction was assayed for nitrate reductase activity by the method of Naik and Nicholas (15) in which a transhydrogenase from *Azotobacter* is used to generate reduced benzyl viologen from NADH in evacuated Thunberg tubes with nitrate as the acceptor. The following reaction mixture was used: main vessel of Thunberg tube : phosphate buffer (pH 7.5), 0.05 M, 1 ml; benzyl viologen, 0.1 M, 0.01 ml; azotobacter enzyme, 0.05 ml; nitrate reductase, 0.5 ml : side arm : KNO_3, 0.1 M, 0.1 ml; NADH 2 μmoles, 0.4 ml. After incubating for 30 minutes the reaction was stopped by adding zinc acetate and ethanol (10). Nitrite was determined in the supernatant solution using the sulfanilamide method (12).

Protein was assayed by the Folin method (14) or by the microbiuret procedure (13). Bovine serum albumen was used as a standard protein.

Nitrogen-fixation was determined by suspending washed cells in a fresh culture solution deficient in sodium without combined nitrogen and exposing them in Warburg flasks to a gas mixture containing 0.20 atm. N_2 enriched with 31.4 % $^{15}N_2$, 0.20 atm. O_2 and 0.60 atm. He. The flasks were shaken in the Warburg bath at 30° at a light intensity of about 400 ft-c from warm-white fluorescent lamps and 0.2 ml Pardee buffer in the side-arm provided 0.2 % CO_2 (17).

Incorporation of ^{15}N nitrogen into cell protein from $^{15}NO_3$ or $^{15}NO_2$ was determined by incubating the appropriate ^{15}N compound with washed cells suspended in fresh culture solutions without nitrate or sodium at 30°.

After incubation, at times specified in the tables, cells were thoroughly mixed in a teflon glass homogenizer to break down clumps of cells before withdrawing samples into chilled centrifuge tubes. These were washed several times in distilled water and centrifuged at 10,000 × g at 4°. They were then plunged into 95 % (v/v) ethanol, homogenized and the protein precipitated with trichloroacetic acid to a final concentration of 10 %. After standing at 4° for 15 minutes the precipitate was centrifuged at 10,000 × g for 10 minutes resuspended in water and reprecipitated with trichloroacetic acid and centrifuged. The nitrogen content of this fraction was determined by a micro-Kjeldahl method with a mercury catalyst. Ammonia was distilled into a borate buffer which was then titrated with sulfuric acid. The samples were acidified with small quantities of sulfuric acid. The ammonium nitrogen was converted into elementary nitrogen by adding alkaline hypobromite in evacuated Rittenberg tubes (16). The enrichment of samples with ^{15}N nitrogen was determined in an MS_2 mass spectrometer.

The assimilation of glutamate was followed by adding U-^{14}C-glutamic acid (supplied by the Radiochemical Centre, Amersham, Buckinghamshire) to washed algae suspended in combined nitrogen and sodium free fresh culture solutions and the incorporation of ^{14}C in the cell protein fraction determined after various incubation times. The algal suspension was chilled immediately after incubation and washed with distilled water and then centrifuged at 10,000 × g for 5 minutes at 4°. This was repeated 3 times. Protein extracted by the procedure used in ^{15}N studies described above was dissolved in 0.1 ml 40 % NaOH and made up to 5 ml with distilled water. Samples were pipetted on to planchets and counted in a Beckman Sharp Laboratories Lowbeta II Gas flow unit. Self absorption was found to be negligible so that no corrections were necessary.

Results and Discussion

Dry-Weight Yields. The dry-weight yields increased with sodium supply reaching a maximum

Table I. *Effect of Sodium on Dry-weight, Chlorophyll and Phycocyanin Contents of* Anabaena cylindrica *Grown with Nitrate Nitrogen and Without Combined Nitrogen*

Cultures harvested 13 days after inoculating.

Amount of NaCl in culture solution	Dry weight: No combined nitrogen	Dry weight: 10 meq/l KNO_3	Chlorophyll: No combined nitrogen	Chlorophyll: 10 meq/l KNO_3	Chlorophyll: No combined nitrogen	Chlorophyll: 10 meq/l KNO_3	Phycocyanin: No combined nitrogen	Phycocyanin: 10 meq/l KNO_3	Phycocyanin: No combined nitrogen	Phycocyanin: 10 meq/l KNO_3
meq/l	g/100 ml culture		mg/100 ml culture		mg/g dry wt		uints*/100 ml culture		units*/g dry wt	
None	0.058	0.041	6.59	3.07	112	76	0.286	0.008	4.900	0.185
0 004	0.060	0.067	9.36	3 25	155	52	0.317	0.044	5.250	0.650
0.010	0.066	0.063	8.55	2.66	130	43	0.315	0.032	4.800	0.515
0.040	0.067	0.070	7.30	7.69	110	110	0.268	0.169	4.02	2.420
0.400	0.064	0.053	8.68	5.18	136	98	0.339	0.152	5.30	2.870
2.000	0.092	0.118	20.50	24.00	223	204	0.600	0.645	6.50	5.500
4.000	0.097	0.094	20.20	20.50	210	220	0.670	0 496	6.90	5.300

* Arbitrary unit = E_{620}^{1cm} of phycocyanin in 100 ml.

in culture solutions containing approximately 2 meq/l NaCl either with or without combined nitrogen (table I). Cultures with nitrate nitrogen containing less than 0.04 meq/l NaCl appeared chlorotic whereas those without combined nitrogen were normal even at 0.004 meq/l NaCl. The chlorophyll contents of the affected cells were markedly reduced in sodium deficient cultures containing nitrate nitrogen (table I). Algal cultures with or without combined nitrogen had similar chlorophyll

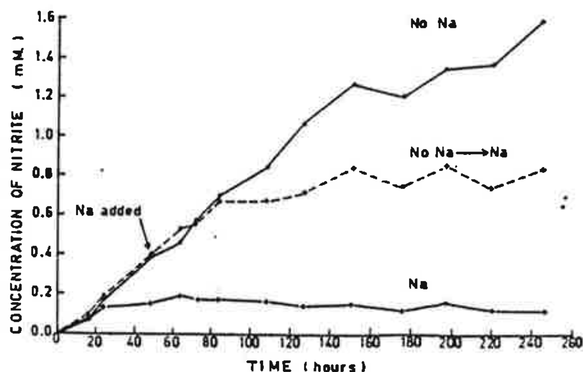

FIG. 1. Effect of sodium on nitrite accumulation by *Anabaena cylindrica* grown in culture solution containing 10 mM KNO_3. No addition 0 – 0; 4.0 meg/l NaCl at onset of experiment, X – X; 4.0 meq/l NaCl added to deficient cultures 45 hours after inoculation.

Table III. *Effect of Nitrite on Dry Weight, and Chlorophyll Content of* Anabaena cylindrica

Harvest 15 days after inoculation.

Treatment*	Final conc of nitrite in the culture	Mean dry wt	Chlorophyll/ culture
	µM	g/l	mg
Normal culture	235	0.795	1000
Normal culture + 0.2 mM KNO_2	910	0 660	600
Normal culture + 0.4 mM KNO_2	1050	0.665	720
Normal culture + 1.0 mM KNO_2	2750	0.789	500
Normal culture + 3.0 mM KNO_2	5100	0.600	370
Sodium-deficient culture	1700	0.680	500

* Nitrite added to cultures before inoculation. Normal cultures contained 4 meq/l NaCl.

Table II. *Effect of Adding Sodium to Na-deficient Cultures containing Nitrate*

Time after inoculation	7: Nitrite	7: Dry wt	7: Chlorophyll	18: Nitrite	18: Dry wt	18: Chlorophyll	18: Phycocyanin
Days	mM	g/culture	mg/culture	mM	g/culture	mg/culture	Units*/culture
Normal (4 meq/l NaCl at inoculation)	140	0.026	1200	170	0.112	1790	0.645
4 meq/l NaCl added to unwashed cells on 7th day	740	0.014	332	970	0.081	606	0 180
4 meq/l NaCl added to washed cells on 7th day	850	0.008	330	210	0.092	1470	0.540
Omit sodium	940	0.010	325	1130	0.046	252	0.090

* Arbitrary unit = E_{620}^{1cm} of phycocyanin in 100 ml.

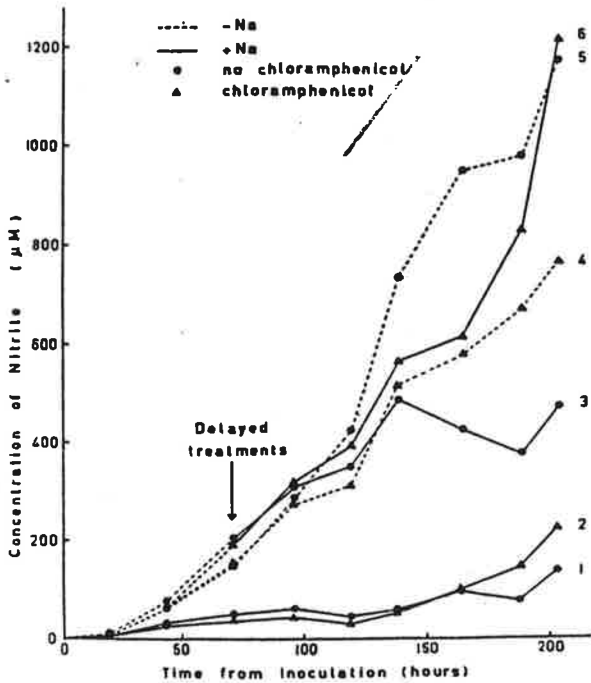

Fig. 2. Effects of various combinations of sodium (4 meq/l NaCl) and chloramphenicol (0.005 mg/ml) on nitrite production by *Anabaena cylindrica* grown with 10 mM KNO_3.

	70↓hr	
+ Na from inoculation	●—●—●	1
+ Na from inoculation to 70 hr then chloramphenicol	●—▲—▲	2
— Na from inoculation to 70 hr then + Na	●---●—●	3
— Na from inoculation to 70 hr then — Na + chloramphenicol	●---▲---▲	4
— Na from inoculation to 70 hr	●---●---●	5
— Na from inoculation to 70 hr then + Na + chloramphenicol	●---▲—▲	6

levels when supplied with more than 0.4 meq/l NaCl. It is unlikely that the reduced chlorophyll content of cells given nitrate and containing less than 0.04 meq/l NaCl restricted dry-weight production since cells grown without combined nitrogen had more than twice the concentration of chlorophyll at similar levels of sodium (table I). Phycocyanin concentrations were also much lower in cells grown with nitrate than in those without combined nitrogen at levels below 0.4 meq/l NaCl (table I).

Toxic Effects of Nitrite in Cultures Containing Nitrate. Nitrite increased rapidly in sodium-deficient cultures as shown in figure 1. After 45 hour growth, sodium was added to some of the deficient cultures and after a further 40 hour period the nitrite content levelled off (fig 1). The chlorotic cells in these cultures however did not recover fully, presumably because nitrite was still present in toxic amounts.

When sodium deficient cells were harvested 7 days after inoculation and washed with distilled water to remove nitrite, then resuspended in a full

Table IV. *Concentrations of Nitrite in Culture Solutions of Normal and Sodium-deficient Cells and the Nitrate Reductase Activity of Cell Extracts. Incorporation of $^{15}NO_3$ and $^{15}NO_2$ into Protein*

Cells collected after 6 days growth. Complete cultures contained 4 meq/l NaCl and 10 mM KNO_3. Cells incubated in 10 mM $K^{15}NO_3$ (30.8 % atom percent excess ^{15}N) and 10 mM $Na^{15}NO_2$ (27.5 % atom percent excess ^{15}N) respectively.

Cultural treatment	Concentration of nitrite in culture solution	Incorporation of ^{15}N into cell-protein: From ^{15}N-labelled nitrate — Corrected enrichment	Protein nitrogen	Uptake of nitrogen	Specific enrichment	From ^{15}N-labelled nitrite — Corrected enrichment	Protein nitrogen	Uptake of nitrogen	Specific enrichment	Nitrate reductase activity — Enzyme activity	Total protein**	Specific activity of enzyme
	% μmole/100 ml culture	atom % ^{15}N excess	mg	μg/hr	μgN/mg protein/1 hr	atom % ^{15}N excess	mg	μg/hr	μgN/mg protein/1 hr	Total units*/100 ml culture	mg/100 ml culture	Units*/mg protein
Complete	65	0.068	0.730	2.16	2.96	0.102	0.650	3.22	4.92	588	12.2	48.4
Omit sodium	240	0.202	0.520	4.55	8.70	0.248	0.410	4.92	12.00	2270	8.5	269.0

* Enzyme unit (mμmoles NO_2^- formed/1 hr)
** Determined by the modified Biuret Method [Itzhaki, R. F. and D. M. Gill, Anal. Biochem. 9: 401 (1964)].

Table V. *Effect of Sodium on Nitrate Reductase Activity in Cell-extracts of* Anabaena cylindrica *Grown in Culture Solution Containing Nitrate*

Cultures were harvested 13 days after inoculation. Cells broken by ultrasonication for enzyme assay.

Amounts of NaCl	Nitrate reductase activity		
	Enzyme activity	Dry wt	Specific activity
meq/l culture solution	Total Units*/ 100 ml culture	g/100 ml culture	Units*/g dry wt
None	173	0.040	4300
0.004	139	0.067	2080
0.4	51	0.053	960
4.0	36	0.093	385

* Enzyme unit (mμmoles NO_2^- formed/1 hr).

Table VI. *Incorporation of* $^{15}N_2$ *into Cells of* Anabaena cylindrica

Cells collected after 6 days growth. Algae suspended in 5 ml nitrogen-free culture medium, incubated at 30° for 2 hours with continuous agitation. Pardee buffer in side-arms provided 0.2 % CO_2 for flasks. Gas phase: 0.2 atm. N_2 enriched with 31.4 % $^{15}N_2$, 0.20 atm. O_2 and 0.60 atm He. Suspensions were illuminated at an intensity of approximately 400 ft-c.

Cultural treatment	Incorporation of ^{15}N into cellular material			
	Corrected enrichment	Total N	Uptake	Specific enrichment
	Atom % ^{15}N excess	mg	μg/hr	μg N/mg Cell N/hr
No combined nitrogen in culture solution				
Complete (4 meq/l NaCl)	0.454 %	0.563	4.00	6.05
Omit sodium	0.264 %	0.330	1.36	4.13
Nitrate nitrogen (0.5 mM) in culture solution				
Complete (4 meq/l NaCl)	0.270 %	0.675	2.85	4.21
Omit sodium	0.166 %	0.287	0.75	2.60

Table VII. *Incorporation of* $^{15}NH_3$ *into Cells of* Anabaena cylindrica

Cells collected after 6 days growth. Cells incubated for 10 minutes in 1 mM $^{15}NH_4NO_3$ (95.65% atom percent excess ^{15}N) in fresh medium.

Cultural treatment	Incorporation of ^{15}N into cellular protein			
	Corrected enrichment	Protein N	Uptake	Specific enrichment
	Atom % ^{15}N excess	mg	μg/hr	μg N/mg protein/hr
No combined nitrogen in culture solution				
Complete (4 meq/l NaCl)	0.162	1805	16.44	9.10
Omit sodium	0.271	745	12.90	17.30
Nitrate nitrogen (0.5 mM) in culture solution				
Complete (4 meq/l NaCl)	0.147	2350	21.66	9.24
Omit sodium	0.217	1965	26.70	13.56

culture solution containing nitrate and sodium, the chlorophyll and phycocyanin contents of the cells were restored almost to those of normal cultures as shown in table II.

When this experiment was done with unwashed cells, however, their chlorophyll and phycocyanin contents remained at a low level because of the excess nitrite present.

When nitrite was added to normal cultures at levels found in sodium-deficient ones, then the alga developed a chlorosis within a few days. The results in table III show a reduction in chlorophyll content with increasing nitrite concentration but the dry-weight yields were unaffected.

Nitrate and N_2 Assimilation. Tracer techniques were used to follow the incorporation of $^{15}NO_3$ and $^{15}NO_2$ into normal and sodium-deficient cells grown with nitrate. It is clear from the results in table IV that the incorporation of ^{15}N into cell protein from either nitrate or nitrite was more markedly increased in sodium-deficient cells. Since the reduction of nitrate is usually more rapid than that for nitrite, the effect of sodium deficiency would result in an accumulation of nitrite as shown in table IV. The enzyme nitrate reductase is far more active in extracts of sodium-deficient cells when expressed either on a culture basis or as a specific activity. (table IV). The effect of graded amounts of sodium in culture solutions on enzyme activity is presented in table V. Thus increased sodium supply results in decreased enzyme activity.

When chloramphenicol was added to sodium-deficient cultures (70 hr growth) together with sodium, nitrate reductase activity was not depressed whereas the addition of sodium alone resulted in a marked reduction in nitrate reductase activity (fig 2). This suggests that a newly formed protein induced by sodium may be involved in controlling nitrate reduction.

The incorporation of $^{15}N_2$ gas into the alga was followed in cells grown with and without nitrate nitrogen. The results in table VI show that in the absence of sodium the enrichment from N_2 gas is markedly reduced. It is of interest that N_2 fixation proceeds even with nitrate at 10 mM in the culture solution as found by Allen (1). In contrast to this result the incorporation of $^{15}NH_3$ (table VII) or ^{14}C glutamate (table VIII) into cell-protein was more rapid in sodium-deficient

Table VIII. *The Incorporation of U-^{14}C Glutamate into Protein of* Anabaena cylindrica

Algae suspended in 10 ml nitrogen-free culture medium at 30°. At the commencement of the incubation period, ^{14}C-glutamate (63,000 cpm) was added. Cells were illuminated with an intensity of approximately 400 ft-c.

Incubation cultural conditions	5: cpm/Total protein per culture	5: Protein/culture	5: cpm/mg Protein	15: cpm/Total protein per culture	15: Protein/culture	15: cpm/mg Protein
min		mg			mg	
No combined nitrogen						
Complete (4 meq/l NaCl at inoculation)	8700	348	2500	8200	397	20.7
Omit sodium	11,100	277	4000	26,700	370	72.0
Nitrate nitrogen (0.5 mM)						
Complete (4 meq/l NaCl at inoculation)	12,800	410	3120	19,500	464	42.0
Omit sodium	17,000	333	5100	23,500	343	68.5

Table IX. *Effect of Sodium and Nitrogenous Compounds on Nitrate Production by the Alga Grown with Nitrate Nitrogen (Nitrite μM)*

Time after inoculation	20 hrs	48 hrs	91 hrs
		μM	
Normal culture	12	44	50
Deficient culture	16	80	148
" " , 0.1 mM NH_4Cl	1	1	5
" " , 0.1 mM Arginine	7	15	75
" " , 0.1 mM Citrulline	9	18	50
" " , 0.1 mM Ornithine	12	15	69
" " , 0.1 mM Glutamic acid	16	70	125
" " , 0.1 mM Glutamine	5	50	95
" " , 0.1 mM Asparagine	3	34	100
" " , 0.1 mM Proline	9	58	108

cells whether grown with nitrate or without combined nitrogen.

Since sodium-deficiency enhances the incorporation of ammonia and glutamate into protein, and these may well be key intermediates in this process, then the effect of the deficiency in depressing nitrogen fixation may be due to its primary effect on the conversion of N_2 gas to ammonia. The addition of ammonia, glutamine, asparagine or some amino acids at low levels (0.1 mM) to the sodium-deficient cultures decreased the nitrite content within 20 hours. Ammonia, arginine, citrulline, ornithine were the most effective as shown in table IX. Similar effects of these compounds on nitrate reductase have been reported in cultured tobacco cells (11).

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SHORT COMMUNICATION

Sodium as an Essential Micronutrient Element for some Higher Plants

Introduction

Sodium was first shown to be essential for *Anabaena cylindrica* Lemm., a blue-green alga [2]. The finding that sodium is essential for *Atriplex vesicaria* Heward ex Benth, (Bladder Salt Bush) [5] suggested that sodium might be an essential micronutrient element for other higher plants. Trials have been carried out on a diverse selection of species to obtain an indication of whether the requirement for sodium by higher plants is general. Species were chosen for these trials after consideration of the following: suggestions in the literature of responses to sodium; suitability for culturing; low sodium content per seed; taxonomic or ecological relationship with *Atriplex vesicaria* or unusual features in common with *Atriplex vesicaria*, such as vesicles arising from the leaf epidermis.

Methods

Techniques used to grow plants in a low sodium environment have been described [4]. Less than 0.07 μe/l of sodium were derived from the salts of the culture solution as an impurity and silica-distilled water used contained less than 0.0087 μe Na/l giving a total sodium concentration of approximately 0.08 μe/l. Culture vessels were of sodium-free plastic material. Atmospheric contamination of plants by sodium was minimized by growing them in a small growth cabinet supplied continuously with filtered air which maintained it at a slightly positive pressure, thus preventing the entry of dust particles which could be a source of sodium to the plants. Seeds were thoroughly washed several times with distilled water before placing them in germination cultures.

The sodium content per seed of *Lycopersicum esculentum* Mill. cv. 'Grosse Lisse' (tomato) was reduced from 0.095 μg to 0.030 μg by washing in four changes of distilled water for a period of 3½ hours. A similar washing procedure was adopted for all species examined in these experiments.

Further details of experimental conditions are given in Table 1.

Results

The dry-weight yields of some species grown with and without the addition of sodium to their cultures and the significance of their responses to

TABLE 1

Yield responses by various higher plants to sodium

Species*	Sodium supplied (days after germination)	Harvested (days after germination)	Yield (dry weight per plant, g) No addition to culture	Yield (dry weight per plant, g) 0.2 me Na_2SO_4 per 2-l culture	Significance of difference†	Remarks
Gramineae						
Hordeum vulgare L. c.v. 'Pallidium' (barley) (1)	11	26	0.77	0.99	*	
(2)	10	45	3.82	4.01		
Chenopodiaceae						
Chenopodium capitatum (L.) Aschers	17	48	12.25	14.37	N.S.	
Beta vulgaris L. (sugar beet)	11	61	3.86	5.07	N.S.	75 μg Na/see
Atriplex nummularia Lindl. (Old man, Giant salt bush)	15	43	0.166	0.830	***	
Atriplex paludosa R. Br. (Marsh salt bush)	10	56	0.215	2.789	*	
Atriplex quinii F. v. M.	10	51	0.116	0.815	*	
Atriplex semibaccata R. Br. (Berry salt bush)	15	42	0.104	0.653	**	
Atriplex inflata F. v. M. (1)	—	—	0.202	9.745	***	
(2)	8	65	0.036	1.865	***	
Atriplex leptocarpa F. v. M.	14	55	0.050	1.329	**	
Atriplex lindleyi, Moq.	14	54	0.093	0.560	*	
Atriplex spongiosa F.v.M. (Pop salt bush)	18	41	0.570	12.472	***	
Atriplex semilunalaris (Aellen)	—	—	0.098	0.526	***	
Atriplex hortensis L. var. *atrosanguinea* Garden orache	12	39	2.849	3.677	*	
Atriplex angustifolia Sm.	14	45	0.531	0.377	N.S.	
Atriplex glabriuscula Edmondston	19	43	24.804	24.100	N.S.	
Atriplex albicans Ait. (1)	23	49	8.818	11.688	N.S.	
(2)	17	49	9.237	9.401		
Kochia pyramidata Benth.	—	—	35.390	33.990	N.S.	
Exomis axyrioides Fenzl. ex Moq.	22	54	0.864	0.925	N.S.	
Cruciferae						
Brassica oleracea L. cv. 'Savoy' (Cabbage)	17	44	14.244	17.766	N.S.	1 seed contai 0.25 μg Na.
Leguminosae						
Trifolium repens L. cv. 'Palestine' (White Clover)	19	43	2.299	2.897	N.S.	
Solanaceae						
Lycopersicum esculentum Mill. cv. 'Grosse lisse' (Tomato) (1)	20	47	4.84	4.76	N.S.	1 seed contai 0.03 μg Na a washing.
(2)	16	42	6.93	7.83		
Compositae						
Lactuca sativa L. cv. 'Great Lakes' (Lettuce) (1)	17	46	3.871	6.113	N.S.	1 seed contai 0.1 μg Na
(2)	14	32	0.822	1.228		
(3)	9	37	2.230	2.444		
Aster tripolium L.	17	50	0.282	0.458	N.S.	

† Level of significance: Not significant – N.S. : 5% * : 1% ** : 0.1% ***.
* Names given in this table are those under which the seeds were received.

sodium are shown in Table 1. Analyses of variance were carried out on the data from each species. In the species having more than one trial, viz *Lycopersicum esculentum*, *Atriplex albicans*, *Hordeum vulgare* and *Lactuca sativa*, the data were combined, after suitable transformation, for analysis.

In all the Australian species of *Atriplex* examined, there were marked increases in dry-weight yields following the application of 0.1 me/l Na_2SO_4 to their cultures. Leaves of deficient plants showed distinct symptoms of chlorosis and in severe cases, necrosis. *Atriplex hortensis*, a species not indigenous to Australia gave a significant, but much smaller response to sodium than that of the Australian species. No deficiency symptoms were detected. Another species, *Atriplex albicans*, not indigenous to Australia, responded significantly to sodium in one out of two trials. This inconsistency was possibly due to uncontrolled differences between the conditions of the individual experiments. The other two species of *Atriplex*, *Atriplex glabriuscula* and *Atriplex angustifolia*, did not respond to sodium.

Hordeum vulgare responded to sodium, significantly, when the data from both trials were combined for analysis. *Lactuca sativa* gave a significant response in only two out of three trials, the third trial being inconsistent with that of the others, possibly due to uncontrolled differences between the conditions of the individual trials as with *Atriplex albicans*.

Discussion

The most striking feature of the results of these experiments is the extremely restricted group of species for which sodium is apparently essential. Even within the genus *Atriplex*, there is remarkable specialisation in the requirement for sodium. Only the Australian species of *Atriplex* developed symptoms due to sodium deficiency. One other species of *Atriplex*, *Atriplex hortensis* significantly increased its yield on receiving sodium but it did not develop Na-deficiency symptoms. Species of other genera of Chenopodiaceae, viz. *Beta*, *Kochia* and *Chenopodium* do not appear to require sodium. Even *Beta vulgaris* (sugar beet) which has been reported to increase its yield when supplied with sodium salts [1 6] showed no significant response in these trials. Possibly, very much larger amounts of sodium are needed to induce yield responses in sugar-beet than in *Atriplex*, suggesting that the roles of sodium in the nutrition of sugar-beet and in species of *Atriplex* responding to sodium, differ. *Hordeum vulgare* cv. 'Pallidium' which showed a small significant response to sodium is a salt-tolerant variety of barley.

Significant responses to sodium by *Lycopersicum esculentum* cv. 'Grosse Lisse' were not consistently obtained in these trials whereas Woolley [8] reported a 12% increase in the mean dry-weight of tomatoes ($P < 0.01$) on the addition of 1 me/l NaCl to the basal culture solution which contained 0.25 μmoles/l, Na as an impurity. However, no symptoms due to a deficiency of sodium were observed.

Other species which did not respond to sodium either have no requirement for sodium, or they require it in amounts so small that the plants obtained adequate sodium under the conditions of the experiment, *i.e.* less than approximately 0.07 μe/l, Na in their cultures which is still greater than the

minimum concentration of Mo (0.005 μe/l) needed to give maximum yield in tomatoes (W. R. Meager, see Hewitt [7], p. 163–65).

Summary

Twenty-three species of higher plants were tested for their responses to 0.1 me/l Na_2SO_4 when added to their culture solutions which contained only 0.08 μe/l Na after purification of the salts and water. All the 9 Australian species of *Atriplex* developed characteristic sodium-deficiency symptoms similar to those previously observed in *Atriplex vesicaria* and their dry-weight yields increased significantly on receiving sodium.

Sodium increased the dry-weight significantly in one of the four species of *Atriplex* not indigenous to Australia but deficiency symptoms were not observed.

A significant dry-weight response to sodium was obtained in *Hordeum vulgare* cv. 'Pallidium'.

No other species, even in Chenopodiaceae, responded to sodium under the conditions of the experiments. It is concluded that they either have no sodium requirement or their requirement is so small that they obtained adequate sodium for normal growth and development in these trials.

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The Requirement for Sodium as a Micronutrient by Species Having the C_4 Dicarboxylic Photosynthetic Pathway

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ABSTRACT

Six species having characteristics of plants with the C_4 dicarboxylic photosynthetic pathway, *Echinochloa utilis* L. Ohwi et Yabuno (Japanese millet), *Cynodon dactylon* L. (Bermuda grass), *Kyllinga brevifolia* Rottb., *Amaranthus tricolor* L. cv. Early splendour, *Kochia childsii* Hort., and *Portulaca grandiflora* Hook (rose moss), responded decisively to 0.1 milliequivalent per liter NaCl supplied to their culture solutions initially containing less than 0.08 microequivalent per liter Na. Chlorosis and necrosis occurred in leaves of plants not receiving sodium. *Portulaca* failed to set flower in the sodium-deficient cultures. Under similar conditions *Poa pratensis* L. (Kentucky blue grass) having characteristics of the C_3 photosynthetic pathway made normal growth and did not respond to the addition of sodium. It is concluded from these results and previously reported work that sodium is generally essential for species having the C_4 pathway but not for species with the C_3 pathway.

Sodium was first shown to be essential for the blue-green alga *Anabaena cylindrica* Lemm. in 1954 (1), when it was demonstrated that small amounts of the element are specifically required for growth. Subsequently, sodium was shown to be essential for the angiosperm, *Atriplex vesicaria* Heward ex Benth (Bladder saltbush) (6), and more recently other species have been shown to respond to sodium (3, 16). Significant responses to sodium have been reported in barley (3) and tomatoes (17), but the responses were small and no deficiency symptoms were observed.

Only certain species of *Atriplex* responded, decisively, to small quantities of sodium whereas other species of the same genus showed no response (3). Subsequently, it was observed that the species of *Atriplex* which required sodium had characteristics of plants with the C_4 photosynthetic pathway whereas those not requiring sodium had features of the C_3 photosynthetic pathway (9–11).

This communication gives results of experiments in which six species from five families (*Echinochloa utilis, Cynodon dactylon, Kyllinga brevifolia, Amaranthus tricolor, Kochia childsii,* and *Portulaca grandiflora*) with features of C_4 plants (7, 9) were shown to have a sodium requirement whereas *Poa pratensis*, a species with C_3 characteristics, showed no response to sodium. These results in conjunction with previously published data are discussed to demonstrate a correlation between the essentiality of sodium and the possession of the C_4 dicarboxylic photosynthetic pathway.

MATERIALS AND METHODS

Seeds of *Echinochloa utilis* L. Ohwi et Yabuno (Japanese millet), *Cynodon dactylon* L. (Bermuda grass), *Poa pratensis* L. (Kentucky blue grass), *Amaranthus tricolor* L. cv. Early splendour, *Kochia childsii* Hort., and *Portulaca grandiflora* Hook (rose moss) were obtained from commercial seed suppliers. Seeds of *Kyllinga brevifolia* Rottb. were collected locally. The procedures for germination and growth of plants under conditions of low sodium have been described previously (2). The basal culture solution had the following composition expressed in μmoles/liter: KNO_3, 5,000; $Ca(NO_3)_2$, 4,000; KH_2PO_4, 1,000; $MgSO_4$, 1,000; $(NH_4)_2HPO_4$, 1,000; H_3BO_3, 46; $MnSO_4 \cdot 7H_2O$, 9.1; $CuSO_4 \cdot 5H_2O$, 0.31; $ZnSO_4 \cdot 7H_2O$, 0.76; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1; NH_4Cl, 350. Iron was supplied as ferric ammonium ethylenediaminetetraacetate at 90 μmoles/liter in the basal culture solution. Less than 0.07 μeq/liter sodium was derived from the purified salts of the culture solution as an impurity, and silica-distilled water contained less than 0.0087 μeq/liter, giving a total sodium concentration of approximately 0.08 μeq/liter. Culture vessels of 2-liter capacity were of sodium-free plastic material. Plants were grown in a naturally illuminated cabinet, slightly pressurized by a continual supply of filtered air. This prevented the entry of dust particles and other atmospheric contaminants which could be

Table I. *Responses of Various Plants to Sodium*

Species[1]	Age at Harvest	Lesions in Plants Not Receiving Sodium	Yield		Significance of Difference
			No addition	0.1 meq/liter NaCl	
	days		*g dry wt/plant*		%
Gramineae					
Poa pratensis L. (Kentucky blue grass)	52	None	0.0236	0.0206	NS[2]
Echinochloa utilis L. Ohwi et Yabuno (Japanese millet)	22	Chlorosis and necrosis	0.404	0.713	0.1
Cynodon dactylon L. (Bermuda grass)	47	Chlorosis	0.178	0.337	1
Cyperaceae					
Kyllinga brevifolia Rottb.	57	Chlorosis	0.628	1.245	1
Amaranthaceae					
Amaranthus tricolor L. cv. Early Splendour	40	Chlorosis	0.884	2.099	0.1
Chenopodiaceae					
Kochia childsii Hort.	21	Chlorosis	0.125	0.442	1
Portulacaceae					
Portulaca grandiflora Hook (rose moss)	29	Chlorosis, necrosis, and failure to set flower	0.242	0.789	1

[1] Names given in this table are those under which the seeds were received.
[2] Not significant.

Table II. *Sodium Requirement in Relation to C_4 Pathway Characteristics*

Species	Lesions in Plants Not Receiving Sodium	Yield		Significance of Difference	Reference	C_4 Pathway Characteristics[1]	Reference	Probable Pathway
		No addition	0.1 meq/liter NaCl or Na_2SO_4					
		g dry wt/plant		%				
Gramineae								
Hordeum vulgare L. cv. Pallidium (barley)	None	0.77 3.82	0.99 4.01	5	(3)	N,H	(8)	C_3
Poa pratensis L. (Kentucky blue grass)	None	0.024	0.021	NS		N		C_3
Echinochloa utilis L. Ohwi et Yabuno (Japanese millet)	Chlorosis and necrosis	0.404	0.713	0.1		K		C_4
Cynodon dactylon L. (Bermuda grass)	Chlorosis	0.178	0.337	1		K,$H^{14}C_4$	(7)	C_4
Cyperaceae								
Kyllinga brevifolia	Chlorosis	0.628	1.245	1		K		C_4
Amaranthaceae								
Amaranthus tricolor L. cv. Early Splendour	Chlorosis and necrosis	0.884	2.099	0.1		K		C_4
Chenopodiaceae								
Chenopodium capitatum L. Aschers	None	12.25	14.37	NS	(3)	N	(15)	C_3
Beta vulgaris L. (sugar beet)	None	3.86	5.07	NS	(3)	N,H,$L^{14}C_4$	(8)	C_3
Atriplex nummularia Lindl. (oldman, giant saltbush)	Chlorosis	0.166	0.830	0.1	(3)	K,L,$H^{14}C_4$, $L^{13}C$	(11, 13, 14)	C_4
Atriplex paludosa R.Br. (marsh saltbush)	Chlorosis and necrosis	0.215	2.789	5	(3)	K		C_4
Atriplex quinii Fv.M.	Chlorosis and necrosis	0.116	0.815	5	(3)	K		C_4
Atriplex semibaccata R.Br. (berry saltbush)	Chlorosis and necrosis	0.104	0.653	1	(3)	K,$L^{13}C$	(13, 15)	C_4
Atriplex inflata Fv.M.	Chlorosis and necrosis	0.202	9.745	0.1	(3)	K,$H^{14}C_4$	(11, 14)	C_4
Atriplex leptocarpa Fv.M.	Chlorosis and necrosis	0.050	1.329	1	(3)	K		C_4
Atriplex spongiosa Fv.M. (pop saltbush)	Chlorosis and necrosis	0.570	12.472	0.1	(3)	K,L,P.carb	(10, 14)	C_4
Atriplex semilunalaris Aellen	Chlorosis and necrosis	0.098	0.526	0.1	(3)	Unknown		...
Atriplex lindleyi Moq.	Chlorosis and necrosis	0.093	0.560	5	(3)	Unknown		...
Atriplex vesicaria Heward ex Benth (bladder saltbush)	Chlorosis and necrosis	0.013	0.129	0.1	(2)	K,$H^{14}C_4$,P. carb,$L^{13}C$	(11, 13)	C_4
Atriplex hortensis L. var. Atrosanguineae (garden orache)	None	2.849	3.677	5	(3)	N,H,$L^{14}C_4$, $H^{13}C$	(14, 15)	C_3
Atriplex angustifolia Sm.	None	0.531	0.377	NS	(3)	Unknown		...
Atriplex glabriuscula Edmonton	None	24.804	24.100	NS	(3)	N,H,$H^{13}C$	(14)	C_3
Atriplex albicans Ait.	None	8.818 9.237	11.688 9.401	NS	(3)	Unknown		...
Kochia pyramidata Benth.	None	35.390	33.990	NS	(3)	N,$L^{14}C_4$	(11)	C_3
Kochia childsii Hort.	Chlorosis and necrosis	0.125	0.442	1		K,L,$L^{13}C$	(13, 14)	C_4
Exomis axyrioides Fenzl ex Moq.	None	0.864	0.925	NS	(3)	Unknown		...
Halogeton glomeratus (Bieb) Meyer	Smaller curved leaves, tendency to wilting	0.285	0.800	1	(16)	K	(15)	C_4
Cruciferae								
Brassica oleracea L. cv. "Savoy" (cabbage)	None	14.244	17.766	NS	(3)	N		C_3
Leguminosae								
Trifolium repens L. cv. "Palestine" (white clover)	None	2.299	2.897	NS	(3)	N		C_3
Solanaceae								
Lycopersicum esculentum Mill. cv. "Grosse Lisse"	None	4.84 6.93	4.76 7.83	NS	(3)	N		C_3
cv. "Marglobe"	None	13.76	15.40[2]	1	(17)	N		C_3

Table II—*Continued*

Species	Lesions in Plants Not Receiving Sodium	Yield		Significance of Difference	Reference	C_4 Pathway Characteristics[1]	Reference	Probable Pathway
		No addition	0.1 meq/liter NaCl or Na_2SO_4					
		g dry wt/plant		%				
Compositae								
Lactuca sativa L. cv. "Great Lakes" (lettuce)	None	3.871	6.113					
		0.822	1.228	NS	(3)	N		C_3
		2.230	2.444					
Aster tripolium L.	None	0.282	0.458	NS	(3)	Unknown		...
Portulacaceae								
Portulaca grandiflora Hook (rose moss)	Chlorosis, no flowers	0.242	0.789	1		K		C_4

[1] Leaf anatomy: N, normal (bifacial); K, Kranz. CO_2 compensation: L, low; H, high. ^{14}C in C_4 compounds (malate, aspartate): $L^{14}C_4$, low; $H^{14}C_4$, high. Phosphoenol pyruvate carboxylase activity: P.carb, high. ^{13}C discrimination: $L^{13}C$, low; $H^{13}C$, high.

[2] Cultures contained 1 meq/liter NaCl.

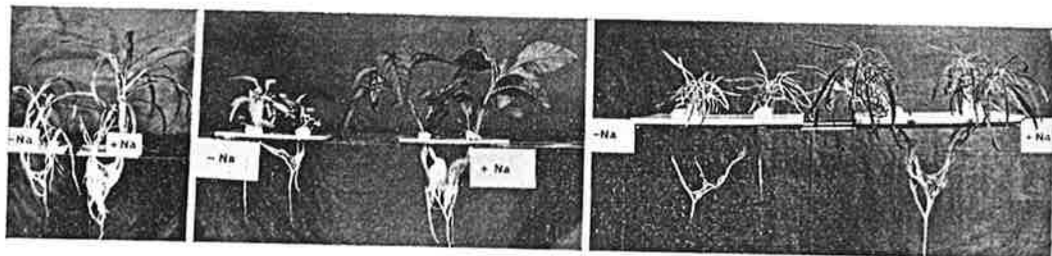

FIG. 1. Comparisons between plants of *Echinochloa utilis* (left), *Amaranthus tricolor* (center), and *Kochia childsii* (right) which received no addition (−Na) and 0.10 meq/liter sodium chloride (+Na).

a source of sodium to the plants. Seeds were thoroughly washed many times with distilled water before placing them in germination cultures. The seedlings were transferred to their cultures when sufficiently developed, and the sodium chloride treatment was supplied to the appropriate cultures.

RESULTS

Echinochloa utilis, Cynodon dactylon, Kyllinga brevifolia, Amaranthus tricolor, Kochia childsii, and *Portulaca grandiflora* showed significant dry weight responses when 0.1 meq/liter NaCl was supplied to their culture solutions (Table I). Plants of each species growing in cultures not receiving sodium made little growth compared to those supplied with sodium and had the characteristic leaf lesions described previously in sodium-deficient *Atriplex* species (Fig. 1) (2, 3, 6). Only the *Portulaca grandiflora* plants which received the sodium treatment set flower. Plants of *Poa pratensis* grew normally in "sodium-free" cultures and did not respond to the sodium treatment.

DISCUSSION

In Table II, the responses of 32 species to 0.10 meq/liter of odium chloride or sodium sulfate are summarized. Only certain species of *Echinochloa, Cynodon* (Gramineae), *Kyllinga* Cyperaceae), *Amaranthus* (Amaranthaceae), *Atriplex, Kochia, Ialogeton* (Chenopodiaceae), and *Portulaca* (Portulacaceae) ave been shown to respond to sodium by marked increases n dry weight. Plants grown in sodium-free cultures exhibited he leaf lesions previously described (2, 3, 6). These species ave characteristics of plants with the C_4 dicarboxylic pathway hich include the "Kranz type" specialized leaf anatomy (9), a w CO_2 compensation value, and reduced ^{13}C discrimination (13). The known C_4 pathway characteristics for each species are shown in Table II. No data relating to the C_4 pathway was available for certain species, including *Atriplex semilunalaris, Atriplex lindleyi, Atriplex angustifolia, Atriplex albicans, Exomis axyrioides,* and *Aster tripolium,* since their requirement for sodium was determined before the discovery of the C_4 dicarboxylic pathway and material is currently unavailable.

Three other species, barley (3), *Atriplex hortensis* (3), and tomato (17), with characteristics of C_3 pathway plants responded to sodium, but only marginally. The plants of these species in "sodium-free" cultures did not exhibit sodium deficiency lesions.

Within *Atriplex* and *Kochia,* which include both C_3 and C_4 species, only the C_4 species have been shown to respond to sodium; no decisive response was obtained in their C_3 species.

It appears from these data that species having characteristics of the C_4 photosynthetic pathway generally have a requirement for sodium.

Little is known of the role of sodium in plant metabolism. The respiration of leaves of sodium-deficient *Atriplex nummularia* is markedly depressed and restored within a few hours of adding sodium to the deficient culture solution, whereas the leaf symptoms are not alleviated until the 5th day. The output of CO_2 in the dark under anaerobic conditions was restored when sodium was fed to the deficient plants (4). In *Anabaena cylindrica,* nitrate reductase activity was greatly increased in cells grown without sodium. Addition of sodium controlled the activity of this enzyme. Nitrogen fixation was reduced in sodium-deficient cells (5). Sodium chloride compared to potassium chloride at 1 mM concentration has been shown to stimulate markedly the uptake of phosphate in the unicellular green alga, *Ankistrodesmus braunii* (12).

The correlation between the possession of the C_4 pathway and the essentiality of sodium could contribute to the under-

standing of the role of sodium in the metabolism of plants for which it is essential. It would be expected to function in a metabolic system unique to species with the C_4 dicarboxylic photosynthetic pathway.

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Publication 7

Short Communication

Growth Responses to Sodium by *Bryophyllum tubiflorum* under Conditions Inducing Crassulacean Acid Metabolism

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ABSTRACT

The dry weight yield of plants of *Bryophyllum tubiflorum* Harvey, a species with Crassulacean acid metabolism characteristics, increased significantly ($P < 1\%$) in response to added sodium (0.1 milliequivalents per liter NaCl was supplied to the culture solution initially containing less than 0.08 microequivalents per liter of Na) when grown under short day (8 hours) conditions but not when grown under long day conditions (16 hours).

From results of other work with Crassulacean acid metabolism species, it appears likely that under long day conditions, the plants assimilate CO_2 by the C_3 pathway but under short day conditions by the Crassulacean acid metabolism pathway in which metabolic processes common to those operating in the C_4 dicarboxylic pathway are active. It is suggested that sodium is involved in plants assimilating CO_2 with the C_4 and Crassulacean acid metabolism pathways.

Recent evidence suggests that species having the C_4 dicarboxylic photosynthetic pathway require small quantities of Na in their nutrition (3). Plants in cultures not supplied with Na made little growth compared with those receiving 0.1 meq/l NaCl and acquired leaf lesions of chlorosis and necrotic leaf tips, whereas species having C_3 (Calvin cycle) fixation grew normally in culture solutions to which no Na was supplied. The mechanisms for CO_2 fixation by species undergoing Crassulacean acid metabolism resemble those of species having the C_4 dicarboxylic photosynthetic pathway in certain respects (1, 4–6). These similarities suggested that CAM[1] species might also require small amounts of Na.

In preliminary experiments conducted in a greenhouse under long day conditions, *Bryophyllum tubiflorum* did not show any growth responses to small amounts of Na added to the cultures from which Na had been carefully eliminated. This lack of response to added Na was attributed to the possibility that the plants already contained sufficient Na for normal growth. However, under these conditions it is likely that the plants would have fixed CO_2 by the C_3 system and that the CAM system would not have been involved. Ting (7), reviewing previous literature, concluded that CAM activity is most pronounced when night temperatures are low and day temperatures are high and that short day conditions were also conducive to the CAM type of metabolism. Recent evidence (4) suggests that the activities of the enzymes specifically involved with CAM metabolism in *Kalanchoe blossfeldiana*, a short day plant, are phytochrome-controlled. In short days there was a progressively rapid increase in the activity of all the enzymes of the CAM pathway whereas in long days or in short days with nights interrupted by red light, the pathway was not operative, presumably due to low activity of PEP carboxylase.

In several experiments we found that growth was greatest under conditions of long days and small diurnal temperature variation. Under these conditions there was no growth response to Na. When plants were grown under conditions of short day length and large diurnal temperature variation, significant responses to Na were obtained although the overall growth was still less than that of the former plants.

Plantlets collected locally from plants of *Bryophyllum tubiflorum* Harvey were grown in low Na culture solution under conditions of small diurnal variation in temperature and with long days (approximately 14 hr/day). The plantlets from these plants were thoroughly washed in several changes of silica-distilled water and transferred to cultures (eight plants per culture) for subsequent experiments. The procedures for growth of plants under conditions of low Na have been previously described (2). The basal culture solution had the following composition expressed in μmoles/l: KNO_3, 5,000; $Ca(NO_3)_2$, 4,000; KH_2PO_4, 1,000; $MgSO_4$, 1,000; $(NH_4)_2HPO_4$, 1,000; H_3BO_3, 46; $MnSO_4 \cdot 7H_2O$, 9.1; $CuSO_4 \cdot 5H_2O$, 0.31; $ZnSO_4 \cdot 7H_2O$, 0.76; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1; NH_4Cl, 350. Iron was supplied as ferric ammonium ethylenediaminetetraacetate at 90 μmoles/l in the basal culture solution.

Less than 0.07 μeq/l Na was derived from the purified salts of the culture solution as an impurity, and silica-distilled water contained less than 0.0087 μeq/l, giving a total Na concentration of approximately 0.08 μeq/l. Culture vessels of 2-l capacity were of Na-free plastic material. Plants under long day conditions were grown in a naturally illuminated cabinet slightly pressurized by a continual supply of filtered air. This prevented the entry of dust particles and other atmospheric contaminants which could be a source of Na to the plant. Short day conditions were obtained in an artificially illuminated growth cabinet situated in an air-conditioned building with filtered air entering it continuously. The air for culture aeration was passed through an 8μm air-filter. NaCl was supplied to the appropriate cultures to give a final concentration of 0.1 meq/l.

The results of these experiments (Table I and Fig. 1) suggest that Na is not required by plants grown under conditions

[1] Abbreviation: CAM: Crassulacean acid metabolism.

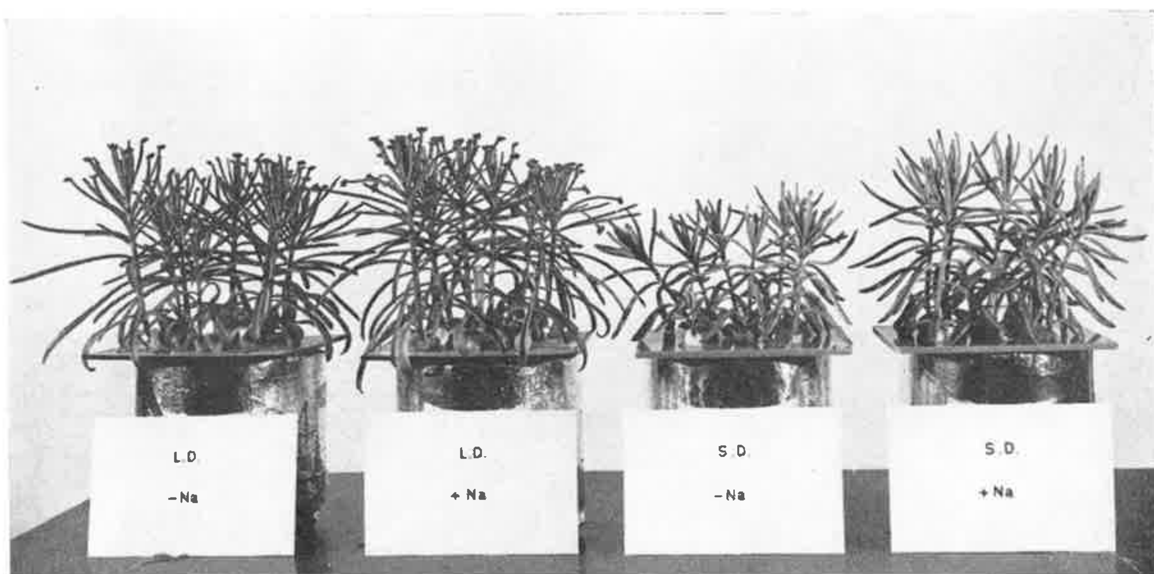

FIG. 1. Comparisons between plants of *Bryophyllum tubiflorum* receiving the following treatments. Left to right: no addition, 0.1 meq/l NaCl (16-hr light period; over-all temperature range, 15 to 38 C); no addition, 0.1 meq/l NaCl (8-hr light period; light temperature 33 C, dark temperature 13 C.

Table I. *Responses by Bryophyllum tubiflorum to NaCl Grown under Different Conditions*

Plants were transferred on day 24 from seedling cultures to culture vessels and received differential NaCl additions under the following conditions. Short days: in artificially illuminated growth cabinet with an 8 hr light period at approximately 2,800 ft-c and 16 hr dark period. Temperature during the light period was 33C and in the dark period 13 C. Long days-short days: in a naturally illuminated cabinet with the normal day length of 11 hr extended to 16 hr per day by a 100 w incandecsent lamp giving an intensity of approximately 100 ft-c. The over-all temperature range was 15 to 38 C. On day 65, cultures were transferred to short day conditions in the artificially illuminated growth cabinet. Long days: in the naturally illuminated cabinet with the normal day length of 11 hr extended to 16 hr per day. Plants were harvested on day 100.

Conditions of Growth	Mean Dry Wt per Plant		Significance of Difference
	No addition	0.1 meq/l NaCl	
	g		%
Short days	0.123	0.176	<1
Long days-short days	0.175	0.218	<1
Long days	0.548	0.521	NS

conducive to the C_3 mode of photosynthesis, *i.e.* under long day periods. Plants grew more actively under these conditions but showed no growth response to Na. Plants taken from the same population, however, when grown under conditions conducive to CAM photosynthesis, *i.e.* under conditions of short days and large diurnal temperature variation, responded significantly to small amounts of Na. Under these conditions of growth, metabolic processes common to those operating in C_4 dicarboxylic photosynthesis are active and this observation would suggest that Na is involved in this area of metabolism both in species having the C_4 photosynthetic system and in members of CAM carbon fixation. This supports the hypothesis that Na may be required for the primary dicarboxylic CO_2-fixation system characteristic of C_4 and CAM plants.

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C_4 Photosynthesis in Sodium-deficient Plants

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Abstract

The C_4 plants *Kochia childsii* Hort. and *Chloris barbata* Sw. showed symptoms characteristic of sodium deficiency. The $\delta^{13}C$ value, CO_2 compensation point and percentage of ^{14}C label in C_4 dicarboxylic acids in short-term photosynthesis were similar in sodium-deficient and normal plants. This is consistent with the operation of the C_4 pathway.

Introduction

Sodium has been shown to be an essential micronutrient element for C_4 plants, but not for C_3 plants (Brownell and Crossland 1972). In addition, a growth response to sodium has been reported in the crassulacean acid metabolism plant *Bryophyllum tubiflorum* Harvey (Brownell and Crossland 1974). This requirement is discussed in a recent review (Brownell 1979). This correlation between the requirement for sodium and the possession of the C_4 pathway prompted us to examine the effects of sodium nutrition upon photosynthetic metabolsim. We report here some early results which show that the fundamental character of C_4 photosynthesis remains unaltered in sodium-deficient plants.

Materials and Methods

Plant Material

Seeds of *Kochia childsii* Hort. were obtained commercially and seeds of *Chloris barbata* Sw. were collected locally. The procedures for the germination and growth of plants under conditions of low sodium have been described previously (Brownell 1965; Brownell and Crossland 1972). The total sodium concentration of the complete culture solution as an impurity was approximately 0·08 μM. Sodium chloride was supplied to appropriate cultures to give a final concentration of 0·1 mM.

Plants were grown in controlled environment chambers under either of the following conditions:
(*a*) 16 h light (500–600 $\mu E\ m^{-2}\ s^{-1}$, 400–700 nm) at 27°C, 8 h darkness at 19°C.
(*b*) 16 h light (180–210 $\mu E\ m^{-2}\ s^{-1}$, 400–700 nm) at 33°C, 8 h darkness at 21°C.

Plants of *K. childsii* and *C. barbata* used in experiments were 21 days old (17 days after differential sodium treatment) and 25 days old (18 days after differential sodium treatment), respectively.

Chlorophyll Determination

Chlorophyll was determined by extracting a weighed amount of leaf tissue with 80% acetone in the dark at 0–5°C. Total chlorophyll, chlorophyll *a* and chlorophyll *b* were determined using the equations of Mackinney (1941).

$\delta^{13}C$ Determination

Shoots of four plants were harvested, dried in an oven at 80°C, and a sample of this material was used for the determination of carbon isotope composition. The relative abundance of ^{13}C is expressed as $\delta^{13}C$ in ‰ with respect to a PDB standard as follows:

$$\delta^{13}\text{C value } (‰) = \left(\frac{^{13}\text{C}/^{12}\text{C sample}}{^{13}\text{C}/^{12}\text{C standard}} - 1\right) \times 10^3.$$

Isotope measurements were made using an isotope ratio mass spectrometer located at the Australian National University.

Determination of CO_2 Compensation Point

Compensation point determinations were carried out using a closed gas-exchange system. The air was circulated through a leaf chamber and the CO_2 concentration was continuously monitored using a Maihak infrared gas analyser. Illumination was from a 500-W mercury vapour lamp giving a quantum flux density at the leaf surface of 700–800 $\mu E\ m^{-2}\ s^{-1}$ (400–700 nm). Leaf temperature was measured with a fine wire thermocouple appressed to the underside of a leaf. The temperature of the assimilation chamber was controlled to give a leaf temperature of 35 ± 2°C during compensation point determinations. Shoot material was excised under water and quickly transferred to the assimilation chamber. The cut end was immersed in water and the plant material was pre-equilibrated by passing humidified air through the system until steady-state conditions were achieved. The system was then closed until the CO_2 compensation point was reached.

Short-term Labelling of C_4 Dicarboxylic Acids

$^{14}CO_2$ feeding experiments were conducted using a 3-litre chamber as described by Caldwell *et al.* (1977). However, the chamber was illuminated with two 500-W incandescent lamps which irradiated the leaves at 900 $\mu E\ m^{-2}\ s^{-1}$ (400–700 nm), and leaves were preconditioned for 45 min. The temperature within the chamber was maintained at 30°C and the plant material was killed after 10 s exposure to $^{14}CO_2$ by plunging into boiling 80% (v/v) ethanol. The tissue was further extracted with 40% (v/v) ethanol and boiling water. The extracts were pooled and pigments removed with chloroform. The aqueous phase was evaporated in a rotary evaporator, redissolved in a small volume of water and then separated into an amino acid fraction and an organic acid–phosphorylated compounds–neutral compounds fraction by passing through Amberlite IR-120.

An aliquot of the amino acid fraction was subjected to two-dimensional thin-layer chromatography using water-saturated phenol in the first, and propan-1-ol–H_2O–n-propyl acetate–acetic acid–pyridine (120:60:20:40:1, v/v) in the second dimension. Thin-layer plates were prepared by the method of Bieleski and Turner (1966).

The second fraction was subjected to two-dimensional thin-layer chromatography using ethanol–ammonia–water (4:1:1, v/v) in the first, and diethyl ether–formic acid (98%)–water (7:2:1, v/v) in the second dimension. Thin-layer plates were prepared by the method of Schurmann (1969).

Labelled compounds were located by autoradiography, excised and counted in scintillation fluid. The ^{14}C in each compound was expressed as a percentage of the total ^{14}C fixed.

Results

A comparison of dry weight yield, chlorophyll content, chlorophyll *a*/*b* ratio, $\delta^{13}C$ value, CO_2 compensation point and percentage of label in C_4 dicarboxylic acids for the two species studied is shown in Table 1.

Discussion

Sodium-deficient plants of *K. childsii* and *C. barbata* showed greatly reduced dry weight production and chlorophyll concentration. However, the chlorophyll *a*/*b* ratio, $\delta^{13}C$ value, CO_2 compensation point and percentage of ^{14}C in C_4 dicarboxylic acids after short-term labelling were unaffected by sodium nutrition and were consistent

with the operation of the C_4 pathway (Hatch and Slack 1970; Black 1973). Downton and Törökfalvy (1975) showed that photosynthetic pathways remained unchanged when the halophytic C_4 grass *Aeluropus litoralis* was grown at low and high levels of NaCl. Our studies show further that the response of C_4 plants to micronutrient levels of sodium (Brownell and Crossland 1972) does not involve changes in the pathway of photosynthetic carbon assimilation.

Table 1. Comparison of dry weight yield, total chloropyll, chlorophyll *a*/*b* ratio, $\delta^{13}C$ value, CO_2 compensation point and percentage ^{14}C in C_4 dicarboxylic acids of sodium-deficient and normal C_4 plants

Species and treatment	*K. childsii*		*C. barbata*	
	0·1 mM NaCl	No addition	0·1 mM NaCl	No addition
Mean dry wt/plant (g)[A,C]	0·710	0·090	0·238	0·071
Chlorophyll (mg g^{-1} fresh wt)[A,D]	0·53	0·25	2·00	1·10
Chlorophyll *a*/*b* ratio[A,D]	3·8	3·8	3·3	3·4
$\delta^{13}C$ value (‰)[B,D]	−13·3	−13·3	−12·1	−13·4
CO_2 compensation point (vpm)[B,D]	1	0	4	2
% ^{14}C in C_4 dicarboxylic acids[A,E]	73	78	73	77

[A] Plants grown in controlled environment (*a*) (See Methods).
[B] Plants grown in controlled environment (*b*) (See Methods).
[C] Significance of difference for both *K. childsii* and *C. barbata* ($P < 0{\cdot}001$).
[D] All values are means of two independent determinations.
[E] Leaves were exposed to $^{14}CO_2$ for 10 s in the light and then killed. Values refer to combined label of malate and aspartate.

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Sodium as an Essential Micronutrient Element for Plants and its Possible Role in Metabolism

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I. INTRODUCTION

A. SCOPE

This article is concerned almost entirely with the effects of sodium on plants including microorganisms for which it can be considered essential according to the criteria of Arnon and Stout (1939). These plants include those angiosperms having the C_4 dicarboxylic photosynthetic pathway (Brownell and Crossland, 1972) and at least some members of the Cyanophyta (Allen and Arnon, 1955; Kratz and Myers, 1955). Concentrations of sodium of about 0·1 mol m^{-3} (2·3 ppm) and below, support optimum growth in these species and its effects at these concentrations on growth and metabolism are discussed. References are also made to certain bacteria and fungi, largely of marine origin, for which sodium has been shown to be essential. Sodium is generally required at relatively high concentrations by these organisms but reference to them is considered to be justified due to their specific requirement for sodium. A recent review by Jennings (1976) has dealt with the effects of sodium at considerably higher concentrations i.e. 50 mol m^{-3} (1150 ppm) and above on plants having the C_3 as well as on plants with the C_4 pathway. Although sodium at these concentrations may stimulate growth in certain species and affects the plants in other ways such as by increasing their succulence (Jennings, 1968), it is apparently not essential for them. Plants with C_3 photosynthesis have been found to develop and grow normally with an almost complete absence of sodium viz. less than 0·256 mmol m^{-3} (0·006 ppm) with tomatoes (Woolley, 1957), less than 0·17 mmol m^{-3} (0·004 ppm) for cotton (Pleunneke and Joham, 1972) and less than 0·07 mmol m^{-3} (0·0016 ppm) for more than 15 species with C_3 photosynthesis (Brownell, 1968).

It is difficult to discuss sodium as a nutrient without making some reference to its effects at high concentration on C_3 plants but the main emphasis in this article is on the consideration of the role of sodium as a nutrient in plants for which it is essential.

Reviews dealing with effects of sodium at high concentrations on plants include those by Wybenga (1957), Hewitt (1963), Rains (1972), Marschner

(1971), Waisel (1972), Marschner (1975), Flowers (1975) and Flowers *et al.* (1977).

Comprehensive descriptions of the effects of sodium salts on *Salicornia herbacea* and *Aster tripolium* were made by van Eijk (1939) and on sugar beet by Tullin (1954) and reviews have been published by Lehr (1953, 1957).

This article comprises a brief history of the understanding of sodium as a nutrient element for plants. The methods for obtaining sodium-free conditions are discussed in the second section and in a further section, the evidence for sodium being an essential element for various plants is given and its known physiological effects are described. Finally, tentative schemes are proposed to explain its possible involvement in plant metabolism.

B. HISTORICAL PERSPECTIVE – 1860 TO 1977

Between 1860 and 1890, it was indicated from experimental work by Knop, Nobbe and Pfeffer that ten elements were absolutely essential for plant life. They were carbon, hydrogen, oxygen, phosphorus, potassium, nitrogen, magnesium, calcium, sulphur and iron. The other elements found in plants were considered of little or no physiological importance and their presence was thought to be due to them entering the cell passively. If large amounts were present, it was attributed to a specific property of the plant to accumulate them.

It was not until early in the twentieth century that the essentiality of further elements was demonstrated. Until then it was generally assumed that the essential elements were all needed in relatively large amounts and little attempt had been made to eliminate impurities from salts used in nutrient solutions.

Due to the general abundance of sodium, it was thought that if it were essential for plants it would be needed in relatively large amounts. There was a tendency to regard sodium which resembled potassium in its chemical properties as possibly having a similar role to that of potassium in the nutrition of plants. Birner and Lucanus (1866) had shown potassium to be essential for oats in water culture but Nobbe, Schroeder and Erdmann (1871) (cited by Hartt, 1934) concluded from their work on potassium nutrition of plants that sodium was not needed by plants. Hellriegel and Willfarth (1898) (cited by Dorph-Petersen and Steenbjerg, 1950) from their observations of growth increases under conditions of potassium deficiency suggested that sodium should be regarded as a beneficial element.

During the next decade, Osterhout (1909, 1912) found sodium to be necessary for certain marine algae. The replacement of sodium by ammonium, calcium, magnesium, potassium, barium, strontium, caesium, rubidium or lithium was "distinctly injurious" to the plants.

Much of the research on sodium as a possible nutrient element for higher

plants was conducted on a field scale from the late nineteenth century until the mid twentieth century. In many instances, growth responses have been reported but the interpretation of the results of these experiments is often difficult. When massive amounts of sodium salts were applied, the responses could have been due to indirect effects of the treatment and not necessarily to the direct involvement of sodium in the metabolism of the plants.

In experiments with complicated media such as sand, vermiculite, ion exchangers or soils, the treatment application could affect differentially a wide array of conditions including the availability of other nutrient elements, the pH, or the growth of microorganisms which could, in turn, affect the growth of plants indirectly.

In experiments conducted before the discoveries of the requirements for micronutrients, increases in yield could have been brought about by the supply of a limiting micronutrient associated with the sodium treatment salt as an impurity.

The designs of some experiments can be criticized for the lack of control treatments. When only one salt of sodium was supplied, the effects observed could have been due to the anion of the salt and not necessarily to the sodium. In other experiments, a salt of sodium e.g. sodium nitrate has been substituted for the salt of the same anion and a different cation e.g. calcium nitrate. Any differences between such treatments could be ascribed to the associated change in the ratio of monovalent to divalent cations in the medium and not specifically to the sodium.

However, despite these possible limitations in experimental procedures, it appears that sodium at high concentrations (10 mol m^{-3} and above) increases growth in many instances.

Perhaps the most puzzling feature of reported responses is their apparent inconsistency. Some workers have obtained no response to sodium even when potassium was limiting. Hartt (1934) found that sodium at 2·25 mol m^{-3} (51·75 ppm) was unable to substitute for potassium in the nutrition of sugarcane. No special measures were taken to grow the plants in a low-sodium medium in this work. Similarly, Montasir *et al.* (1966) obtained no growth response to sodium in sesame when potassium was limiting.

There are many cases of responses to sodium when potassium is limiting. Early publications from the Rhode Island Agricultural Research Station by Hartwell and Pember (1908) and Hartwell and Damon (1919) showed that sodium could act as a partial substitute for potassium for the growth of wheat in water culture. In barley, sodium has been shown to replace potassium to some extent (Mullison and Mullison, 1942; Lehr and Wybenga, 1958; Montasir *et al.* 1966). Many other species have also responded to sodium in a deficiency of potassium including cotton (Joham, 1955; Joham and Amin, 1965) and oats (Truog *et al.*, 1953). Ulrich and Ohki (1956) observed growth responses with sugar beet only under low potassium conditions.

It has also been shown that sodium at 10 mol m^{-3} (230 ppm) has restored the translocation of carbohydrates in calcium-deficient cotton plants to the levels obtained with non-limiting levels of calcium present (Joham and Gossett, 1974; Joham and Johanson, 1973; Whitenberg and Joham, 1974). Partial substitution of sodium for calcium in the growth of excised cotton roots was also demonstrated (Johanson and Joham, 1971).

There have been numerous reports of growth responses to sodium at high concentrations (10 mol m^{-3} and above) even when other nutrient elements are present at optimal levels. These responses have been obtained generally (but not always) in species of Chenopodiaceae, including sugar beet, Swiss Chard, spinach, *Atriplex hastata* and mangolds (Harmer and Benne, 1945; Harmer *et al.*, 1953; Kushizaki and Yasuda, 1964; El-Sheikh *et al.*, 1967; El-Sheikh and Ulrich, 1970; Montasir *et al.*, 1966), *Salicornia herbacea* and the Composite species, *Aster tripolium* (Van Eijk, 1939; Baumeister and Schmidt, 1962). Connor (1969) showed that sodium chloride at 256 mol m^{-3} (5897 ppm) sodium added to a complete culture solution gave a 69% increase in yield, whereas all levels of potassium chloride or calcium chloride suppressed the yield of *Avicennia marina* (grey mangrove) by 12% and 78%, respectively. No visual signs of deficiency were noticeable in the plants not receiving sodium.

Hewitt (1963) concluded that the response to sodium is dependent upon the species and the composition of the culture solution. This is shown clearly in the experiments of Montasir *et al.* (1966) who examined a number of plants for the effect of sodium with different levels of potassium in the culture solutions. *Atriplex hastata*, alfalfa, barley, spinach and flax all responded by increased dry weight production on receiving sodium chloride when potassium was limiting, but sesame did not respond to sodium at any levels of potassium. Alfalfa and flax showed medium responses with adequate potassium levels while *Atriplex* and spinach showed large responses with ample potassium present. It was suggested that sodium has a specific function in the nutrition of *Atriplex* and spinach as it increased growth even at the highest level of potassium while in sesame it was detrimental. The calcium concentration on a dry weight basis decreased with decreasing sodium chloride in the solution. The treatments were applied in such a manner that the sums of concentrations (on an equivalence basis) of sodium and potassium were the same in all treatments.

Harmer and Benne (1945) and Harmer *et al.* (1953) surveyed investigations on the effects of sodium on plant growth and placed crops into one of two classes each of which was subdivided into two tentative groups with regard to their response to sodium:

A. Benefited by sodium in a deficiency of potassium

Group 1. None to slight benefit

e.g. buckwheat, corn, lettuce.

Group 2. Slight to medium benefit
e.g. asparagus, barley, flax, wheat.

B. Benefited by sodium in sufficiency of potassium

Group 3. Slight to medium benefit
e.g. cabbage, mustard, radish, rape.

Group 4. Large benefit
e.g. celery, mangold, sugar beet, turnip.

Lehr (1953) in a similar scheme classified crops according to the ability of sodium to replace their potassium requirements and to the independent effect of sodium on its yield.

The discussion to this point has been concerned with the effects of sodium at high concentrations in increasing dry weight yields. Sodium nutrition also appears to affect the plant qualitatively. There have been many observations of increased sugar concentration in sugar beet fertilized with sodium (El-Sheikh and Ulrich, 1970; Palladina and Bershtein, 1974). The quality of fibre crops including flax and cotton has been influenced by sodium treatment. Flax has been found to have optimal fibre quality when 6% of the exchangeable ions were sodium (Lehr and Wybenga, 1955; Wybenga and Treggi, 1958; Treggi, 1961). Szymanek (1952) found the straw yield was unaffected but that there were more, stronger fibres in flax receiving nitrogen as sodium nitrate than as calcium nitrate. Moscolov and Aleksandrovskaya (1962) obtained increased yields of flax and fat content of seeds with increased synthesis of sucrose in the leaves in response to supplying sodium chloride instead of potassium.

With low substrate levels of calcium or potassium, both the dry weight production and the bolls : leaves plus stem ratio of cotton were reduced. Sodium at 10 mol m^{-3} (230 ppm) partly restored these values in calcium-deficient plants and fully restored them in potassium-deficient plants (Joham, 1955).

Little is known about the way in which sodium is involved in increasing yields and bringing about qualitative changes in response to high concentrations of sodium in certain plants. Sodium may act as a monovalent cation activator of enzymes. It appears that although K^+ is specifically required by some enzymes and appears to be the most effective activator of the monovalent cations in many other enzymes there are no known instances where a higher plant enzyme is specifically activated by Na^+ (Evans and Sorger, 1966). Evans and Sorger (1966) considered that the effectiveness of K^+, NH_4^+, Rb^+, Na^+ and Li^+ as activators of many enzymes was consistent with their effectiveness to stimulate growth. In many of the 46 enzymes they listed from animals, higher plants and microorganisms which require monovalent ions for maximal activity their effectiveness as activators decreases in the following order: K^+, Rb^+, NH_4^+, Na^+, Li^+. Na^+, for example, is only about 20% as effective as Rb^+ as a monovalent cation activator of pyruvate kinase and K^+

and both NH_4^+ and Rb^+ were more effective than Na^+ (Miller and Evans, 1957; McCollum *et al.*, 1958). Particulate starch synthetase has been shown to have an absolute requirement for K^+ but Rb^+, Cs^+ and NH_4^+ were 80% and Li^+ 21% and Na^+ only 8% as effective as K^+ as monovalent cation activators (Nitsos and Evans, 1969). Hawker *et al.* (1974) studied the effects of sodium and potassium on starch synthesis in leaves of *Spinacia oleracea* and *Beta vulgaris*. Growth was decreased with low levels of potassium but the addition of sodium increased the growth to that of plants with adequate potassium. By comparison *Phaseolus vulgaris* grew poorly when part of the potassium was replaced by sodium. They found that whereas sugar beet plants grown with adequate levels of potassium contained nine times more starch than leaves of plants grown on high sodium low potassium culture solutions, the starch content in spinach was not affected. Potassium, but not sodium stimulated, by about 100%, the activity of starch granule bound ADP–glucose starch synthetase from sugar beet, bean and *Atriplex nummularia* but not from spinach leaves. Neither potassium nor sodium caused marked stimulation in other enzymes associated with carbohydrate metabolism.

Oji and Izawa (1969) obtained evidence that potassium ions stimulated the formation of nitrate reductase and nitrite reductase in rice seedlings. Sodium could partially substitute for potassium in the enzyme formation.

Potassium and the other monovalent cations are required to be present in very high concentrations as activators of enzymes compared to those required for other mineral cations (with the exception of magnesium) which act as physiological activators of plant enzymes. For maximal activity often concentrations of 50 to 100 mol m^{-3} are required. Epstein (1972) suggests that such loose binding between potassium and the enzymes activated by it may not have been a limiting factor in the early marine phases of evolution when high concentrations of potassium were readily available in the sea water. The other monovalent cations are also required at high concentrations for their optimum activity.

Evans and Wildes (1971) suggest the following mechanisms to explain the activation by univalent cations of certain enzymes for which they function as activators: (i) subunit structure of some enzymes may depend on univalent cations; (ii) capacity of some enzymes to bind a particular coenzyme is ion dependent; (iii) univalent cations may act as alloteric effectors; (iv) univalent cations may influence the conformation of some enzymes without causing gross changes in physical structure; (v) univalent ions may stabilize reaction intermediates during enzyme catalysis by mechanisms not yet fully understood.

The finding that sodium was an essential micronutrient element for certain, but not all plants, came as a surprise. Here was a ubiquitous element, closely resembling the macronutrient element potasssium in many of its chemical

properties being needed in only very small quantities for the plants requiring it. The first suggestion that sodium might be needed as a micronutrient element was probably due to Pfeffer (1899) who stated:

> "... it is not always easy to decide with certainty whether an element is essential or not. Moreover, if but a little substance is required, the presence of the merest trace as impurities in the water or the salts employed, or dissolved from the walls of the glass vessel containing the culture fluid, may produce a marked effect especially in the fungi, & although the traces may be so small that the tests employed failed to reveal them. Nor has a flowering plant ever been developed in the complete absence of silicon or sodium, a condition which, however, could only be secured by a cultivation in a fluid which was not in contact with the glass. Both seeds and spores always contain a certain amount of the essential elements . . ."

Little attention was given to the suggestion of Pfeffer (1899) that sodium could have a role as an essential micronutrient until the early 1950s. The late Professor J. G. Wood, of the University of Adelaide suggested the possibility that sodium and/or chlorine could be essential for plants in very small amounts since at that time no growth experiments had been reported in which these elements had been carefully excluded from the plants' environment. He suggested a Ph.D. project to the author to examine these elements as possible micronutrients for plants. Two developments greatly assisted the investigation. The first was the introduction of the emission flame photometer (followed soon after by the invention of the atomic absorption spectrophotometer (Walsh, 1955)), which enabled sodium to be determined rapidly at low concentrations. The second was the use of apparatus of plastic materials virtually free of sodium which had recently become available. Professor Wood suggested that we work with *Atriplex vesicaria* (bladder salt bush) as one of the species to be investigated. He had shown that it had a propensity to accumulate large quantities of sodium and chlorine (Wood, 1925) and hence had a possible need for larger amounts of these elements than other plants. He was also interested in its possession of the bundle sheath in its leaf anatomy which he had described previously (Wood, 1925). This he thought might have some special physiological significance! Dry weight responses to sodium chloride were obtained in preliminary water culture experiments in this species as observed by Ashby and Beadle (1957) with *Atriplex inflata* and *Atriplex nummularia.*

During the course of this work Broyer *et al.* (1954) demonstrated the essentiality of chlorine in tomatoes and subsequently the requirement for chlorine was shown for ten further species by Johnson *et al.* (1957). There now seems little doubt that chlorine is an essential micronutrient for all higher plants. Although the requirement for chlorine was considerably higher than for the other micronutrients, its essentiality was not readily demonstrated until the chlorine (probably partly in dust and HCl vapour) was filtered from

the air surrounding the plants. The first clear evidence of sodium being essential for plant life was produced by Allen and Arnon (1955) who demonstrated a specific requirement for the blue-green alga *Anabaena cylindrica*. Only 0·22 mol m^{-3} (5 ppm) sodium were needed and no other monovalent cation would substitute for sodium. Two years later, *Atriplex vesicaria* (bladder salt bush) was also shown to have a specific requirement for sodium (Brownell and Wood, 1957).

Plants not receiving sodium made only little growth and showed signs of sodium-deficiency including chlorosis and necrosis of leaves. Optimal growth was restored on the addition of 0·1 mol m^{-3} (2·3 ppm) of sodium to the culture solution. From the pattern observed with the other essential elements, it was expected that sodium would be required by all plants. However, the range of species for which sodium was shown to be essential was surprisingly restricted to Australian species of *Atriplex*. None of the other species examined could be shown to have a requirement for sodium (Brownell, 1968). Even when great care was taken to eliminate sodium from the environment of the plants, the other 23 species examined including other species of *Atriplex* grew normally without addition of sodium salts to their culture (Brownell, 1968). It was concluded that these species either had an extremely small requirement for sodium compared to that of the Australian species of *Atriplex* or that they did not require it at all. Since no distinguishing features were then known between the Australian and other species of *Atriplex* which could explain the difference in their responses, it still seemed possible that all higher plants might require sodium but those plants which had grown normally without added sodium might require only extremely small amounts compared to the Australian species.

When great care was taken to further eliminate sodium from the salts of the culture solution, the concentration of sodium in the basal culture solution was reduced to 0·0685 mmol m^{-3} (0·0016 ppm). However, all plants examined with the exception of the Australian species of *Atriplex*, still grew normally without added sodium even though the Australian species of *Atriplex* needed approximately 0·1 mol m^{-3} (2·3 ppm) sodium for maximum growth. This is 1460 times the concentration of sodium in the purified basal solution which supported normal growth of the other species.

With the discovery of the C_4 dicarboxylic acid photosynthetic pathway (Hatch and Slack, 1970), the correlation between the requirement for sodium and the possession of the C_4 pathway was established (Brownell and Crossland, 1972). It is, of course, still possible that C_3 plants have a requirement for sodium but it would have to be less than about 0·0685 mmol m^{-3} (0·0016 ppm).

Responses to small amounts of sodium were obtained in *Bryophyllum tubiflorum*, a Crassulacean acid metabolizing plant when grown under short-day conditions with a large diurnal variation in temperature but not under

long-day conditions with small diurnal temperature variation (Brownell and Crossland, 1974; Boag, 1976).

At the present time, little is known of the physiological role of sodium in plants. The respiration rate of leaves of C_4 *Atriplex* species is depressed under low sodium conditions but was rapidly restored on the addition of sodium but no other monovalent cation (Brownell and Jackman, 1966). In the blue-green alga, *Anabaena cylindrica*, sodium appears to control the activity of nitrate reductase; its activity increases up to twenty times in sodium-deficient compared to normal nitrate-grown cells. The nitrogen-fixing activity is decreased in sodium-deficient compared to normal cells (Brownell and Nicholas, 1967). A similar response of nitrate reductase to sodium has not been observed in a higher plant with the C_4 decarboxylic acid photosynthetic pathway. The similarity of the C_4 decarboxylic acid systems in C_4 and CAM plants and its absence in C_3 plants immediately suggests that this could be the area of metabolism in which sodium is involved. Some recent work, however, suggests that sodium may affect a part of metabolism not yet defined which occurs in C_4 and CAM but not in C_3 plants. Present studies indicate that sodium may have a role in preventing a "leakage" of carbon compounds from the metabolic system of plants for which it is essential. A scheme is presented in this article to explain this hypothesis.

II. LOW-SODIUM CULTURE CONDITIONS

A. DEFINITION

It is not possible to specify an exact concentration of sodium at which growth is limited in plants for which it is essential. In *Atriplex vesicaria* (bladder salt bush), concentrations of less than 0·05 mol m^{-3} (1·25 ppm) sodium may limit growth but the concentration would be expected to vary according to the species and conditions of the experiment (Brownell and Wood, 1957). In *Anabaena cylindrica*, a concentration of 0·22 mol m^{-3} (5 ppm) Na was needed for optimum growth (Allen and Arnon, 1955). Although these concentrations are high compared to those of some other elements required for optimal growth e.g. Mn, 0·01 mM (0·55 ppm); Cu, 0·001 mM (0·0635 ppm); Mo, 0·0005 mM (0·048 ppm) (Hewitt and Smith, 1975) special methods are needed to obtain culture media with concentrations of sodium low enough to produce lesions resulting from sodium-deficiency in plants, consistently, due to the ubiquity of sodium. Untreated salts even of analytical reagent grade, often contain large, variable amounts of sodium. Brownell (1965) prepared a culture solution composed of unpurified analytical reagent salts containing 4·63 mmol m^{-3} (0·106 ppm) sodium compared to 0·069 mmol m^{-3} (0·0016 ppm) sodium in the solution made up from salts especially prepared to have only low sodium concentrations. The culture solution of

Williams (1960) containing reagent grade chemicals and deionized water had concentrations of sodium between 52·2 and 78·3 mmol m^{-3} (1·2 to 1·8 ppm). Plants of *Halogeton glomeratus* showed a marked decline in yield when grown on this culture solution compared to those grown on the same solution to which 1 mol m^{-3} (23 ppm) sodium was supplied. Woolley (1957) prepared a culture solution containing 0·256 mmol m^{-3} (0·006 ppm) sodium in experiments designed to determine if sodium were essential for tomatoes.

In experiments in which sodium was shown to be essential for *Anabaena cylindrica*, Allen and Arnon (1955) prepared culture solutions by twice re-crystallizing the macronutrient salts but did not state the concentration of sodium in their purified medium. The addition of sodium chloride to give the solution a sodium concentration of 217 mmol m^{-3} (5 ppm) was sufficient for optimal growth of the alga. In a study of the effects of sodium on nitrogen metabolism in the same organism, the concentration of sodium was reduced to 0·174 mmol m^{-3} (0·004 ppm) (Brownell and Nicholas, 1967) by methods described by Brownell (1965). Ward and Wetzel (1975) estimated a concentration of 10·26 mmol m^{-3} (0·236 ppm) sodium in their basal solution in a study on the effects of sodium on blue-green algal growth. They used unpurified salts.

The plants for which sodium has been shown to be essential, including certain blue-green algae, C_4 and CAM plants appear to require less than about 217 mmol m^{-3} (5·00 ppm) of sodium for optimum growth. Therefore, it is necessary to purify the culture solution salts to free them from sodium and thus obtain decisive signs of sodium-deficiency in these plants.

It is obvious that these levels of sodium are very much less than those of about 10 mol m^{-3} (230 ppm) and above needed to elicit growth responses in certain other species including sugar beet (Harmer and Benne, 1945).

B. DETERMINATION OF SODIUM

Early work on the effects of sodium in plant nutrition was hampered by the lack of suitable methods for determining sodium. Indirect methods employed were too inaccurate and insensitive to enable quantitative determination of small amounts of sodium in plants and their culture solutions. Bertrand (1929) studied the sodium contents of plant material by the uranyl acetate precipitation method. This was satisfactory for determinations of tissues with high concentrations of sodium but was not sufficiently sensitive for estimating sodium at the lower concentrations. The method was time-consuming and tedious making it difficult to carry out determinations on many samples.

From 1940–1950 the use of the emission flame photometer became general. Jones (1960) describes an early version of flame photometer constructed by Klemperer in 1910. In this instrument, spray-charged oxyacetylene flames carrying unknown and standard solutions (which were varied) were com-

pared visually in a divided spectroscope eye-piece. Lundegardh in the 1930s used a monochromator, vacuum photocell and amplifier with an air-acetylene flame. During the 1930–1950 period, monochromators were replaced by optical filters transmitting only narrow ranges of the optical spectrum. This became the basis for the modern emission photometer which provided a rapid, sensitive method for determining the sodium concentrations in water and extracts of plant tissues. Vogel (1961) has described the method and some of the main types of instruments employed.

Wallace *et al.* (1948) used the emission flame photometer to determine the sodium contents of 300 samples from over 100 native and cultivated plants growing in New Jersey. They ranged from 0·00 to 3·00% on a dry weight basis.

Brownell (1965) found the flame photometer to be suitable for the determination of sodium in distilled water by carefully evaporating it down to one-thousandth of its original volume in a silica beaker and determining the sodium it contained with the flame photometer but large interferences occurred when determining sodium at low concentrations in solutions of nutrient salts and ashed plant material. To overcome these interferences Woolley (1957), using a flame spectrophotometer, made readings at wavelengths of 586 nm and 594 nm as well as at 589·5 nm. Interpolation between the wavelengths of 586 nm and 594 nm gave values representing sodium-free luminescence which was subtracted from the value obtained at the sodium emission line at 589·5 nm. The method of Pleunneke and Joham (1972) was similar except that they estimated the background radiation at 586 nm and 592 nm. They used a 1% (v/v) purified HCl solution as a reagent blank in plant tissue analysis. When estimating sodium with a filter flame photometer in solutions of nutrient salts, particularly in those of calcium and potassium, the relative errors due to the interferences of other ions increased as the salts were progressively purified (Brownell, 1965). This is shown in Fig. 1 in which the estimated concentration of sodium in 2·4 M KNO_3 as determined by the flame photometer decreased after three successive recrystallizations to a constant level. Further recrystallizations did not reduce the apparent concentration of sodium. This apparent level of sodium could have been due either to a small quantity of sodium which was not eliminated by repeated recrystallization or to interference by potassium.

Estimations of sodium in the same solutions with an atomic absorption instrument (Box and Walsh, 1959) were virtually free of interference (Fig. 1). However, when estimating sodium in highly concentrated solutions of nutrient salts and digests of plant material, the response of the atomic absorption instrument to sodium was reduced. Under these conditions, it was necessary to prepare calibration curves showing the response of the atomic absorption instrument to known concentrations of sodium in solutions similar to those in which sodium was to be estimated. Calibration curves

were prepared for determining sodium in solutions containing potassium and calcium (Fig. 2).

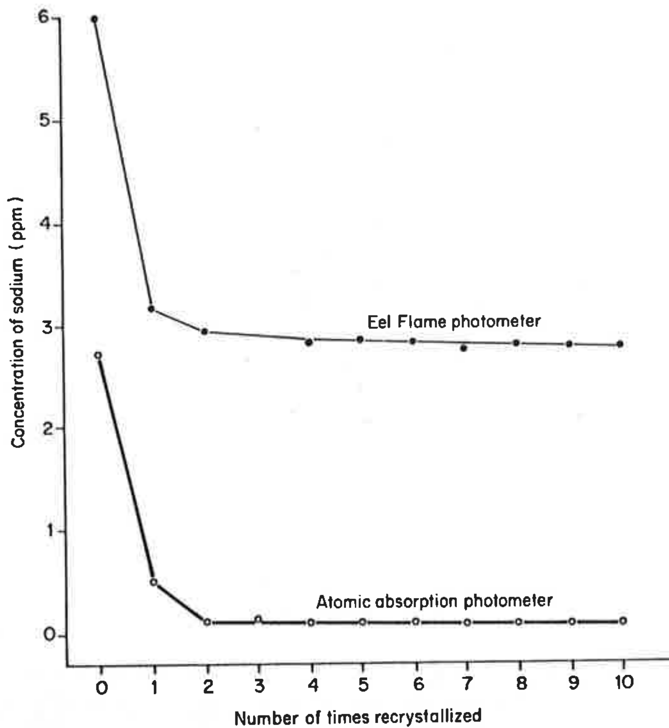


Fig. 1. The apparent concentration of sodium in 2·4 mol l^{-1} KNO_3 determined with the Eel flame photometer and by an atomic absorption photometer (Box and Walsh, 1959) (P. F. Brownell, unpublished work).

The estimates of sodium concentrations in solutions of purified salts used for making up culture solutions when made with the Eel filter flame photometer, the Bechman DU flame spectrophotometer and the atomic absorption instrument showed poor agreement, particularly in the calcium nitrate data (Table I, Brownell, 1959). Emission methods were generally unsatisfactory due to the interference from certain ions. Furthermore, results obtained with the filter flame photometer varied according to the filter used in the instrument (Fig. 3).

Neutron activation analysis has been used to determine sodium in plant material (Singh and Dieckert, 1973). These determinations are subject to interference from magnesium if the sample contains relatively more magnesium than sodium and the thermal neutron used has an appreciable fast neutron component. In peanut flour containing 3·3 $\mu g\ g^{-1}$ dry weight Na and

3·7 mg g^{-1} Mg the proportion of apparent sodium contributed by magnesium was 42 % but this was an unusually critical test for the method as the tissue contained an abundance of magnesium and only traces of sodium.

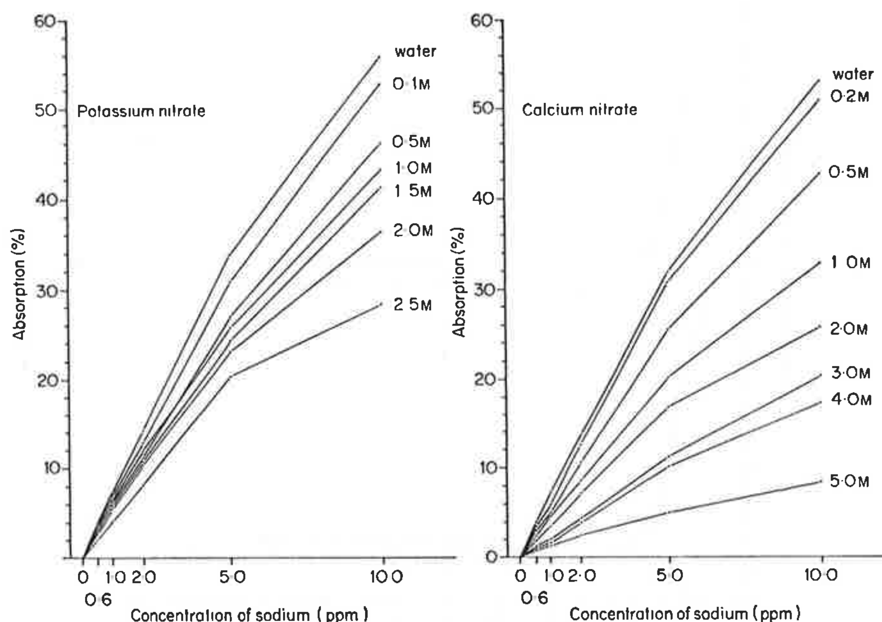


Fig. 2. Calibration curves showing the absorption by sodium in solutions of potassium nitrate and calcium nitrate at varying concentrations (P. F. Brownell, unpublished work).

Special care is required for the determination of sodium in plant tissues at low concentrations. Woolley (1957), at harvest, placed material from tomato plants in perforated polyethylene bags which were in turn placed in paper bags and left in an 80°C blower-oven for several days. The dried material was ground in a Wiley mill to pass a 40-mesh sieve. A polythene plate was substituted for the glass plate normally supplied as a part of the Wiley mill. Samples were stored in new, but unwashed polyethylene bags. They were ashed at 700°C in platinum crucibles and after cooling made up in 0·16 M HNO_3. With the sample sizes and dilutions used, it was possible to determine sodium down to concentrations of 0·005 μmol g^{-1} dry weight (0·12 ppm (dry basis)).

Pleunneke and Joham (1972) dry-ashed 2 g samples of dried plant material from cotton plants in nickel crucibles for 4 h at 225°C and then overnight at 550°C. The ash was dissolved in purified dilute HCl and the crucibles were rinsed with four 20 ml aliquots of warm water. The dissolved sample was

TABLE I

Concentrations of Sodium in the Culture Solution

Salt	Concentration of salt in culture solution	Estimated concentration of sodium in culture solution (ppm)		
		Eel flame photometer	Beckman D.U. and spectro-photometer	SI-RO-SPEC and atomic absorption
$Ca(NO_3)_2$	0·004 mol l^{-1}	0·10	0·03	0·0013
KNO_3	0·006 mol l^{-1}	0·007	0·0054	0·00075
KH_2PO_4	0·001 mol l^{-1}	0·0024	0·0017	0·00025
$MgSO_4$	0·001 mol l^{-1}	0·00002	0·00002	no response
$FeCl_3$	0·006 mmol l^{-1}	0·002	0·00001	no response
H_3BO_3	0·46 μmol l^{-1}	0·00032	0·00008	0·00001
$MnSO_4$	0·182 μmol l^{-1}	0·00028	0·0002	0·000005

From Brownell (1959).

collected in a polyethylene beaker and filtered and brought to volume for the sodium determination. Concentrations of sodium as low as 0·1 μmol g^{-1} dry weight (2·3 ppm (dry basis)) were determined by this method. Both Woolley (1957) and Pleunneke and Joham (1972) determined the sodium in the extracts by emission flame photometry described above.

Brownell (1965) determined sodium in acid-digested material by atomic absorption spectroscopy in the following method:

> From finely ground plant material two representative fractions were taken of less than 0·5 g. These were dried at 95° until they had reached constant weight and then placed in a desiccator. When at room temperature, they were re-weighed and placed in quartz Kjeldahl tubes of approximately 15 ml capacity and digested with 1 cm^3 H_2SO/ (S.G. 1·86), 1 cm^3 $HClO_4$ (S.G. 1·70) and 5 cm^3 HNO_3 (S.G. 1·42) (which had been redistilled in silica). When the digestion was complete, the digest was made up to a suitable volume with distilled water, and the concentration of sodium determined with the atomic absorption instrument. Digests of material containing only very low concentrations of sodium were made up to small volumes so that the sodium concentration would be high enough to be determined with the atomic absorption instrument. It was found that the response of the instrument to known concentrations of sodium in these highly concentrated solutions was less than in water. The depression in the response to sodium in the solution appeared to be due to the sulphuric acid used in the digestion of the plant material and calibration curves were prepared using similar amounts of sulphuric acid (Fig. 4).

It is possible to determine concentrations of sodium as low as 0·043 μmol g^{-1} dry weight (1 ppm (dry basis)) by this method.

Longitudinal profiles of sodium and potassium ions have been made in roots of *Hordeum distichon* L. and *Atriplex hortensis* by flameless atomic absorption spectroscopy by Jeschke and Stelter (1976). Concentrations of

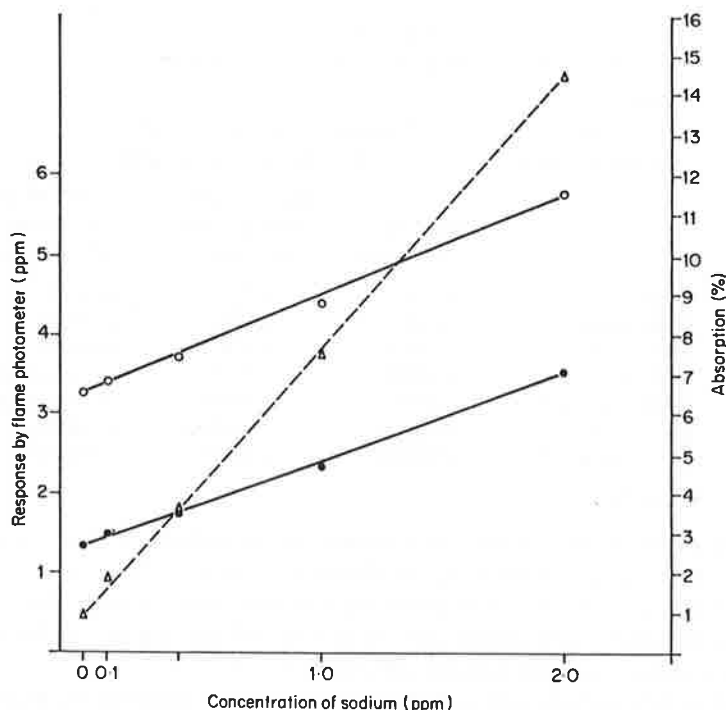


Fig. 3. Comparison of responses of an Eel flame photometer fitted with different filters and the atomic absorption photometer (Box and Walsh, 1959) to sodium in a 0·02 mol l^{-1} calcium nitrate solution. Response by the flame photometer with filter A ○——○, with filter B ●——● and the atomic absorption photometer (Box and Walsh, 1959) △-----△ (P. F. Brownell, unpublished work).

sodium as low as 0·1 μmol g^{-1} (2·3 ppm) on a fresh weight basis were determined in 0·5 mm sections of single roots equilibrated or grown in potassium-free, 1 mM sodium solution. The roots were injected into a flameless ionization chamber on the tip of a pipette.

C. CULTURE TECHNIQUES

The techniques for obtaining low sodium culture conditions are critical in these studies as the sodium levels must be extremely low to obtain clear signs of its deficiency in experimental plants. Sodium is very abundant in water, most reagents and in the atmosphere as dust or sea spray and although the requirements by plants for sodium appear to be much greater than for some of the other micronutrients, it can be as difficult to demonstrate the essential nature of sodium as for some of the other micronutrients.

The known sources of sodium to plants include water, the air surrounding the plants and that used for aeration of cultures, seeds, the salts of the culture solution and the culture apparatus.

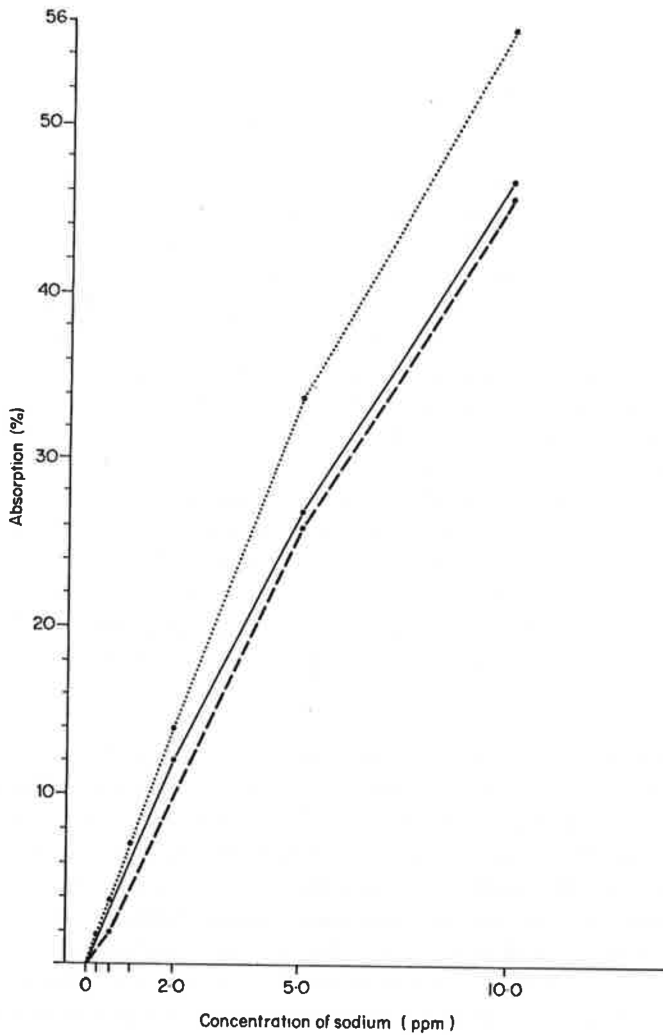


Fig. 4. Responses to known concentrations of sodium in water ●---------●, the acid digest of plant material ●——● and 3·6 N sulphuric acid (approximately the concentration of sulphuric acid in the acid digest ●– – –● (P. F. Brownell, unpublished work).

1. Water

Sodium must be carefully removed from water for it to be suitable for growth experiments in which sodium is studied as a micronutrient. Water distilled in pyrex glass contains too much sodium for decisive signs of sodium deficiency to be obtained in plants. The use of deionizers does not appear to be completely satisfactory either. In experiments carried out in Adelaide,

South Australia rainwater containing approximately 87 mmol m^{-3} (2 ppm) sodium was passed through a commercial deionizer consisting of columns of cation and anion exchange resins arranged in series. The treated water contained 3·5 mmol m^{-3} (0·08 ppm) sodium (Brownell 1965). However, Williams (1960) obtained evidence of a sodium requirement by *Halogeton glomeratus* using resin deionized water. The sodium content was 52 to 78 mmol m^{-3} (1·2 to 1·8 ppm) and unpurified reagent grade nutrients were used.

Pleunneke and Joham (1972) in their study of the effects of sodium upon the free amino acid content of cotton leaves passed distilled water through two Barnstead mixed-bed demineralizer cartridges. It was then stored in polyethylene vessels and contained less than 0·078 μmol (0·0018 ppm) sodium. Woolley (1957) using a polyethylene condenser for his study on the sodium and silicon requirements of plants obtained water containing 0·026 mmol m^{-3} (0·0006 ppm) sodium. Hewitt (1966) suggests that some of this sodium could have been derived from the polyethylene which is manufactured by a process involving alkali. Brownell and Wood (1957) redistilled water three times in tinned metal stills and stored it in polyethylene containers. It contained 0·0218–0·0348 mmol m^{-3} (0·0005–0·0008 ppm) sodium. Redistilling demineralized rainwater from a silica still reduced its sodium content from 3·5 to 0·0087 mmol m^{-3} (0·08–0·0002 ppm) (Brownell, 1965). Storage in large polyethylene containers appears to be satisfactory.

2. Air Purification

Relatively large accessions of sodium occur particularly in coastal areas. Hutton (1953) and Turton (1953) presented data showing that approximately 20 lb/acre/year (22 kg ha^{-1} $year^{-1}$) of sodium are deposited in rain over areas including Yorke Peninsula and Keith in South Australia and at Coolup in Western Australia. Brownell (1965) estimated accessions of sodium in a conventional glasshouse by placing filter papers horizontally in different sites within the glasshouse and determining the increase in sodium of each paper at the end of each week of exposure when the papers were replaced by another set. The mean amount of sodium collected per cm^2 per week was 0·02 μmol. This is of the same order as the accession of chlorine (0·022 μmol cm^{-2}) collected by Johnson *et al.* (1957) at Berkeley, California. It was calculated that this rate of accession of sodium (approximately 210 μmol sodium per 780 cm^2 of plant cover in 93 days) was almost sufficient to account for the increase (294 μmol) observed in the amount of sodium recovered in the culture solution and the plant material above that originally present as an impurity of the culture solution, seed and water at the beginning of the experiment (Table II). In subsequent experiments, Brownell and Wood (1957) and Brownell (1965) grew plants in a small greenhouse designed to prevent contamination of plants and their cultures by sodium from the atmosphere (Fig. 5). A slightly positive pressure was maintained

TABLE II
Sodium Supplied: Sodium Recovered

Conditions of experiment	Amount supplied (μmol)				Amount recovered (μmol)				
	Seeds	Solution	Water[d]	Total	Leaves	Stems and petioles	Roots	Remaining in culture solution	Total
In conventional glasshouse[a]	3 (10 seeds)	43[c]	80	126	153	33	117	117[c]	420
In pressurized cabinet[b]	1·07 (4 seeds)	5[c]	0·04	6·11	1·52	1·64	1·66	1·13[c]	5·95

[a] Ten plants of *Atriplex vesicaria* were grown in 4·5 litres of basal culture solution to which no sodium had been intentionally added. Experiment was of 93 days duration.

[b] Four plants of *Atriplex vesicaria* were grown in 2 litres of basal culture solution to which no sodium had been intentionally added. The experiment was of 48 days duration.

[c] These data were obtained using emission flame photometry. Due to positive interference from ions in the culture solution, these values are generally higher than they should be.

[d] Water used in the experiment in the glasshouse contained 4 mmol Na m^{-3} and water used in the pressurized cabinet contained 0·010 mmol Na m^{-3}. Amounts of sodium were calculated on the total volume of water supplied to the culture during the experiment.

From Brownell (1965).

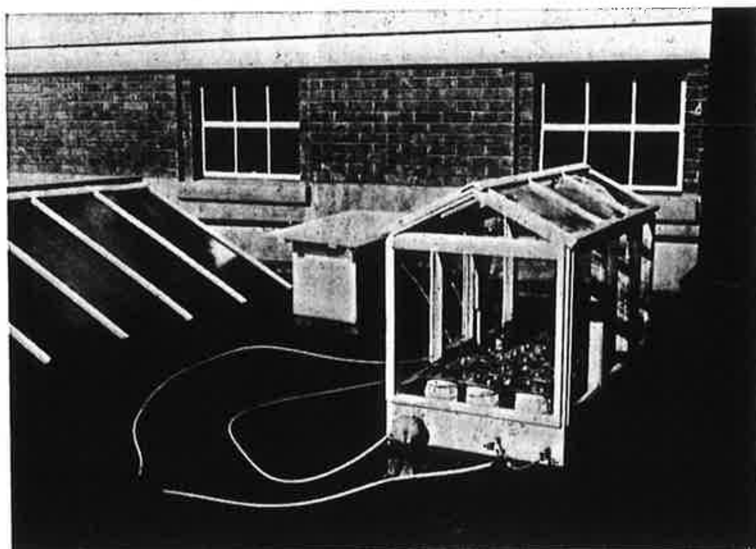


Fig. 5. The pressurized greenhouse. Note the housing of the air compressor on the left, which pumped air through a series of cotton-wool filters to a T-piece at this end of the greenhouse from which part of the air passed directly into the greenhouse and part was used for aeration of cultures. Windows were of polyvinylchloride and access was through a screw-clamped door on the left. The polythene tube which entered the greenhouse on the left carried wiring for electrical heating and the thermostat controlling it. The cloth-covered disc on the immediate right of this tube was the outlet for surplus air. From Brownell and Wood (1957).

within the greenhouse by a compressor which supplied air continuously to both cultures for aeration and to the greenhouse itself through Whatman No. 1 filter papers and washed absorbent cotton wool contained in metal cylinders.

The amount of sodium known to have been added intentionally was approximately the amount of sodium recovered in the culture solution and plant organs at the end of the experiment in this greenhouse (Table II). No increase in the amount of sodium could be detected after the cultures had remained in the experimental glasshouse for 48 days.

All air entering the compressor was drawn through Whatman No. 1 filter papers which were changed at 24-hourly intervals and the sodium they had trapped determined. The amounts of sodium trapped per day (from about 10^5 litres of air) rose and fell periodically (Fig. 6).

It was found that the amounts were greatest when strong winds blew from the west (the seaward side). Under these conditions opening of the cabinet and manipulation of cultures was avoided.

Woolley (1957) in experiments designed to determine if sodium and silicon were essential for tomatoes had a room in a greenhouse supplied with air at a

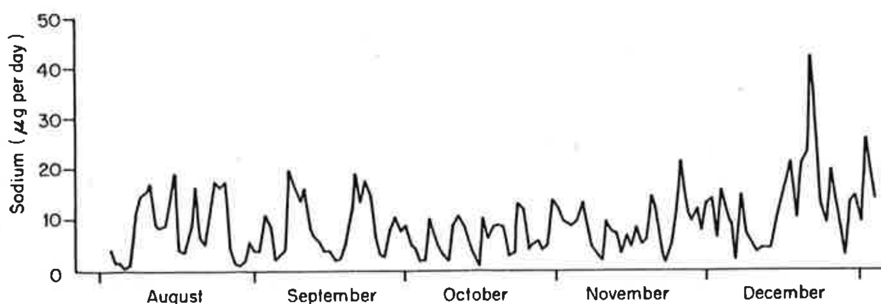


Fig. 6. Amounts of sodium trapped per day from air drawn through a Whatman No. 1 filter paper; about 10^5 litres of air passed through this filter per day. From Brownell (1965).

slight positive pressure which had been passed through four filters in the following order:

1. A charcoal and limestone filter to trap smog and acid gases.
2. A fibreglass filter to trap macroscopic dust, including particles of carbon from the smog filter.
3. A Mine Safety Appliance paper filter designed to stop particulate matter as small as 0·5 μm diameter.
4. An excelsior pad, continuously wet with distilled water.

It was found in the sodium supply–recovery balance that the "minus" treatment plants received significant amounts of sodium from unknown sources and the recovery of sodium from the plants and that remaining in the culture solution was 3·15 μmol which is almost twice that known to have been supplied (2·9 μmol) in the culture solution salts, water, seeds, dacron, polyethylene bag and polyethylene container (Table III). It is extremely unlikely that all the sodium (2 μmol) in the polyethylene container would have been supplied to the system so that the contamination of sodium from unknown sources must have been large.

Pleunneke and Joham (1972) grew cotton plants in a clear polyvinyl chloride-covered box located in a greenhouse. All inner wood surfaces were impregnated with plastic spray and coated with mineral oil. Air circulation was provided by a fan mounted in a metal plenum chamber attached to one end of the growth chamber. Air entering the chamber passed through a distilled, deionized wash and a series of three filters separated by mineral oil coated compartments.

Most of the sodium present in the harvested plants could be accounted for by that supplied in the seed and the culture solution.

Singh and Diekert (1975) described a simple system for the production of low-sodium peanut seedlings to seven days of age. During this period they picked up only 2 μg of extraneous sodium per seedling.

Brownell (1965) could not detect any increase in the concentration of

TABLE III
Sodium Supply–Recovery Balance

Treatment	Known Na supplied	Av Na in plants: Shoots	Roots	Av Na remaining in solutions	Na recovered
		μg atoms/culture			
Minus	2·9[a]	2·0	0·88	0·27	3·15
Na	2000	740	640	510	1890
Na, Si, Al	2000	770	640	470	1880
Elkhorn sand	6·6[a]	2·1	9·2	0·29	11·6

[a] These values are probably excessive, since they include the entire 2 μg atoms that could possibly have been supplied by the polyethylene container. From Woolley (1957).

sodium in water contained in open vessels after a fortnight in the greenhouse pressurized with filtered air. However, the concentration of sodium in water in a culture vessel after a fortnight of continuous aeration even when covered, increased by 0·4 mmol m^{-3} (0·01 ppm). At this rate of contamination, the amount of sodium in the most highly purified solution (0·069 mmol m^{-3}) (0·00158 ppm) would be increased many times in even a short experiment. In subsequent experiments the air for aeration of cultures was effectively freed from sodium by passing it through distilled water contained in a train of plastic vessels.

Pleunneke and Joham (1972) filtered air for aeration of nutrient solutions through two 12·5 cm plugs of acid-washed cotton and four distilled, doubly deionized water washes (changed daily). Woolley (1957) inserted a Dacron plug at the top of each aeration tube to act as filter.

3. Salts of the Culture Solution

Responses to sodium have been reported in experiments in which unpurified salts were used. Ashby and Beadle (1957) obtained significant increases in yield in *Atriplex inflata* and *Atriplex nummularia* grown on Solution 1 of Hoagland and Arnon (1938) prepared from A.R. salts and distilled water when sodium was added to 50 mol m^{-3} as either NaCl or Na_2SO_4. Tomatoes did not respond to this sodium treatment in the same experiment. Black (1960) observed similar significant growth responses in *Atriplex vesicaria* receiving up to 20 mol m^{-3} NaCl. Further increase in concentration of NaCl decreased growth. Williams (1960) obtained significant responses to 1 mol m^{-3} NaCl, $NaNO_3$ and 4 mol m^{-3} Na_2SO_4 applied to *Halogeton glomeratus* grown in the solution of Hoagland and Arnon (1938) using unpurified salts.

In establishing the essentiality for sodium by the blue-green alga, *Anabaena cylindrica*, Allen and Arnon (1955) twice recrystallized the macronutrient salts but used untreated A.R. salts for the micronutrients.

In attempting to determine if tomatoes had a sodium or silicon require-

ment, Woolley (1957) prepared all the nutrient salts by two recrystallizations except molybdic acid. The recrystallizations consisted of two operations; a rapid crystallization from a hot supersaturated solution, and a slow crystallization as the supernatant from the first operation gradually cooled. It was suggested that most impurities would be associated with the first small crystals rapidly formed at high temperatures by adsorption or occlusion. These crystals were discarded and the larger crystals slowly formed at lower temperatures were used in the preparation of nutrient solutions. The molybdic acid was prepared by the prolonged heating at 70° of a 10% solution of ammonium molybdate. The sodium concentration of the final culture solution was 0·511 mmol m^{-3} (0·012 ppm).

In their study of the effect of low sodium levels upon the free amino acid content of cotton leaves, Pleunneke and Joham (1972) purified the macronutrient salts by recrystallization in stainless steel and polyethylene vessels. Three recrystallization cycles were employed with potassium salts and four cycles were used for the purification of $MgSO_4{\cdot}7H_2O$ and $Ca(NO_3)_2{\cdot}4H_2O$. Micronutrients apart from $FeSO_4{\cdot}7H_2O$ were not recrystallized. The EDTA used in the preparation of iron chelate was dissolved in polyethylene distilled NH_4OH and precipitated with purified HCl. The process was repeated three times and the EDTA then reacted with recrystallized $FeSO_4{\cdot}7H_2O{\cdot}$ The final culture solution contained less than 0·17 mmol Na m^{-3} (0·004 ppm).

Brownell (1965) prepared a culture solution containing less than 0·069 mmol m^{-3} (0·0016 ppm) sodium. The concentrations of sodium in solutions of purified salts and untreated salts are compared (Table IV).

The following methods were used to obtain salts with low sodium content. Potassium nitrate, potassium dihydrogen phosphate, magnesium sulphate and manganese sulphate were recrystallized in silica vessels up to six times. Alternatively, diammonium phosphate was prepared by first distilling phosphorous oxychloride from silica, cautiously hydrolysing it in water, boiling off hydrochloric acid and adding two equivalents of redistilled ammonium hydroxide.

Calcium nitrate was obtained by preparing calcium salicylate from calcium carbonate and salicylic acid, recrystallizing it several times and then ashing. The oxide was dissolved in nitric acid redistilled from silica. Sodium contamination of the resulting purified salt was $\frac{1}{250}$ of that of the analytical reagent salt.

Boric acid was freed from sodium by making a saturated solution of boric acid in ethanol in the boiling flask of a silica still. The boron was volatilized as the ethyl ester of boron and the distillate collected in a platinum vessel. After drying the distillate in a waterbath, boric acid remained. This was placed in a desiccator until its weight was constant then made up into a stock solution 10 000 times as concentrated as it was in the final solution.

The concentration of sodium was reduced from 435 mmol kg^{-1} (10 000 ppm)

TABLE IV

Sodium Contributed to the Culture Solution as Impurities of Component Salts Before and After Purification

Salt	Conc. of salt in culture solution (μM)	Sodium contributed to culture solution by component salts (μmol. 2l^{-1}) Untreated analytical reagent salts	Prepared salts
Calcium nitrate	4000	4·35	0·0174
Potassium nitrate	5000	0·52	0·0109
Potassium dihydrogen phosphate	1000	2·18	0·0174
Diammonium sulphate	1000	0·52	0·0347
Magnesium sulphate	1000	0·26	0·00174
Boric acid	46	0·0026	0·00087
Manganese phosphate	9·1	0·0252	0·01320
Copper sulphate	0·31	0·00026	0·00026
Zinc sulphate	0·76	0·0065	0·000435
Ammonium molybdate	0·10	0·00022	0·000218
Ferric ammonium ethylene tetra acetate	90	1·39	0·0347
Ammonium chloride	350	0·00569	0·00565
Total sodium in culture solution due to sodium impurities of all component salts		9·26	0·137

From Brownell (1965).

in boric acid (to which sodium had been intentionally added) to less than 218 μmol kg^{-1} (5 ppm) in boric acid purified in this way. Solutions of copper sulphate, zinc sulphate and ammonium molybdate were made up from A.R. grade salts without purification as the amounts of sodium they contributed to the culture solutions were extremely small.

Iron was supplied to cultures in a single addition of ferric ammonium ethylene tetra acetic acid (EDTA). This was prepared by a method similar to that of Jacobson (1951) except that ammonium hydroxide was used instead of potassium hydroxide in equivalent amount. Potassium hydroxide, which contained much sodium as an impurity would have been difficult to purify, whereas the ammonium hydroxide redistilled in silica contained an amount of sodium too small to be detected. Ferrous sulphate was recrystallized six times from solutions acidified by small quantities of sulphuric acid, and the resulting crystals were dried in an oven at 50°. EDTA was dissolved in 2 mol l^{-1} ammonium hydroxide (redistilled in silica) and then precipitated by the addition of 2 mol l^{-1} HCl (redistilled in silica). This procedure was repeated four times and the resulting precipitate was washed in several changes of distilled water and dried in an oven at 50°.

Ammonium chloride was formed by the addition of ammonium hydroxide to an equivalent amount of hydrochloric acid (both redistilled in silica). The resulting solution was concentrated by boiling, cooled to room temperature, and placed in a refrigerator overnight. The ammonium chloride crystals formed were dried in a desiccator to constant weight and made up in a stock solution 20 000 times the concentration required in the full concentration culture solution.

4. Culture Apparatus

To obtain clear-cut signs of sodium-deficiency, the choice of culture apparatus is critical. The use of glassware even borosilicate glass should be avoided and in its place materials including platinum, silica-ware, stainless-steel and selected plastics have been found to give minimum contamination.

Ashby and Beadle (1957) obtained significant responses to sodium in *Atriplex nummularia* and *Atriplex inflata* seedlings grown in vermiculite which were transferred to cultures in which the plants were supported by cotton-wool plugs in holes of masonite tops previously impregnated with paraffin wax supported over four-litre enamelled pots of nutrient solution. Woolley (1957) germinated tomato seeds on moist acid-washed cheesecloth in polyethylene containers. The plants were cultured in two-litre polyethylene freezer containers with holes drilled in the lids, and were supported by Dacron wool which had previously been washed with 10% HNO_3 and 10% HF, followed by repeated rinsing with water until the pH of the rinse water remained constant. Aeration of the culture solutions was by a continuous flow of air bubbling from a polyethylene tube fitted with a Dacron plug to

act as a filter into each culture vessel. The total amount of sodium per container was estimated to be 2·0 μmol (46 μg). Probably very little of this sodium would have been available. Hewitt (1966) considered that this high sodium figure for the polyethylene could have been due to the use of alkali in manufacture. Pleunneke and Joham (1972), in studying the influence of sodium nutrition upon the free amino acid content of cotton leaves, germinated the seeds on cheesecloth platforms in sterile polypropylene beakers covered with Saran wrap. Seedlings were transferred to eight-litre polyethylene nutrient solution containers containing three plants each. The outside of the containers was painted black and coated with aluminium foil to prevent light penetration. Seedlings were initially supported by rinsed cotton plugs and later by plugs on ring stands. Plastic tubing was attached to the base of each solution container passing through the growth chamber wall thus providing a means by which the solutions were changed without opening the chamber.

Brownell (1965) germinated seeds on nylon gauze sewn into a circle of polyethylene tubing to form a flat disc supported by polystyrene legs in a circular polythene vessel. Water or culture solution in this vessel was aerated through a fine-bore silica tube. Culture vessels of two-litre capacity were made from half-gallon polythene containers by cutting off their tops. The vessels had covers of grey Perspex which held four evenly spaced plants, secured by white terylene (equivalent to Dacron) fibres washed in many changes of silica distilled water, clamped between the longitudinally split halves of polythene tubing. Cultures were aerated continuously, with air filtered through cotton-wool and bubbled through frequently changed distilled water and filter papers, by means of centrally placed silica tubes dipping to the bottom of culture vessels. Paper, black on one side and white on the other was wrapped around the culture vessels to exclude light from the culture solution and the roots of plants.

Samples of all materials associated with the cultures were boiled in small amounts of concentrated HNO_3 (redistilled in silica); the amounts of sodium extracted by this treatment were small in all cases.

5. Seeds

The amounts of sodium per seed vary widely. A seed of *Beta vulgaris* (sugar beet) contained 13·04 μmol (300 μg) sodium whereas a lettuce seed 0·0043 μmol (0·1 μg) sodium (Brownell, 1968). Many other species have seeds with amounts of sodium between these limits. *Lycopersicum esculentum* tomato cv. "Marglobe" contained 0·008 μmol (0·18 μg) sodium per unwashed seed (Woolley, 1957).

Cabbage contained 0·0109 μmol (0·25 μg) per seed and *Atriplex vesicaria* (bladder salt bush) had a sodium content of 0·27 μmol (6·21 μg) per seed after washing (Brownell, 1968).

A two-litre culture solution prepared from purified salts and silica-distilled water contains only approximately 0·16 μmol (3·68 μg) sodium as an impurity (Brownell, 1965). The contribution of sodium by the seeds could be a relatively important source under these conditions.

Methods of reducing the amounts of sodium in the seed include harvesting the seeds from plants grown under conditions of low sodium. By this method, Woolley (1957) reduced the sodium per seed in tomatoes cv. "Marglobe" to 0·002 μmol (0·046 μg) which was approximately one quarter of the amount present in the original seed.

Another method of reducing the sodium per seed is by washing. Large amounts of sodium were found in the water which had been used to wash seeds of sugar beet, *Atriplex vesicaria* and *Kochia childsii* (Brownell, 1958, 1968).

The sodium content per seed of tomato cv. "Grosse Lisse" was reduced from 0·004 μmol (0·095 μg) to 0·001 μmol (0·031 μg) by washing in four changes of distilled water over a period of 3½ hours (Brownell, 1968). The amounts of sodium in the water which had been used to wash the seeds of other species including barley, cabbage, white clover, tomato, *Chenopodium capitatum*, *Exomis axyrioides* and *Aster tripolium* were low in all cases (Brownell, 1958).

Johnson *et al.* (1957) could only show losses of chlorine from carrot seeds when the seeds of lettuce, tomato, cabbage, carrot, sugar beet, barley, alfalfa, buckwheat, corn, beans and squash were washed; the washed carrot seed contained five times less chlorine in the unwashed seeds.

Vigorous washing may damage the seed by removing other mineral nutrients or organic substances essential for the normal development of the plant. Although the mineral nutrients could be replaced by soaking the seed in a nutrient solution, the organic substances would not be replaced.

By growing plants to a large size, the sodium present is distributed over a large amount of metabolizing tissue and the relative contribution of sodium from the seed becomes less. However, long-term experiments are open to objections arising from the depletion of other nutrient elements from the culture solution, the possible infection of the culture solution by micro-organisms, or the extra risk of contamination from manipulation and the greater area of leaves capable of absorbing sodium from the atmosphere.

As a general procedure, Brownell (1965) washed seeds in many changes of distilled water until the amount of sodium in the wash water could not be detected with the flame photometer adjusted to its maximum sensitivity.

6. Composition of Culture Solution

The culture solution must provide all the elements known to be essential for plant growth with the exception of sodium at concentrations which support vigorous growth when sodium is added to the solution. It is an advantage

to select a culture solution composed of salts which are amenable to purification procedures. The basal culture solution used by Brownell and Wood (1957) and Brownell (1965) was similar to that of Broyer *et al.* (1954) and had the following composition expressed in μmol l^{-1}: KNO_3, 5000; $Ca(NO_3)_2$, 4000; $MgSO_4$, 1000; $(NH_4)_2HPO_4$, 1000; KH_2PO_4, 1000; H_3BO_3, 46; $MnSO_4.7H_2O$, 9·1; $CuSO_4.5H_2O$, 0·31; $ZnSO_4.7H_2O$, 0·76; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0·1; NH_4Cl, 350. Iron was supplied as the ferric ammonium EDTA at 90 μmol l^{-1} in the basal culture solution by a method similar to that of Jacobson (1951).

For the growth of the blue-green alga, *Anabaena cylindrica*, Brownell and Nicholas (1967) used culture media similar to that described by Allen and Arnon (1955) with the following composition (mol m^{-3}): $MgSO_4$, 1; KH_2PO_4/K_2HPO_4 (pH 7·2), 2; $CaCl_2$, 0·5; and KCl, 10 (in the combined nitrogen-free medium), or KNO_3, 10 (in the nitrate-containing medium). Micronutrients were supplied (μg cm^{-3}) as follows: Fe (as the EDTA complex) 5, Mn (as $MnSO_4.4H_2O$) 0·5, Mo (as $(NH_4)_6Mo_7O_{24}.4H_2O$) 0·1, Zn (as $ZnSO_4.7H_2O$ $4H_2O$) 0·05, Cu (as $CuSO_4.5H_2O$) 0·02, B (as H_3BO_3) 0·05, and Co (as $Co(NO_3)_2$), 0·01.

III. RESPONSES TO SODIUM AT LOW CONCENTRATIONS

A. LOWER PLANTS

1. Blue-green Algae

There have been several suggestions in the early literature of blue-green algae having a requirement for sodium. In 1898, Benecke (cited by Allen and Arnon, 1955) described a species of *Oscillatoria*, which grew in a medium in which all potassium salts had been replaced by sodium salts. Although it is likely that some potassium would still have been present in the medium as an impurity, the work suggested that sodium was acting as an independent nutrient element. Emerson and Lewis (1942) found *Chroococcus* supplied with 0·026 mol m^{-3} (1 ppm) potassium made only "very poor growth" without the addition of sodium. Allen (1952) grew 23 out of 30 cultures of Myxophyceae without added potassium salts but found they required sodium for growth in the presence of potassium. Some potassium probably would have been present in the cultures as an impurity of the ordinary C.P. grade chemicals which were used. Kratz and Myers (1955) obtained a decisive growth response to small additions of sodium in *Anacystis nidulans* even though no attempt had been made to purify the salts to eliminate their sodium contamination. The growth of *Anabaena flos aquae* A37 was shown to be severely limited by the absence of either Na or K from the culture medium (Bostwick *et al.*, 1968). Optimal growth was achieved with 4·81 mol m^{-3} (188 ppm) potassium and 1·76 mol m^{-3} (40·5 ppm) sodium. To obtain optimum

growth of *Nostoc muscorum* Eyster (1970) found it necessary to increase the sodium concentration of the medium with increasing concentration of potassium in the medium. These observations suggested a possible nutrient effect of sodium in small amounts. Allen and Arnon (1955) critically examined sodium as a nutrient for a blue-green alga, *Anabaena cylindrica*. They carefully obtained low sodium conditions in their cultures. Glass was washed with 3 mol l^{-1} HCl followed by thorough rinsing with glass distilled water. The C.P. grade salts of the culture solution, $MgSO_4$, KCl, NaCl, KH_2PO_4 and Na_2HPO_4 were purified by three successive recrystallizations from hot water. $CaSO_4$ was prepared by the addition of H_2SO_4 to a solution of calcium chloride, followed by thorough washing of the $CaSO_4$ precipitate. When these precautions were taken to exclude sodium contamination, normal development of the alga did not occur in the absence of sodium but was restored by the addition of sodium. Five parts per million of sodium or higher was required for optimal development of the alga. Potassium at 8·0 mol m^{-3} was supplied to all cultures to ensure that the response to sodium occurred in the presence of adequate potassium. It was also found that potassium was essential in the presence of adequate sodium. The elements lithium, rubidium and caesium at 1 or 10 ppm in the culture were unable to replace the requirement for sodium in these cultures. Sodium-deficient cultures were shown to have less phycocyanin than those with adequate sodium but their chlorophyll content was not affected. Brownell and Nicholas (1967) found a marked difference in ratio (1 : 62) between the amounts of phycocyanin in sodium-deficient cultures containing less than 0·17 mmol m^{-3} (0·004 ppm) sodium as an impurity and normal cultures supplied with 4 mol m^{-3} (92 ppm) sodium in cultures containing nitrate. On the other hand, the cultures containing no combined nitrogen showed relatively small differences (1 : 2·3) in the ratio of phycocyanin between sodium-deficient and normal cultures. The ratio between the amounts of chlorophyll in sodium-deficient and normal cultures were (1 : 6·7) for nitrate containing cultures and (1 : 3·07) for cultures containing no combined nitrogen. The lack of agreement between the chlorophyll observations made by Allen and Arnon (1955) and Brownell and Nicholas (1967) could be due to the differences in the amounts of sodium in the cultures. Furthermore, the effects of sodium nutrition on the amounts of phycocyanin and chlorophyll in cultures of blue-green algae may well be conditioned by other properties of the cultures including the age of cells, light intensity and presence or absence of combined nitrogen.

Brownell and Nicholas (1967) found that dry weight yields increased with sodium supply when the cells were grown with nitrate nitrogen or without combined nitrogen (Table I). It is noteworthy that cells grown with nitrate, at suboptimal levels of sodium, contained much less chlorophyll and phycocyanin than those grown without combined nitrogen (Table V; Fig. 7).

TABLE V

Effect of Sodium on Dry Weight, Chlorophyll and Phycocyanin Contents of Anabaena cylindrica *Grown With Nitrate Nitrogen and Without Combined Nitrogen*

Amount of NaCl in culture (mol m^{-3})	Dry weight (g/100 cm^3 culture)		Chlorophyll (μg/100 cm^3 culture)		Phycocyanin [a](units/100 cm^3 culture)	
	No combined nitrogen	10 mol m^{-3} KNO_3	No combined nitrogen	10 mol m^{-3} KNO_3	No combined nitrogen	10 mol m^{-3} KNO_3
None	0·058	0·041	6·59	3·07	0·268	0·008
0·10	0·066	0·063	8·55	2·66	0·315	0·032
2·00	0·092	0·118	20·50	24·00	0·600	0·645
4·00	0·097	0·094	20·20	20·50	0·670	0·496

Cultures harvested 12 days after inoculating.

[a] Arbitrary Unit = $E_{620}^{1\,cm}$ of phycocyanin in 100 ml.

From Brownell and Nicholas (1967).

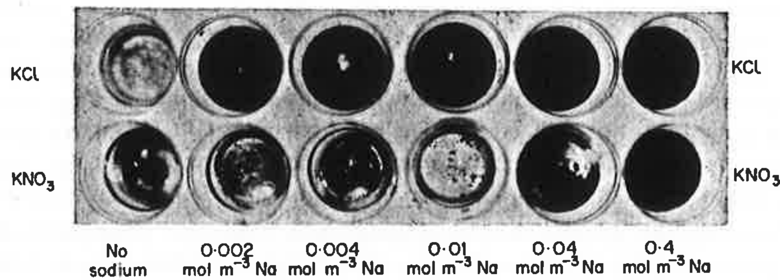


Fig. 7. Effect of sodium on *Anabaena cylindrica* grown without combined nitrogen (KCl) or with nitrate (KNO_3). Note darker algae (more chlorophyll and phycocyanin) without combined nitrogen (KCl) to the paler ones in nitrate (KNO_3) grown in cultures at equivalent rates of sodium below 0·4 mol m^{-3} (9·2 ppm). From Brownell and Nicholas (1967).

2. *Other Algae*

There appears to be little firm evidence for the essentiality of sodium in algae other than in members of the Cyanophyta. There have been reports (Osterhout, 1909, 1912) that sodium was necessary for the growth of the green alga, *Ulva* and red algae in the genera *Gigartina*, *Ptilota*, *Iridaea* and *Prionitis*. The evidence was based on experiments in which little or no growth was obtained when the sodium of sea water was replaced by other cations including ammonium, calcium, magnesium, potassium, barium, strontium, caesium, rubidium or lithium. The best growth occurred with magnesium, calcium and potassium as the substitutes for sodium. It is not surprising that the cations substituting for the sodium were unable to support normal growth. The substitution of an element normally present at a concentration

of 500 mol m^{-3} by another element would be expected to have severe effects on the algae in addition to any possible effect caused by the lack of sodium.

The range of tolerance to the concentration of sodium for some euryhaline species including *Hemiselmis virescens*, *Monochrysis lutheri*, *Phaeodactylon tricornutum*, *Nannochloris oculata* and *Skeletonema costatum* was studied by Droop (1958). The organisms generally tolerated concentrations of sodium between 13 mol m^{-3} (300 ppm) and 522 mol m^{-3} (12 000 ppm) with maximum growth occurring between 87 mol m^{-3} (2000 ppm) and 261 mol m^{-3} (6000 ppm). The results suggest that sodium has an independent role as a nutrient element for these species and is generally required in high concentrations. McLachlan (1960) showed that sodium chloride at 80 mol m^{-3} (1840 ppm) supported optimal growth in another euryhaline algae, *Dunaliella tertiolecta*. He found the minimum requirement for sodium to be much greater than for any other element and it was not possible to substitute other monovalent cations for the minimum requirements. The alga could tolerate high concentrations of sodium chloride.

Treatments of sodium chloride have been shown to stimulate the uptake of phosphate by the unicellular green alga, *Ankistrodesmus braunii* (Simonis and Urbach, 1963). The enhancement of phosphate uptake by sodium was much greater than by potassium or calcium. The stimulation of phosphate binding in the organisms by sodium was rapid occurring after five seconds (Ullrich-Eberius, 1973). There does not appear to be any evidence that sodium is essential for the growth of *Ankistrodesmus braunii*.

3. Bacteria

The literature describing the salt requirements of marine bacteria was reviewed recently by Pratt (1974).

Sodium has been shown to be required specifically for the growth of certain marine bacteria and MacLeod (1965) suggested that the possession of a sodium requirement distinguishes marine bacteria from most non-marine species. Most marine bacteria which have been examined require 0·2–0·3 mol l^{-1} sodium for optimal growth. Such organisms require the addition of sodium salts or sea water even to the complex laboratory media commonly used for isolation, though such media are usually contaminated with appreciable amounts of sodium. Lithium, rubidium and caesium were not able to replace the requirement of sodium for the growth of the organisms. The requirement of marine bacteria for sodium at high concentrations apparently can be lost when cultured on low-sodium media. MacLeod and Onofrey (1963) trained a marine pseudomonad to grow on a medium prepared without added sodium salts by streaking cultures serially on to the surface of plates of the medium containing progressively lower concentrations of sodium, down to 0·028 mol l^{-1} sodium. The organism still required sodium for growth when the adapted culture was tested in a chemically defined

medium containing less than $6{\cdot}5 \times 10^{-5}$ mol l^{-1} sodium. The optimal requirements of sodium for the marine bacteria so far examined are in the range of 0·005 to 0·2 mol l^{-1} depending upon the species.

Later observations have shown some non-marine bacteria also to require sodium. MacLeod (1968) found two strains of the non-halophilic *Rhodopseudomonas* to have small but specific requirements for sodium. Kodama and Taniguchi (1976) demonstrated the growth rate of *Pseudomonas stutzeri*, a non-halophytic bacteria, to be a sigmoidal function of sodium concentration from 2 to 50 mol m^{-3} (46–1150 ppm). The growth rate was half-maximal at 0·5 mol m^{-3} (11·5 ppm).

It was found that five strains (including isolate S85) of an important cellulolytic species in the bovine rumen, *Bacteriodes succinogenes*, required 84 mol m^{-3} (1932 ppm) sodium when potassium was present at concentrations between 3 mol m^{-3} (117 ppm) and 50 mol m^{-3} (1955 ppm) in the medium for maximal growth (Bryant *et al.*, 1959). With lower levels of sodium, higher levels of potassium were needed and the reverse was also true suggesting that the role of these ions was to act as an osmoticum; little growth occurred when the calculated total ion concentration was outside the range of 0·6 to 1·2% solution of sodium chloride. However, sodium appeared also to have a specific role in the nutrition of the organism as only poor growth occurred if sodium was omitted regardless of the potassium concentration in the medium.

A non-marine photosynthetic bacterium, *Rhodopseudomonas spheroides* has a specific requirement for sodium. Maximum growth was obtained with 1·74 mol m^{-3} (40 ppm) sodium (Sistrom, 1960).

4. Fungi

There have been few studies on the sodium requirement of fungi. Siegenthaler *et al.* (1967) showed sodium to be specifically required for the uptake of phosphate in *Thraustochytrium roseum*, a lower marine Phycomycete; its growth rate was greatest with 200–400 mol m^{-3} (4600–9200 ppm) sodium. Vishniac (1960) also obtained increased phosphorus and oxygen uptake in *Thraustochytrium globosum* when receiving 200 mol m^{-3} (4600 ppm) sodium as sodium chloride. Smaller or negligible increases of phosphorus or oxygen uptake were obtained when the chlorides of lithium, potassium and rubidium and sucrose at the same molarity were supplied. This suggested the specific involvement of sodium in the processes of phosphorus and oxygen uptake.

The marine yeast, *Candida marina* when grown in a carefully purified nutrient medium to remove sodium responded to sodium at 1% level of significance (W. A. Shipton and K. Watson, unpublished data), (Table VI).

Many more lower plants may be shown to have a requirement for sodium at low concentrations when they are examined. There are extra technical difficulties encountered when preparing the organic media needed to support these heterotrophic organisms free of sodium.

TABLE VI

Effect of Sodium and Potassium Salts on the Growth of Candida marina. *The Medium Contained* $4 \cdot 8 \times 10^{-7}$ *mol* l^{-1} Na^+ *and* 10^{-2} *mol* l^{-1} K^+ *Before Addition of Specific Ions*

Treatment	Cations added (mol l^{-1})	Yield (g dry weight/50 cm^3 culture medium)
Nil	0	0·047 b[a]
NaCl	$6 \cdot 7 \times 10^{-3}$	0·108 a
KCl[b]	$6 \cdot 7 \times 10^{-3}$	0·069 c
$KCl + Na_2SO_4$ ($K^+:Na^+ = 1:1$)	$13 \cdot 4 \times 10^{-3}$	0·106 a

[a] Figures followed by the same letter are not significantly different ($P < 0 \cdot 05$).
[b] Level of Na^+ contamination of KCl was 5×10^{-7} mol l^{-1}.
(From unpublished data of W. A. Shipton and K. Watson)

B. *Atriplex vesicaria* HEWARD EX BENTH.

Before 1954, no serious attempts were known to have been made to remove sodium and chlorine from the environment of plants before adding the treatment salts containing sodium and/or chlorine. It was therefore possible that plants could have a requirement for these elements so small that it had always been satisfied under the conditions in which plants had been grown. The late Professor J. G. Wood suggested using the Australian bladder salt bush (*Atriplex vesicaria*) as one species in a trial as it had interesting features including the possession of a bundle-sheath in leaves (Wood, 1925; Black, 1954) and the ability to accumulate large amounts of sodium and chlorine compared to other species growing in similar habitats.

During the course of this work, chlorine was demonstrated to be essential for tomatoes by Broyer *et al.* (1954) and it was subsequently shown to be essential for a wide range of species (Johnson *et al.*, 1957; Ozanne *et al.*, 1957). This led to the acceptance of chlorine as an essential micronutrient element for higher plants. During the same period Allen and Arnon (1955) discovered the requirement for sodium as a specific micronutrient by the blue-green alga.

In preliminary experiments in an ordinary glasshouse, plants of *Atriplex vesicaria* growing in the basal culture solution showed significant increases in dry weight when sodium salts (but not when additional potassium salts in equivalent amounts) were supplied at levels of 0·35, 1·75 and 7·0 mol m^{-3} of sodium. When the plants were relatively large and had acquired tertiary branches, the leaves of all plants not receiving sodium appeared paler green than those of plants with added sodium. Analysis of plant organs and culture solutions at the end of the experiment showed an increaae of 300 μmol sodium above the amount supplied in the culture solutions and seeds. This

suggested that plants had received sodium, presumably as cyclic salt, from the atmosphere (Brownell and Wood, 1957) (see also Section II.C, this chapter). To preclude this possibility, a cabinet was constructed which was maintained at a slightly positive pressure to prevent entry of dust. Other precautions to prevent contamination are described in Section II.C.

Bracteoles were removed from seeds, which were then washed in distilled water and germinated on nylon gauze over silica-distilled water; after emergence of the radicle, the distilled water was replaced by basal culture solution of one-fifth full concentration. When 11 to 14 days old, the seedlings which had acquired cotyledons and apical buds were selected for uniformity and transferred to the polythene culture vessels containing two litres of culture solution. Differential treatments were supplied at this stage and the following experiments performed (Brownell, 1965):

1. Effect of Small Graduated Amounts of Sodium on Growth

Different treatments were applied to cultures in each of four blocks (Table VIII). The concentration of sodium in the full concentration culture solution due to the sodium contributed by potassium sulphate was reduced from 7·1 to 0·039 mmol m^{-3} by recrystallizing the potassium sulphate five times. The cultures within each block were placed in random positions at the beginning of each experiment.

By the twenty-fifth day after germination plants which had not received sodium sulphate could be distinguished from those which had by their yellow colour and fewer leaves each of smaller area. White necrotic areas appeared along tips and margins of the cotyledons and older leaves on the thirtieth day (Fig. 8). Some plants died by the thirty-fourth day. A plant which showed symptoms just described and another which had died, were examined by plant pathologists at the Waite Agricultural Research Institute for the presence of pathogenic organisms. None were found in these plants. When harvested on the forty-eighth day, plants which had received sodium sulphate appeared markedly different from those which had not, having many more leaves of darker green colour which showed no necrosis (Fig. 9).

It should be noted that these symptoms were easily distinguished from those of chlorine-deficiency in the same species. Chlorine-deficiency was manifested by sudden wilting in leaf-tips at a relatively late stage of growth in plants which otherwise resembled normal plants. There was no sign of chlorosis which occurs at an early stage of growth in sodium-deficient plants.

The difference between the root systems of plants grown with and without the addition of sodium sulphate was observable at an early stage and was pronounced by the forty-eighth day (Fig. 10).

From the results in Table VII obtained when the plants were harvested on the forty-eighth day, the yield is seen to have increased asymptotically with increasing sodium sulphate. As plants which received 0·60 mol m^{-3} of potassium

TABLE VII

Yields of Atriplex vesicaria *Following the Application of the Sulphates of Sodium and Potassium*

Treatment	Fr wt/vessel (g) Leaf-blades	Stems and petioles	Roots	Total	Dry wt/vessel (g) Leaf-blades	Stems and petioles	Roots	Total
I No addition	0·301	0·021	0·221	0·543	0·0324	0·0042	0·0153	0·0519
II 0·02 mol m^{-3} Na_2SO_4	2·101	0·144	1·441	3·686	0·2354	0·0212	0·0913	0·3479
III 0·10 mol m^{-3} Na_2SO_4	2·926	0·224	2·148	5·298	0·3468	0·0324	0·1357	0·5149
IV 0·60 mol m^{-3} Na_2SO_4	2·940	0·228	2·940	6·108	0·3475	0·0355	0·1286	0·5116
V 0·60 mol m^{-3} K_2SO_4	0·436	0·019	0·326	0·781	0·0433	0·0031	0·0206	0·0670

All values are the means of yields from four 2-litre cultures of four plants each. The statistical treatment of total dry weight data was as follows: II > I at 1% level of significance; III > I at 0·1% level of significance; III > II at 5% level of significance.

From Brownell (1965).

TABLE VIII

Fresh and Dry Weight Changes After Recovery of Sodium Deficient Plants of Atriplex vesicaria *Following the Application of Sodium Sulphate Treatments*

	Treatment	Fr wt/vessel (g) Leaf-blades	Stems and petioles	Roots	Total	Dry wt/vessel (g) Leaf-blades	Stems and petioles	Roots	Total
I	No sodium sulphate	0·877	0·078	0·671	1·626	0·0894	0·0124	0·0481	0·1499
II	0·10 mol m^{-3} Na_2SO_4 applied on day 16	8·139	1·007	6·923	16·069	1·0236	0·1619	0·4810	1·6665
III	0·10 mol m^{-3} Na_2SO_4 applied on day 31	3·713	0·334	2·800	6·847	0·4713	0·0544	0·2133	0·7390

All values are the means of yields of two vessels of four plants each. The statistical treatment of total dry weight data was as follows: III > I at 1% level of significance; II > I at 0·1% level of significance; II > III at 5% level of significance.

From Brownell (1965).

Fig. 8. A plant of *Atriplex vesicaria* grown in a basal culture solution from which sodium was eliminated. Note the few yellow-green leaves of small area possessing, in some cases, white necrotic patches at their tips and the absence of secondary shoots. This plant had a height of 1·0 inch and was photographed on the 48th day. From Brownell and Wood (1957).

sulphate, a concentration equivalent to the highest concentration of sodium sulphate treatment in their cultures, could not be distinguished from the plants grown in the "no addition" cultures, it was evident that the increase in yield with increasing sodium sulphate was not due to the sulphate but to the sodium of the sodium sulphate treatment. This also showed that the part played by sodium in the nutrition of *Atriplex vesicaria* could not be performed by additional potassium when supplied in an amount equivalent to the highest sodium sulphate treatment. The lowest sodium sulphate treatment for maximum dry weight production was about 0·2 mmol/2-litre culture and

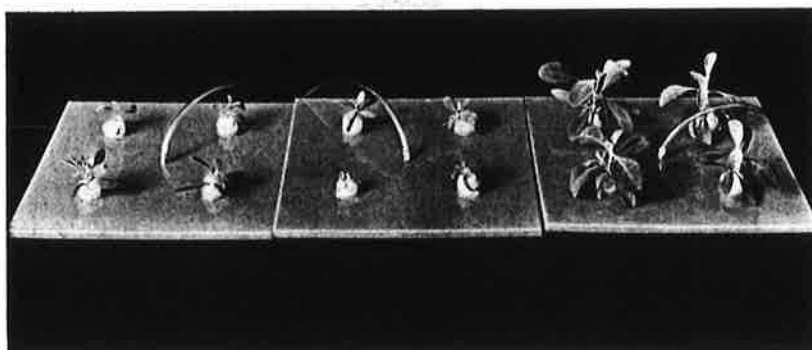


Fig. 9. Comparison between the growth of tops of plants of *Atriplex vesicaria* growing in the basal culture solution with addition of 0·60 mol m^{-3} potassium sulphate (left), with no addition (centre) and with 0·02 mol m^{-3} sodium sulphate (right). The plants had a height of approximately 2·5 cm (left), 2·5 cm (centre) and 5·1 cm (right). From Brownell (1965).

the leaf material contained about 295 μmol/g (6785 ppm) (dry basis). Although these data would be expected to vary markedly according to the conditions of the experiment, the sodium requirements of *Atriplex vesicaria* were high in comparison with the requirements of micronutrients by plants of other species (W. R. Meager, see Hewitt (1966) pp. 163–165).

Fig. 10. Comparison between the tops and root growth of plants of *Atriplex vesicaria* grown in the basal solution which received no addition (left), and 0·60 mol m^{-3} sodium sulphate (right). The heights of the tops of the plants were approximately 2·5 cm (left) and 6·4 cm (right). The photograph was taken on the 48th day. From Brownell (1965).

2. Recovery of Sodium-deficient Plants Following the Application of Sodium

Plants, 14 days old, were transferred to culture vessels containing the basal culture solution without added sodium. On the sixteenth day sodium sulphate (0·1 mol m^{-3}) was added to one set of culture vessels and seven days later plants growing in these solutions could be distinguished from the controls by their darker green colour. Signs of deficiency, similar to those described, again appeared in cultures that had not received sodium, and by the thirty-first day, when a second set of deficient cultures received a treatment of 0·1 mol m^{-3} of sodium sulphate, symptoms were severe.

Four days after receiving this delayed sodium treatment, plants showed signs of recovery by a progressive change of colour in older leaves (and in some cases cotyledons) from yellow to green; greening commenced at tips and around midribs and gradually spread over the lamina. Plants growing in the set of cultures which received no sodium treatment throughout the experiment became progressively more chlorotic making little further growth. On the other hand, vigorous growth occurred in both sets of cultures which received added sodium. When harvested on the forty-ninth day, the results in Table VIII were obtained. The complete recovery of plants growing in cultures which received a small addition of sodium sulphate (even though they were adequately supplied with sulphate), is convincing evidence for sodium being an essential nutrient element for *Atriplex vesicaria.*

3. Effects of Lithium, Sodium, Potassium, Rubidium and Caesium on Sodium-deficient Plants

Seedlings were grown by the techniques described above in the previous experiments and transferred to culture vessels on the fifteenth day after germination. On the twenty-second day when the symptoms of sodium deficiency were clearly recognizable, four cultures each containing four plants were harvested. The mean dry weight per culture was 0·0187 ± 0·0012 g. On the same day differential treatments shown in Table IX were applied to the cultures of each of four blocks. The concentration of sodium in the culture solution due to the sodium associated with the treatment application is also given and was not greater than 0·035 mmol m^{-3} (0·0008 ppm) in any of the treatments other than that of sodium sulphate.

On the twenty-seventh day, plants which had received the sodium sulphate treatment showed signs of recovery. Plants growing in cultures which received no sodium sulphate became progressively more chlorotic, making little further growth. On the other hand marked growth occurred in the set of cultures which had received sodium.

Plants in untreated, and in lithium, potassium and rubidium sulphate treated cultures were indistinguishable. By the thirty-third day, some plants had died in the cultures which had not received sodium. The mean dry

TABLE IX

Effects of Equivalent Amounts of the Sulphates of Lithium, Sodium, Potassium or Rubidium When Applied to Cultures of Sodium Deficient Plants of Atriplex vesicaria

Treatment	Sodium/2-litre culture (μmol) As impurity of basal culture solution	Due to treatment salts	Total	Yield (Each value is the mean of 4 replications) Mean dry wt/culture (g) Leaf-blades	Stems and petioles	Roots	Total
I Control	0·14	(No addition)	0·14	0·179	0·030	0·061	0·270
II Li_2SO_4 0·10 mol m^{-3}	0·14	0·052 (Impurity)	0·192	0·163	0·027	0·051	0·241
III Na_2SO_4 0·10 mol m^{-3}	0·14	200 (Treatment)	200·14	0·761	0·183	0·288	1·232
IV K_2SO_4 0·10 mol m^{-3}	0·14	0·017 (Impurity)	0·157	0·169	0·024	0·049	0·242
V Rb_2SO_4 0·10 mol m^{-3}	0·14	0·069 (Impurity)	0·209	0·220	0·045	0·071	0·336

Plants were harvested on the 44th day. The statistical treatment of total dry weight data was as follows: III > I, II, IV, V at 0·1% level of significance; I, II, IV, V indistinguishable.

From Brownell (1965).

weights per culture obtained for each treatment on the forty-fourth day are shown in Table IX.

These experiments showed sodium to be an essential nutrient element for *Atriplex vesicaria* according to the criteria of Arnon and Stout (1939). Plants, protected from atmospheric contamination of sodium, grown in culture solutions containing only small amounts of sodium showed characteristic deficiency symptoms by the yellowing of their leaves and development of white, necrotic patches on their tips and margins.

Plants developed few or no secondary shoots and in some cases died at an early stage; no pathogenic organisms could be found in their tissues. The first criterion of Arnon and Stout (1939) that "a deficiency of it makes it impossible for the plant to complete its life cycle", was satisfied.

It was also shown that only sodium of the group 1 elements, lithium, potassium and rubidium brought about the recovery of sodium-deficient plants. It therefore appeared that the second criterion that "such deficiency is specific to the element in question and can only be prevented by supplying this element", has almost certainly been satisfied.

The fulfilment of the third of the criteria, that "the element is directly involved in the nutrition of the plant quite apart from its possible effects in correcting some unfavourable microbiological or chemical condition of the soil medium" is difficult to achieve. However, plants in these experiments were grown in solution culture so that the sodium supplied in the treatment was more likely to have affected the nutrition of the plant directly than if more complicated media had been used. Even so the possibility still exists that the sodium corrected an unfavourable chemical or microbiological condition of the culture solution, and this possibility cannot be dismissed until a specific role of sodium in the metabolism of the plant has been demonstrated. These experiments were of short duration so that the possible complicating effects due to the depletion of nutrients in the culture solution were avoided, and the risk of heavy infection of the cultures by organisms such as algae, fungi and bacteria was minimized.

C. OTHER SPECIES HAVING THE C_4 DICARBOXYLIC PHOTOSYNTHETIC PATHWAY

The finding that sodium is essential for *Atriplex vesicaria* suggested that sodium might be an essential micronutrient element for other higher plants. Trials were carried out on a diverse selection of species to find out if the requirement for sodium was general for higher plants (Brownell, 1968). Species were chosen for these trials after consideration of the following: suggestions in the literature of responses to sodium; suitability for culturing; low sodium content per seed; taxonomic or ecological relationship with *Atriplex vesicaria* or unusual features in common with *Atriplex vesicaria* such as vesicles arising from the leaf epidermis.

The techniques used to grow plants in a low sodium environment are

described in Section II.C. The culture solution had a sodium concentration of about 0·08 mmol m^{-3} (0·0018 ppm) derived from impurities of the salts and silica-distilled water. Seeds were thoroughly washed in distilled water before germination. The dry weight yields of species grown with and without the addition of sodium to their cultures and the significance of their responses to sodium are shown in Table X.

The most striking feature of the results of these experiments was the extremely restricted group of species for which sodium is apparently essential. Even within the genus *Atriplex*, there was remarkable specialization in the requirement of sodium. Only the Australian species of *Atriplex* developed symptoms due to sodium deficiency. One other species of *Atriplex*, *Atriplex hortensis* significantly increased its yield on receiving sodium but it did not develop sodium-deficiency symptoms. Species of other genera of Chenopodiaceae viz. *Beta*, *Kochia*, *Exomis* and *Chenopodium* did not appear to require sodium. Even *Beta vulgaris* (sugar beet) which has been reported to increase its yield when supplied with sodium salts (Adams, 1961; Harmer and Benne, 1945) showed no significant responses in these trials. Possibly, very much larger amounts of sodium are needed to induce yield responses in sugar beet than in *Atriplex*, suggesting that the roles of sodium in the nutrition of sugar beet and species of *Atriplex* responding to sodium differ. *Hordeum vulgare* cv. "Pallidium" which showed a small significant response to sodium is a salt-tolerant variety of barley. Significant responses to sodium by *Lycopersicum esculentum* cv. "Grosse Lisse" were not obtained in these trials whereas Woolley (1957) reported a 12% increase in the mean dry weight of tomatoes ($P < 0{\cdot}01$) on the addition of 1 mol m^{-3} NaCl to the basal solution which contained 0·25 mmol m^{-3} (0·0058 ppm) sodium as an impurity. However, no symptoms due to a deficiency of sodium were observed.

Other species which did not respond to sodium either have no requirement for sodium, or they require it in amounts so small that the plants obtained adequate sodium under the conditions of the experiment, i.e. less than approximately 0·08 mmol m^{-3} (0·00184 ppm) sodium in their cultures.

Subsequently, with the discovery of the C_4 dicarboxylic photosynthetic pathway (Hatch and Slack, 1966; Hatch and Slack, 1970; Osmond, 1969a; Osmond, 1969b; Slack and Hatch, 1967), it was observed that the species of *Atriplex* which required sodium had characteristics of plants with the C_4 pathway whereas those not requiring sodium had features of the C_3 photosynthetic pathway. This suggested that all species with the C_4 pathway might require sodium as a micronutrient and that all species with C_3 photosynthesis might have no requirement.

In addition to the ten Australian species of *Atriplex* which have been shown to need sodium (Table X), the requirement for sodium has now been demonstrated in a further nine C_4 species belonging to five families (Gramineae, Cyperaceae, Amaranthaceae, Chenopodiaceae and Portulacaceae) but no re-

sponses to sodium were detected in two C_3 species of Gramineae, *Poa pratensis* and *Panicum milioides* (Table XI). The latter species has certain features of a C_4 plant including the possession of Kranz anatomy and reduced photorespiration (Quebedeaux and Chollet, 1977).

In Table XII, the responses of 37 species to 0·10 mol m^{-3} of sodium chloride or sodium sulphate are summarized. Only certain species of *Echinochloa*, *Cynodon*, *Chloris*, *Panicum*, *Eleusine* (Gramineae), *Kyllinga* (Cyperaceae), *Amaranthus* (Amaranthaceae), *Atriplex*, *Kochia*, *Halogeton* (Chenopodiaceae) and *Portulaca* (Portulacaceae) were shown to respond to sodium by marked increases in dry weight. Plants of these species grown in "sodium-free" cultures had the characteristic leaf lesions of chlorosis and necrosis observed previously in sodium-deficient *Atriplex vesicaria*. These species have characteristics of plants with the C_4 dicarboxylic pathway which include the "Kranz type" specialized leaf anatomy (Moser, 1934; Black, 1954; Hatch and Slack, 1970; Smith and Brown 1973), a high activity of phosphoenol pyruvate carboxylase (Hatch and Slack, 1970), a low CO_2 compensation value and a reduced ^{13}C discrimination (Smith and Epstein, 1971). The known C_4 pathway characteristics for each species are shown in Table XII. No data relating to the C_4 pathway were available for certain species, including *Atriplex semilunalaris*, *A. lindleyi*, *A. angustifolia*, *A. albicans*, *Exomis axyrioides* and *Aster tripolium* as their responses to sodium had been determined before the discovery of the C_4 dicarboxylic pathway and the material has not been available since. Three other species, barley, *Atriplex hortensis* (Brownell, 1968) and tomato (Woolley, 1957) responded to sodium but only marginally. The plants of these species in "sodium-free" cultures did not exhibit sodium-deficiency lesions.

Within *Atriplex* and *Kochia* which include both C_3 and C_4 species, only the C_4 species have been shown to respond to sodium; no decisive response was obtained in the C_3 species. It appears from these data that species having characteristics of the C_4 photosynthetic pathway generally have a requirement for sodium.

D. RESPONSE BY A SPECIES HAVING CRASSULACEAN ACID METABOLISM

The finding that species having the C_4 photosynthetic pathway require sodium as a micronutrient (Brownell and Crossland, 1972) suggested that CAM species might also require small amounts of sodium due to the similarities of their mechanisms for CO_2 fixation.

In preliminary experiments conducted by Brownell and Crossland (1974) in a greenhouse under long-day conditions, *Bryophyllum tubiflorum* failed to respond to small amounts of sodium added to the cultures from which sodium had been carefully eliminated. The absence of a response to sodium was attributed to the possibility that the plant already contained sufficient sodium for normal growth. However, under these conditions it appeared

TABLE X

Yield Responses by Various Higher Plants to Sodium

Species[a]		Sodium supplied (days after germination)	Harvested (days after germination)	Yield (dry weight per plant, g) No addition to culture	0·2 mmol Na_2SO_4 per 2-litre culture	Significance of difference[b]	Remarks
Gramineae							
Hordeum vulgare L. cv. "Pallidium"	(1)	11	26	0·77	0·99	*	
(barley)	(2)	10	45	3·82	4·01		
Chenopodiaceae							
Chenopodium capitatum (L.) Aschers		17	48	12·25	14·37	N.S.	
Beta vulgaris L. (sugar beet)		11	61	3·86	5·07	N.S.	75 μg Na seed^{-1}
Atriplex nummularia Lindl. (Old man, Giant salt bush)		15	43	0·166	0·830	***	
Atriplex paludosa R.Br. (Marsh salt bush)		10	56	0·215	2·789	*	
Atriplex quinii Fv.M.		10	51	0·116	0·815	*	
Atriplex semibaccata R.Br. (Berry salt bush)		15	42	0·104	0·653	**	
Atriplex inflata Fv.M.	(1)	—	—	0·202	9·745	***	
	(2)	8	65	0·036	1·865	***	
Atriplex leptocarpa F.v.M.		14	55	0·050	1·329	**	
Atriplex lindleyi Moq.		14	54	0·093	0·560	*	
Atriplex spongiosa Fv.M. (Pop salt bush)		18	41	0·570	12·472	***	
Atriplex semilunalaris (Aellen)		—	—	0·098	0·526	***	
Atriplex hortensis L. var. *atrosanguinea* Garden orache		12	39	2·849	3·677	*	

Atriplex angustifolia Sm.		14	45	0·331	0·377	N.S.	
Atriplex glabriuscula Edmondston		19	43	24·804	24·100	N.S.	
Atriplex albicans Ait.	(1)	23	49	8·818	11·688	N.S.	
	(2)	17	49	9·237	9·401		
Kochia pyramidata Benth.		—	—	35·390	33·990	N.S.	
Exomis axyrioides Fenzl. ex Moq.		22	54	0·864	0·925	N.S.	
Cruciferae							
Brassica oleracea L. cv. "Savoy" (Cabbage)		17	44	14·244	17·766	N.S.	1 seed contained 0·25 μg Na
Leguminosae							
Trifolium repens L. cv. "Palestine" (White Clover)		19	43	2·299	2·897	N.S.	
Solanaceae							
Lycopersicum esculentum Mill. cv. "Grosse Lisse" (Tomato)	(1)	20	47	4·84	4·76	N.S.	1 seed contained 0·03 μg Na after washing
	(2)	16	42	6·93	7·83		
Compositae							
Lactuca sativa L. cv. "Great Lakes" (Lettuce)	(1)	17	46	3·871	6·113	N.S.	1 seed contained 0·1 μg Na
	(2)	14	32	0·822	1·228		
	(3)	9	37	2·230	2·444		
Aster tripolium L.		17	50	0·282	0·458	N.S.	

[a] Names given in this table are those under which the seeds were received.
[b] Levels of significance: Not significant—N.S.; 5%*; 1%**; 0·1%***.
From Brownell (1968).

TABLE XI

Responses of Various Plants to Sodium

Species[a]	Age at harvest (days)	Lesions in plants not receiving sodium	Yield: No addition (g dry wt/plant)	Yield: 0·1 mol m^{-3} NaCl (g dry wt/plant)	Significance of difference %
Gramineae					
Poa pratensis L. (Kentucky blue grass)[c]	52	None	0·0236	0·0206	NS[b]
Echinochloa utilis Ohwi et Yabuna (Japanese millet)[c]	22	Chlorosis and necrosis	0·404	0·713	0·1
Cynodon dactylon L. (Bermuda grass)[c]	47	Chlorosis	0·178	0·337	1
Chloris barbata Swartz (Purple top Chloris)[d]	23	Chlorosis	0·071	0·239	0·1
Chloris gayana Kumpth (Rhodes grass)	21	Chlorosis	0·018	0·271	0·1
Panicum maximum Jacq. (Guinea grass)	17	Chlorosis	0·335	0·427	
Panicum milioides Nees ex. Trin.	50	None	0·539	0·533	NS[b]
Eleusine indica (L.) Gaertn. (Crowsfoot grass)[d]	21	Chlorosis	0·221	0·435	0·1
Cyperaceae					
Kyllinga brevifolia Rottb.[c]	57	Chlorosis	0·628	1·245	1
Amaranthaceae					
Amaranthus tricolor L. cv. "Early Splendour"[c]	40	Chlorosis	0·884	2·099	0·1
Chenopodiaceae					
Kochia childsii Hort.[c]	21	Chlorosis	0·125	0·442	1
Portulacaceae					
Portulaca grandiflora Hook (Rose moss)[c]	29	Chlorosis, necrosis and failure to set flower	0·242	0·789	1

[a] Names given in this table are those under which the seeds were received.
[b] Not significant.
[c] Data from Brownell and Crossland (1972).
[d] Data from unpublished work of T. S. Boag.

TABLE XII

Sodium Requirement in Relation to C_4 Pathway Characteristics

Species	Lesions in plants not receiving sodium	Yield: No addition (g dry wt/plant)	Yield: 0·1 mol m^{-3} NaCl or Na_2SO_4 (g dry wt/plant)	Significance of difference %	Reference	C_4 pathway characteristics[1]	Reference	Probable pathway
Gramineae								
Hordeum vulgare L. cv. "Pallidium" (barley)	None	0·77 3·82	0·99 4·01	5	c	N, H	f	C_3
Poa pratensis L. (Kentucky blue grass)	None	0·024	0·021	N.S.	d	N	c	C_3
Echinochloa utilis L. Ohwi et Yabuno (Japanese millet)	Chlorisis and necrosis	0·404	0·713	0·1	d	K	c	C_4
Cynodon dactylon L. (Bermuda grass)	Chlorosis	0·178	0·337	1	d	K, $H^{14}C_4$	e	C_4
Chloris barbata Swartz (purple top chloris)	Chlorosis	0·071	0·239	0·1	a	K	a	C_4
Chloris gayana Kumpth (Rhodes grass)	Chlorosis	0·108	0·271	0·1	a	K, P. carb	h	C_4
Panicum maximum Jacq. (Guinea grass)	Chlorosis	0·335	0·427	N.S.	a	P. carb	h	C_4
Panicum milioides Nees ex. Trin.	None	0·539	0·533	N.S.	a	L, $L^{14}C_4$	k	C_3
Eleusine indica (L.) Gaertn. (Crowsfoot grass)	Chlorosis	0·221	0·435	0·1	a	K	g	C_4
Cyperaceae								
Kyllinga brevifolia Rottb.	Chlorosis	0·628	1·1245	1	d	K	c	C_4

continued

TABLE XII—*continued*

Species	Lesions in plants not receiving sodium	Yield: No addition (g dry wt/plant)	Yield: 0·1 mol m^{-3} NaCl or Na_2SO_4 (g dry wt/plant)	Significance of difference %	Reference	C_4 pathway characteristics[1]	Reference	Probable pathway
Amaranthaceae								
Amaranthus tricolor L. cv. "Early Splendour"	Chlorosis and necrosis	0·884	2·099	0·1	d	K	c	C_4
Chenopodiaceae								
Chenopodium capitatum L. Aschers	None	12·25	14·37	N.S.	c	N	n	C_3
Beta vulgaris L. (sugar beet)	None	3·86	5·07	N.S.	c	N, H, L$^{14}C_4$	f	C_3
Atriplex nummularia Lindl. (oldman, giant saltbush)	Chlorosis	0·166	0·830	0·1	c	K, L, H$^{14}C_4$, L^{13}C	j, l, m	C_4
Atriplex paludosa R. Br. (marsh saltbush)	Chlorosis and necrosis	0·215	2·789	5	c	K		C_4
Atriplex quinii Fv. M.	Chlorosis and necrosis	0·116	0·815	5	c	K		C_4
Atriplex semibaccata R.Br. (berry salt bush)	Chlorosis and necrosis	0·104	0·653	1	c	K, L^{13}C	l, n	C_4
Atriplex inflata Fv.M.	Chlorosis and necrosis	0·202	9·745	0·1	c	K, H$^{14}C_4$	j, m	C_4
Atriplex leptocarpa Fv.M.	Chlorosis and necrosis	0·050	1·329	1	c	K		C_4

Atriplex spongiosa Fv.M. (pop salt bush)	Chlorosis and necrosis	0·570	12·472	0·1	c	K, L, P. carb	j, m	C_4
Atriplex semilunalaris Aellen	Chlorosis and necrosis	0·098	0·526	0·1	c	Unknown		
Atriplex lindleyi Moq.	Chlorosis and necrosis	0·093	0·560	5	c	Unknown		...
Atriplex vesicaria Heward ex Benth. (bladder salt bush)	Chlorosis and necrosis	0·013	0·129	0·1	b	K, $H^{14}C_4$, P. carb, $L^{13}C$	j, l	C^4
Atriplex hortensis L. var. *atrosanguineae* (garden orache)	None	2·849	3·677	5	c	N, H, $L^{14}C_4$, $H^{13}C$	m, n	C_3
Atriplex angustifolia Sm.	None	0·531	0·377	N.S.	c	Unknown		...
Atriplex glabriuscula Edmonton	None	24·804	24·100	N.S.	c	N, H, $H^{13}C$	m	C_3
Atriplex albicans Ait.	None	8·818 9·237	11·688 9·401	N.S.	c	Unknown		...
Kochia pyramidata Benth.	None	35·390	33·990	N.S.	c	N, $L^{14}C_4$	j	C_3
Kochia childsii Hort.	Chlorosis and necrosis	0·125	0·442	1		K, L, $L^{13}C$	l, m	C_4
Exomis axyrioides Fenzl ex Moq.	None	0·864	0·925	N.S.	c	Unknown		...
Halogeton glomeratus (Bieb) Meyer	Smaller, curved leaves, tendency to wilting	0·285	0·800	1	n	K	n	C_4
Cruciferae								
Brassica oleracea L. cv. "Savoy" (cabbage)	None	14·244	17·766	N.S.	c	N		C_3

continued

TABLE XII—*continued*

Species	Lesions in plants not receiving sodium	Yield: No addition (g dry wt/plant)	Yield: 0·1 mol m^{-3} NaCl or Na_2SO_4 (g dry wt/plant)	Significance of difference %	Reference	C_4 pathway characteristics[1]	Reference	Probable pathway
Leguminosae								
Trifolium repens L. cv. "Palestine" (white clover)	None	2·299	2·897	N.S.	c	N		C^3
Solanaceae								
Lycopersicum esculentum Mill. cv. "Grosse Lisse"	None	4·84 6·93	4·76 7·83	N.S.	c	N		C_3
cv. "Marglobe"	None	13·76	15·40[2]	1	p	N		C_3
Compositae								
Lactuca sativa L. cv. "Great Lakes"	None	3·871 0·822 2·230	6·113 1·228 2·444	N.S.	a	N		C_3
Aster tripolium L.	None	0·282	0·458	N.S.	a	Unknown		...
Portulacaceae								
Portulaca grandiflora Hook (rose moss)	Chlorosis no flowers	0·242	0·789	1		K		C_4

[1] Leaf anatomy: N, normal (bifacial); K, Kranz. CO_2 compensation: L, low: H, high. ^{14}C in C_4 compounds (malate, aspartate): $L^{14}C_4$, low; $H^{14}C_4$ high. Phosphoenol pyruvate carboxylase activity: P. carb, high. ^{13}C discrimination: $L^{13}C$, low; $H^{13}C$, high. [2] Cultures contained 1 mol m^{-3} NaCl.

a T. S. Boag, unpublished work
b Brownell and Wood (1957); Brownell (1965)
c Brownell (1968)
d Brownell and Crossland (1972)
e Chen *et al.* (1971)
f Downton and Tregunna (1968)
g Guttierrez *et al.* (1974)
h Hatch *et al.* (1975)
i Osmond (1969a)
j Osmond (1969b)
k Quebedeaux and Chollet (1977)
l Smith and Epstein (1971)
m Tregunna *et al.* (1970)
n Welkie and Caldwell (1970)
o Williams (1960)
p Woolley (1957)

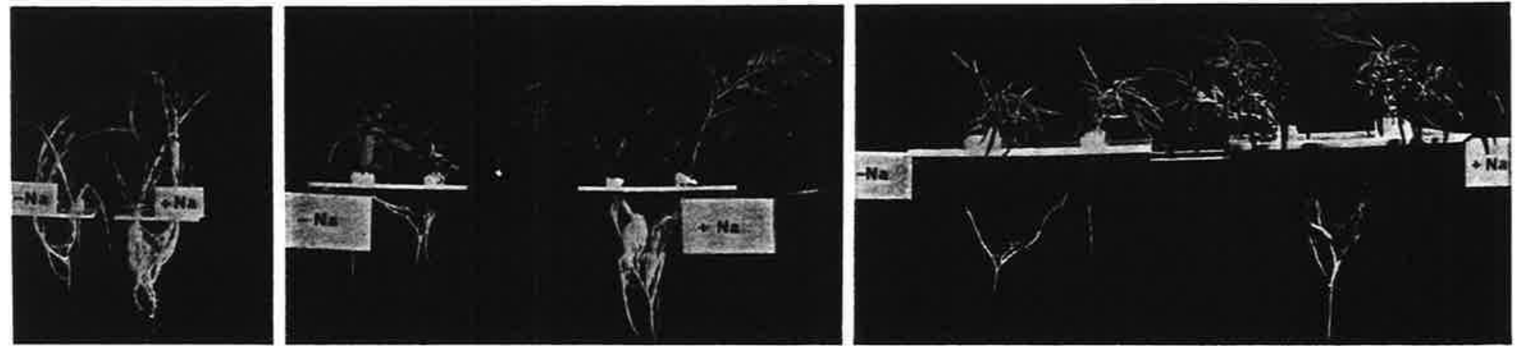


Fig. 11. Comparison between plants of *Echinochloa utilis* (left), *Amaranthus tricolor* (centre) and *Kochia childsii* (right) which received no addition (−Na) and 0·10 mol m^{-3} sodium chloride (+Na). From Brownell and Crossland (1972).

possible that the plants would have assimilated CO_2 almost entirely by the C_3 option with little involvement of the CAM option. Ting (1970), reviewing previous literature, concluded that CAM activity is most pronounced when night temperatures are low and day temperatures are high and that short-day conditions were also conducive to CAM. Brulfert *et al.* (1973) found the activities of the enzymes specifically involved with CAM metabolism in *Kalanchoe blossfeldiana*, a short-day plant, to be phytochrome-controlled. In short days there was a progressively rapid increase in the activity of all the enzymes of the CAM pathway whereas in long days or in short days with nights interrupted with red light, the pathway was not operative, presumably due to low activity of PEP carboxylase.

In several experiments, Brownell and Crossland (1974) found that growth of *Bryophyllum tubiflorum* was greatest under conditions of long days and small diurnal temperature variation. Under these conditions there was no growth response to sodium. When plants were grown under conditions of short day length and large diurnal temperature variation, significant responses to sodium (0·1 mol m^{-3} (2·3 ppm)) were obtained although the overall growth was still less than that of the former.

The results of these experiments (Table XIII, Fig. 12) suggested that sodium is not required by plants grown under conditions thought to be conducive to the C_3 option of photosynthesis, i.e. under long day periods. Plants grew more actively under these conditions but did not respond to sodium. Plants taken from the same population, however, when grown under conditions conducive to CAM photosynthesis, i.e. under conditions of short days and large diurnal temperature variation, responded significantly to small amounts of sodium. Under these conditions of growth, metabolic processes common to those operating in C_4 dicarboxylic photosynthesis are active and this observation would suggest that sodium is involved in this area of metabolism both in species having the C_4 dicarboxylic photosynthetic system and in members of CAM carbon fixation. This supported the hypothesis that sodium may be required for the primary dicarboxylic CO_2-fixation system characteristics of C_4 and CAM plants.

E. DISCUSSION

There is little doubt that sodium is specifically required in small amounts for the blue-green algae, *Anabaena cylindrica* and *Anacystis nidulans*. It may be generally essential for blue-green algae but this is still open to conjecture as few other blue-green algae have been tested critically for their sodium requirement.

Sodium, at similar levels, is also specifically required by *Atriplex vesicaria* according to the criteria of Arnon and Stout (1939) (Brownell, 1965) and evidence from further experiments (Brownell and Crossland, 1972) now makes it likely that all plants with the C_4 dicarboxylic photosynthetic pathway

TABLE XIII

Responses by Bryophyllum tubiflorum *to NaCl Grown Under Different Conditions*

Conditions of growth	Mean dry wt per plant: No addition	Mean dry wt per plant: 0·1 mol m^{-3} NaCl	Significance of difference
	g		%
Short days	0·123	0·176	<1
Long days–short days	0·175	0·218	<1
Long days	0·548	0·521	N.S.

Plants were transferred on day 24 from seedling cultures to culture vessels and received differential NaCl additions under the following conditions. Short days: in artificially illuminated growth cabinet with an 8 h light period at approximately 2800 ft-c and 16 h dark period. Temperature during the light period was 33°C and in the dark period 13°C. Long days–short days: in a naturally illuminated cabinet with the normal day length of 11 h extended to 16 h per day by a 100 w incandescent lamp giving an intensity of approximately 100 ft-c. The overall temperature range was 15° to 38°C. On day 65, cultures were transferred to short day conditions in the artificially illuminated growth cabinet. Long days: in the naturally illuminated cabinet with the normal day length of 11 h extended to 16 h per day. Plants were harvested on day 100.

From Brownell and Crossland (1974).

have a requirement for sodium although it has been shown to be essential for species in only five families of C_4 plants to the present time.

Responses to sodium at low levels were also obtained in a CAM species, *Bryophyllum tubiflorum* only when grown under certain conditions i.e. with short days and high diurnal variation in temperature but not with long days with low diurnal temperature variation (Brownell and Crossland, 1974).

The question of whether or not sodium is essential in small amounts for species with the C_3 photosynthetic pathway has not been resolved. It can be stated, however, that if the C_3 species examined by Brownell (1968) do require sodium, it would be at a concentration less than 0·08 mmol m^{-3} (0·0018 ppm) since the plants grew normally in this purified culture solution. On the other hand, plants with C_4 photosynthesis required a concentration of sodium of about 0·1 mol m^{-3} (2·3 ppm) for optimal growth—about 1250 times the concentration of that remaining as an impurity in the basal culture solution which supported optimal growth of the C_3 species. The possibility that sodium is essential for all species, but in extremely small amounts, cannot be disproved until the amount of sodium available to the plant is reduced to a minute quantity; Steinberg (1937) estimated tentatively (without divulging the basis of the estimation) that if an element is present in greater quantities than 1 part per billion (1 ppb (U.S.) = 0·001 ppm), one cannot be reasonably certain that it is not essential. Hewitt and Smith (1975) consider that the maximum concentration of molybdenum required by *Scenedesmus obligans* with ammonia or urea as nitrogen sources could be as low as 0·0000001 ppm. On this basis, since the concentration of sodium used in this

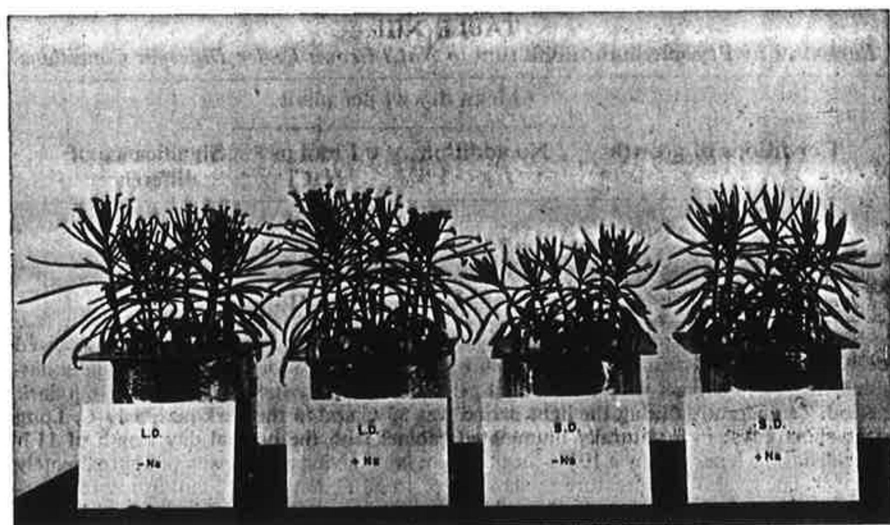


Fig. 12. Comparison between plants of *Bryophyllum tubiflorum* receiving the following treatments. Left to right: no addition, 0·1 mol m^{-3} NaCl (16-h light period; overall temperature range, 15° to 38°); no addition, 0·1 mol m^{-3} NaCl (8-h light period; light temperature 33°, dark temperature 13°). From Brownell and Crossland (1974).

study was approximately 0·08 mmol m^{-3} (0·0018 ppm) and the possibility existed of sodium also reaching the plant from other sources, it would have been necessary to further reduce the level of sodium in the culture solution before sodium could have been considered non-essential for the species examined in this work.

The discoveries that plants with C_4 photosynthesis require sodium and that CAM plants (employing under certain conditions photosynthetic machinery resembling that of C_4 plants) may respond to sodium suggest a common metabolic basis not shared by C_3 species to explain their sodium requirement. It, therefore, seems unlikely that C_3 species have a requirement for sodium even at a minute concentration.

It is not possible to explain the sodium requirement by the blue-green algae in similar terms as there is little evidence of them having the C_4 dicarboxylic carbon fixation pathway except under specialized conditions of high temperature and CO_2 tension which do not normally occur (Döhler 1974). Sodium-deficient cells of *Anabaena cylindrica* growing in nitrate-containing solutions have much higher nitrate reductase activity than that of normal cells (Brownell and Nicholas, 1967). No such effect of sodium deficiency on nitrate reductase activity was observed in *Echinochloa utilis*, a C_4 plant (P. F. Brownell, unpublished data). Thus, no common basis has been discovered to explain the sodium requirement of the blue-green algae on the

one hand and the C_4 and CAM species on the other. They may require sodium for entirely different functions.

In conclusion, it appears that sodium is almost certainly only essential for at least certain blue-green algae, C_4 and CAM species but not for C_3 species of higher plants. This would make sodium a unique essential element in that it would only be needed by certain species.

Reports have been made of sodium chloride treatments having changed the photosynthetic options in certain species. It was found (Winter and von Willert, 1972) that *Mesembryanthenum crystallinum*, normally a C_3 species, grown in the presence of 350 mol m^{-3} sodium chloride, showed typical gas exchange reactions of CAM plants, exhibiting a clear CO_2 uptake in the dark accompanied by an increase in malate content of leaves. Neither CO_2 uptake in the dark nor increase in malate content was found in plants grown in the absence of sodium chloride. It appeared to undergo typical C_3 photosynthesis under these conditions. The leaves of sodium chloride treated plants also underwent cytological changes by the formation of new vacuole-like spaces under the chloroplasts between the plasmalemma and cell wall (von Willert and Kramer, 1972). These changes in metabolism and leaf cytology occurred following the application of an extremely high concentration of sodium chloride (350 mol m^{-3}). This concentration would cause water-stress, one of the factors proposed by Osmond (1975, 1976) and Ting (1970, 1976) that induces the CAM option. It is probable that a similar water stress from droughting treatments would have given a similar response. It seems most unlikely that this is a specific response to sodium.

Sodium chloride (50 mol m^{-3} (1150 ppm Na)) was claimed to have affected the balance between C_3 and C_4 pathways of carbon fixation in young leaves of a grass, *Aeluropus litoralis* Parl (Shomer-Ilan and Waisel, 1973). This grass normally fixes CO_2 by the C_3 photosynthetic pathway, having no detectable PEP carboxylase activity, but when exposed to 50 mol m^{-3} sodium chloride solution, a high level of PEP carboxylase activity was observed and further evidence for the fixation of CO_2 by the C_4 pathway was the ^{14}C labelling of aspartate in leaves fixing $^{14}CO_2$ in the light. The C_4 species, *Zea mays* and *Chloris gayana* were not consistently affected by the 100 mM sodium chloride treatment. It seems likely that the high levels of sodium chloride involved in this response were also due to water stress imposed on the plants and not to a specific effect of the sodium ion.

Kennedy (1977) observed similar effects in the C_4 plants, *Zea mays* and *Portulaca oleracea*, from NaCl-, polyethylene glycol- or naturally induced water stress. With increased water stress, irrespective of its cause, the percentage of C_4 acids decreased and the C_3 acid percentage increased. The CO_2 compensation point increased and the photosynthetic rates were reduced. Clearly the effects of the NaCl treatment could not be ascribed to the sodium it supplied.

IV. METABOLIC AND PHYSIOLOGICAL EFFECTS OF SODIUM AT LOW CONCENTRATIONS

A. GENERAL STRATEGIES

The almost complete lack of information on the involvement of sodium at low concentrations in any biological system makes it difficult to find effective approaches to defining its role in plants for which it is essential.

One approach has been to look for an early response by sodium-deficient plants to sodium, a response that precedes the obvious signs of recovery shown by the greening of chlorotic leaves and increased growth. A metabolic response to sodium detected near the beginning of this period may represent a primary step in recovery and hence contribute to an understanding of the function of sodium in the nutrition of the plant. The cascade of measurable responses which follow in the longer-term ranging from increased production of chlorophyll, changes in the soluble and insoluble nitrogen fractions and increases in carbohydrate content are probably indirect effects of the sodium treatment. An early response detected in the C_4 species of *Atriplex* was an increased rate of respiration which occurred within a few hours of applying sodium whereas dry weight responses were observed only after six days (Brownell and Jackman, 1966). This response was studied in detail and is described later in this section.

A further approach was to study metabolic responses to sodium in the blue-green alga, *Anabaena cylindrica* (Brownell and Nicholas, 1967). This alga has a specific requirement for sodium and it seemed likely that the function of sodium in the alga could be similar to that in *Atriplex*. The alga has advantages over higher plants for metabolic studies in having a short generation time, being easy to sample and manipulate in metabolic experiments.

Following the discoveries of the correlation between the possession of the C_4 pathway and the requirement for sodium (Brownell and Crossland, 1972) and the response to sodium by a plant undergoing CAM (Brownell and Crossland, 1974), it appeared likely that sodium might play a role within the C_4 dicarboxylic acid system common to C_4 and CAM but not to C_3 plants. This area of metabolism is being investigated currently in this laboratory.

B. *Anabaena cylindrica*

1. Nitrogen Metabolism

It was found that *Anabaena cylindrica* had a higher requirement for sodium when grown with nitrate than without combined nitrogen (Fig. 7), (Brownell and Nicholas, 1967). When nitrate was supplied, nitrite increased markedly in sodium-deficient cultures as shown in Fig. 13. The addition of sodium to deficient cultures, after 45 h growth, depressed nitrite production after a further 40 h period, compared with the deficient cultures.

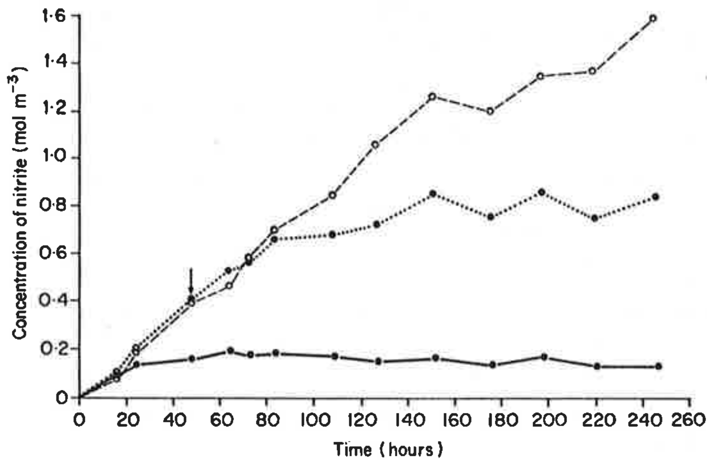


Fig. 13. Effect of sodium on nitrite production by *Anabaena cylindrica* grown in culture solution containing 10 mM KNO_3. No addition, ○--○--○; 4·0 mol m^{-3} NaCL at onse tof experiment, ●—●—●; 4·0 mol m^{-3} Na Cl added to deficient cultures 45 h after inoculation, ●...●....●. From Brownell and Nicholas (1967).

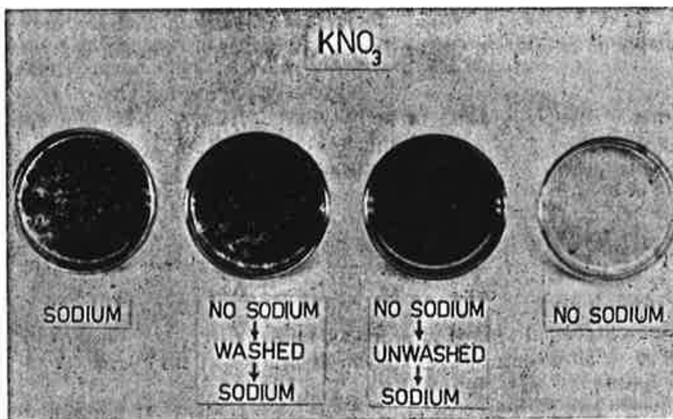


Fig. 14. Eighteen day old cultures containing nitrate. From left to right: dark culture which received 4 mol m^{-3} NaCl at inoculation; dark culture which received 4 mol m^{-3} NaCl 7 days after inoculation after washing and resuspending in a fresh medium; pale culture which received 4 mol m^{-3} NaCl without washing; pale sodium-deficient culture. From Brownell and Nicholas (1967).

The effect of washing deficient cells in water, to remove nitrite, on their subsequent recovery on adding sodium is shown in Fig. 14. Unless nitrite was removed, the addition of sodium did not reconstitute chlorophyll and phycocyanin. Graded amounts of nitrite when added to normal cultures resulted in a chlorosis of the cells within a few days, as shown in Table XIV.

TABLE XIV

Effect of Nitrite on Chlorophyll Content of Anabaena cylindrica

Treatment[a]	Final concentration of nitrite in the culture (mmol m^{-3})	Chlorophyll/culture (mg)
Normal culture	235	1000
Normal culture + 0·2 mol m^{-3} KNO_2	910	600
Normal culture + 0·4 mol m^{-3} KNO_2	1050	720
Normal culture + 1·0 mol m^{-3} KNO_2	2750	500
Normal culture + 3·0 mol m^{-3} KNO_2	5100	370
Sodium-deficient culture	1700	500

Harvest 15 days after inoculation.
[a] Nitrite added to cultures before inoculation. Normal cultures contained 4 mol m^{-3} NaCl.
From Brownell and Nicholas (1967).

TABLE XV

Effect of Sodium on Nitrate Reductase Activity in Cell Extracts of Anabaena cylindrica *Grown in Culture Solution Containing Nitrate*

Amounts of NaCl (mol m^{-3} culture solution)	Nitrate reductase activity: Enzyme activity (total units[a]/100 cm^3 culture)	Dry wt (g/100 cm^3 culture)	Specific activity (units[a]/g dry weight)
None	173	0·040	4300
0·004	139	0·067	2080
0·4	51	0·053	960
4·0	36	0·093	385

Cultures were harvested 13 days after inoculation.
Cells broken by ultrasonication for enzyme assay.
[a] Enzyme unit (nmol NO_2^- formed h^{-1}).
From Brownell and Nicholas (1967).

Extracts of normal cells and of those given suboptimal amounts of sodium were assayed for nitrate reductase and the results are given in Table XV. The enzyme had markedly increased activity when the element was limiting. This accounted for the accumulation of free nitrite in deficient cultures which in turn resulted in a chlorosis of the cells. The effect of adding sodium aseptically *in vivo* to sodium-deficient cells is shown in Table XVI. It is clear that the addition of sodium reduced the enzyme activity to normal levels. When chloramphenicol was added at the same time as sodium to sodium-deficient cells, nitrate reductase was not repressed to normal levels (Fig. 15). This

TABLE XVI
Nitrate Reductase Activity of Extracts of Sodium Deficient and Normal Cells of Anabaena cylindrica *and those Recovering from the Deficiency*

Cultural treatment	Enzyme activity (total units[a]/ 100 cm^3 culture)	Total protein (mg/100 cm^3 culture)	Specific activity of enzyme (units[a]/mg protein)
Complete	540	23·6	11·5
Omit sodium	10 800	12·6	860
Sodium added to deficient cultures, 50 h after inoculation and enzyme assayed 135 h later	1240	20·0	31

[a] Enzyme unit (nmol NO_2^- formed h^{-1}).
P. F. Brownell and D. J. D. Nicholas (unpublished data).

suggests that a newly-formed protein factor(s) dependent on sodium may exert a control on the enzyme.

Not only was the reduction of nitrate to nitrite enhanced by sodium deficiency but also the rates of incorporation of nitrogen-15, labelled nitrate-ammonia and ^{14}C-glutamate into protein were greatly increased as shown in Table XVII. Thus a sodium-deficiency appears to decontrol the assimilation of nitrate via nitrite, ammonia and glutamate into cell-protein. This was also found for the incorporation of $^{15}NH_3$ and ^{14}C-glutamate by sodium-deficient cells grown without combined nitrogen and therefore relying solely on nitrogen gas as a nitrogen source.

Smith (1977) by observing the rates of nitrate disappearance also found the rate of nitrate reduction by *Anabaena cylindrica* to be greater in sodium-deficient than in sodium-sufficient cultures both on a protein and on a culture basis (Fig. 16).

The sodium-deficient cultures with nitrate supplied (Fig. 17) or without combined nitrogen (Fig. 18) had less cellular nitrogen but increased extra-cellular organic nitrogen per culture compared to normal cultures. There was a greatly increased amount of extracellular organic nitrogen on a protein basis in sodium-deficient compared to normal cultures.

Although the findings of Smith (1977) and Brownell and Nicholas (1967) agree on the enhanced rate of nitrate utilization in the sodium-deficient compared to normal cells, Smith (1977) did not observe the accumulation of nitrite in sodium-deficient cultures reported by Brownell and Nicholas (1967). This anomaly could be explained by the system operating differently due to differences between the growth conditions of these studies or a possible physiological difference between the strains of algae used. However, it is apparent that nitrate reduction was accelerated under conditions of sodium-deficiency in both studies.

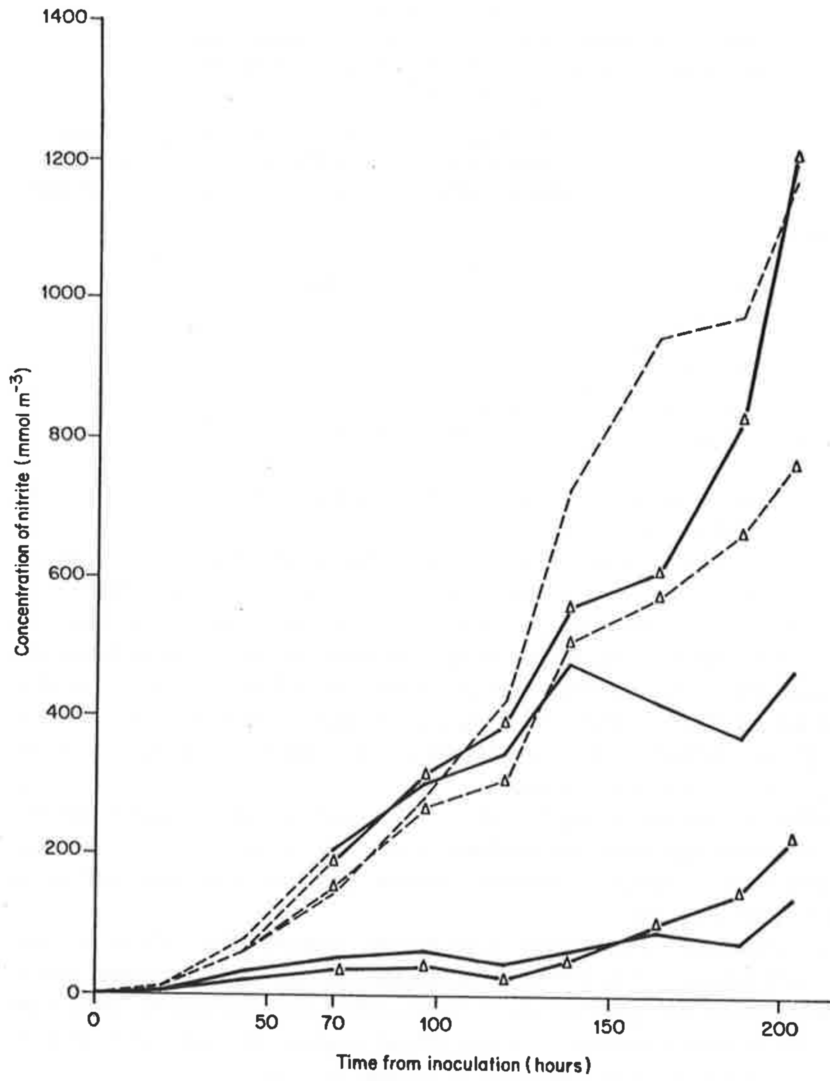


Fig. 15. Effects of sodium and chloramphenicol on nitrite production by *Anabaena cylindrica* grown with 10 mM KNO_3.
4 mol m^{-3} NaCl ——
No sodium supplied -----
Chloramphenicol (0·005 mg cm^{-3}) (△)
From Brownell and Nicholas (1967).

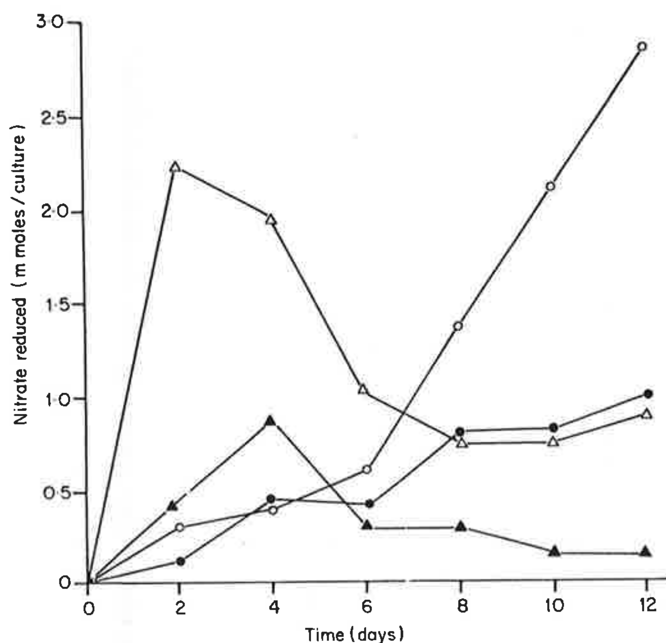


Fig. 16. Nitrate reduction by *Anabaena cylindrica* grown with 10 mol m^{-3} KNO_3.
No added sodium (○) per culture; (△) per mg protein.
0·26 mol m^{-3} NaCl (●) per culture; (▲) per mg protein.
From Smith (1977).

Brownell and Nicholas (1967) found that $^{15}N_2$ fixation into cell protein was reduced in sodium-deficient cells compared with the normal ones (Table XVII). Ward and Wetzel (1975) observed decreased rates of acetylene reduction in cells grown in cultures to which no sodium was added. Smith (1977) also found the rate of acetylene reduction to be lower in sodium-deficient than normal cultures ($P < 0{\cdot}01$) but on a cellular basis there was no significant difference. Cultures with added nitrate had decreased rates of acetylene reduction (Fig. 19). These observations suggest that sodium is required for the reduction of nitrogen gas to ammonia assuming the latter to be a key intermediate in nitrogen-fixation.

The addition of either ammonia, glutamine, asparagine, citrulline or ornithine at low levels (0·1 mM) to sodium-deficient cultures reduced nitrite content within 20 h. Ammonia, arginine, citrulline and ornithine were the most effective as shown in Table XVIII. Similar effects of these compounds on nitrate reductase have been reported in isolated tobacco cells (Filner, 1966).

No similar effect of sodium-deficiency in decontrolling nitrate reductase in *Echinochloa utilis* (a C_4 species) could be detected.

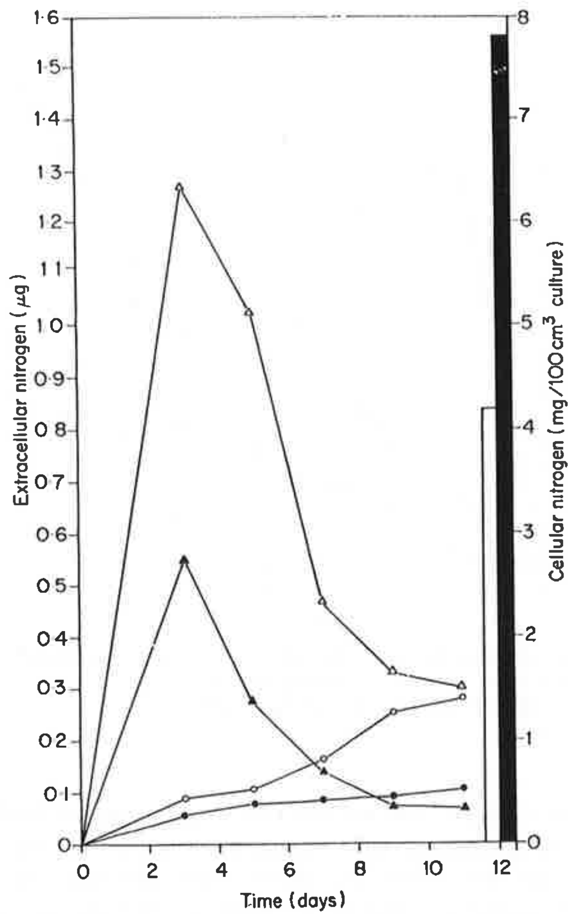


Fig. 17. Cellular and extracellular nitrogen of *Anabaena cylindrica* grown with 10 mM KNO_3.
Cellular nitrogen of 12-day old culture without added sodium (open bar).
Cellular nitrogen of 12-day old culture with 0·26 mol m^{-3} NaCl (closed bar).
Extracellular nitrogen:
No added sodium (○) per culture; (△) per mg protein.
0·26 mol m^{-3} NaCl (●) per culture; (▲) per mg protein.
From Smith (1977).

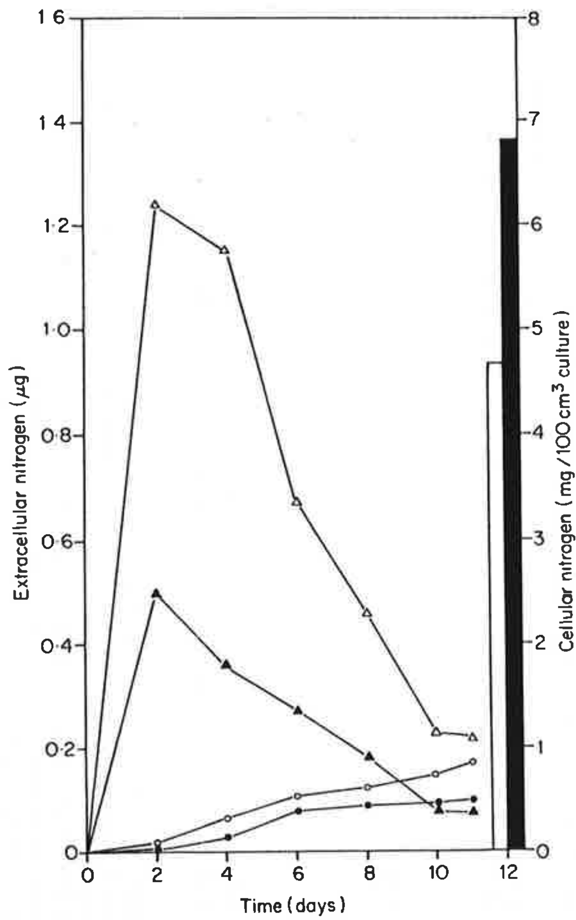


Fig. 18. Cellular and extracellular nitrogen of *Anabaena cylindrica* grown without combined nitrogen.
Cellular nitrogen of 12-day old cultures without added sodium (open bar).
Cellular nitrogen of 12-day old cultures with 0·26 mol m^{-3} NaCl (closed bar).
Extracellular nitrogen:
No added sodium (○) per culture; (△) per mg protein.
0·26 mol m^{-3} NaCl (●) per culture; (▲) per mg protein.
From Smith (1977).

TABLE XVII

Incorporation of $^{15}N_2$ into Cellular Material and $^{15}NO_3$, $^{15}NO_2$, $^{15}NH_4$ and U^{14}C-glutamate into Protein

	Specific enrichment				
	From $^{15}N_2$[a]	From $^{15}NO_3$	From $^{15}NO_2$	From $^{15}NH_4$	From U^{14}C-glutamate
	μg N/mg cell N/h	μg N/mg protein/h	μg N/mg protein/h	μg N/mg protein/h	cpm/mg protein
No combined nitrogen in culture solution					
Complete (4 mol m^{-3} NaCl)	6·05			9·10	20·7
Omit sodium	4·13			17·30	72·0
Nitrate nitrogen in culture solution (10 mol m^{-3} KNO_3)					
Complete	4·21	2·96	4·92	9·24	43·0
Omit sodium	2·60	8·70	12·00	13·56	68·5

Cells collected after 6 days growth.

[a] Cells suspended in 5 cm³ nitrogen and sodium-free culture solution incubated at 30° for 2 h in a Warburg flask with continuous agitation. Pardee buffer in side-arm provided 0·2% CO_2. Gas phase: 0·2 atm. N_2 enriched with 31·4% $^{15}N_2$, 0·20 atm. O_2 and 0·60 atm. He. Suspensions were illuminated at an intensity of approximately 400 foot-candles.

Cells incubated in 5 cm³ nitrogen and sodium-free culture solution at 30° as follows: 10 mol m^{-3} $^{15}NO_3$ (31 atom % excess); 10 mol m^{-3} $^{15}NO_2$ (27·5 atom % excess) for 1 h or 0·1 mol m^{-3} $^{15}NH_4NO_3$ (95·65 atom % excess) for 10 min. ^{14}C-glutamate (63 000 cpm) added to cells suspended in 10 cm³ of the nitrogen and sodium-free culture solution for 10 min. Cells illuminated at 400 foot-candles.

From Brownell and Nicholas (1967).

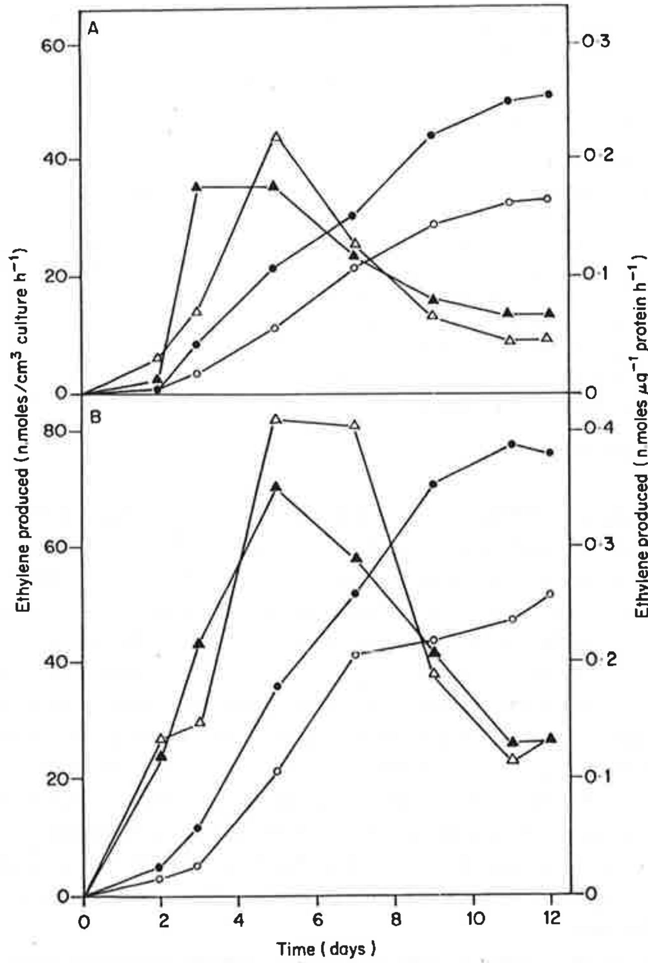


Fig. 19. Effect of sodium on acetylene reduction by *Anabaena cylindrica* grown with 10 mM KNO_3 (A); without combined nitrogen (B).
No added sodium (○) per cm^3; (△) per mg protein.
0·26 mol m^{-3} NaCl (●) per cm^3; (▲) per mg protein.
From Smith (1977).

2. Carbon Metabolism

It has been found that cells of *Anabaena cylindrica* grown in low-sodium media (0·0103 mol m^{-3} (0·236 ppm)) have decreased ^{14}C assimilation and released extracellularly a higher proportion of previously fixed carbon as organic carbon (Ward and Wetzel, 1975). The carbon uptake (as ^{14}C) in cultures without added sodium was about one-third that of cultures grown at concentrations of sodium of 0·22 mol m^{-3} (5 ppm) to 2·2 mol m^{-3} (50 ppm). There

TABLE XVIII
Effect of Sodium and Nitrogenous Compounds on Nitrite Production by the Alga Grown With Nitrate Nitrogen
(Nitrite mmol m^{-3})

Time after inoculation (h)	20	48	91
Normal culture	12	44	50
Deficient culture	16	80	148
Deficient culture, 0·1 mol m^{-3} NH_4Cl	1	1	5
Deficient culture, 0·1 mol m^{-3} arginine	7	15	75
Deficient culture, 0·1 mol m^{-3} citrulline	9	18	50
Deficient culture, 0·1 mol m^{-3} ornithine	12	15	69
Deficient culture, 0·1 mol m^{-3} glutamic acid	16	70	125
Deficient culture, 0·1 mol m^{-3} glutamine	5	50	95
Deficient culture, 0·1 mol m^{-3} asparagine	3	34	100
Deficient culture, 0·1 mol m^{-3} proline	9	58	108

From Brownell and Nicholas (1967).

was no significant difference between cultures with these higher levels of sodium. Rates of excretion of organic carbon in cultures with no added sodium were about half those of cultures receiving the sodium treatments but a greater proportion of carbon assimilated (as inorganic ^{14}C) was excreted as organic carbon in the cultures with no added sodium. The actual percentages of excreted carbon were small (2–3 %) but they suggest reduced efficiency in carbon utilization in low sodium cultures. These increased losses of organic carbon occurred even though the concentration of sodium in the lower sodium culture solutions was 0·0103 mol m^{-3} (0·236 ppm) which is relatively high compared to the level needed to produce the definite signs of sodium deficiency obtained when only about 0·174 mmol m^{-3} (0·004 ppm) of sodium are present as an impurity (Brownell and Nicholas, 1967). It is possible that a much greater loss of carbon would occur in cells subjected to an extreme sodium deficiency.

The quantity of organic matter present in cultures receiving from 0·22 mol m^{-3} (5 ppm) to 2·2 mol m^{-3} (50 ppm) was twice that present in cultures not receiving sodium. As the rates of carbon assimilation were over three times greater in cultures receiving sodium than in sodium-deficient cultures, more carbon appears to be lost after assimilation in cultures with added sodium than can be accounted for by excreted organic carbon. Ward and Wetzel (1975) found that the particulate carbon and rates of ^{14}C assimilation did not vary with increases in nitrate concentration. Particulate organic carbon in cultures receiving 0·22 mol m^{-3} (5 ppm) sodium was approximately twice that of cultures not receiving sodium. The proportion of ^{14}C assimilated and then released as organic carbon increased by approximately 25 % and 58 % with increasing nitrate concentrations of 0·0323 mol m^{-3} (2 ppm) and 0·323 mol m^{-3} (20 ppm) in sodium-deficient cultures.

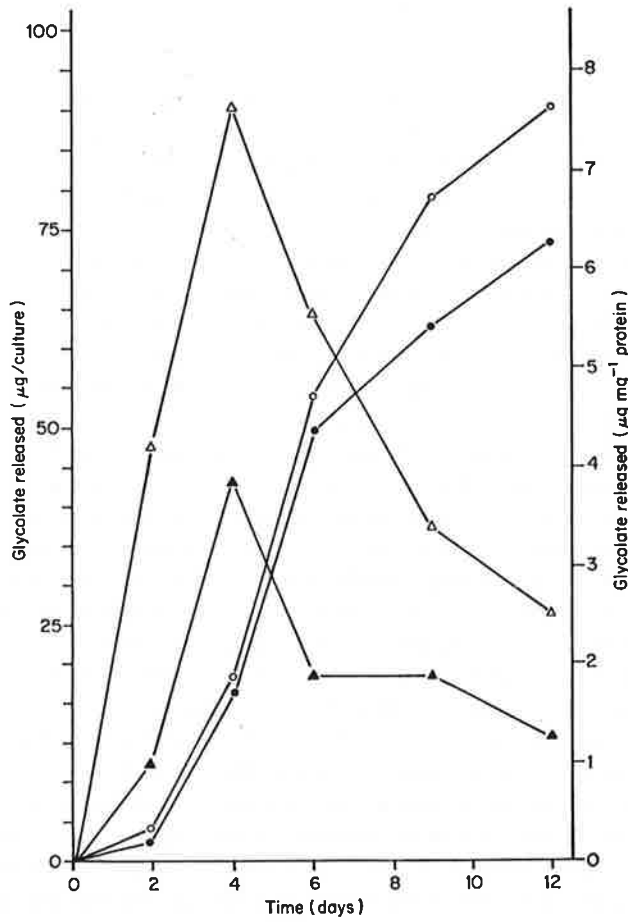


Fig. 20. Effect of sodium on glycolate release by *Anabaena cylindrica* grown without combined nitrogen.
No added sodium (○) per culture; (△) per mg protein.
0·26 mol m^{-3} NaCl (●) per culture; (▲) per mg protein.
From Smith (1977).

Smith (1977) also showed that sodium-deficient cultures of *Anabaena cylindrica* released greater proportions of previously fixed ^{14}C as organic carbon than sodium-sufficient cells. Significantly more glycolate was released extracellularly in sodium-deficient cultures not containing added combined nitrogen in the media (Fig. 20). Glycolate determinations could not be made on nitrate-containing cultures due to nitrate interference.

C. OTHER LOWER PLANTS

1. *Bacteria*

The function of sodium in the metabolism of bacteria and fungi is considered here even though the concentrations of sodium involved are generally much higher than those required for the growth of blue-green algae and C_4 plants. The role of sodium is of interest in these organisms as its effects were shown to be specific and decisive. Furthermore, there is evidence that at least some of these organisms, after a number of stages of subculturing on media with progressively lower concentrations of sodium, have a greatly decreased but an absolute requirement for sodium (MacLeod and Onofrey, 1963).

MacLeod (1965) suggested that the role of sodium is in the transport of substrates into the bacterial cell. Drapeau and MacLeod (1963) obtained evidence for the function by dissociating the uptake of the substrates from their subsequent metabolism with the use of non-metabolizable analogues of metabolizable substrates. When washed cells of a marine pseudomonad B–16 were incubated with ^{14}C-α-aminoisobutyric acid, the analogue of one of the naturally occurring amino acids, it accumulated inside the cells but could not be metabolized. Its uptake required the presence of sodium in the suspending medium. As the uptake took place without a lag period from an incubation medium containing chloramphenicol, it seemed unlikely that the accumulation was due to the preliminary induction of a penetration mechanism. Lithium, potassium, rubidium, ammonium or sucrose could not substitute for sodium in the transport process (Table XIX) and equivalent amounts of the sulphate or chloride of sodium were equally effective.

The process was an active one as the substrate was accumulated at about 3000 times its concentration in the medium and the uptake was stimulated by the presence of an oxidizable substrate (e.g. galactose). MacLeod (1965) concluded that since galactose needed less sodium for the maximum rate of

TABLE XIX

Specificity of the Requirement for the Marine Pseudomonad B–16 for Na^+ for the Uptake of α-Aminoisobutyric Acid (From Drapeau and MacLeod, 1963)

Addition to suspending medium[a]	^{14}C activity of cells (cpm)
0	66
NaCl	6467
KCl	4
RbCl	22
NH_4Cl	27
LiCl	29
Sucrose	44

[a] At concentration of 200 mol m^{-3}. Incubation time 45 minutes.

oxidation than was needed for the optimal rate of uptake of the amino acid analogue, sodium had a role in the uptake process in addition to any possible role in oxidative metabolism. A non-metabolizable analogue of galactose, D-fucose also required sodium for uptake suggesting that the requirement for sodium for galactose oxidation actually represented a requirement for its transport. The uptake of the amino acid analogue, α-aminoisobutyric acid by cells of the marine luminous bacterium *Achromobacter* (*Photobacterium*) *fischeri* has also been found to be a sodium-dependent process.

MacLeod (1965) using washed-cell preparations of two marine pseudomonads found different concentrations of sodium to be required for the maximum rate of oxidation of different substrates. For the maximal rate of oxidation of acetate, butyrate, propionate or an oxidizable sugar 50 mol m^{-3} (1150 ppm) sodium was required. For malate, citrate and succinate 150–200 mol m^{-3} (3450–4600 ppm) sodium was necessary. These differences could be explained by assuming the presence of a number of permeases in the cell membrane with quantitatively different requirements for sodium.

Kahane *et al.* (1975) demonstrated sodium-dependent glutamate transport in membrane vesicles of *Escherichia coli* K-12. Mutants of *E. coli* K-12 are able to utilize glutamate as a major carbon source and transport glutamate more effectively than wild type strains which are unable to grow on this amino acid. A marked similarity was observed between the effects of sodium on glutamate transport in membrane vesicles and in whole cells of *E. coli* K-12. On the basis of the data, it was concluded that the glutamate "carrier" of *E. coli* is in the plasma membrane and that it is possible that a glutamate-binding protein is involved in transport.

Kodama and Taniguchi (1977) found in the presence of potassium, that sodium rapidly enhanced respiration and activated cellular motility and transport of potassium, amino acids and phosphate in the non-halophilic bacterium, *Pseudomonas stutzeri*. Inorganic phosphate was taken up rapidly and esterified to nucleoside triphosphates and diphosphates. Respiration and phosphate transport responded to sodium which increased with temperature. Respiration was more sensitive to polymyxin B in the presence of sodium. Since dinitrophenol stimulated respiration when either potassium or sodium was limiting, sodium appears to be required for the cytoplasmic membrane of *Pseudomonas stutzeri* to perform energy-linked functions coupled to respiration. It appears that sodium exerts its primary effect on the membrane so that energy stored as the energized state of the membrane can be used to drive ATP synthesis, active transport, cellular motility and other energy-linked processes. Approximately 1 mol m^{-3} (2·3 ppm) of sodium was needed for full activity.

2. Fungi

Vishniac (1960) showed 49 isolates of non-filamentous phycomycetes of

marine origin to require sodium chloride for optimum growth. In one species, *Thraustochytrium globosum*, it was found that the optimum concentration of sodium chloride was unchanged after five serial cultures using large inocula. In *Thraustochytrium roseum* uptake of phosphate and oxygen was much greater in the presence of 0·2 mol l^{-1} sodium chloride than in the presence of 0·2 mol l^{-1} of the chlorides of lithium, potassium, rubidium or magnesium or sucrose. Siegenthaler *et al.* (1967) observed that phosphate uptake and transport in *Thraustochytrium roseum* was maximally stimulated by 200–400 mol m^{-3} sodium chloride. They suggested that the effectiveness of sodium chloride in phosphate transport was not related to its osmotic pressure but that the increase in respiration obtained with the sodium chloride treatment was due to its osmotic pressure as sucrose at the same concentration also increased the respiration rate without affecting the ability of the cells to take up phosphate.

D. C_4 AND CAM PLANTS

1. Changes Following the Supply of Sodium in Sodium-deficient C_4 Atriplex *Plants*

A study was made of the changes in leaf respiration rate, chlorophyll content, soluble and insoluble nitrogen fractions, and sugar and starch contents upon the addition of sodium to sodium-deficient *Atriplex* plants (Brownell and Jackman, 1966). The sequence of changes that follow the supply of sodium to sodium-deficient plants of *Atriplex nummularia* is shown in Fig. 21. For at least five days, the growth of plants which received sodium (expressed as fresh weight of shoots) was similar to that of plants to which no sodium was added (Fig. 21A). After seven days, however, the growth of the sodium-fed plants began to increase rapidly compared with that of untreated controls. This delay in growth response occurred despite a rapid uptake of sodium into the shoots from the time of application (Fig. 21B). By the fifth day the plants had taken up most of the sodium supplied in the culture solution, thus the concentration in the leaves remained steady until the seventh day when it showed a slight decrease presumably due to the onset of rapid growth.

The respiration rate per unit fresh weight of shoots (Fig. 21C) increased rapidly (for about three days) after receiving sodium, reaching about twice that of the sodium-deficient plants. Thereafter, there was little further change in rate.

The concentration of chlorophyll in the leaves increased rapidly to almost double the initial concentration by the third day after the addition of sodium (Fig. 21D) and continued to increase throughout the ten days of the experiment.

The concentrations of both sugars and starches in sodium-deficient plants were low (Fig. 22). During recovery, however, these levels gradually rose to several times those found in deficient plants.

Even though, for at least five days, the growth of plants which received

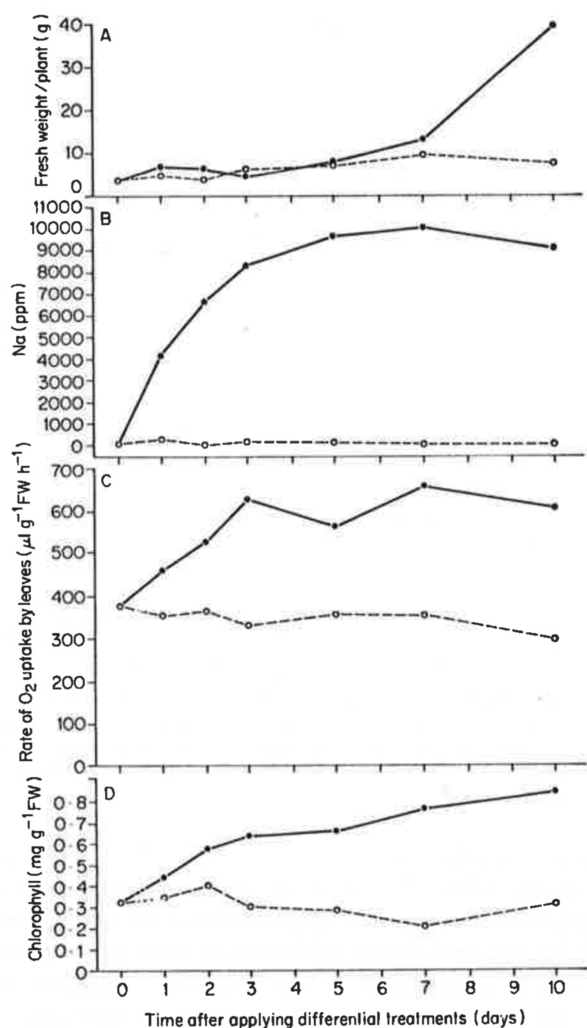


Fig. 21. Changes in fresh weight of shoots (A), sodium concentration (B), rate of oxygen uptake by leaves (C), and chlorophyll concentration (D) following the addition of sodium sulphate (0·6 mol m^{-3}), to 32-day old sodium-deficient *Atriplex nummularia* (old man salt bush) plants. All harvests were made at 9 a.m. Each point is derived from two cultures of eight plants of which six plants were used for chlorophyll determinations, and the remainder for measurements of sodium concentration and respiration rate. Total fresh weights of all 16 plants are recorded. No addition, ○-----○; 0·60 mol m^{-3} Na_2SO_4, ●——●. From Brownell and Jackman (1966).

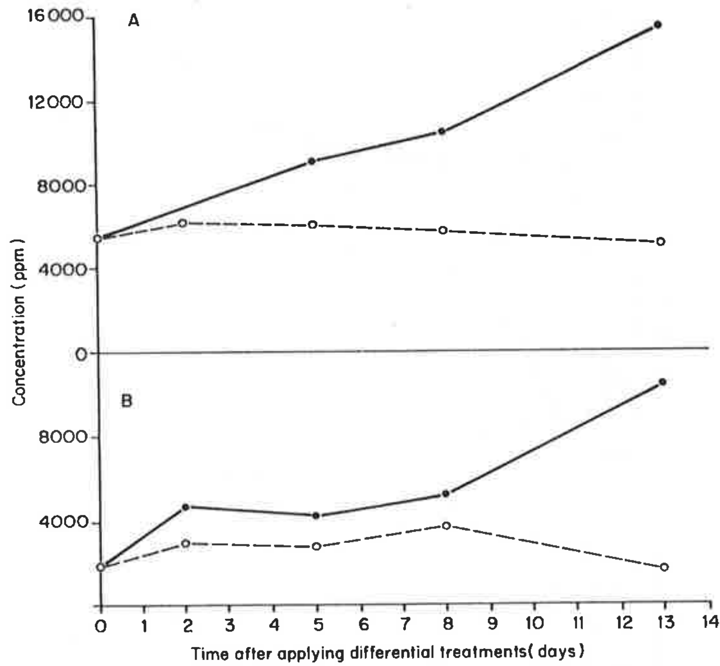


Fig. 22. Changes in concentration of sugars (A) and starch (B) following the addition of sodium sulphate (0·6 mol m^{-3}) to 45-day old sodium-deficient *Atriplex nummularia* (old man salt bush) plants. Points are the means of determinations of six samples each containing the leaves of two plants. No addition, ○------○; 0·60 mol m^{-3} Na_2SO_4, ●——●. From Brownell and Jackman (1966).

sodium (expressed as fresh weight of shoots) was similar to that of plants to which no sodium was added, there is evidence of an increase in the production of photosynthate during this period. Sugar concentrations increased steadily in the plants which received sodium compared to those not receiving sodium. The levels of starch also increased in the sodium-treated plants compared to those not receiving sodium after a lag period of about eight days (Fig. 22).

2. Uptake and Distribution of Sodium and Potassium

Numerous studies have been made of the uptake and transport of sodium in plants but these generally have been with C_3 plants for which sodium does not appear to be essential or with C_4 plants growing in cultures with concentrations of sodium too high to limit growth. It seems that it is more relevant in terms of attempting to understand the function of sodium as a nutrient to work with C_4 plants with the amount of sodium available ranging from deficiency to optimum levels. A few studies have been made with C_4 plants under these conditions. Brownell (1965) supplied graded amounts of sodium sulphate to plants of *Atriplex vesicaria.* The dry weight production and the

TABLE XX

Effects of Treatments of Sodium Sulphate on Dry Weight Production and Concentrations of Sodium and Potassium in Leaves, Stems and Roots of Atriplex vesicaria

Treatment		Dry wt (g) All values are the mean of 5 cultures of 4 plants each				Conc. of sodium and potassium (mmol/kg) All values are the means of duplicate samples taken from 5 replicated cultures of each treatment					
						Sodium			Potassium		
		Leaves	Stems	Roots	Total	Leaves	Stems	Roots	Leaves	Stems	Roots
I	No sodium sulphate	0·0560	0·008	0·022	0·086	10·0	7·1	7·1	2834	1913	1547
II	0·01 mol m^{-3} Na_2SO_4	0·257	0·043	0·098	0·398	47·8	6·5	6·5	4450	2583	1442
III	0·02 mol m^{-3} Na_2SO_4	0·377	0·066	0·138	0·581	78·3	11·7	7·0	2504	2197	1563
IV	0·06 mol m^{-3} Na_2SO_4	0·461	0·088	0·173	0·722	213·0	20·2	11·7	2476	2169	1540
V	0·10 mol m^{-3} Na_2SO_4	0·489	0·089	0·193	0·771	295·7	51·0	29·1	2225	2205	1683
VI	0·60 mol m^{-3} Na_2SO_4	0·685	0·149	0·267	1·101	1129	338·7	257·8	1688	1934	1445

The statistical treatment of total dry weight data was as follows: VI, V, IV, III, II > I at 0·1% level of significance: VI > V at 5% level of significance: II, III; III, IV; IV, V indistinguishable.

Seedlings were transferred to cultures 9 days after germination, the different treatments applied on day 10, and the plants harvested on day 51.

From Brownell (1965).

concentrations of sodium and potassium in leaves, stems and roots of *Atriplex vesicaria* obtained are shown in Table XX.

The dry weight production increased asymptotically with increasing applications of sodium sulphate. The concentrations of sodium increased strikingly in all fractions, especially in the leaves where the increase was more than 100-fold when 0·60 mol m^{-3} (13·80 ppm) of sodium sulphate was supplied although their dry weight production increased only 12 times.

The concentrations of potassium in leaves, stems and roots increased when 0·01 mol m^{-3} (0·23 ppm) sodium sulphate was supplied, but decreased when the amounts of sodium sulphate were further increased to 0·60 mol m^{-3}.

Similar results were obtained by Ashby and Beadle (1957) in which they showed significant increases in dry weight yield in the C_4 species, *Atriplex inflata* and *Atriplex nummularia* following the addition of either sodium chloride, sodium nitrate or sodium sulphate at 10 mol m^{-3} (230 ppm Na) to the culture solution. The concentration of sodium in the leaves increased from 93- to 227-fold when 10 mol m^{-3} (230 ppm) Na was supplied. Williams (1960) using another C_4 species, *Halogeton glomeratus*, also obtained significant dry weight increases on the supply of 1·0 mol m^{-3} (23 ppm) sodium as either the chloride, nitrate or sulphate. The concentration of sodium in the leaves increased from 110 mmol kg^{-1} (2·530 ppm) to 2800 mol kg^{-1} (64 400 ppm) on the addition of 1 mol m^{-3} of sodium chloride to the culture solution. In both studies, potassium concentrations in leaves were markedly depressed by the sodium treatments.

The uptake of sodium by *Atriplex nummularia*, a C_4 species, was found to be rapid. Brownell and Jackman (1966) calculated that after five days little sodium remained in culture solutions initially containing 0·6 mol m^{-3} sodium sulphate. The uptake was calculated by the increase in the amounts of sodium determined in the leaves.

It appears that the species of *Atriplex* used in these studies and *Halogeton glomeratus* may not be typical in their ability to accumulate sodium in their tissues at high concentrations in relation to the concentration of sodium in the cultures. Contrasting data have been obtained in the C_3 species, tomato (Woolley, 1957) and cotton (Pleunneke and Joham, 1972) and in the C_4 species *Echinochloa utilis* and *Kochia childsii* (Table XXI) (D. W. Mill and P. F. Brownell, unpublished work). It is obvious that the ability to accumulate sodium at high concentrations in the tissues from low substrate sodium levels is not general for all C_4 species.

The high concentration of ions in the leaves of some *Atriplex* species has been explained by the active transport of certain ions including chlorine and sodium into epidermal vesicles or bladders which they possess (Osmond *et al.*, 1969).

Foliar applications of sodium have brought about localized recovery in sodium-deficient plants (P. F. Brownell and D. W. Mill, unpublished work).

TABLE XXI
Concentration of Sodium in Leaves (Dry Wt Basis)

	No addition		Added sodium		Added Na
	ppm	μmol/kg	ppm	μmol/kg	concn
Atriplex vesicaria[a] (0·08 mmol m^{-3} sodium)	230[e]	10 000	6801	295 700	0·1 mol m^{-3}
Kochia childsii[b] (0·08 mmol m^{-3} sodium)	10·17[f]	442	233	10 110	0·1 mol m^{-3}
Gossypium hirsutum[c] cv. "Stonefield" 2B–S9 (0·17 mmol m^{-3} sodium)	2·3[g]	100	4·14	180	0·0047 mol m^{-3}
Lycopersicum esculentum[d] cv. "Marglobe" (0·35 mmol m^{-3} sodium)	2·05[h]	89	667	29 000	1·0 mol m^{-3}

[a] Brownell (1965).
[b] D. W. Mill and P. F. Brownell, unpublished work.
[c] Pleunneke and Joham (1972).
[d] Woolley (1957).
[e] Culture solution contained 0·08 mmol m^{-3} sodium as an impurity.
[f] Culture solution contained 0·08 mmol m^{-3} sodium as an impurity.
[g] Culture solution contained 0·17 mmol m^{-3} sodium as an impurity.
[h] Culture solution contained 0·35 mmol m^{-3} sodium as an impurity.

One microlitre of a solution containing 5 μg Na as sodium chloride and 40% ethanol to facilitate infiltration was applied to the upper epidermis of one leaf of a sodium-deficient *Kochia childsii* plant. Within two or three days the leaf receiving the sodium solution greened-up and made rapid growth. Even after two weeks the other leaves of the same plant remained small and chlorotic. Similar results have been obtained with *Atriplex vesicaria.* Mill (1977) applied ^{22}NaCl to leaves of sodium-deficient and sodium-treated *Kochia childsii* plants. Even after seven days of applying the treatment over 80% of the radioactivity was retained in the young treated leaves and over 60% in the old treated leaves (Table XXII). It therefore appears that small amounts of sodium applied to the leaves are relatively immobile and are able to bring about the observed localized recovery of a single leaf while the other leaves still show signs of the deficiency.

3. *Respiration*

Since it was one of the first detected, the respiratory response by *Atriplex inflata* and *A. nummularia* was studied more fully by examining the effects of different salts of sodium, other univalent cations, varying concentrations of sodium, feeding sodium to cut shoots, feeding sucrose, and the effects of sodium on anaerobic CO_2 production (Brownell and Jackman, 1966).

Whether supplied as chloride or sulphate, equivalent amounts of sodium brought about similar increases in the rate of O_2 uptake (Fig. 23). It appeared, therefore, that these increases depended upon the sodium ion and

TABLE XXII

Distribution of Sodium in Plants of Kochia childsii

	Radioactivity (percentage)			
	Sodium-deficient plants[a]		Sodium-treated plants[b]	
Part of plants	Young leaf application	Old leaf application	Young leaf application	Old leaf application
Leaf receiving sodium treatment	85·1 ± 0·66	63·1 ± 0·57	80·6 ± 0·65	80·3 ± 0·68
Leaves above treated leaf	10·2 ± 0·19	19·5 ± 0·28	8·3 ± 0·21	12·4 ± 0·23
Leaves below treated leaf	3·0 ± 0·15	8·3 ± 0·22	4·1 ± 0·19	5·1 ± 0·17
Stems and roots	1·7 ± 0·10	9·1 ± 0·23	7·0 ± 0·17	2·3 ± 0·15

[a] Plants were grown under low-sodium culture conditions and harvested 27 days after germination.

[b] Plants received 0·1 mol m^{-3} NaCl in their cultures 7 days after germination.

[c] 5 μg Na as NaCl with ^{22}Na activity of 0·05 μCi in 40% ethanol was applied in a total volume of 1 μl to the upper epidermis 21 days after germination.

Data are the means of values from four plants.

From Mill (1977).

were not due to excess cation absorption (Jacobson, 1955; Jacobson and Ordin, 1954), as with the latter phenomenon, different rates of oxygen uptake would be expected as a result of the different mobilities of chloride and sulphate ions.

The rates of O_2 uptake by leaves of sodium-deficient plants 48 hours after receiving sulphates of univalent cations (0·1 mol m^{-3}) in culture solutions are shown in Fig. 24. Sodium sulphate increased the rate of oxygen uptake by 73% above that of the control whereas the sulphates of lithium, potassium, rubidium and caesium did not increase the rate. It seems unlikely that this is salt-stimulated or anionic respiration (Lundegardh, 1955; Robertson and Turner, 1945) for two reasons; first, the salt-stimulated response generally occurs only in tissues initially containing low concentrations of ions, whereas the concentrations of ions in leaf tissues of *Atriplex* are high compared with the low concentrations of sodium required to elicit the response; second, salt-stimulated respiration may be brought about by various ions, unlike this respiratory response in *Atriplex* which requires sodium, specifically.

Both respiration rate (O_2 uptake) and growth (fresh weight) tend towards a maximum value with similar concentrations of sodium in the culture solution (Fig. 25).

From these results it appeared that lack of sodium limited the rate of respiration of *Atriplex* leaf tissues. If this brought about a reduction in phosphorylation, growth could conceivably be restricted by the decreased amounts of high energy phosphate compounds available for cellular work and syntheses.

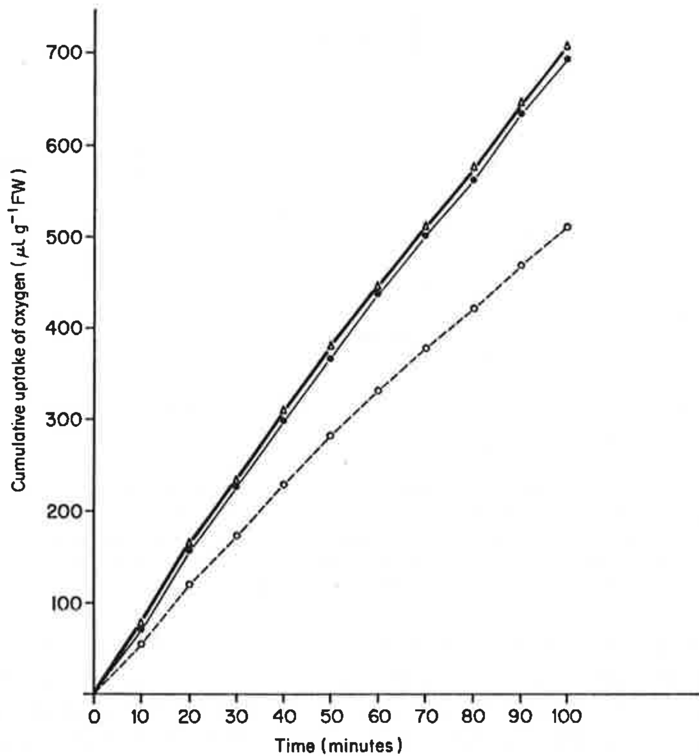


Fig. 23. Effect of sodium either as chloride or sulphate on the rate of O_2 uptake by leaves of sodium-deficient *Atriplex nummularia* (old man salt bush). Sodium chloride or sodium sulphate (0·22 mol m^{-3}) was added to the culture solution 48 hours before harvesting. Points are the means of O_2 uptake of four Warburg flasks each containing the leaves of two plants. No addition, O——O; NaCl, Δ——Δ; Na_2SO_4, ●——●. From Brownell and Jackman (1966).

When sodium was introduced to the tissue through cut stems, the rate of O_2 uptake by the leaves was 20% greater than that of untreated leaves (Table XXIII) and analyses showed that there had been an increase in the sodium concentration. This respiration response, only 1½ h after the application of sodium, was detected in a considerably shorter time than in other experiments when sodium was added to the culture solution. It is difficult to determine precisely the time required for this response to sodium, first because it is not known when sodium arrives at the actual site of action, and second because it would not be expected to reach all cells in a tissue at the same time.

Since the concentrations of sugars and starch were low in leaves of sodium-deficient plants (Fig. 22), it seemed possible that lack of respiratory substrates could have limited their respiration rates. Hence cut leaves were supplied with sucrose and their respiration rates measured. The results

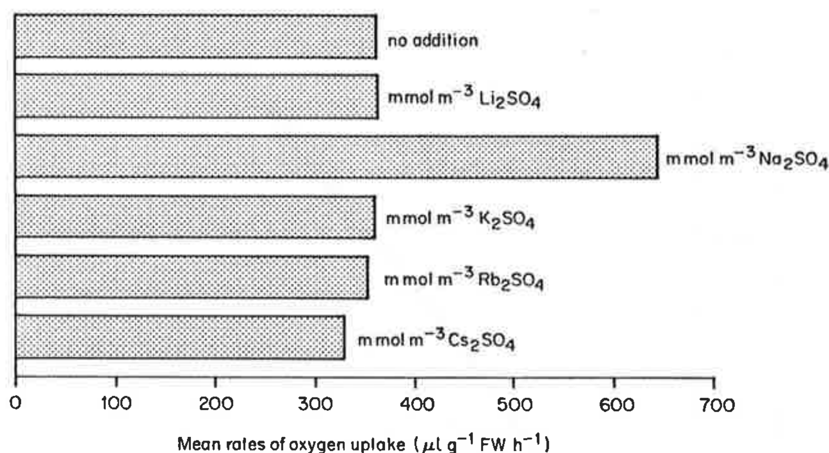


Fig. 24. The rates of O_2 uptake by sodium-deficient plants of *Atriplex nummularia* (old man salt bush) which received 0·1 mol m^{-3} of the sulphate of lithium, sodium, potassium, rubidium or caesium 48 hours before harvesting compared with the control plants which received no addition. Points are the means of O_2 uptake of four Warburg flasks each containing the leaves of two plants. From Brownell and Jackman (1966).

(Table XXIV) showed that addition of sucrose increased the respiration rates of leaves from both sodium-deficient and normal plants suggesting that the small amounts of endogenous substrate were limiting the rate of respiration.

The supply of sodium to the culture solution 43 hours before harvesting also caused an increase in the respiration of leaves which received no additional sucrose. This could have been due to either a direct effect of sodium on the respiratory system or alternatively to an indirect effect, for example by increasing the amount of substrate derived from photosynthesis during the period after addition of sodium. When sucrose was fed in an attempt to remove the substrate limitation, there was still a response to sodium, suggesting that its effect is a direct one on some part of the respiration process. If however, the substrate limitation was not entirely removed by the exogenous sucrose, the apparent effect of sodium on respiration rate could be through its effect on photosynthesis.

In all experiments, sodium was supplied in the light when the high rates of transpiration needed for the rapid uptake of the treatment solutions would be favoured. Even so, it is unlikely that in the short-term experiments (1½ h) there would have been sufficient time for differences in the photosynthate produced between treatments to be great enough to affect the results of the subsequent respiration experiment. Thus, it seems that the effect of the sodium treatment on respiration in these experiments was direct and not due to its possible effect on photosynthesis.

In an initial attempt to locate the part of the respiratory system in which

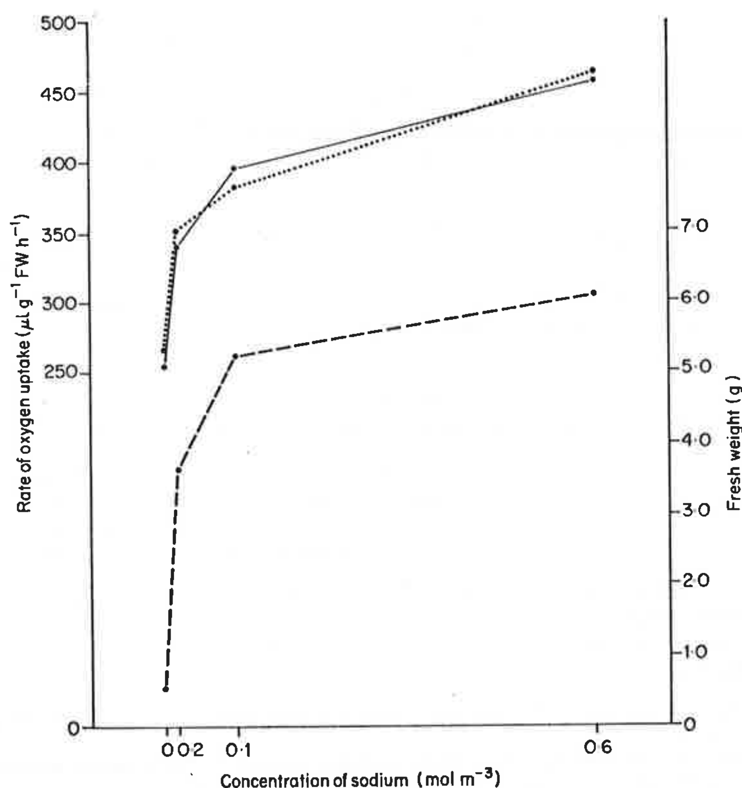


Fig. 25. Effect of concentration of sodium in culture solution on fresh weight (●----●) and rate of oxygen uptake in *Atriplex nummularia* (old man salt bush). Fresh weight data are means of five cultures of four plants each, sodium being supplied 16 days after germination and the plants harvested 32 days later. The rates of O_2 uptake were determined 28 hours ●.........● and 76 hours (●——●) after supplying sodium to 46-day old plants. From Brownell and Jackman (1966).

sodium might be involved, rates of CO_2 output of sodium-deficient and sodium-fed plants were compared. The results obtained (Table XXV) showed that the addition of sodium to sodium-deficient plants stimulated the rate of anaerobic CO_2 production and that the rate of O_2 uptake when leaves were returned to air was unaffected by the anaerobic treatment. Hence, the increase in rate of CO_2 production under anaerobic conditions, suggests that sodium acts in the glycolytic stages of respiration. However, an earlier effect of sodium on some other system leading to this respiratory response is still possible.

This effect of sodium in increasing the respiratory rate in leaves does not appear to be general for all C_4 species. Mill (1977) was not able to obtain similar results when sodium was supplied to sodium-deficient plants of *Kochia*

TABLE XXIII

Effect of Feeding Sodium to Cut Shoots on Rate of O_2 Uptake and Sodium Concentration of Leaves

	O_2 uptake[b] ($\mu l\ g^{-1}$ fr. wt h^{-1})	Sodium conc.[c] (mmol Na per kg dry wt)
No sodium added	211	12·1
Added sodium[a]	256	18·5

[a] Cut shoots were placed in sodium sulphate (2·2 mol m^{-3}) for 1½ h in the light.
[b] Values are mean rates of uptake of six Warburg flasks each containing the leaves of 2 to 3 plants.
[c] Values are the means of two determinations on the leaves from six Warburg flasks.
From Brownell and Jackman (1966).

TABLE XXIV

Effect of Feeding Sucrose to Cut Leaves on Rate of O_2 Uptake

	O_2 uptake ($\mu l\ g^{-1}$ fr. wt h^{-1})		Percentage increase due to sucrose
	Without sucrose	With sucrose[b]	
No sodium added	244	322	32
Sodium added[a]	302	400	32
Percentage increase due to sodium	24	24	

Data are the mean rates of O_2 uptake of three Warburg flasks each containing the leaves of 2 or 3 plants.
[a] 0·60 mol m^{-3} sodium sulphate added to culture solution 43 hours before harvesting.
[b] Cut petioles were immersed in 0·1 mol l^{-1} sucrose for 1½ h in light.
From Brownell and Jackman (1966).

TABLE XXV

Effect of Sodium on Rate of Anaerobic CO_2 Production of Sodium Deficient Atriplex nummularia *Leaves*

	Anaerobic phase CO_2 output	Aerobic phase O_2 uptake
No sodium added	85	316
Added sodium[a]	105	426

Data are the mean rates of gas exchange ($\mu l\ g^{-1}$ fr. wt h^{-1}) of sixteen Warburg flasks each containing the leaves of 2 to 3 plants.
[a] 0·1 mol m^{-3} sodium sulphate was supplied to the culture solution 24 hours before harvesting.
From Brownell and Jackman (1966).

childsii. At present it is not possible to explain this difference in the respiratory responses of these species. As already pointed out, there is a large difference between these C_4 plants in their ability to accumulate sodium from a lower-sodium substrate and it is possible that the response described for the *Atriplex* species could be attributable in some way to their propensity for accumulating sodium from low substrate concentrations. The respiratory response may not necessarily be a sequential step in the events taking place from when the sodium treatment was applied to when the signs of recovery appear.

4. Nitrogen Fractions

Brownell and Jackman (1966) found marked differences in the ratios of 80% ethanol-soluble nitrogen to total nitrogen between the leaves of sodium-deficient and normal plants of *Atriplex* by 20 days after supplying sodium but no difference was detectable at two days after the addition of sodium (Table XXVI) although by this time there was a definite respiratory increase. Hence, the apparent long-term effect of sodium in decreasing the ratio of soluble nitrogen to total nitrogen is probably indirect and only one of the many changes likely to take place in the later stages of recovery.

5. Photosynthetic Responses

The finding that plants having the C_4 photosynthetic pathway require sodium as a micronutrient (Brownell and Crossland, 1972) and that a CAM plant, *Bryophyllum tubiflorum*, responds to sodium only when grown under certain conditions (Brownell and Crossland, 1974) suggested that sodium is involved in the first carboxylation and the decarboxylation of the resulting C_4 dicarboxylic acid. This appeared likely since this metabolic system operates only in C_4 and CAM plants but not in C_3 plants.

Three types of C_4 plants have been described on the basis of their C_4 acid decarboxylating systems and ultrastructural features (Gutierrez *et al.*, 1974; Hatch *et al.*, 1975). They comprise:

1. Malate type
   NADP-malic enzyme-type
2. Aspartate type
   NAD-malic enzyme-type
3. Aspartate type
   Phosphoenol pyruvate carboxykinase type.

Species for which sodium has been shown to be essential fall into all these categories. *Echinochloa utilis* which has relatively high NADP malic enzyme activity (P. F. Brownell, unpublished work), *Kochia childsii* and *Portulaca grandiflora* (rose moss) (Gutierrez *et al.*, 1974) would be in type 1. Type 2 includes *Amaranthus tricolor* (Gutierrez *et al.*, 1974), *Atriplex spongiosa* (pop salt bush) (Hatch *et al.*, 1975) and *Cynodon dactylon* (Bermuda grass) (Chen *et al.*, 1971). *Chloris gayana* (Hatch *et al.*, 1975) and *Eleusine indica* (Gutierrez

TABLE XXVI

Concentrations of Soluble and Insoluble Nitrogen in Leaves of Atriplex

	Atriplex nummularia [a] two days after receiving sodium				*Atriplex inflata* [b] twenty days after receiving sodium			
		Nitrogen content (% fr. wt)				Nitrogen content (% fr. wt)		
	Fr. wt per plant (g)	ethanol extract	Residue	Sol N / Total N	Fr. wt per plant (g)	ethanol extract	Residue	Sol N / Total N
No sodium added	1·33	0·29	0·42	0·41	0·11	0·32	0·39	0·45
Sodium added [c]	1·76	0·25	0·39	0·39	0·50	0·17	0·46	0·27

[a] Data are the means of determinations on five individual plants.
[b] Data are the means of determinations on four samples of two plants when no sodium was added and one plant when sodium was supplied.
[c] 0·60 and 0·10 mol m^{-3} sodium sulphate to *Atriplex nummularia* and *Atriplex inflata*, respectively.
From Brownell and Jackman (1966).

et al., 1974) are in type 3 and have been shown to have a sodium requirement (T. S. Boag, unpublished work).

If sodium is needed for the system operating in C_4 and CAM plants comprising the first carboxylation and the subsequent decarboxylation, it would be expected to affect a part of the system common to types 1, 2 and 3 species. Of the enzymes highly active in the three types of C_4 species but not in C_3 species, only the four enzymes, phosphoenol pyruvate carboxylase, pyruvate orthophosphate dikinase, adenylate kinase and pyrophosphatase have similar activities in all types of C_4 plants (Hatch, 1976). These enzymes also appear to be active in the group of CAM species defined by Dittrich *et al.* (1973) as having high activities of NADP malic enzyme but not detectable phosphoenol pyruvate carboxykinase activity. This group includes *Bryophyllum tubiflorum*, the species which responded by increased dry weight yield to sodium when grown under conditions of short day and large diurnal temperature fluctuations (Brownell and Crossland, 1974; Boag, 1976).

The possible requirement of sodium for the formation or activation of the other enzymes indicated by Hatch (1976) to be present only in the individual types of C_4 species would not explain the general requirement of sodium for all the three types of C_4 species.

The other CAM species described by Dittrich *et al.* (1973) contained high activities of phosphoenol pyruvate carboxykinase but low activities of NADP malic enzyme. The only enzyme occurring in plants of this group common to the C_4 and the other CAM group of species but not active in C_3 species would be phosphoenol pyruvate carboxylase. No CAM species in this group has yet been examined for a sodium requirement.

If sodium is involved in the primary carboxylation (phosphoenol pyruvate carboxylase) and the subsequent decarboxylation steps of photosynthesis in C_4 and CAM plants, it could be affecting the activity of phosphoenol pyruvate carboxylase or the formation of phosphoenol pyruvate, the carbon dioxide acceptor. In both C_4 and the CAM species, *Bryophyllum tubiflorum*, the enzyme, pyruvate orthophosphate dikinase, is involved in the conversion of pyruvate (derived from the decarboxylation) to phosphoenol pyruvate. This is the CO_2 acceptor in C_4 plants but is converted by reverse glycolysis to sugars and starches in *Bryophyllum tubiflorum.* These compounds are the sources of the PEP, the CO_2 acceptor in the dark.

The other enzymes implicated in the supply of PEP would be adenylate kinase required in the following reaction:

$$ATP + AMP \rightarrow 2\ ADP$$

and pyrophosphatase required in the reaction:

$$PPi \rightarrow 2\ Pi.$$

It seemed possible that sodium could be involved in these reactions.

It appears that sodium nutrition in C_4 plants does not affect the formation of phosphoenol pyruvate carboxylase. *Echinochloa utilis* plants were harvested at four times in an experiment to determine the effect of sodium on the enzyme

(P. F. Brownell, unpublished work). Although there was a marked growth response to the sodium addition, there was no significant difference between the specific activity of PEPC extracted from sodium-deficient and normal plants at any harvest (Fig. 26). These results indicate that sodium does not affect the formation of PEPC but it is still possible that sodium may be necessary for the activation of the enzyme *in vivo*. This would not have been revealed in this experiment as no measures were taken to obtain sodium-free assay conditions. If the enzyme requires sodium for its activation, there would probably have been sufficient sodium present in the assay medium not to have limited its activity in the *in vitro* assay. Holtum (1975) detected no difference in PEPC activity on a fresh weight basis in leaves of sodium-deficient and normal plants of *Kochia childsii* but the assay was not carried out under sodium-free conditions.

No activation effect of sodium was observed on pyruvate orthophosphate dikinase, phosphoenol pyruvate carboxylase or adenylate kinase in assay media containing about 0·2 mol m^{-3} (4·6 ppm) sodium (M.D. Hatch, personal communication). It is still possible that sodium is needed for activation of these enzymes in lower concentrations.

Evidence for the lack of an effect of sodium on the activity of PEPC *in vivo* was obtained from preliminary ^{14}C labelling experiments with *Kochia childsii* exposed to $^{14}CO_2$ in the light (Holtum, 1975; Webb, 1977). No consistent differences were observed between the labelling patterns of sodium-deficient and normal plants which could suggest an effect of sodium nutrition on the activity of phosphoenol pyruvate carboxylase.

Further indirect evidence that phosphoenol pyruvate carboxylase activity is not influenced *in vivo* is presented by Boag (1976) who followed the patterns of carboxylation and decarboxylation (determined by changes in the titratable acidity of leaves) in the CAM plant, *Bryophyllum tubiflorum*. It is possible to dissect out the processes of carboxylation and decarboxylation which are separated in time and to observe the possible effects of sodium nutrition on them. It was found that the rate of carboxylation in the dark in plants grown under both short- and long-day regimes was not affected by the sodium treatment (Fig. 27A and B). This suggests that sodium had not affected the *in vivo* activity of PEPC, the enzyme responsible for the increase of acid (as malic acid in the vacuoles). Similarly, there was no evidence for differences in the rates of decarboxylation in the light between sodium-deficient and normal plants. It thus appears that this system which collects carbon in the dark in CAM plants to be converted eventually to photosynthate by the Calvin cycle in the light is not affected by sodium nutrition even though there are significant increases in dry weight in response to sodium under certain conditions e.g. short days and large diurnal temperature variations. It is probably justified to extrapolate this finding to C_4 plants which have similar systems for the primary carboxylation and to postulate

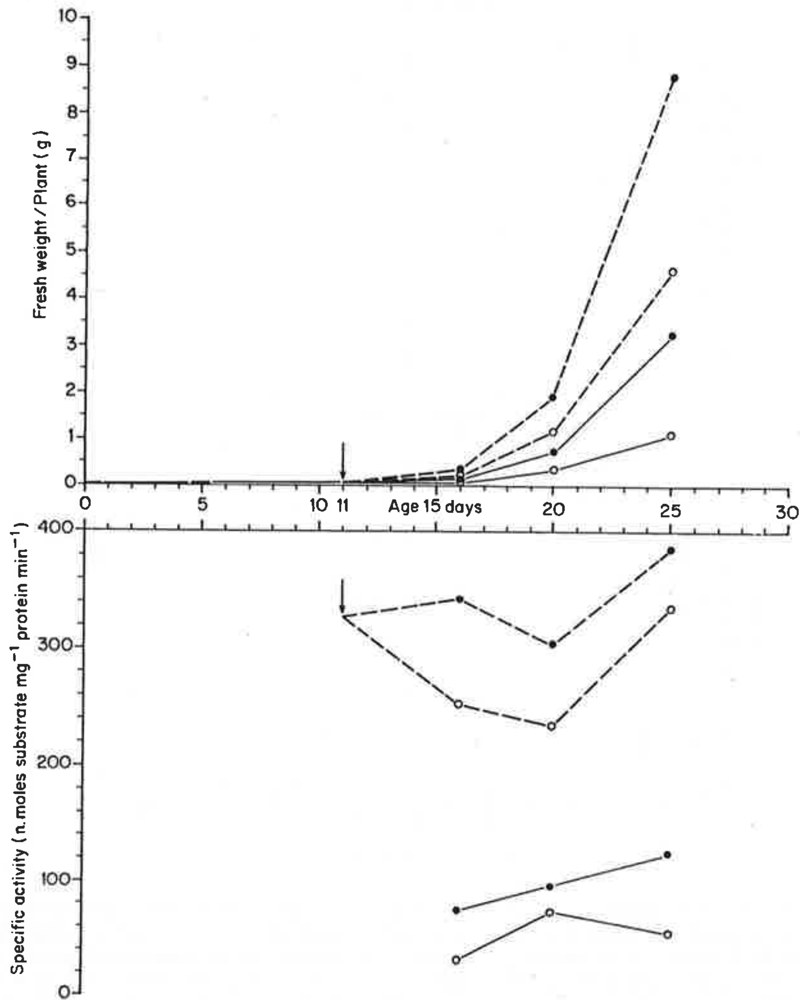


Fig. 26. Changes in fresh weight (*upper*) and specific activity of phosphoenol pyruvate carboxylase (*lower*) of shoots, (– – –) and roots (——) of *Echinochloa utilis* L. following the addition of 0·1 mol m^{-3} of sodium chloride to eleven-day old sodium-deficient plants. Fresh weight data are means of 24 plants in the first and 12 plants in the subsequent harvests. Activities of phosphoenol pyruvate carboxylase are the means of values from four determinations in extracts of tops and roots. The extraction of the enzyme was by the method of Slack and Hatch (1967) and the assay according to Osmond (1969a). No addition, (○); 0·1 mol m^{-3} NaCl, (●) (P. F. Brownell, unpublished work).

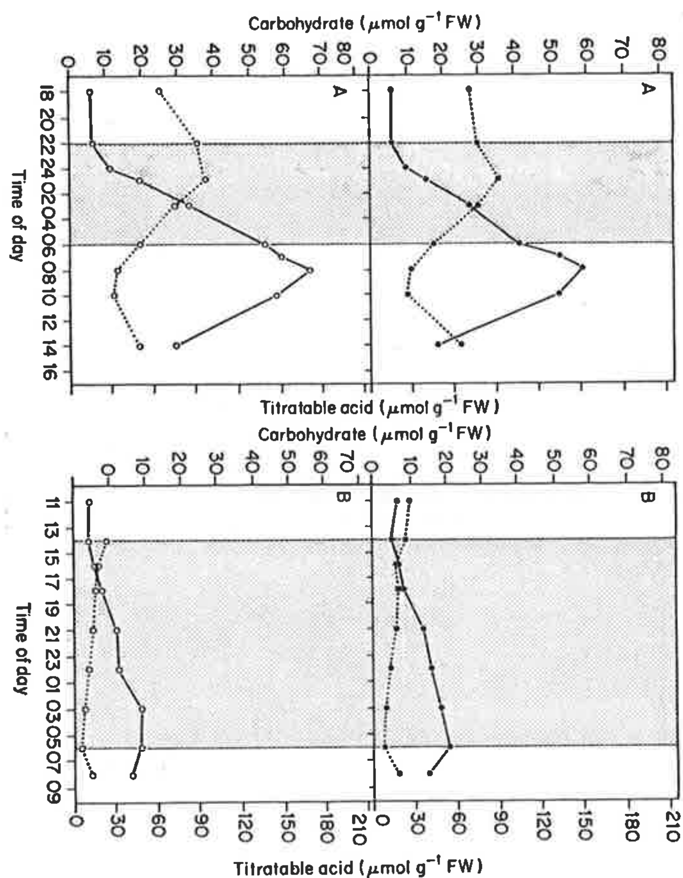


Fig. 27. Diurnal changes in titratable acidity (——) and acid-soluble carbohydrates (..........) in leaves of sodium-deficient (○) and normal (0·1 mol m^{-3} sodium chloride treated) (●) *Bryophyllum tubiflorum* grown under (A) a sixteen-hour light period, temperature 33°–36°C/eight-hour dark period, temperature 24°–26°C. Illumination was 183 μE m^{-2} sec^{-1} (9480 lux) and (B) under an eight-hour light period, temperature 33°C/sixteen-hour dark period, temperature 13°C. Illumination was 255 μE m^{-2} s^{-1} (10 200 lux). The plants were 72 days old. From Boag (1976).

that sodium is not acting in this system but in a later stage possibly in the photosynthetic carbon reduction cycle.

There is ample evidence for sodium increasing the rate of photosynthesis when supplied to sodium-deficient C_4 plants and the CAM plant, *Bryophyllum tubiflorum* grown under conditions of short days and large diurnal temperature variation. The simplest evidence is from marked increases in growth as fresh or dry weight yields which are now well established (Brownell and

Wood, 1957; Williams, 1960; Brownell and Crossland, 1972; Brownell and Crossland, 1974).

Evidence for short-term differences is suggested from calculation of data from Brownell and Jackman (1966) in which the respiration of leaves of sodium-deficient and normal plants of *Atriplex nummularia* (old man salt bush) was expressed as glucan equivalents respired per day. These quantities, added to the glucan equivalents in the glucose and starch (Fig. 21) produced by sodium-deficient and normal plants, respectively, give an estimate of their relative photosynthetic performance during the early period of recovery prior to a growth response (Fig. 28). By calculation, it can be shown that the combined glucan equivalents of glucose plus starch production and respiration per day would be about 10% of the dry weight for sodium-deficient plants compared to 20% for normal plants.

The ^{14}C-labelling of amino acid, organic acid and sugar fractions extracted from leaves of *Atriplex nummularia* (old man salt bush) when exposed to

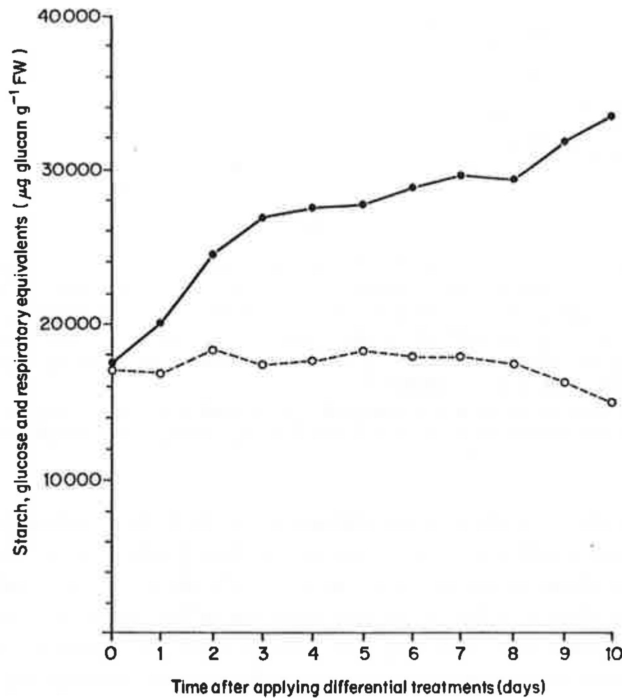


Fig. 28. Increase in "photosynthate" in leaves of *Atriplex nummularia* (old man salt bush) during the recovery from sodium-deficiency following the addition of 0·60 mol m^{-3} sodium sulphate to their cultures. "Photosynthate" was calculated indirectly as glucans from glucose and starch data added to glucan equivalents respired per day on the assumption that the respiratory quotient = 1 and that 0·75 μl O_2 (STP) = 1 μg glucan. No addition, ○----○; 0·60 mol m^{-3} sodium sulphate, ●——●. From Brownell and Jackman (1966).

$^{14}CO_2$ illuminated at approximately 1000 ft-c intensity for periods of two, five and ten minutes are shown in Fig. 29 (P. F. Brownell, unpublished work).

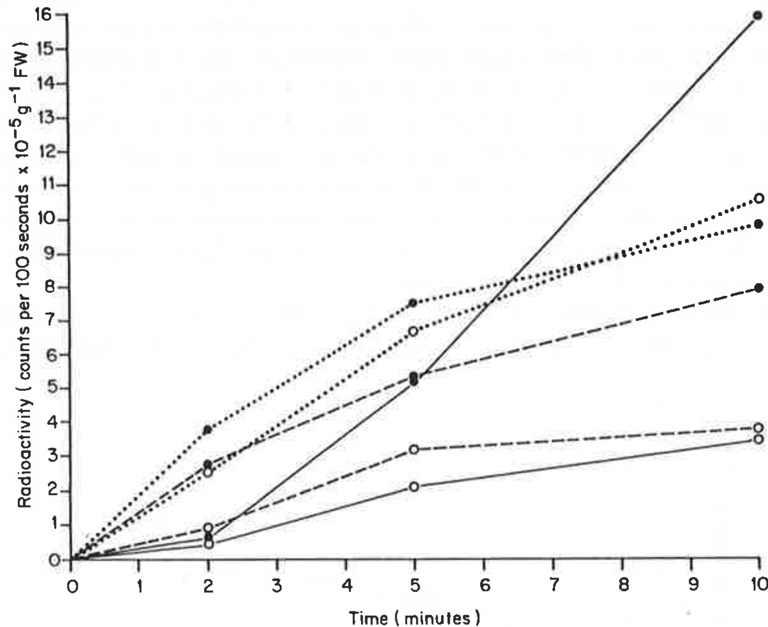


Fig. 29. The effect of sodium on the ^{14}C labelling of amino acid, ...; organic acid, ----; and sugar, ——, fractions from leaves of *Atriplex vesicaria.* One set of leaves (approximately 0·5 g fresh weight) from sodium-deficient, (○), and the other from plants which had received 0·60 mol m^{-3} sodium sulphate in their cultures four days previously, (●), were simultaneously exposed for each period (2, 5 and 10 minutes) in a 200 ml chamber to 10 μCi $^{14}CO_2$ at a light intensity of 2000 ft-c.
Extractions and separations of carbon fractions were carried out according to the method of Canvin and Beevers (1961) (P. F. Brownell and S. E. Knowles, unpublished work).

The radioactivity of organic acids and amino acids from leaves of sodium-treated plants was greater than from sodium-deficient plants after two minutes, but the labelling of amino acids after fifteen minutes was similar for both treatments. On the other hand, the labelling of the sugar fraction from sodium-deficient and normal leaves was similar after two minutes but the difference between the labelling of sodium-deficient and normal leaves became greater with time. After fifteen minutes of exposure, the radioactivity of sugars extracted from leaves of normal plants was almost four times greater than that from sodium-deficient plants. The greater amount of ^{14}C from the $^{14}CO_2$ being incorporated into the sugar fraction in normal than sodium-deficient plants indicates that they were photosynthesizing more rapidly.

6. Transport

There have been numerous reports of adenosine triphosphatase (ATPase) systems using ATP as the energy source for the active transport of ions in plants. An active mechanism for the extrusion of Na^+ in *Scenedesmus* was described by Kylin (1966) similar to the phenomenon observed in tissues and cells of animals (Skou, 1964). ATPase activity has been demonstrated in homogenates from higher plant tissues including roots of sugar beet (Hansson and Kylin, 1969), oats (Fisher and Hodges, 1969; Leonard *et al.*, 1973), oats, wheat, barley and maize (Fisher *et al.*, 1970), sugar beet cotyledons (Karlsson and Kylin, 1974) and in leaves of mangroves (Kylin and Gee, 1970).

Hodges *et al.* (1972) purified an ion-stimulated ATPase from oat roots by discontinuous sucrose density gradient centrifugation and showed it to be substrate specific and associated with the plasma membranes. The plasma membrane-associated ATPase of oat roots was activated by divalent cations $Mg^{2+} = Mn^{2+} > Zn^{2+} > Fe^{2+} > Ca^{2+}$ and further stimulated by KCl and other monovalent salts both inorganic and organic (Leonard and Hodges, 1973). Fisher *et al.* (1970) established correlations between ion fluxes and ion-stimulated ATPase activity in roots of barley, oats, wheat and maize. According to Jennings (1976) only roots of sugar beet (Hansson and Kylin, 1969; Karlsson and Kylin, 1974) and leaves of the mangrove, *Avicinnia nitida* have been shown to have ($Na^+ + K^+$)-activated ATPase with an activity specifically related to the ratio of Na^+: K^+ in the assay medium as shown in the animal system (Skou, 1964). The ATPase prepared from homogenates of sugar beet roots had highest activity with the ratio of Na^+: K^+ at 1 : 1 in homogenates at constant ionic strength (Hansson and Kylin, 1969). In homogenates from leaves of mangroves with varying ratios of Na^+ : K^+, Kylin and Gee (1970) demonstrated salt-stimulation at three peaks.

Attempts have been made to correlate activities of ATPases with physiological functions. Kylin and Gee (1970) suggest that the occurrence of ($Na^+ + K^+$) activated ATPases might be correlated with salt tolerance in certain species including *Avicennia marina* in which optimum absorption of K^+ is a function of the concentration of Na^+ in the medium. They interpret this dependence on Na^+ as a coupling between uptake of potassium and extrusion of sodium. Palladina and Bershtein (1974) postulate that both Na^+- and K^+-activated ATPases exist in sugar beet petioles. The ATPase has its highest activities at certain Na^+: K^+ ratios. They suggest that the highest dry weight yields and sugar contents occurred when the K^+: Na^+ ratio of the tissues was near these ratios.

The demonstration of the activity of ($Na^+ + K^+$) ATPase in certain plants could suggest that they may require sodium as an essential element. However, it would be necessary to have evidence that the ($Na^+ + K^+$) ATPase is functional in such plants and that it is absolutely dependent on sodium for its activity. From data presented by Palladina and Bershtein (1974), Kylin and

Gee (1970) and Leonard and Hodges (1973) it appears that K^+ in the absence of Na^+ supports high activities of the ATPases. It therefore follows that the ATPases are not absolutely dependent upon sodium for their function. Furthermore, the ATPases dependent upon ($Na^+ + K^+$) for their activity, occur in plants such as sugar beet and mangroves for which sodium does not seem to be essential.

The concentrations of monovalent ions required for maximum activation of ATPases are high, approximately 50 mol m^{-3} K^+ in oat roots (Leonard and Hodges, 1973), 100 mol m^{-3} ($Na^+ + K^+$) in mangroves (Kylin and Gee, 1970) compared to the concentrations of sodium in the tissues of C_4 plants receiving sufficient sodium for maximum growth (maximum growth was obtained in *Kochia childsii* when its leaves contained only 10·1100 mmol kg^{-1} sodium on a dry basis). It thus appears unlikely that sodium is required solely for the activation of ATPases in a C_4 species.

It is possible that sodium might be involved in some way with the transport of metabolites at intra- and extracellular levels between structures having specialized functions in plants for which sodium is essential. In blue-green algae with heterocysts, there is good evidence that the heterocyst is the site of nitrogen-fixation and the vegetative cells the site of carbon assimilation (Fay *et al.*, 1968; Stewart *et al.*, 1969). The heterocysts supply combined nitrogen to the vegetative cells which are the source of assimilated carbon compounds for the heterocysts (Wolk, 1968). Similarly, in plants with the C_4 photosynthetic pathway, there is rapid shuttling of metabolites between organelles and cytoplasm and between mesophyll and bundle-sheath cells (Osmond and Smith, 1976). Although concentration gradients are considered to be sufficient to explain observed rates of transport without invoking an active process (Hatch and Osmond, 1976), it is still possible that there may be situations where active processes are involved.

From work with leaf discs of *Amaranthus paniculatus*, an aspartate NAD-malic enzyme type of C_4 plant, Raghavendra and Das (1977) suggest a possible light-dependent active transport of amino acids into the bundle-sheath, mediated by Na^+-dependent ATPase activity. They reported alanine production by illuminated discs when infiltrated with asparate which was accelerated by additions of α-oxoglutarate and glutamate. Alanine production was also supported by oxaloacetate but was only stimulated by glutamate. The aspartate-dependent alanine production was increased in the light or by the addition of ATP and sodium ions. They interpret these results as indicating a light-dependent transport of alanine into the bundle-sheath involving an Na^+-dependent ATPase. It would be of interest to know if this effect is specific to sodium ions and if a similar phenomenon occurs in the PEP carboxykinase aspartate type of C_4 plants. The concentration of sodium needed to give maximum rates of alanine production was high (approximately 100 mol m^{-3}) compared to the concentrations in leaves of plants

receiving sufficient sodium for maximum growth. However, the actual concentration of sodium in the leaf discs may have been considerably lower than those of the incubating solutions.

In certain bacterial cells, sodium has been shown to play a role in the transport of substrates (MacLeod, 1965; Kahane *et al.*, 1975), (see Section IV. C.1.). There seems to be little information on the mechanisms involved in the transport of metabolites in these organisms.

If these organisms, blue-green algae, C_4 plants and sodium-requiring bacteria have active transport systems for metabolites, it is possible that sodium may be involved in their functions.

V. TENTATIVE SCHEMES FOR THE ROLE OF SODIUM IN C_4 AND CAM PLANTS AND IN BLUE-GREEN ALGAE

Two schemes are suggested to summarize our present ideas on the role of sodium in plants for which it is essential. The first scheme applies to C_4 and CAM plants and the second to *Anabaena cylindrica* and possibly to other members of Cyanophyta. Both schemes must be regarded as extremely speculative due to the present lack of information but they appear to be consistent with the data available. At this time, it appears unlikely that sodium has a similar role in the two groups of plants.

A. C_4 AND CAM PLANTS

As sodium is probably essential for all C_4 plants and increases growth of CAM plants (under conditions where they are largely dependent upon dark-fixation of carbon dioxide) but does not appear to be essential for C_3 plants, it seemed likely that sodium might function within the C_4 dicarboxylic acid system operating in C_4 and CAM plants but not in C_3 plants.

However, it has not been possible to obtain evidence for sodium having a direct role in the C_4 dicarboxylic acid system of either C_4 or CAM plants. Neither has sodium been found to affect the enzymes specifically associated with this system nor the operation of the overall system.

The enzymes involved in the C_4 dicarboxylic acid system include phosphoenol pyruvate carboxylase, pyruvate orthophosphate dikinase, adenylate kinase and pyrophosphatase (Hatch, 1970; Dittrich *et al.*, 1973). Sodium nutrition does not appear to affect the formation of phosphoenol pyruvate carboxylase extracted from leaves of C_4 plants (Holtum, 1975; P. F. Brownell, unpublished work). Furthermore, sodium did not increase the activity of phosphoenol pyruvate carboxylase, pyruvate orthophosphate dikinase, or adenylate kinase in low-sodium assay media (M. D. Hatch, personal communication).

The overall C_4 dicarboxylic acid system does not appear to be specifically dependent upon sodium nutrition. The pattern of ^{14}C labelling of C_4 acids in

leaves of the C_4 species, *Kochia childsii*, exposed to $^{14}CO_2$ in the light was similar in sodium-deficient and normal plants (Holtum, 1975; Webb, 1977). In the CAM species, *Bryophyllum tubiflorum*, the rates of carboxylation in the dark and decarboxylation in the light were not affected by sodium nutrition (Boag, 1976).

Despite the lack of evidence for any effects of sodium on this C_4 dicarboxylic system which supplies CO_2 to the photosynthetic carbon reduction cycle with the ultimate production of photosynthate, the sodium-deficient C_4 and short-day CAM plants have lower rates of photosynthesis than normal plants as shown in ^{14}C-labelling and growth experiments (see section IV.D). Therefore, it appears sodium is in some way essential for efficient photosynthetic assimilation of endogenous CO_2 produced by decarboxylation of malic or aspartic acids in the light. It would follow that sodium is involved in an area of metabolism (possibly not yet defined) associated with the PCR/PCO cycles and not with the C_4 dicarboxylic acid system common to C_4 and CAM plants.

The following scheme proposed by Boag (1976) (Fig. 30) summarizes our present ideas on the role of sodium in C_4 and CAM plants primarily based on work with *Bryophyllum tubiflorum*. The assumption is made that sodium has the same role in the nutrition of C_4 and CAM plants since they have similar primary carboxylation and decarboxylation systems. There are differences in the origin of the phosphoenol pyruvate and in the separation of the carboxylation and decarboxylation processes being spatial in C_4 plants and temporal in CAM plants. Plants with C_4 photosynthesis appear to have only one photosynthetic option whereas CAM plants are able to utilize a dark CO_2-fixation system or direct CO_2 assimilation by the PCR cycle in the light in varying proportions depending upon the conditions of growth.

In Fig. 30, the effects of sodium nutrition are compared in *Bryophyllum tubiflorum* under short- and long-day regimes. The processes occurring in the dark, the primary carboxylation of phosphoenol pyruvate to form oxaloacetate and its reduction to malate are shown in the stippled portion of the diagrams. The processes occurring in the light, the decarboxylation of malate and the reduction of the CO_2 to photosynthate by the PCR cycle are shown in the unstippled areas. The CO_2 enters the PCR cycle (represented as a cube with two halves). Two sources of CO_2 are shown; one from the decarboxylation of malate generated in the dark-fixation process (supplying the left-hand half of the cube) and the other exogenous atmospheric CO_2 (supplying the right-hand half of the cube) where it is assimilated directly by RuDP carboxylase via the C_3-like option.

It is postulated, that under conditions of sodium-deficiency (Fig. 30a and b, lower diagrams) there is a loss of carbon derived from the C_4 dicarboxylic CO_2 fixation system in the PCR/PCO systems under both short- and long-day conditions. Evidence for this loss is that compared to normal plants sodium-deficient plants produce less photosynthate despite the apparently similar

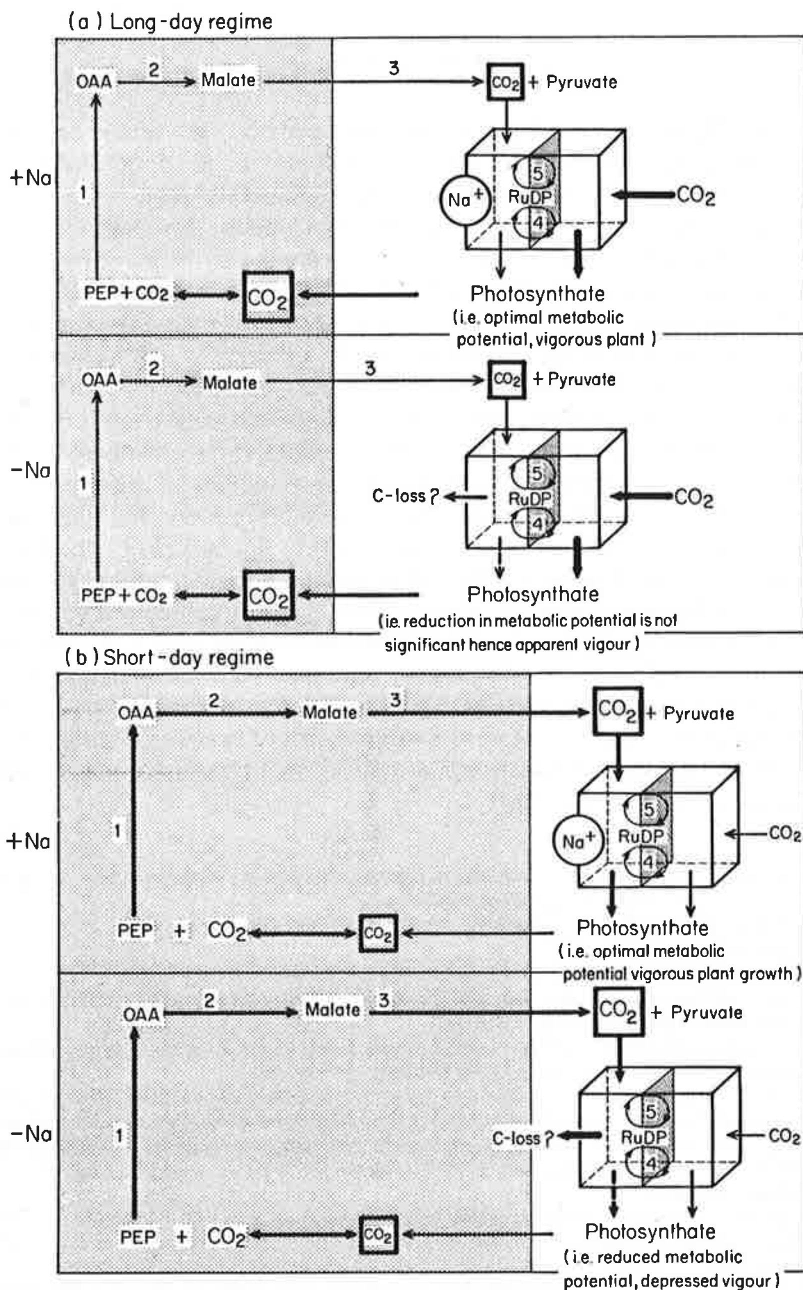


Fig. 30. Proposed scheme to explain the responses to sodium by plants of *B. tubiflorum* maintained under different growth regimes. This scheme indicates the possible pathways of carbon assimilation by +Na (i.e. plants supplied with 0·1 mmol NaCl l^{-1}) and —Na (i.e. no addition) plants grown under;

(a) long-days (16 h; 34°C)/short-warm-night (8 h, 24°C) regime.

(b) short-days (8 h; 33°C)/long-cool-night (16 h; 13°C) regime.

Thickness of line is a quantitative indication of significance of alternate pathways, CO_2 produced endogenously is shown enclosed by a box and exogenous or atmospheric CO_2 is not enclosed by a box.

1. PEP-carboxylase; 2. MDH; 3. Malic Enzyme; 4. RuDP-carboxylase; 5. RuDP-oxygenase. From Boag (1976).

supply of CO_2 from dark CO_2 fixation. This carbon is represented as being lost or "escaping" (through a "hole" in this part of the cube). Sodium is indicated preventing loss of carbon (as a "plug" filling this hole).

Depending upon the growth regime of CAM plants, this loss of carbon would be expected to have a variable effect upon overall plant growth. If plants are grown under long-day conditions (Fig. 30a), a large proportion of the CO_2 assimilated by the plant will be derived directly from the exogenous atmospheric source to be assimilated by RuDP carboxylase: only a small part will be supplied by the decarboxylation of malate, produced in the dark-CO_2 fixation process. Under these conditions the decreased conversion of endogenous CO_2 to phosphate will be relatively unimportant compared to the overall rate of photosynthesis and the effect of sodium nutrition on the growth of the plants will not be readily detected. However, the situation in CAM plants under a short-day regime is very different (Fig. 30b). Little atmospheric carbon dioxide will be fixed directly by RuDP carboxylase and the plant will be thus largely dependent upon the photosynthetic assimilation of CO_2 produced by the decarboxylation of malate from dark CO_2-fixation. Under these conditions, this decreased conversion of endogenous CO_2 to photosynthate in sodium-deficient plants will be manifested by reduced growth and in severe cases, chlorosis and necrosis of the leaves. On the other hand, plants receiving sodium will not suffer this loss of carbon and will make normal, vigorous growth.

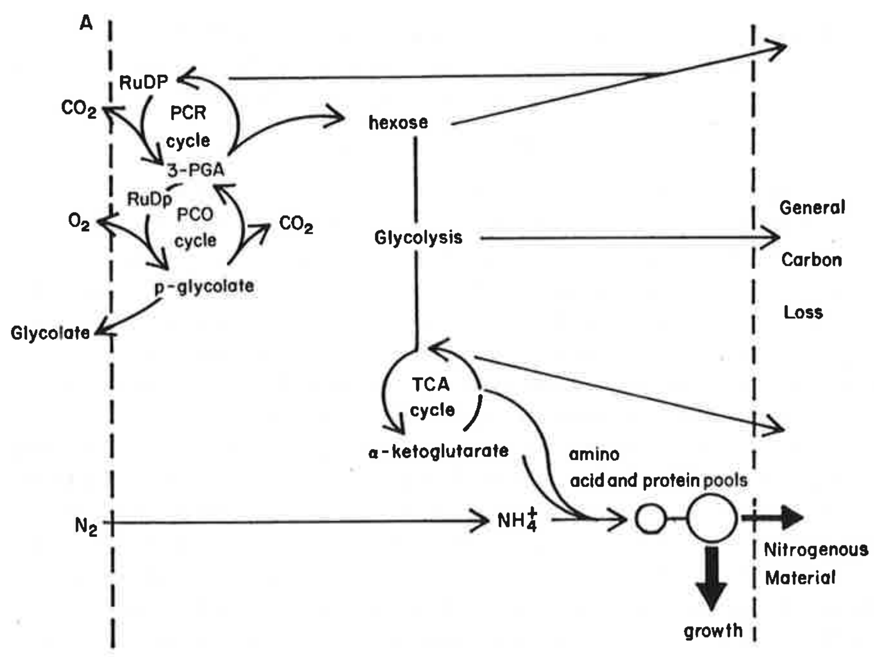
Fig. 31. Proposed scheme to explain the role of sodium in the blue-green alga, *Anabaena cylindrica* grown in nitrate-containing cultures.

The following suggestions are made:

A. Sodium-deficient cells:
1. The permeability of the membrane increases but the cell remains intact.
2. Nitrate diffuses rapidly into the cells inducing nitrate reductase activity which leads to the more rapid reduction of nitrate.
3. The subsequent steps of nitrate assimilation leading to the formation of protein are accelerated (indicated by the relative thickness of lines).
4. When ^{15}N-labelled nitrite, nitrate or ammonia is supplied to sodium-deficient cells, the protein becomes more rapidly labelled than in normal cells. However, the increase in the amount of protein is less rapid in deficient than in normal cells suggesting the loss of nitrogenous compounds to the medium. Therefore, growth is retarded in deficient cultures.
5. A greater proportion of the carbon assimilated is also lost into the medium in deficient cultures in compounds including glycolate and certain nitrogenous compounds.

B. Normal cells:
1. The membrane integrity is maintained permitting the uptake of nitrate at the usual lower rate.
2. Nitrate assimilation proceeds at a normal rate.
3. Larger pools of protein accumulate with only a low rate of loss of nitrogenous compounds, thus more material is available for growth.
4. Loss of carbon through normal release processes is moderate.
5. The rate of nitrogen-fixation on a protein basis is similar for both sodium-deficient and normal cells.

From Smith (1977).



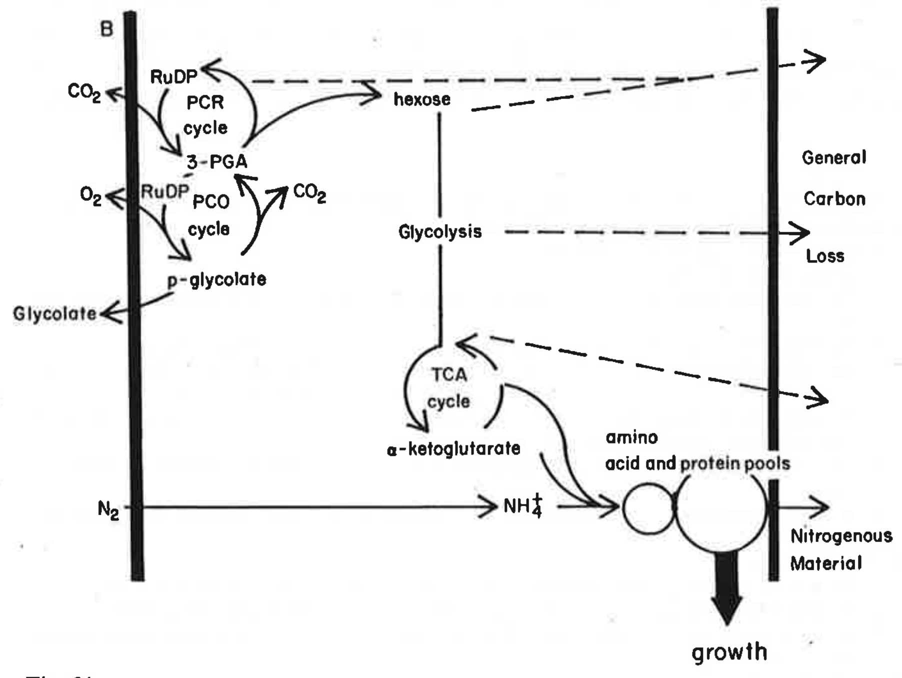


Fig. 31

The way in which carbon could be lost from the PCR/PCO system is not known. However, it is possible that it is lost as CO_2; perhaps sodium nutrition in some way affects the mode of action of RuDP carboxylase/oxygenase.

B. *Anabaena cylindrica*

The following tentative schemes (Figs 31 and 32) proposed by Smith (1977) summarize our present view of the involvement of sodium nutrition in the carbon and nitrogen metabolism of *Anabaena cylindrica* and possibly other members of Cyanophyta for which sodium is an essential element. Two schemes are presented, one for algae supplied with nitrate (Fig. 31) and the other for algae not supplied with combined nitrogen and thus dependent upon nitrogen-fixation for their source of nitrogen (Fig. 32). (In each figure, A represents sodium-deficient cells and B normal cells.)

Sodium-deficient cells incorporate nitrogen from nitrate more rapidly than normal cells (indicated by the relative thickness of arrows in Fig. 31A and B). The evidence for this is the increased rates of ^{15}N enrichment of protein of cells fed $K^{15}NO_3$ in their cultures, the enhanced specific activity of nitrate reductase (Brownell and Nicholas, 1967) and the greater rate of nitrate uptake in sodium-deficient compared to normal cultures (Smith, 1977). Smith (1977) also observed a more rapid release of nitrogenous compounds by sodium-deficient algae (Fig. 17). This was 33% of the total cellular nitrogen in sodium-deficient cultures compared to only 6% for normal cultures supplied with nitrate. While the same trend occurred in cultures not supplied with combined nitrogen (Figs 18, 32), the overall release of combined nitrogen was less, being only 17% of the total cellular nitrogen in sodium-

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Fig. 32. Proposed scheme to explain the role of sodium in the blue-green alga, *Anabaena cylindrica* grown without combined nitrogen.

The following suggestions are made:

A. Sodium-deficient cells:
1. Nitrate assimilation does not occur, therefore sodium nutrition is not involved in this system.
2. Assimilation of nitrogen occurs exclusively via nitrogen fixation at overall rates less than for cells receiving nitrate. There is a smaller pool of protein and release of extracellular nitrogen compounds compared to those in nitrate-grown cultures.
3. Nitrogenous compounds needed for growth are lost more rapidly into the medium and growth is therefore reduced.
4. Glycolate and other carbon compounds are released in higher amounts by deficient cells.
5. It is suggested that the membranes are affected in a similar manner as in nitrate-grown cells.

B. Normal cells:
1. Similar to nitrate-grown cultures but the nitrate assimilation system is not operative.
2. Nitrogen fixation on a protein basis is similar in sodium-deficient and normal cells.
3. Release of nitrogen and carbon-containing compounds is less by normal than sodium deficient cells.

From Smith (1977).

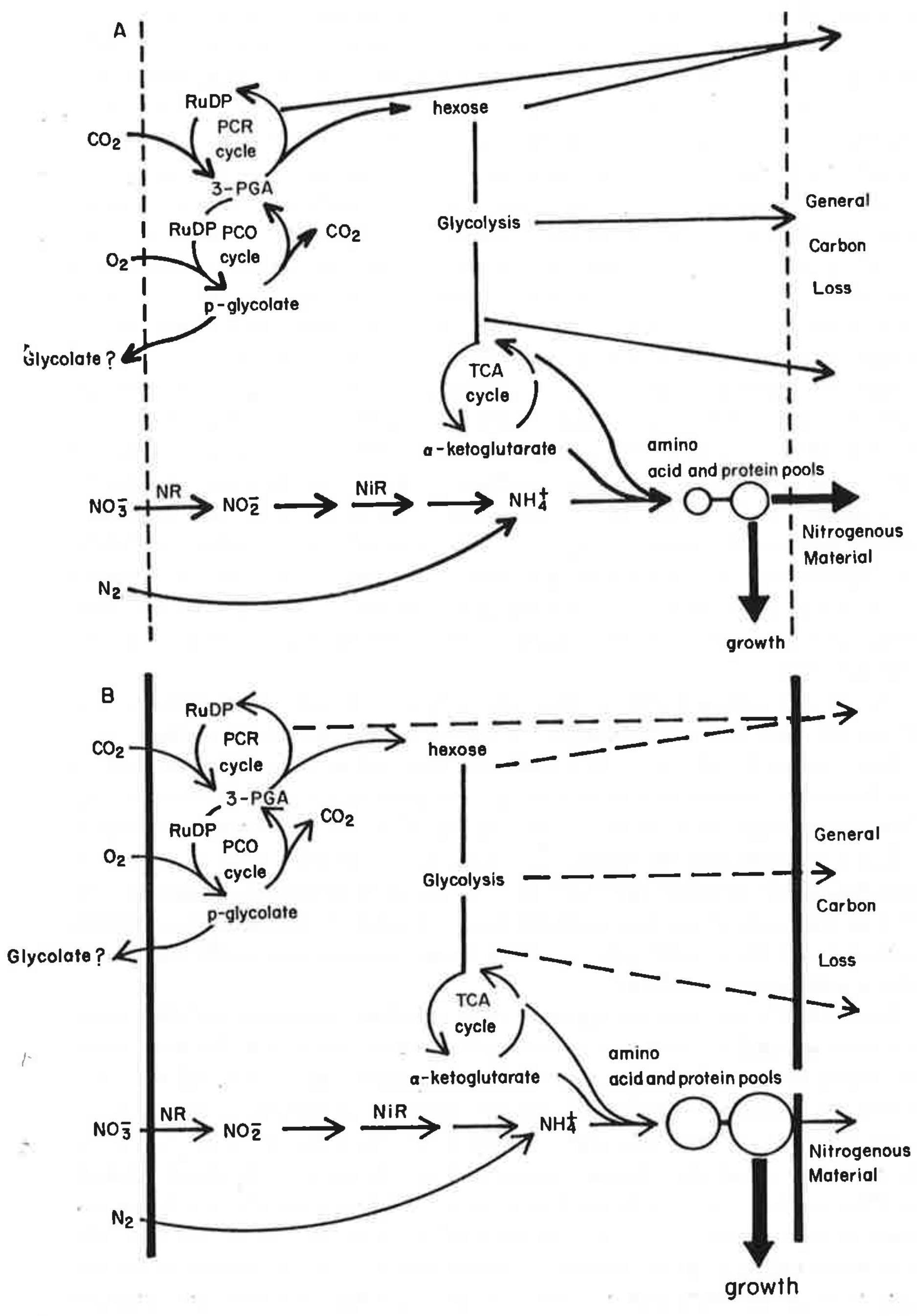

Fig. 32

deficient cultures compared to 7% in normal cultures. The production of extracellular nitrogenous compounds by blue-green algae has been recorded by Fogg (1952) who found increased production of mainly polypeptide and lesser amounts of amide in cultures deficient in iron and molybdenum. The amounts also increased with age of culture. Jones and Stewart (1969) also found greater losses of extracellular nitrogen in *Calothrix scopulorum* under suboptimal conditions. They suggested that the peptides and traces of amino acids found may have possibly originated from the mucilaginous sheath surrounding the alga. The increased release of nitrogenous compounds may be a general effect of suboptimal growth conditions and not a specific effect of sodium deficiency. However, the greater rate of uptake and reduction of nitrate appears likely to be a specific effect of sodium deficiency.

Sodium-deficient cultures of *A. cylindrica* released increased organic carbon, expressed as a percentage of the total carbon assimilated (Ward and Wetzel, 1975; Smith, 1977). Sodium-deficient cells in cultures not supplied with combined nitrogen released significantly more glycolate into the media than normal cells (Smith, 1977). Glycolate is one of the more common substances excreted by algae (Fogg, 1952; Cheng *et al*., 1972; Hellebust, 1965). The production and release of glycolate is of interest in that its possible source could be the PCO cycle (Cheng and Colman, 1974; Ingle and Colman, 1976) which would indicate a higher rate of photorespiration in sodium-deficient cells.

The loss of carbon would be expected to be closely associated with the loss of nitrogen as much of the nitrogen compounds would contain carbon.

Brownell and Nicholas (1967) suggested that sodium may exert its control specifically on nitrate reductase through the production of a protein factor. They based their suggestion on the increased levels of nitrite in sodium-deficient cultures and the failure of sodium to control the activity of nitrate reductase when supplied simultaneously with chloramphenico, an inhibitor of protein synthesis. However, evidence from ^{15}N and ^{14}C labelling experiments indicated that the overall activity of the nitrate assimilation system increased with a deficiency of sodium.

Smith (1977) also observed greater rates of nitrate reduction but they were not accompanied by the marked increases in nitrite levels in sodium-deficient compared to normal cultures that had been found by Brownell and Nicholas (1967). To explain the increased rates of nitrate assimilation, it was initially suggested that sodium deficiency may have the multiple effect of increasing the activities of all the enzymes concerned in this process. However, Smith (1977) considered it more likely that sodium could be needed to maintain the integrity of membranes. In a deficiency of sodium, the membranes would offer little resistance to the uptake of nitrate which would be available for the induction of the inducible enzyme, nitrate reductase. Ohmori and Hattori (1970) found that nitrate reductase is induced in *Anabaena cylindrica* by its

substrate, nitrate, and its activity depends upon the rate of entry of nitrate into the cells. This report is consistent with the model proposed by Butz and Jackson (1977) for the transport and reduction of nitrate by a membrane-located nitrate reductase enzyme. With the increased availability of nitrate at the sites of nitrate reductase induction, both the nitrate reductase activity and the rate of nitrate reduction would increase in sodium-deficient cells. The rate of the subsequent steps of nitrate assimilation would also be expected to increase in response to the higher substrate levels in the sodium-deficient compared to normal cells.

The leaky membranes in sodium-deficient cells could also explain the observed losses of nitrogenous compounds including polypeptides, amides and amino acids and the carbon compounds including glycolate. Under these conditions, compounds of nitrogen and carbon, which in normal cells would contribute to growth would be lost to the surrounding medium.

In cells grown without nitrate, there could also be losses of carbon- and nitrogen-containing compounds due to the defective membranes (Fig. 32).

Loss of compounds containing carbon and nitrogen would reduce the amount of metabolites available for growth and this would account for the poorer growth in sodium-deficient cultures. Losses of these compounds from cells receiving sodium would be minimal and normal growth would result.

With the data available, it is not possible to ascertain at what stage of assimilation the losses occur. Assimilated carbon could be released from the PCR/PCO system as glycolate or it may be released with nitrogen in amino acids, amides, peptides or proteins. Carbon and nitrogen could be released at steps prior to the formation of proteins or they may be derived from the breakdown of protein.

This scheme for the involvement of sodium in the nutrition of blue-green algae requires firm evidence for sodium being needed for the integrity of the cell membranes. The evidence for sodium having this role is based only on physiological data; there is no histological evidence for this possible effect of sodium on membrane integrity. No obvious differences between membranes of sodium-deficient and normal cells have been revealed by light microscopy. If sodium is involved in the maintenance of membrane structure, this may become apparent following studies of ultrastructure by electron microscopy.

VI. SUMMARY AND CONCLUSIONS

1. This article concentrates on sodium as an essential micronutrient element for certain higher plants and microorganisms.
2. A clear distinction is drawn between the roles of sodium as an essential micronutrient element for some species and as a beneficial element (at much higher concentrations) for some others.
3. Results of many experiments to determine the effect of sodium on

growth have been difficult to interpret due to the lack of control treatments, the use of complicated media and to the omission of micronutrients in the culture media.

4. Sodium, at high levels above 10 mol m^{-3} (230 ppm) has stimulated growth in certain species (mainly members of Chenopodiaceae and other species including *Aster tripolium* and some mangroves) even when other essential elements were supplied at optimum concentrations.
5. Other species responded to high levels of sodium by increased growth only in a deficiency of a nutrient element. Wheat, barley, oats and cotton have been shown to respond to sodium in a deficiency of potassium and cotton in a deficiency of calcium.
6. Some species do not respond to high levels of sodium even when other nutrient elements including potassium are limiting.
7. Sodium, at high levels, has been shown to affect some plants qualitatively. Increases in the concentration of sugar have been recorded in sugar beet and the quality of fibre crops improved by application of salts of sodium.
8. Following the suggestion that sodium may be required in very small amounts as an essential element, experiments were conducted in which sodium was carefully removed from the environment of the plants. Critical techniques for the determination of sodium in low concentrations, sometimes in the presence of high concentrations of other substances were needed to assess the relative sources of sodium to the plant. Initially, emission flame photometry was used but this method was largely replaced by the atomic absorption spectroscopy method in which interferences were minimal.
9. Potential sources of sodium to the cultures were the culture solution salts, the water, the culture apparatus, the seeds, air for aeration of cultures and the air surrounding the cultures. Methods are described which greatly reduce the magnitude of these sources.
10. *Atriplex vesicaria* was the first higher plant for which sodium was shown to be essential as a micronutrient. Following this discovery, it was suggested that sodium might be required by other species which are halophytes or which accumulate sodium at high concentrations in their tissues. However, it must be stressed that there is no clear correlation between the possession of halophytic or sodium-accumulating properties and the requirement of sodium as a micronutrient.
11. Sodium has been shown to be essential for some blue-green algae including *Anabaena cylindrica*, and for C_4 species in the families, Gramineae, Cyperaceae, Amaranthaceae, Chenopodiaceae and Portulacaceae. In two genera of Chenopodiaceae, *Atriplex* and *Kochia*, which contain both C_3 and C_4 species, sodium was shown to be essential only for the C_4 species. The C_3 species apparently had no requirement for sodium.

12. The minimum concentrations of sodium required for maximum growth of *Atriplex vesicaria* were about 0·05 mol m^{-3} (1·25 ppm) and for *Anabaena cylindrica* 0·22 mol m^{-3} (5 ppm). No other monovalent cation would support growth in the absence of sodium.
13. The Crassulacean acid metabolism species, *Bryophyllum tubiflorum* responded to low concentrations of sodium when grown under conditions of short-days with large diurnal temperature variation. Under these conditions, the bulk of the CO_2 assimilated was taken up in the dark involving the C_4 dicarboxylic system.
14. The signs of sodium deficiency are similar in all species for which it is essential. Leaves become chlorotic and in severe cases the margins and tips become necrotic. Full recovery can be obtained from chlorosis by the addition of a sodium salt at about 0·1 mol m^{-3} (2·3 ppm) to the culture solution. If 5 μg Na in a solution of NaCl or Na_2SO_4 is applied to a single leaf of a sodium-deficient plant of *Kochia childsii*, it will green-up and expand rapidly whereas even after two weeks the remainder of the plant retains its signs of sodium deficiency. When ^{22}NaCl in the same quantity is applied to leaves a large proportion of the radioactivity (63% to 85%) is retained by the leaf after one week.
15. Sodium has been reported to be essential (generally at concentrations of 50 mol m^{-3} and higher) for some marine bacteria and fungi and non-halophilic bacteria.
16. Little is known of the function of sodium in organisms for which it is essential. Sodium-deficient compared to normal *Anabaena cylindrica* produces less dry weight and protein and has lower nitrogen-fixation rates.
17. In nitrate-containing cultures, sodium-deficient compared to normal *Anabaena cylindrica* takes up nitrate nitrogen and incorporates it into protein more rapidly both on a culture and protein basis. Nitrate reductase extracted from sodium-deficient cells has a specific activity many times greater than from normal cells. No similar effect on nitrate reduction was detected in C_4 plants.
18. Sodium-deficient cells, with or without combined nitrogen release nitrogenous compounds more rapidly and a greater proportion of assimilated carbon including glycolate than normal cells.
19. An early response by sodium-deficient leaves of C_4 *Atriplex* to sodium was an increase in the rate of respiration as oxygen uptake or CO_2 output. The concentration of sodium to elicit this response was of the same order as that required for growth responses. Sodium was specifically required; no response was obtained to other monovalent cations. This does not appear to be a general response for C_4 plants as no clear respiratory response to sodium was obtained in *Kochia childsii*. Chlorophyll content increased rapidly following the supply of sodium.

20. It seemed likely that sodium was required for the functioning of the C_4 dicarboxylic CO_2-fixing system operating in C_4 and CAM species (under certain conditions) but was not required in C_3 species which lack this system. However, there is little evidence to support this. Neither the whole system nor its key enzymes appear to depend on sodium for their operation.
21. A tentative scheme is suggested to explain the role of sodium in C_4 and CAM plants. A deficiency of sodium apparently does not affect the rate of CO_2 supply via the C_4 dicarboxylic system for reduction in the PCR/PCO system yet the overall photosynthetic rates are reduced. This suggests that carbon, in some form, is being lost from the system. It is postulated that sodium prevents this loss in a normal plant. One possibility is that sodium may, in some way, control the ribulose diphosphate carboxylase/oxygenase option, allosterically.
22. The role of sodium in the nutrition of *Anabaena cylindrica* and possibly in other blue-green algae is shown in a separate scheme. Sodium is postulated as being needed to maintain membrane integrity. In a deficiency of sodium, the membranes of cells growing in nitrate permit the rapid entry of nitrate resulting in the induction of high nitrate reductase activity. The subsequent steps of nitrate assimilation are accelerated due to high concentrations of substrates. It is suggested that increased losses of compounds containing carbon and nitrogen may be the result of increased permeability of membranes in a deficiency of sodium. There is no electron micrograph evidence for an effect of sodium on the ultrastructure of membranes, at present.
23. It has been suggested that sodium may act as a monovalent cation activator for certain enzymes in plants for which it is essential. This does not seem to be a likely function for sodium as no plant enzymes are known that are specifically activated by sodium. Furthermore, enzymes which are activated by monovalent cations require very high concentrations of the ions for maximum activity. These concentrations are generally higher than the concentrations of sodium in tissues of plants receiving enough sodium for maximal growth.
24. The proposal that sodium is required for the activation of ATPase systems is difficult to support. ATPases stimulated by Na^+ and K^+ have been found in some species but these are not species for which sodium has been shown to be essential. The ($Na^+ + K^+$) ATPases appear to be at least partially activated by K^+, alone. The levels of Na^+ and K^+ required for maximal activity are high compared to the tissue concentrations of sodium necessary for maximal growth.

    It is possible that sodium has a role in the transport of metabolites in plants for which it is essential but no mechanisms have been suggested for this.

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3:15 82

ZINC UPTAKE BY SUGARCANE CELLS IN SUSPENSION CULTURE AND BY EXCISED ROOTS OF TWO CULTIVARS

John E. Bowen, Beaumont Agric Research Center, Univ of Hawaii, Hilo, Hawaii 96720

Zn uptake by suspension-cultured sugarcane cells (cv H57-5174) is reduced by 90 % at 4 C and by 80 % by 10 uM CCCP, 10 uM DNP, and other metabolic inhibitors, as compared to that at 25 C in the absence of inhibitors. Thus, this component of Zn uptake satisfies criteria for energy-dependent transport. A double reciprocal plot manifests 4 phases separated by sharp transitions and is compatible with the concept of multi-phasic uptake mechanisms.

Excised sugarcane roots (cv H57-5174) actively absorb Zn at a rate twice that of cv H53-263 roots when the external Zn concentration is below 0.1 mM. The H57-5174 Zn transport system is saturated at 0.1 mM, though, whereas that of H53-263 remains unsaturated even at 0.3 mM, the highest concentration studied. A Lineweaver-Burk plot shows 3 distinct phases for each cultivar but the two cultivars differ markedly with regard to transition points and kinetic constants for each phase. Also, there was no similarity between transitions and kinetic constants in H57-5174 cells and roots.

3:30 83

Fe-DEFICIENCY STRESS INDUCED DEVELOPMENT OF TRANSFER CELLS IN THE EPIDERMIS OF RED PEPPER ROOTS.

E.-Ch. Landsberg, Institute of Plant Nutrition, Techn.Universi-ty of Berlin, Lentzeallee 55-57, D 1ooo Berlin 33, FRG.

Fe-efficient red pepper (capsicum annuum cv. "Izmir Carliston") responds to Fe-deficiency stress with an accumulation of citric and malic acid in the root tissue, a concomitant proton ex-trusion and an increasing production of reducing substances. These biochemical reactions are closely correlated with a striking morphogenetic change in the roots, that leads to a typical swelling of the cortex close to the root tip and an increasing number of root hairs in this zone. The swelling is caused by isodiametric cell enlargement instead of elongation. A study of the root ultrastructure by transmission electron-microscopy revealed the occurrence of many epidermal transfer cells, which are characterized by an intensive wall labyrinth system on the peripheral cell walls and a dense cytoplasm with numerous mitochondria and cisternae of rough endoplasmic reti-culum. Even young developing root hairs showed similar cytolo-gical features. Epidermal transfer cells were also found in roots of other Fe-efficient dicots like sunflower and tomatoes. A possible relationship between the formation of transfer cells and the induction of biochemical mechanisms of Fe-deficiency stress response will be discussed.

3:45 84

THE EFFECT OF POTASSIUM ON THE TURGOR OF YOUNG LEAVES OF PHASEOLUS VULGARIS

Konrad Mengel & Wolfgang Arneke, Institute of Plant Nutrition, Justus Liebig-University Giessen

Plants were grown in solution culture with two K treatments, one with sufficient K (control) and one insufficient. Leaf turgor was calculated according to the equation: $\Psi_p = \Psi - \Psi_s$. Ψ was measured by means of the Scholander bomb, Ψ_s cryoscopically. Insufficient K supply resulted in a lower turgor in the young leaves (5 bar) as compared with the control leaves (7 bar). The lower turgor was associated with a reduced growth rate, smaller mesophyll cells and an in-creased dry matter content. Besides soluble amino compounds K was the most important osmoticum. It is suggested that K is indispensable in establishing an optimum turgor in young plant tissue.

Monday Afternoon, August 4 Session 6

4:00 85

THE POSSIBLE ROLE OF SODIUM AS A MICRONUTRIENT IN C_4 PLANTS.

Peter F. Brownell, Ross O. Nable and Mark Johnston. Dept. of Botany, James Cook University of North Queensland, Queensland 4811, Australia.

In the C_4 species, Atriplex spongiosa, Amaranthus tricolor and Kochia childsii, the increase in dry-weight yield was greater in sodium-deficient than non-deficient plants when the ambient CO_2 concentration was increased from 330 to 1500 ppm. A C_3 plant, Atriplex hastata resembled the sodium-deficient C_4 plants in its response to CO_2 concentrations and did not respond to sodium. In sodium-deficient C_4 plants, the oper-ation of the Calvin cycle may be limited by a reduced supply of CO_2 via the C_4 appendage. This limitation is largely over-come by an increase in the external CO_2 concentration.

The concentration of alanine in leaves of sodium-deficient plants decreased rapidly upon the addition of sodium at similar concentrations (approximately 0.04 mM) as required for growth responses. Upon illumination, alanine concentrations in leaves of previously dark-treated plants decreased rapidly and were restored when returned to dark conditions. These responses were not detected in tomatoes, a C_3 plant: a scheme is present-ed to explain the possible relationships between sodium nutrit-ion, light treatment and alanine concentration and to suggest the possible role of sodium in C_4 plants.

4:15 86

PROBLEMS AND THEIR CONSEQUENCES IN ATOMIC ABSORPTION DETERMINATION OF COPPER IN FRACTION I PROTEIN

Larry S. Daley, Carlton Wendel, John Talent, & Lee J. Theriot. Dept. of Chem., North Texas State Univ, Denton Tx,76203.

Atomic absorption analysis (AA) subestimates the amount of copper present in a commercial preparation of Fraction I. This effect was found due to masking matrix effects, that are not fully compensated for by standard addition techniques, but require in addition partial degradation of the enzyme. ESR spectra indicate that at least part of the copper is in the form of spin coupled copper, a form more susceptable to volatilization. Fraction I is the protein of the enzyme ribulose-bis-phosphate carboxylase [RuBPCase (E.C.4.1.1.39)]. Previous published information suggests that under certain circumstances copper may effect RuBPCase catalytic activity. However, how much copper is bound to RuBPCase remained controversial. Copper continues bound to Fraction I after gel filtration. When gel filtered Fraction I was disassociated into subunits by treatment with 2% NaOH several things occurred: a) some copper was released from the protein, b) the AA response for copper bound to Fraction I subunits increased considerable over the AA response for copper of undissociated Fraction I, c) the total copper found in the NaOH degraded products of Fraction I was more than one mole of copper per mole.

Abstract Only 87

FURTHER PURIFICATION AND PROPERTIES OF ADENYLYL SULFATE (APS): AMMONIA ADENYLYL TRANSFERASE (APSAT) FROM CHLORELLA.

Heinz Fankhauser and Jerome A. Schiff, Photobiology Institute, Brandeis University, Waltham, MA 02254

APSAT, which catalyzes the formation of adenosine 5' phosphor-amidate (APA) from ammonia and APS (Plant Physiol. 63,162(1979)) has now been purified 1800 fold to homogeneity using ammonium sulfate precipitation, and DEAE, Sephadex and Reactive Blue 2-Agarose chromatography. The purified enzyme shows one band (about 60-65 KD) on polyacrylamide electrophoresis (PE) and 3 subunits on SDS PE of 26, 21, and 17 KD consistent with an en-zyme MW of 64 KD. Isoelectrofocusing yields one band of pI 4.2. The pH optimum of the APSAT-catalyzed reaction is 8.8, the Km for APS is 0.82 mM and the Km for ammonia (assuming NH_3 is the active species) is 10 mM. A large variety of ammonia analogs such as amines, amides, etc. will not replace ammonia. One mole of APS reacts with one mole of ammonia to give one mole each of APA and sulfate; no AMP is found. If APS and ammonia are boiled in the absence of enzyme, both APA and AMP are formed indicating that the enzyme may exclude water from the active site. A biological function for APA, which has a high free-energy of hydrolysis, is being sought.

NSF (PCM 76-21486) support.

Brownell, P.F., Nable, R.C. and Johnston, M (1980)
Plant Ph...

Publication 11

16:00 **818**

PROPERTIES OF SUGARBEET RIBULOSE -1.5- BISPHOSPHATE CARBOXYLASE AS AFFECTED BY SALINITY

Bruria Heuer & Zvi Plaut, Institute of Soils & Water, ARO, The Volcani Center, Bet Dagan 50-200, Israel.

The effect of salinity on activity, properties and structure of sugar beet Ribulose -1.5- bisphosphate carboxylase were investigated. Plants were exposed to NaCl in half strength Hoagland solution for 7 days and their leaves were then used for enzyme isolation and purification. An enhancement in ribulose -1.5- bisphosphate carboxylase activity was found both in crude extracts and in purified preparations following plant exposure to 180 mM NaCl. Kinetic properties of the enzyme were remarkably affected by salinity as determined by a 4.5-fold increase in Km[HCO_3^-] and Km[CO_2], while Vmax was increased by 50 percent only. Data based on polyacrylamide-gel-electrophoresis and a column chromatography suggest that molecular weight of the small subunit of RuBP carboxylase was reduced from 15,500 to 12,500 in plants grown under salinity. The large subunit was much less affected by salinity, and no change was found in the whole enzyme. Similarly, a remarkable change in amino acid composition and the content of titratable SH groups was found for the small subunit. These results indicate that mild salt concentrations induced conformational changes in sugarbeet's RuBP carboxylase structure leading to its semi-salinity tolerance.

16:15 **819**

AUTOTROPHIC NATURE OF CASTOR BEAN ENDOSPERM. J.H.H. Wong & C.R. Benedict, Dept. Plant Sciences, Texas A&M Univ., College Station, TX 77843

We have reported significant levels of RuBPCase along with many of the enzymes of the Calvin cycle in non-photosynthetic endosperms of germinating castor beans. It is possible that glucose-3,4-^{14}C resulting from dark $^{14}CO_2$ fixation can be accounted for by the conversion of CO_2 to sugars by the Calvin cycle functioning with a PGA-triose P shuttle to by-pass the missing NADP-linked TPD step. The level of many of the Calvin cycle enzymes in the endosperm equals the level in green leaves. The mol wt of the endosperm RuBPCase, the large and small subunit are: 560,000, 55,000, and 15,000 daltons. The Km values for RuBP, HCO_3^- (CO_2) and MG^{++} are: 5.4×10^{-4}M, 1.36×10^{-2}M (9.2×10^{-4}M), and 5.67×10^{-4}M which are similar to RuBPCase from green leaves. Dark $^{14}CO_2$ fixation predominantly labels mal and cit without significantly labeling PGA. In $H^{14}CO_3^-$-$H^{12}CO_3^-$ pulse-chase experiments, 87% of the label in cit and mal are transferred to sugars showing dark incorporation of $^{14}CO_2$ into sugars does not occur through the Calvin cycle but through C_4 acids and gluconeogenesis. The nuclear and proplastid genes coding for RuBPCase and other autotrophic enzymes are transcribed and translated in the dark but the Calvin cycle is not operable because of a deficiency of NADP-linked TPD.

16:30 **820**

EFFECTS OF CO_2 AND O_2 LEVELS ON THE REGULATION OF PHOTOSYNTHETIC CARBON METABOLISM BY AMMONIA IN SPINACH MESOPHYLL CELLS.

Arthur L. Lawyer, Karen L. Cornwell, Peder O. Larsen, and James A. Bassham, Lab. of Chemical Biodynamics, Lawrence Berkeley Lab., Univ. of Calif., Berkeley, CA 94720

Photosynthetic metabolism of isolated spinach mesophyll cells was characterized under conditions favoring photorespir-

16:45 **821**

PHOTOSYNTHETIC UNIT DENSITY EFFECTS ON PHOTOSYN

Norman Terry, Plant & Soil Biology Dept., Univ.
Berkeley, CA 94720

The influence of photosynthetic unit (PSU) d
synthesis in 20 light/CO_2 environments was stud
stress as a means of varying PSU density. Net
CO_2 uptake (P) was measured by leaf gas exchang
plants (Beta vulgaris L.) cultured in a standar
Net photosynthesis was positively correlated wi
In environments similar to field conditions (30
CO_2 per liter air and irradiances approximating
P per area increased by 30 percent with increas
sity over the normal range (from 68×10^{12} to 1
per square centimeter). At high irradiances PS
fluenced P per area most by increasing the capa
to photochemically utilize absorbed light energ
of PSU density on absorptance was a relatively
terminant of photosynthetic rate at high irradi
more significant at low irradiance. It is conc
sugar beets photosynthesizing at high irradianc
mal or high ambient CO_2 supply, PSU density is
colimiting factor influencing photosynthetic ra

Abstract Only **822**

CHARACTERIZATION OF C_4 PHOTOSYNTHESIS IN SODIUM
PLANTS

Stuart Boag, Barry Osmond and Peter Brownell, De
mental Biology, RSBS, ANU, PO Box 475, Canberra
Bot.Dept., James Cook Uni of N.Qsld. 4811, Aust

Gas exchange, ^{14}C-labelling kinetics, enzyme
and particular metabolite pool sizes were asses
range of C_4 sodium-deficient plants. Qualita
sodium-deficient plants retain all of the funda
characteristics of C_4 photosynthetic carbon met
However, light-response and CO_2 response curves
plants show earlier saturation in comparison to
Increased labelling of alanine or glycine and
$^{14}CO_2$ fixation in deficient plants were consist
larger free amino acid pool sizes and proportic
aminotransferase activities. These results w
cussed in relation to the nature of steady-stat
synthesis in sodium-deficient plants with spec
on the possible role of sodium in C_4 photosynt
bolism.

PLANT PHYSIOLOGY SUPPLEMEN
146 (1981).

Abstract Only **823**

PHOTOSYNTHETIC ACTIVITY IN ISOLATED CE
IPOMOEA BATATAS L.

CHIN HO LIN & CHIN HWA HU, Department
National Chung Hsing University, Taich

Isolated single cells from Ipomoea
prepared to study the effect of light
and the incubation medium pH on photos
The macerated cells were identified an

Aust. J. Plant Physiol., 1984, **11**, 319–24

Effect of Sodium Nutrition and Light upon the Concentrations of Alanine in Leaves of C_4 Plants

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[B] Present address: Department of Agronomy, University of Kentucky, Lexington, Kentucky 40546, U.S.A.

Abstract

The concentration of alanine in the leaves of representatives of each of the three types of C_4 plants was found to be consistently greater in sodium-deficient than in sodium-sufficient plants. The sodium treatment, however, did not affect the alanine concentration in the leaves of tomato, a C_3 plant or in the roots of *Amaranthus tricolor*, a C_4 plant. The alanine concentration of leaves of *A. tricolor* decreased rapidly upon the addition of sodium to cultures of sodium-deficient plants. The concentration of sodium required to give this response was approximately 50 µM, which was similar to that needed for the growth response. When plants that had been previously illuminated were placed in the dark, the alanine concentration increased rapidly, but was lowered again to the original concentration on return to the light. These changes in the alanine concentrations of leaves of C_4 plants in response to sodium and light treatments are discussed in relation to the C_4 photosynthetic system.

Introduction

Sodium is an essential micronutrient for C_4 but not C_3 plants (Brownell and Crossland 1972), and is probably involved in the C_4 system. Raghavendra and Das (1977, 1978) reported light-dependent active transport of amino acids between the mesophyll and bundle sheath cells of *Amaranthus paniculatus*, an NAD–ME-type C_4 plant, mediated by a sodium-dependent ATPase. When undertaking similar experiments with *Amaranthus tricolor*, also an NAD–ME-type of C_4 plant, we observed that sodium-deficient plants had consistently greater concentrations of alanine in their leaves than those of sodium-treated plants. This effect is described in this paper.

Materials and Methods

Plant Material

The following species were used: *Kochia childsii* Hort., *Echinochloa utilis* Ohwi et Yabuno, *Chloris barbata* Sw., *Amaranthus tricolor* L. (pigmented form), *Atriplex spongiosa* F. Muell. and *Eleusine indica* (L.) Gaertn. These plants were selected as representatives from each of the three types of the C_4 plants described by Gutierrez *et al.* (1974) and Hatch *et al.* (1975). *Lycopersicum esculentum* Miller (tomato) was chosen as a C_3 control species.

Growth Conditions

The procedures for the germination and growth of plants under conditions of low sodium have been described previously (Brownell 1979). Total sodium concentration of the basal culture solution was approximately 0·08 µM. Sodium chloride was supplied to appropriate culture solutions to give a final concentration of 0·1 mM, unless otherwise stated.

Plants were grown in a naturally illuminated glasshouse with a maximum photon irradiance of approximately 1800 µmol m^{-2} s^{-1} in full sunlight.

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Harvesting Procedure

Weights were obtained after drying to constant weight at 90°C. Alanine was determined on representative samples of young fully expanded leaves.

Light Treatment

Prior to extraction, plants were illuminated for 60 min with light from a 500 W Philips flood lamp passing through a 10 cm water filter which acted as a heat absorber. The photon irradiance was 1700 μmol m^{-2} s^{-1}.

Extraction of Alanine

Approximately 1 g of leaf tissue was ground with a mortar and pestle in 80% ethanol and the extract boiled for 5 min. After cooling, the extract was centrifuged at 10 000 *g* for 20 min. The supernatant was then reduced in volume to 1·0 ml and deproteinized according to the method of Grassl (1974) followed by centrifugation at 3000 *g* for 10 min.

Alanine Determination

Alanine was determined by chromatography. A 20-μl sample was applied to Whatman No. 3 chromatography paper and the amino acids separated by 1-dimensional ascending chromatography using the following solvents described by Bieleski and Turner (1966); solvent I, methyl ethyl ketone–pyridine–water–acetic acid (70 : 15 : 15 : 2, v/v); solvent II, n-propanol–water–n–propyl acetate–acetic acid–pyridine (120 : 60 : 20 : 4 : 1, v/v).

Amino acids were detected by spraying the chromatography papers with a solution containing 100 mg cadmium acetate, 10 ml water, 5 ml acetic acid, 100 ml acetone and 1 g ninhydrin. After air-drying at room temperature the chromatograms were heated for 15 min at 60°C. Alanine was identified by cochromatography and the alanine spot was cut out and eluted in a solution comprising methanol–ethyl acetate–water (1 : 1 : 1, v/v), 1% acetic acid and 1% cadmium acetate. Either 3, 6 or 9 ml of eluting solution was used depending on the colour intensity of the alanine spot. The absorbance of the eluate was measured at 505 nm and the concentration of alanine estimated from a standard curve.

Table 1. Effect of sodium nutrition on the concentration of alanine in leaves
NaCl was applied approximately 10 days after germination

Species	Age when harvested (days)	Concn of alanine [μmol (g fresh wt)$^{-1}$] No addition	0·1 mM NaCl
	C_4 species		
NADP–ME-type			
Kochia childsii	33	9·76	4·46
Echinochloa utilis	40	7·56	3·36
PEP–CK-type			
Chloris barbata	35	5·35	2·12
NAD–ME-type			
Amaranthus tricolor	40	8·21	3·36
Atriplex spongiosa	34	7·38	2·95
Eleusine indica	36	6·16	2·23
	C_3 species		
Lycopersicum esculentum (tomato)	36	2·09	2·46

Results and Discussion

In all the C_4 species, the concentration of alanine in the leaves of sodium-deficient plants was at least twice as great as in the leaves of NaCl-treated plants (Table 1) but the

alanine concentrations in the roots of the C_4 species, *A. tricolor* (Fig. 1) and in the leaves of the C_3 species, tomato (Table 1), were not affected by the NaCl-treatment.

Sodium supplied as the chloride (0·1 mM) or sulfate (0·05 mM) gave similar decreases in the alanine concentrations of leaves when added to cultures of sodium-deficient *A. tricolor* (Table 2), demonstrating that the effect was due to sodium and not to the associated anions.

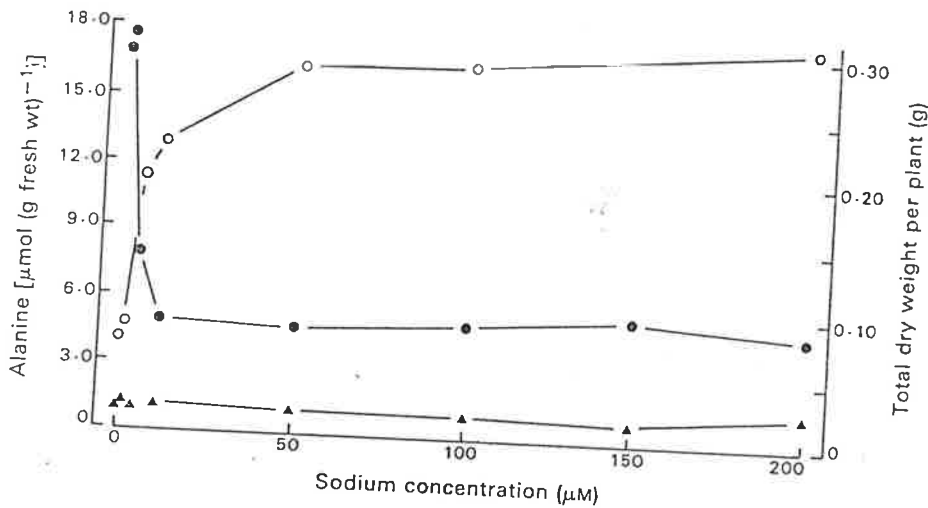

Fig. 1. Responses in growth and alanine concentrations to sodium nutrition by *A. tricolor*. Plants were 12 days old before NaCl treatments were applied and were harvested 22 days later. The dry weight (○) represents the mean weight of four cultures of four plants each. Alanine concentrations were determined in leaves (●) and roots (▲).

Table 2. Effect of different salts of sodium on the concentration of alanine in leaves of *A. tricolor*

Treatment	Concn of alanine [μmol (g fresh wt)$^{-1}$] in leaves of plants: 31 days old	35 days old
No addition	8·42	7·80
0·1 mM NaCl applied		
15 days after germination	3·05	3·42
31 days after germination	7·91	3·94
0·05 mM Na_2SO_4 applied		
31 days after germination	7·85	3·65

When the plants were transferred to the dark, marked increases in the alanine concentrations were observed in the leaves of sodium-deficient and sodium-sufficient *A. tricolor* that had been previously illuminated for 60 min (Fig. 2). When the plants were returned to the light, the alanine concentrations were restored to the initial values. These changes were rapid, occurring within 10 min of changing the light treatment; such rapid

changes suggest that a substantial proportion of the pool of alanine being determined was associated with photosynthesis.

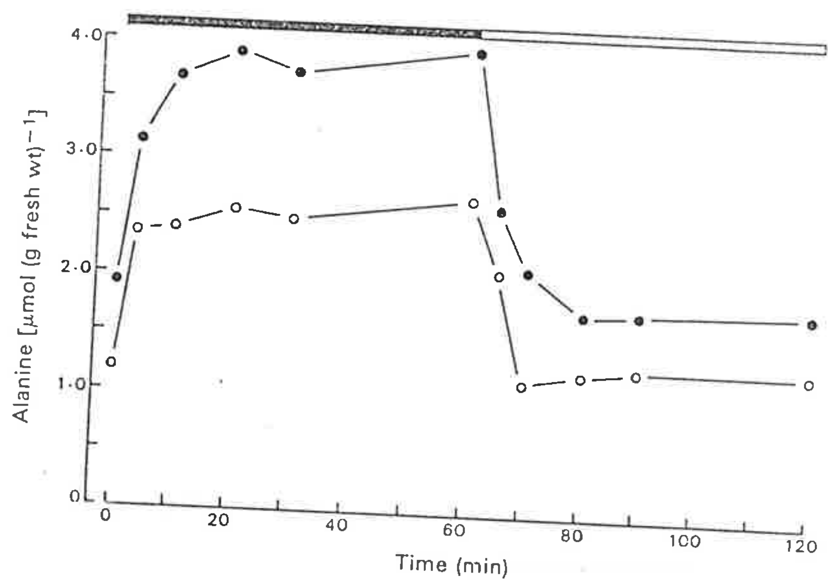

Fig. 2. Effects of light and dark treatments on the concentration of alanine in leaves of previously illuminated (1700 μmol m^{-2} s^{-1}) *A. tricolor*. The concentration of alanine was determined at intervals in 40 day-old sodium-deficient (●) and sodium-treated (○) plants.

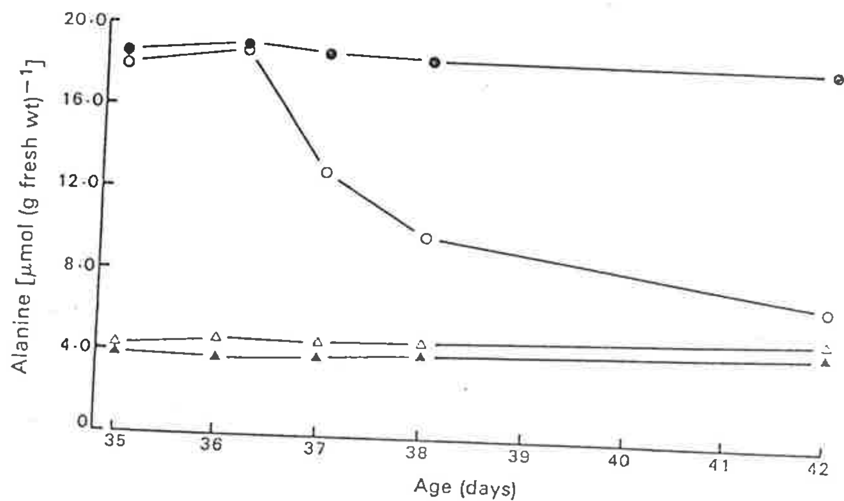

Fig. 3. Changes in alanine concentration with age of *A. tricolor* during recovery from sodium deficiency. ● No NaCl addition throughout. ○ 0·1 mM NaCl addition on day 35. ▲ 0·1 mM NaCl addition on day 10. △ 0·1 mM NaCl additions on days 10 and 35.

The alanine concentration in the leaves of *A. tricolor* decreased rapidly during the recovery from sodium deficiency (Fig. 3). Within 2 days of receiving sodium, the recovering plants had a markedly lower concentration of alanine than that of the sodium-deficient control plants. After a further 5 days, they had an alanine concentration similar to that of the sodium-sufficient control plants. These changes in the alanine concentration of

recovering plants preceded by about 3 days the visual signs of recovery from sodium deficiency, including increased growth and greening.

When *A. tricolor* cultures were supplied with increasing concentrations of sodium chloride, the minimum concentration needed to effect the decrease in the concentration of alanine was similar to that required for the growth response (Fig. 1). With increasing concentrations of sodium chloride to 10 μM, both the alanine concentration and the dry-weight yield changed sharply. At 50 μM sodium chloride concentration, the alanine concentration had fallen to its minimum and the dry-weight yield was at its maximum. As plant yield and the alanine concentrations of leaves respond to similar concentrations of sodium, it appears likely that the alanine is intimately involved in the systems affected by sodium nutrition.

Sometimes large variations between experiments were observed in the concentrations of alanine in leaves of similarly treated plants. These differences may have been due to seasonal variations in light and temperature conditions in the glasshouse. However, confidence in our findings was gained by the repeatability of the results in terms of the trends obtained in response to the sodium and light treatments achieved in many separate experiments.

The observed effects of sodium nutrition and light treatment upon the alanine concentrations in the leaves of C_4 plants may be explained using the scheme of Hatch (1976*a*). Alanine and pyruvate are assumed to be in equilibrium, the reaction being catalysed by alanine aminotransferase (Hatch 1976*b*; Hatch and Osmond 1976). The higher concentration of alanine in the leaves of sodium-deficient than in sodium-sufficient plants may be due to a limitation in the transport or further metabolism of pyruvate or alanine returning from the bundle sheath cells.

In mesophyll chloroplasts, pyruvate is utilized for the regeneration of phospho*enol*pyruvate, the CO_2 acceptor in a reaction catalysed by pyruvate, orthophosphate dikinase (Hatch 1976*a*). This enzyme undergoes changes between active and inactive forms in response to light and darkness, respectively (Hatch and Slack 1969; Yamamoto *et al.* 1974). In the dark, the inactivity of pyruvate, orthophosphate dikinase could lead to an increase in the concentration of pyruvate and the observed increase in concentration of alanine. The high concentrations of alanine found in illuminated leaves of sodium-deficient plants (Table 1) could also be related to reduced activity of pyruvate, orthophosphate dikinase. Although Boag (1981) showed that the *in vitro* activity of this enzyme was not affected by sodium nutrition, it is possible that sodium ions could be necessary for its *in vivo* activity.

In addition, Raghavendra and Das (1977, 1978) suggested that ATPases were involved in active transport of metabolites and that sodium ions may be required for their activation. Although it has been calculated that passive diffusion through the plasmodesmata could account for the observed movements of metabolites between the bundle sheath cells and mesophyll cells (Olesen 1975; Hatch and Osmond 1976), it is possible that ATPases may be involved in organelle membrane transport, for example the transport of pyruvate into the mesophyll chloroplasts. If sodium ions are required for the activation of these ATPases the transport of pyruvate into chloroplasts could be limited, resulting in an increase in the concentration of pyruvate and the observed increase in the concentration of alanine.

Future work will focus on the effects of sodium nutrition on other intermediates involved in C_4 photosynthesis.

Acknowledgments

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Responses to Ambient CO_2 Concentrations by Sodium-deficient C_4 Plants

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Abstract

The signs of sodium deficiency in the C_4 species *Amaranthus tricolor* and *Atriplex spongiosa* were alleviated when the species were grown in conditions of high CO_2 concentration (1500 μl CO_2 l^{-1}). In experiments in which *A. tricolor* was grown in conditions of low CO_2 concentration (15 μl CO_2 l^{-1}), the signs of sodium deficiency were accentuated. The sodium-deficient plants resembled the C_3 species *Atriplex hastata* and tomato in their response to the CO_2 treatments. Growth and chlorophyll concentrations increased in both the sodium-deficient C_4 plants and the C_3 plants when they were grown in conditions of increased CO_2 concentration. Sodium-sufficient C_4 plants were relatively unaffected by the CO_2 treatments.

Introduction

The growth of C_4 plants, unlike that of C_3 plants, is relatively unaffected by the ambient CO_2 concentration (Wong 1979). Estimates of the proportion of CO_2 entering the bundle sheath cells by diffusion without involving the C_4 pathway vary, from negligible (Hatch and Osmond 1976) to about 18% (Black 1973; Rathnam 1978).

Sodium is unique as a micronutrient for higher plants in that it is required only by plants possessing the C_4 appendage (Brownell and Crossland 1972, 1974). If the normal operation of the C_4 appendage depends on sodium, the flow of CO_2 to the bundle sheath cells via this system would be reduced in sodium-deficient plants. Experiments were carried out to determine if the signs of sodium deficiency could be modified by different ambient CO_2 concentrations.

Materials and Methods

Plant Material

The following species were studied: *Amaranthus tricolor* L. (pigmented and non-pigmented forms), *Atriplex hastata* L., *Atriplex spongiosa* F. Muell., and *Lycopersicum esculentum* Mill. cv. 'Red Cloud' (tomato).

Growth Conditions

The procedures for the germination and growth of plants under conditions of low sodium have been described previously (Brownell 1979). The total sodium concentration of the complete culture solution as an impurity was approximately 0·08 μM. Sodium-sufficient plants were obtained by supplying sodium chloride to give a final concentration of 0·1 mM in appropriate cultures.

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Six to eight 2-litre containers, each with four or eight plants, were enclosed in either 150-litre Perspex cabinets (600 × 450 × 550 mm) or 200-litre glass cabinets (728 × 570 × 482 mm), which were naturally illuminated. The maximum photon irradiance in the cabinets was approximately 2500 μmol m^{-2} s^{-1}. The CO_2 treatments are given in Table 1.

Table 1. Composition and supply of gases to growth cabinets

All treatments were applied during the light period only; ambient air was supplied during the dark period. All cultures were aerated with normal air. CO_2-free air was obtained by passing the air through soda lime columns, and CO_2 was obtained from a CIG gas cylinder

CO_2 treatment	Gas type	Gas flow rate (litre min^{-1})	
		Perspex cabinets, 150-litre volume	Glass cabinets, 200-litre volume
Low CO_2 (15 $\mu l\ l^{-1}$)	Ambient air		1·8
	Culture aeration		1·0
	CO_2-free air		58·0
Normal air	Ambient air	25	59·0
	Culture aeration	2	1·0
High CO_2 (1500 $\mu l\ l^{-1}$)	Ambient air	28	59·0
	Culture aeration	2	1·0
	CO_2	0·035	0·07

Harvesting Procedure

The age of plants at harvest was between 21 and 56 days. Dry weights were obtained after drying the plants to constant weight at 90°C.

Determination of Sodium in Leaves

Plant material was dried in aluminium foil trays at 90°C for 24 h. Samples of approximately 0·3 g of powdered plant material were placed in silica crucibles and ashed at 420°C for 14 h. After cooling, 7 ml of silica-distilled 2 M HCl was added. The crucibles were heated over a steam bath until the volume was reduced to about 2 ml. The solutions were made up to volume and the concentration of sodium was determined by atomic absorption photometry.

Determination of Chlorophyll

Chlorophyll was extracted from leaf tissue with 80% (v/v) acetone in the dark at 0–5°C and determined using the equations of Mackinney (1941). Alternatively, 96% (v/v) ethanol was used as the extracting solvent in the presence of approximately 0·4 g of $CaCO_3$ per gram fresh weight of tissue and chlorophyll determined using the equations of Wintermans and De Mots (1965).

Statistical Treatment

Data were analysed using analysis of variance and *t*-test. Log transformations were carried out on dry weight data before analysis to equalize the error variances.

Results

The signs of sodium deficiency include reduced growth, chlorosis and necrosis of leaves (Brownell 1979). These signs were reduced in sodium-deficient C_4 plants grown in atmospheres of 1500 $\mu l\ CO_2\ l^{-1}$ as compared to normal air (330 $\mu l\ CO_2\ l^{-1}$), but were more pronounced in plants grown in atmospheres of 15 $\mu l\ CO_2\ l^{-1}$. The sodium-sufficient C_4 plants appeared similar, irrespective of the CO_2 treatment.

The dry weight yield of sodium-deficient plants of *A. tricolor* and *A. spongiosa* increased significantly with an increase in ambient CO_2 concentration (Figs 1*a*, 1*b* and 1*c*). However, the growth of sodium-sufficient C_4 plants showed little response to different CO_2 concentrations. The differences in the responses of sodium-deficient and sodium-sufficient

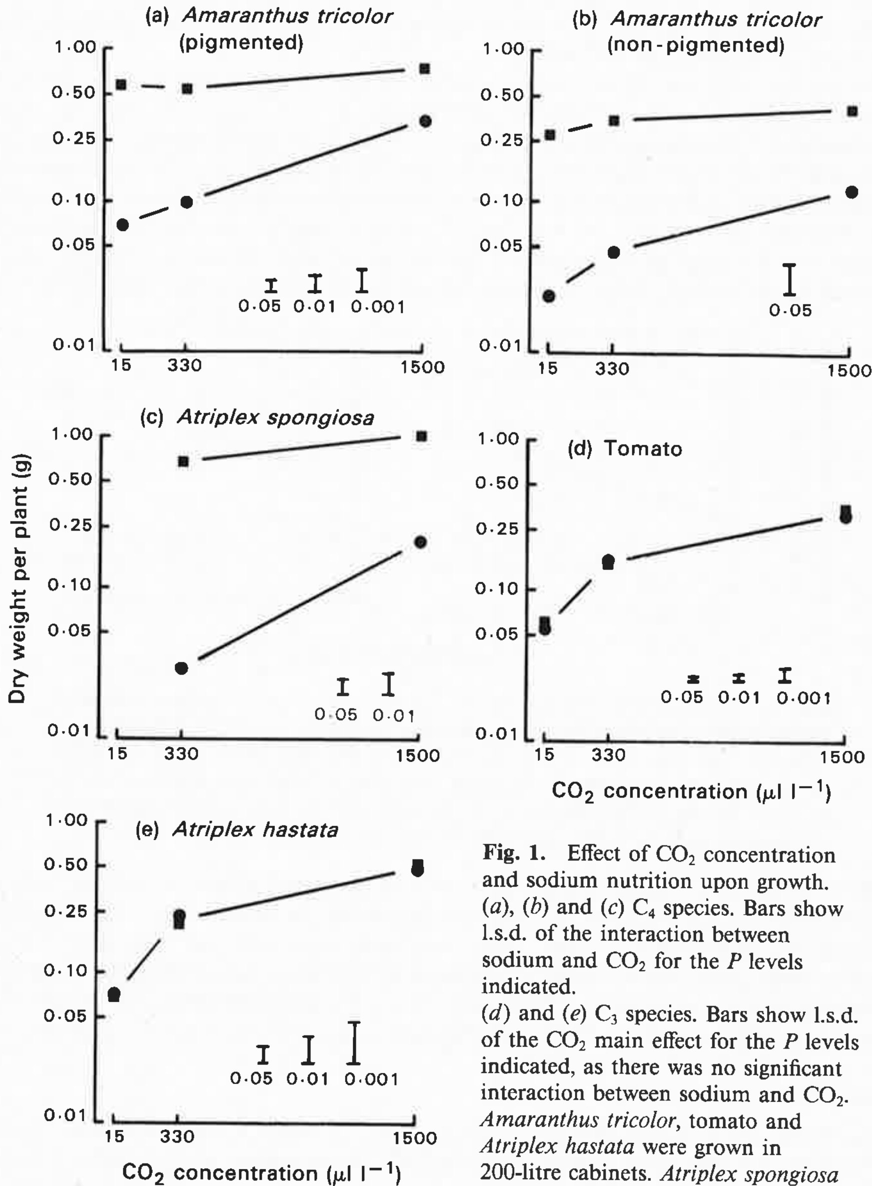

Fig. 1. Effect of CO_2 concentration and sodium nutrition upon growth. (*a*), (*b*) and (*c*) C_4 species. Bars show l.s.d. of the interaction between sodium and CO_2 for the *P* levels indicated.
(*d*) and (*e*) C_3 species. Bars show l.s.d. of the CO_2 main effect for the *P* levels indicated, as there was no significant interaction between sodium and CO_2. *Amaranthus tricolor*, tomato and *Atriplex hastata* were grown in 200-litre cabinets. *Atriplex spongiosa* was grown in 150-litre cabinets.
● No addition of sodium.
■ 0·1 mM NaCl.

C_4 plants to CO_2 is highlighted by the interaction of sodium and CO_2, which was significant ($P<0·05$) in all experiments. In the experiments in which a significant response to CO_2 was evident in sodium-sufficient plants, the relative increase in growth was not nearly as marked as that observed in sodium-deficient plants. In *Atriplex spongiosa*, for example,

both sodium-sufficient and sodium-deficient plants responded significantly to an increase in CO_2 concentration from 330 to 1500 μl CO_2 l^{-1}. The relative increase in growth between 330 and 1500 μl CO_2 l^{-1} was approximately 1·5 times in the sodium-sufficient plants and seven to eight times in the sodium-deficient plants.

The C_3 species *Atriplex hastata* and tomato were also grown under similar concentrations of CO_2. These plants did not respond to sodium but increased in yield with an increase in CO_2 concentration (Figs 1*d* and 1*e*).

The sodium concentrations in the leaves of *A. tricolor* were determined to ensure that the growth response of sodium-deficient plants to CO_2 was not due to sodium contamination. The concentration of sodium in the leaves of sodium-deficient plants grown in atmospheres of 15, 330 and 1500 μl CO_2 l^{-1} was 75, 31 and 13 μg g^{-1} dry wt, respectively. The concentration of sodium in the leaves of sodium-sufficient plants grown in atmospheres of 15, 330 and 1500 μl CO_2 l^{-1} was 251, 271 and 226 μg g^{-1} dry wt, respectively. The decreases in sodium concentration in the leaves of sodium-deficient plants may be attributed to the sodium taken up being diluted by the increased dry matter (Fig. 1*b*).

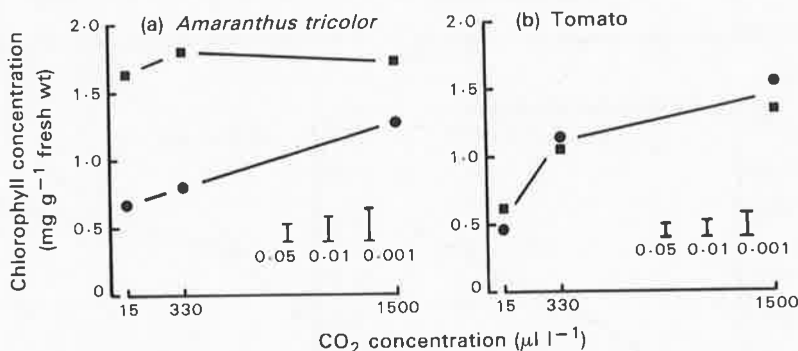

Fig. 2. Effect of CO_2 concentration and sodium nutrition upon chlorophyll concentration. (*a*) *Amaranthus tricolor* (non-pigmented), extracted with 96% (v/v) ethanol. Bars show l.s.d. of the interaction between sodium and CO_2 for the *P* levels indicated. (*b*) Tomato, extracted with 80% (v/v) acetone. Bars show l.s.d. of the CO_2 main effect for the *P* levels indicated, as there was no significant interaction between sodium and CO_2. ● No addition of sodium. ■ 0·1 mM NaCl.

The concentration of chlorophyll in leaves of sodium-deficient *A. tricolor* increased significantly with an increase in CO_2 concentration. In comparison, the chlorophyll concentration of sodium-sufficient plants was relatively unaffected (Fig. 2*a*). In the C_3 species tomato, there was no difference in chlorophyll concentration between those plants grown with or without added sodium within any CO_2 treatment (Fig. 2*b*). However, the chlorophyll concentration increased significantly with an increase in CO_2 concentration.

Discussion

The yield and chlorophyll concentration of the C_3 and sodium-deficient C_4 plants increased significantly with increased ambient CO_2 concentration. By comparison, sodium-sufficient C_4 plants showed little response to similar CO_2 conditions. From a consideration of the sodium concentrations in the leaves, it is clear that the increase in yield of sodium-deficient plants was not due to sodium contamination. Therefore, it is likely that the alleviation of the signs of sodium deficiency was due to the increased ambient CO_2 concentration.

In sodium-sufficient C_4 plants, the bulk of the CO_2 fixed by the Calvin cycle would enter the bundle sheath cells via the C_4 pathway. In these plants, it is assumed that the concentration of CO_2 in the bundle sheath is maintained at near optimal concentrations

for the operation of the Calvin cycle irrespective of ambient CO_2 concentration. However, it appears that in sodium-deficient plants, a greater proportion of the CO_2 fixed by the Calvin cycle would enter by direct diffusion, due to the possibly impaired operation of the C_4 cycle. The growth responses to ambient CO_2 concentrations by sodium-deficient C_4 plants were similar to those obtained with C_3 plants, which depend solely upon diffusion for the CO_2 entering the Calvin cycle.

An alternative explanation that could accommodate the results of this study requires that the C_4 pathway in both the sodium-sufficient and sodium-deficient plants functions at a similar rate. Under normal conditions, the concentration of intermediate pools of $CO_2 + HCO_3^-$ range from 0·6 to 2 mM in the bundle sheath cells of C_4 plants (Hatch 1976; Hatch and Osmond 1976), creating a CO_2 concentration gradient between the bundle sheath and the mesophyll cells. The reduction in yield observed in sodium-deficient plants could be attributed to a possible increased leakiness of the bundle sheath to CO_2. If this were the case, the rate of loss of CO_2 would depend largely upon the concentration of CO_2 in the mesophyll cells, which would be modified by changes in the ambient CO_2 concentration.

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Effect of Sodium Nutrition on Chlorophyll *a/b* Ratios in C_4 Plants

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Abstract

Methods for the determination of chlorophyll were compared in *Amaranthus tricolor*, *Kochia childsii* and *Chloris gayana*. From sequential extraction data, 96% ethanol appeared to be more efficient than 80% acetone in extracting chlorophyll from these plants.

The chlorophyll *a/b* ratio was significantly lower in sodium-deficient compared to normal C_4 plants. Of the group I elements, only sodium, irrespective of the salt supplied to deficient cultures, restored the chlorophyll *a/b* ratios to the value observed in normal plants. The concentration of sodium required to increase the chlorophyll *a/b* ratio in leaves of sodium-deficient plants was similar to that required to bring about the growth responses. The increase of the chlorophyll *a/b* ratio occurred at an early stage during recovery from sodium deficiency preceding the increase in chlorophyll concentration and the growth response. It is therefore likely that the low chlorophyll *a/b* ratio may be intrinsically associated with the condition of sodium deficiency.

Introduction

Sodium is a unique micronutrient in that it is required only by plants possessing the C_4 system (Brownell and Crossland 1972, 1974). Preliminary phaeophytin data suggested that the chlorophyll *a/b* ratio was lower in sodium-deficient than normal C_4 plants. Three types of C_4 plants have been described on the basis of the predominant C_4 acid decarboxylase present (Gutierrez *et al.* 1974; Hatch *et al.* 1975). In the NADP–ME-type species, the chlorophyll *a/b* ratio of the bundle sheath cells is greater than that of the mesophyll cells whereas, in the NAD–ME-type species, the opposite has been found (Mayne *et al.* 1974). In PEP–CK-type species, the mesophyll and bundle sheath cells have similar chlorophyll *a/b* ratios (Mayne *et al.* 1974).

High chlorophyll *a/b* ratios are, in general, associated with low chlorophyll/P_{700} ratios, low delayed light emission and low Hill reaction activity which suggests relatively more cyclic to non-cyclic electron flow and thus a potentially higher ratio of ATP/NADPH production (Edwards *et al.* 1976; Edwards and Huber 1981). It is therefore possible that a lower chlorophyll *a/b* ratio in sodium-deficient plants could result in an imbalance in the energy production of the mesophyll or bundle sheath cells or both.

Although the balance between cyclic and non-cyclic electron flow can be estimated from photochemical data, pseudocyclic electron flow should also be considered. Changes in the balance between cyclic, non-cyclic and pseudocyclic electron flow give chloroplasts flexibility to meet the specific energy requirements for carbon assimilation (Edwards *et al.* 1976).

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The suggestion that the chlorophyll *a/b* ratio is lower in sodium-deficient plants was inconsistent with the findings of Boag and Brownell (1979), who concluded that the chlorophyll *a/b* ratio was not affected by sodium nutrition. Boag and Brownell (1979) reported that the chlorophyll *a/b* ratio was 3·8 in both sodium-deficient and normal plants of *Kochia childsii* and 3·4 in sodium-deficient and 3·3 in normal plants of *Chloris barbata* when extracted with 80% (v/v) acetone. This inconsistency may be due to the different methods used for the chlorophyll determination. This paper compares two widely used methods of chlorophyll determination and describes the effects of sodium nutrition on the chlorophyll *a/b* ratio in C_4 plants.

Materials and Methods

Plant Material

Amaranthus tricolor L. (non-pigmented form), *Chloris gayana* Kunth and *Kochia childsii* Hort. were chosen as representatives of the three types of C_4 plants. *Atriplex hastata* L. and *Lycopersicum esculentum* Miller, cv. Red cloud (tomato) were chosen as representative C_3 species.

Growth Conditions

The procedures for the germination and growth of plants under conditions of low sodium have been described previously (Brownell 1979). The concentration of sodium as an impurity in the complete culture solution was approximately 0·08 μM. Normal plants were obtained by supplying appropriate cultures with NaCl to give a final concentration of 0·1 mM except where otherwise specified. Where the other group I elements were used, they were supplied as chlorides. The sodium as an impurity contributed by these salts gave sodium concentrations less than 0·02 μM in the final culture solution.

Plants were grown in 2-litre culture vessels containing eight plants each. Experiments were carried out in a naturally illuminated growth cabinet supplied continuously with filtered air to minimize sodium contamination. The maximum photon irradiance was approximately 2500 $\mu mol\ m^{-2}\ s^{-1}$.

Harvesting Procedure

The age of plants at harvest was between 20 and 42 days. Dry weights were obtained after drying to constant weight at 90°C.

Determination of Chlorophyll

Chlorophyll was extracted from 1–2 g of young fully expanded leaf tissue with either 80% (v/v) acetone or 96% (v/v) ethanol. After harvesting, the tissue was immediately weighed and ground with 20 ml of solvent in a mortar and pestle in the presence of a small quantity of acid washed sand and approximately 0·4 g of $CaCO_3$ per gram fresh weight of tissue. The homogenates were centrifuged at 10 000 *g* for 10 min and the pellets were re-extracted with 10 ml of solvent. Samples were extracted four or five times, until all colour was removed.

The combined supernatants were made up to volume and appropriately diluted to give a maximum absorbance reading not exceeding 0·8 units. All extraction procedures were carried out in the dark or at very low light intensities at 0–5°C. Prior to spectrophotometric measurements, the samples were equilibrated at room temperature in the dark for about 5 min. Spectrophotometric measurements were made using a Beckman DB-G spectrophotometer which was calibrated against a didymium standard. Occasionally, a Varian series 634 spectrophotometer was used to confirm the results obtained with the Beckman DB-G instrument. Both instruments gave the same results. Chlorophyll was determined using the equations of Mackinney (1941) for the 80% (v/v) acetone extracts and those of Wintermans and De Mots (1965) for the 96% (v/v) ethanol extracts. The absorbance of the samples was measured at 740 nm to correct for any possible turbidity in the extracts. The extraction procedures were carried out rapidly and the spectrophotometric measurements were made within 3 h of harvesting the leaf tissue.

Results and Discussion

With normal plants of *K. childsii*, *A. tricolor* and *C. gayana* the extraction of chlorophyll *a*, and to a lesser extent chlorophyll *b*, was achieved with fewer extractions with 96% ethanol than 80% acetone (Figs 1*a*, 1*b* and 1*c*). The chlorophyll *a/b* ratio of

the cumulative 96% ethanol extracts showed little change with sequential extractions (Figs 1*d*, 1*e* and 1*f*). However, with the cumulative 80% acetone extracts, the chlorophyll *a*/*b* ratio increased up to at least the third extraction. With four extractions the chlorophyll *a*/*b* ratios of the cumulative extracts were significantly greater ($P<0\cdot01$, *t*-test) with 96%

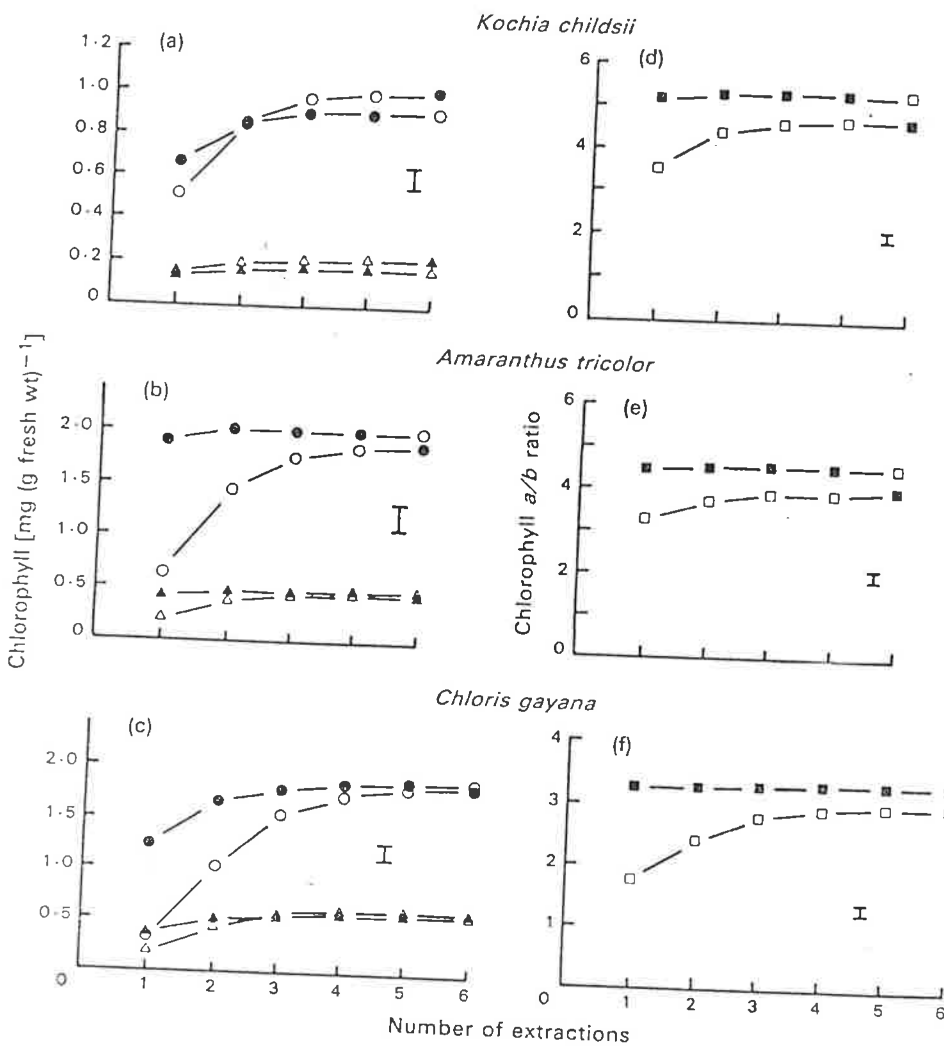

Fig. 1. The comparison of 96% ethanol and 80% acetone for the sequential extraction of chlorophyll from C_4 plants. (*a*), (*b*) and (*c*) The cumulative concentrations of chlorophyll *a* and *b* with sequential extractions. (*d*), (*e*) and (*f*) The chlorophyll *a*/*b* ratio of cumulative extracts obtained from sequential extractions. Bars indicate twice the maximum s.e.m., $n = 4$. The extracting solvents were exchanged for the final extraction. ● Chlorophyll *a* extracted with 96% ethanol. ○ Chlorophyll *a* extracted with 80% acetone. ▲ Chlorophyll *b* extracted with 96% ethanol. △ Chlorophyll *b* extracted with 80% acetone. ■ Chlorophyll *a*/*b* ratio extracted with 96% ethanol. □ Chlorophyll *a*/*b* ratio extracted with 80% acetone.

ethanol than 80% acetone for the three species. This was not due to incomplete extraction of chlorophyll, as substituting the extracting solvents for each other for the final extraction failed to yield any additional chlorophyll or alter the chlorophyll *a*/*b* ratios. Wickliff and Arnoff (1962) and Wintermans (1969) suggested that 80% acetone extracted compounds

that interfere with the determination of chlorophyll *a*. This could account for the differences in the chlorophyll *a/b* ratios observed using the two extraction solvents.

Leaves of sodium-deficient and normal plants of *K. childsii* were extracted with both 96% ethanol and 80% acetone. Both methods gave similar results for the chlorophyll *a/b* ratio in sodium-deficient plants (Fig. 2). However, in normal plants, the chlorophyll *a/b* ratio was significantly greater ($P<0 \cdot 001$, *t*-test) when extracted with 96% ethanol than with 80% acetone. With both methods, the chlorophyll *a/b* ratio was lower ($P<0 \cdot 001$, *t*-test) in sodium-deficient than normal plants.

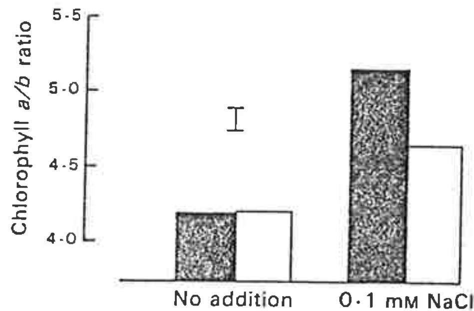

Fig. 2. The chlorophyll *a/b* ratio of sodium-deficient and normal plants of *K. childsii* extracted with 96% ethanol (shaded) and 80% acetone (unshaded). Bar represents twice the maximum s.e.m., $n = 4$.

The above data suggest that 96% ethanol is a more efficient solvent than 80% acetone for the extraction of chlorophyll in these plants. This is in agreement with the findings of Wintermans (1969). Consequently, 96% ethanol was used in subsequent experiments.

A decreased chlorophyll *a/b* ratio in sodium-deficient compared to normal plants was observed in *K. childsii*, *C. gayana* and *A. tricolor* representing each of the three types of C_4 plants (Table 1). This finding is inconsistent with that of Boag and Brownell (1979),

Table 1. Effect of sodium nutrition on chlorophyll *a/b* ratios
Significance of differences between adjacent values: n.s., not significant; *$P<0 \cdot 05$; **$P<0 \cdot 01$; ***$P<0 \cdot 001$

	Chlorophyll *a/b* ratio No addition		0·1 mM NaCl
	C_4 species		
NADP–ME-type			
Kochia childsii	4·19	***	5·16
PEP–CK-type			
Chloris gayana	3·49	*	3·73
NAD–ME-type			
Amaranthus tricolor	3·47	**	4·84
	C_3 species		
Lycopersicum esculentum	3·75	n.s.	3·90
Atriplex hastata	3·66	n.s.	3·69

who reported no significant difference between the chlorophyll *a/b* ratio of sodium-deficient and normal plants of *K. childsii* and *Chloris barbata*. This inconsistency may be attributed to the differences observed between 96% ethanol and 80% acetone as chlorophyll extracting solvents. The C_3 species *L. esculentum* (tomato) and *Atriplex hastata* showed no difference in chlorophyll *a/b* ratio when grown with or without added sodium (Table 1). As only plants possessing the C_4 appendage have been shown to require sodium as a micronutrient (Brownell and Crossland 1972, 1974) and the chlorophyll *a/b* ratio of only the C_4 species was affected by sodium nutrition, it is likely that these differences in chlorophyll *a/b* ratios are related to sodium nutrition.

Equivalent concentrations of sodium supplied to cultures of sodium-deficient plants as the chloride, sulfate, nitrate, dihydrogen orthophosphate or bicarbonate gave significant increases ($P<0 \cdot 01$, t-test) in yield, chlorophyll concentration and chlorophyll a/b ratios (Fig. 3). Of the group I elements supplied as LiCl, NaCl, KCl, RbCl, and CsCl to give a final concentration of 0·1 mM to cultures of sodium-deficient *Amaranthus tricolor*, only sodium significantly increased ($P<0 \cdot 001$, t-test) the yield, chlorophyll concentration and chlorophyll a/b ratio (Fig. 4). These results demonstrate that the increase in chlorophyll a/b ratio is due specifically to sodium.

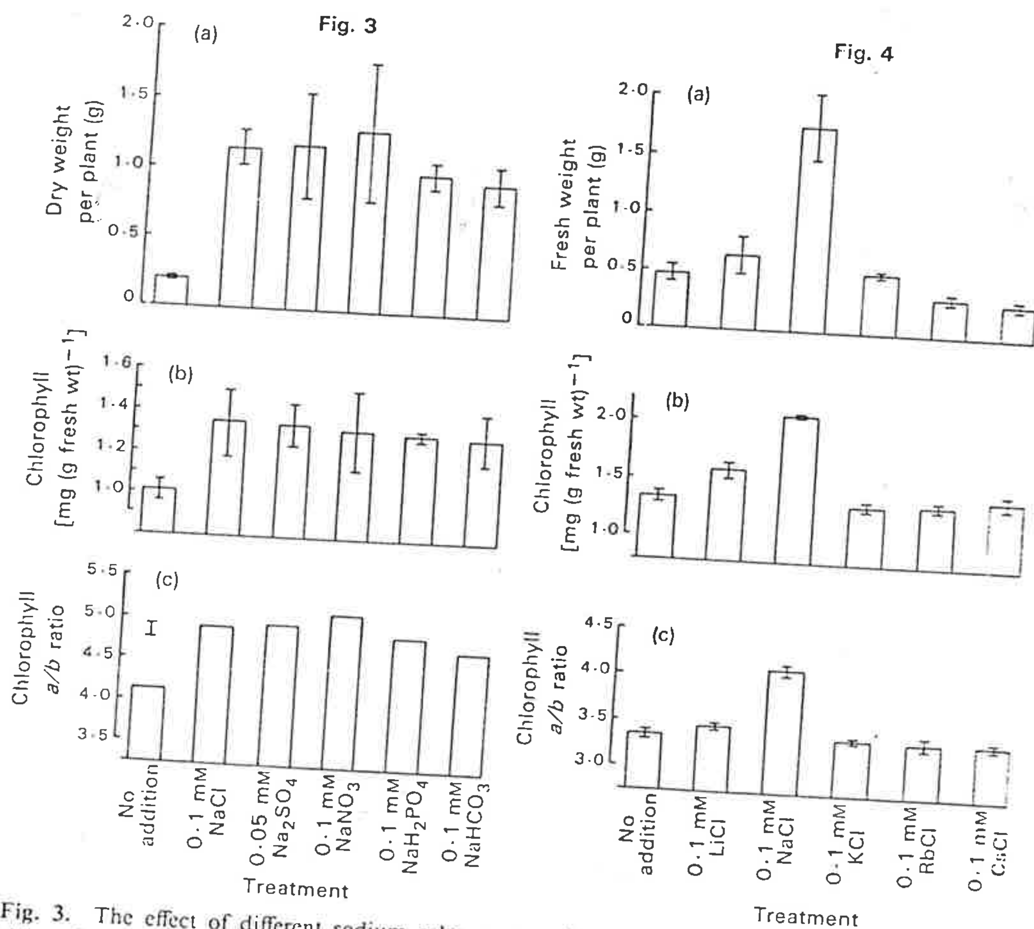

Fig. 3. The effect of different sodium salts on the yield (a), chlorophyll concentration (b) and chlorophyll a/b ratio (c) of *A. tricolor*. (a) Mean = s.e.m., $n = 2$. (b) Mean ± s.e.m., $n = 3$. (c) Bar represents twice the maximum s.e.m., $n = 3$.

Fig. 4. The effect of different group I elements on yield (a), chlorophyll concentration (b) and chlorophyll a/b ratio (c) of *A. tricolor*. (a) Mean = s.e.m., $n = 3$. (b) and (c) Mean ± s.e.m., $n = 4$.

When plants of *A. tricolor* were grown in increasing concentrations of sodium chloride, the yield, chlorophyll concentration and chlorophyll a/b ratio increased sharply, reaching a plateau at approximately 50 μM sodium chloride (Fig. 5). This indicates that the chlorophyll concentration and the chlorophyll a/b ratio are closely involved in the systems affected by sodium nutrition.

The chlorophyll a/b ratio of *A. tricolor* increased rapidly during the recovery from sodium deficiency (Fig. 6a). Within 1 day of receiving sodium, the recovering plants had significantly greater ($P<0 \cdot 01$, t-test) chlorophyll a/b ratios than the sodium-deficient plants.

After an additional day, the chlorophyll *a/b* ratio of the recovering plants did not differ significantly from the normal plants. The increase in chlorophyll *a/b* ratio in recovering plants preceded the increases in chlorophyll concentration and yield. The chlorophyll concentration of recovering plants was not significantly greater than that of sodium-deficient plants until 2 days after the addition of sodium (Fig. 6*b*). Differences in yield between recovering plants and sodium-deficient plants were not apparent until 3–5 days after the addition of sodium (Fig. 6*c*). The rapid increase in chlorophyll *a/b* ratio in response to the sodium treatment may represent a key step in the recovery from sodium deficiency.

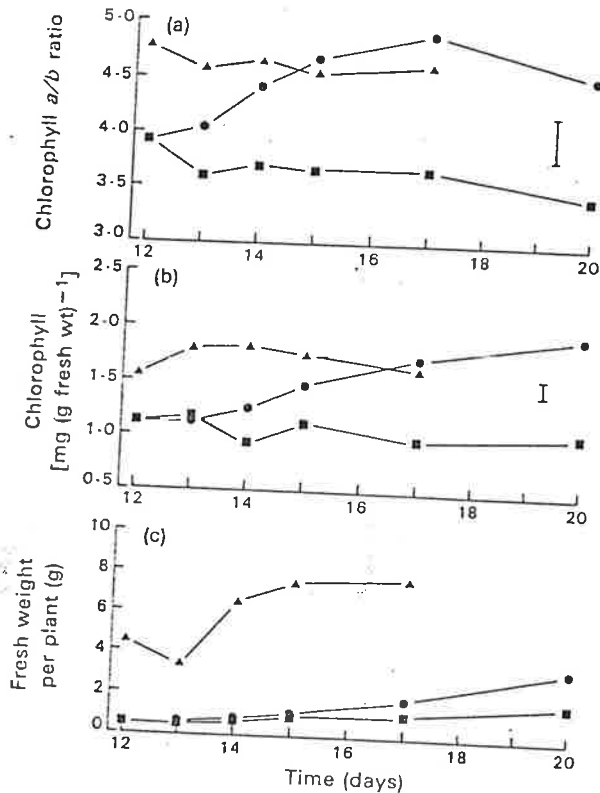

Fig. 5. The effect of concentration of sodium chloride upon yield (*a*), chlorophyll concentration (*b*) and chlorophyll *a/b* ratio (*c*) of *A. tricolor*. (*a*) Mean ± s.e.m., $n = 2$. (*b*) and (*c*) Mean ± s.e.m., $n = 3$.

In general, high chlorophyll *a/b* ratios suggest relatively more cyclic to non-cyclic electron flow and thus a potentially higher ratio of ATP/NADPH production (Edwards *et al.* 1976; Edwards and Huber 1981). The lower chlorophyll *a/b* ratio could result in a lower ratio of ATP/NADPH production in sodium-deficient compared to normal plants. If the chlorophyll *a/b* ratio is lower in the mesophyll cells, this could lead to a decrease in the production of ATP. As the regeneration of phospho*enol*pyruvate from pyruvate in mesophyll chloroplasts requires ATP (Hatch 1976), the rate of this process could be limited in sodium-deficient plants. This would support the suggestion of Nable and Brownell (1984) that the observed increased alanine concentration in sodium-deficient plants is associated with a corresponding increase in the concentration of pyruvate due to a limitation in the conversion of pyruvate to phosph*enol*pyruvate in sodium-deficient plants.

Acknowledgments

The financial support of the Australian Research Grants Scheme is gratefully acknowledged.

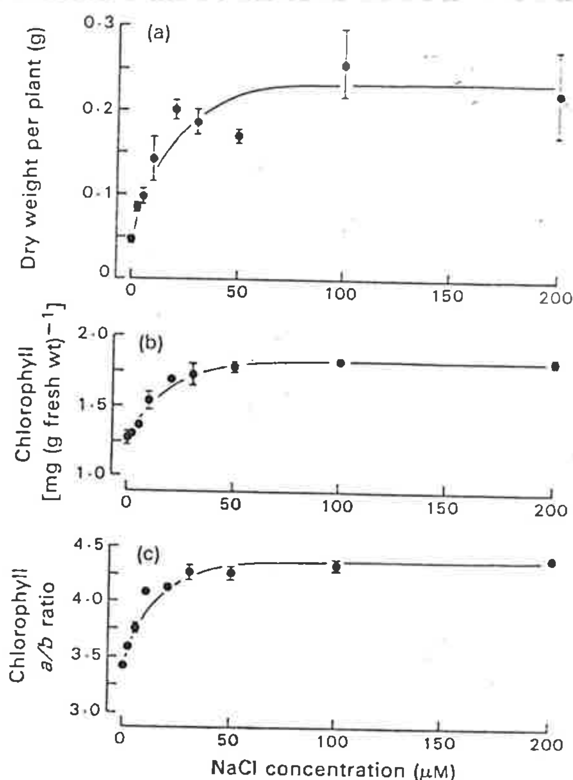

Fig. 6. Changes in chlorophyll a/b ratio (a), chlorophyll concentration (b) and yield during the recovery from sodium deficiency in *A. tricolor* (c). Bars indicate twice the maximum s.e.m. ■ No addition. ▲ 0·1 mM NaCl day 0. ● 0·1 mM NaCl day 12.

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Corrigendum

Volume 11, Number 4

Effect of sodium nutrition on chlorophyll *a*/*b* ratios in C_4 plants.
M. Johnston, C. P. L. Grof and P. F. Brownell
pages 330 and 331. The graphs in Figs 5 and 6 were transposed. The graph in Fig. 6 should be Fig. 5 and *vice versa*.

PHOTOSYNTHETICA 20 (4): 476—479, 1986

BRIEF COMMUNICATION

Sodium Deficiency in the C_4 Species *Amaranthus tricolor* L. Is Not Completely Alleviated by High CO_2 Concentrations*

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Abstract

Increased ambient CO_2 concentration alleviated but did not completely overcome the signs of Na-deficiency in plants of *A. tricolor*. The chlorophyll *a*/*b* ratios of Na-deficient plants which were significantly lower than those of Na-sufficient plants, were not significantly affected by ambient CO_2 concentration indicating that the increased CO_2 concentration alleviated the signs of Na-deficiency by by-passing the Na requiring processes.

Sodium is an essential micronutrient element for C_4 plants (Brownell and Crossland 1972, Brownell 1979). Sodium-deficient C_4 plants have a lower chlorophyll (chl) *a*/*b* ratio than Na-sufficient plants (Johnston *et al.* 1984b). This lower chl *a*/*b* ratio is thought to be intrinsically related to the condition of Na-deficiency in C_4 plants. The signs of Na-deficiency are modified by growing plants in different concentrations of CO_2 (Johnston *et al.* 1984a). At low CO_2 concentrations (15 $cm^3(CO_2)\ m^{-3}$), the signs were accentuated, and at high ones (1500 $cm^3(CO_2)\ m^{-3}$) they were partially elleviated.

Experiments were designed to determine (*1*) if an increase in ambient CO_2 concentration could completely overcome the signs of Na-deficiency, and (*2*) if the alleviation of the signs of Na-deficiency is associated with changes in the chl *a*/*b* ratios. The yield, chl content and chl *a*/*b* ratio were compared in Na-deficient and Na-sufficient plants of *Amaranthus tricolor* L. grown in a range of CO_2 concentrations from 15 to 6000 $cm^3(CO_2)\ m^{-3}$.

Amaranthus tricolor L., (non-pigmented form) was germinated and grown under low-Na conditions as described previously (Brownell 1979). The concentration of Na as an impurity in the complete culture solution was approx. 0.08 μM. Na-sufficient plants were obtained by supplying NaCl to give a final concentration of 0.1 mM in appropriate cultures. Eight 2000 cm^3 containers, each with eight plants were enclosed in 0.2 m^3 glass cabinets (728 × 570 × 482 mm), which were naturally illuminated. The photon irradiance in the cabinets was approx. 2500 $\mu mol\ m^{-2}\ s^{-1}$ (PhAR). The CO_2 treatments were obtained by supplying the gases (Table 1). Three separate experiments of similar design were carried out. For experiments 1, 2 and 3, plants were 13, 10 and 18 d old, respectively, before receiving the Na and CO_2 treatments; these treatments were continued for a further 14, 12 and 18 d, respectively, and then harvested.

Dry matters were obtained after drying to constant mass at 90°C. Chlorophyll was extracted from leaf tissue with 96 % (v/v) ethanol as described by Johnston *et al.* (1984b). Absorption measurements were made using a *Beckman DB-G* spectrophotometer which was calibrated against a didymium standard. Chlorophyll amounts were determined using the equations of Wintermans and De Mots (1965).

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Data were analysed using analysis of variance and t-test. Log transformations were carried out on dry matter data before analysis to equalize the error variances.

In all CO_2 concentrations, the yield of Na-deficient plants of *A. tricolor* was significantly less ($p < 0.001$) than that of Na-sufficient plants (Fig. 1*A*). The yield of Na-deficient plants increased significantly when grown in CO_2 concentrations from 15—3000 $cm^3(CO_2)\ m^{-3}$, but were not

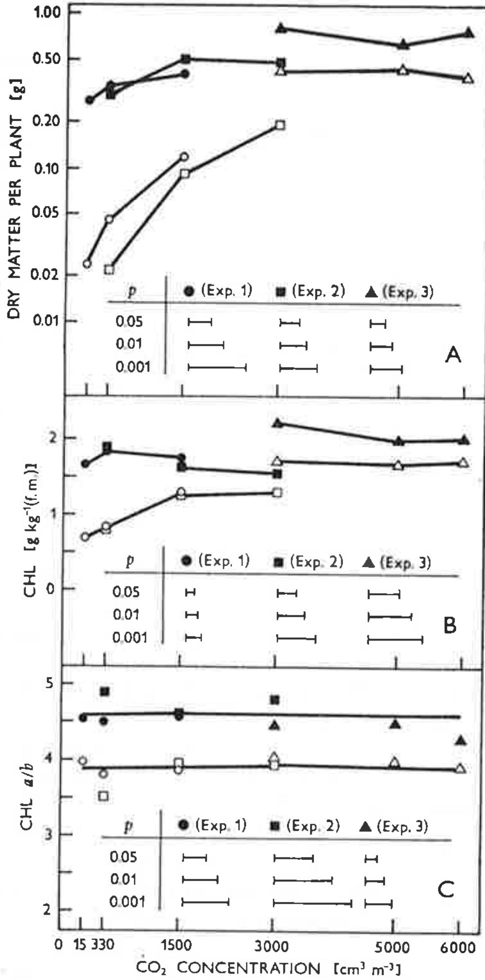

Fig. 1. Effect of CO_2 concentration upon (*A*) yield, (*B*) chlorophyll (chl) content, and (*C*) chl *a/b* ratios of Na-deficient (*open symbols*) and Na-sufficient (*solid symbols*) plants of *A. tricolor*. Log transformations were carried out on dry matter data prior to analysis to equalize the error variances. The bars for yield and chl content, experiment 1 (○ ●) and 2 (□ ■) show LSD of the interaction between sodium and CO_2 for the p levels indicated. For yield and chl content in experiment 3 (△ ▲), and the chl *a/b* ratios in experiments 1, 2 and 3, bars show the LSD of the sodium main effect for the p levels indicated, as there was no significant interaction between sodium and CO_2.

Table 1

Composition and supply of gases to growth cabinets. All treatments were applied during the light period only; normal air was supplied during the dark period. All cultures were aerated with normal air at 16.7 $cm^3\ s^{-1}$. CO_2-free air was obtained by passing the air through soda-lime columns and CO_2 was obtained from *Commonwealth Industrial Gases Ltd.*, Australia.

CO_2 treatment [$cm^3(CO_2)\ m^{-2}$]	Gas type	Gas flow rate [$cm^3\ s^{-1}$]
15	Normal air	30.0
	CO_2-free air	967.0
330	Normal air	983.0
1500	Normal air	983.0
	CO_2	1.2
3000	Normal air	983.0
	CO_2	2.7
5000	Normal air	983.0
	CO_2	4.7
6000	Normal air	983.0
	CO_2	5.7

further increased in 3000—6000 $cm^3(CO_2)\ m^{-3}$. The yield of Na-sufficient plants was relativel unaffected by the ambient CO_2 concentrations.

Although there was variation in the chl content of the leaves between experiments, the ch content in the leaves of Na-deficient plants was significantly less ($p < 0.01$) than in Na-sufficien plants within each experiment (Fig. 1*B*). The chl content in Na-deficient plants increased signi ficantly when grown in CO_2 concentrations from 15—1500 $cm^3(CO_2)\ m^{-3}$. Concentrations of CO above 1500 $cm^3(CO_2)\ m^{-3}$ did not affect the chl content of Na-deficient plants. The chl conten of Na-sufficient plants was relatively unaffected by ambient CO_2 concentrations.

The chl *a*/*b* ratios of both the Na-deficient and Na-sufficient plants were not significantly affecte by the ambient CO_2 concentrations (Fig. 1*C*). As observed by Johnston *et al.* (1984b), the Na deficient plants had a consistently lower ($p < 0.001$) chl *a*/*b* ratio than the Na-sufficient plants.

The yield and chl data indicate that an increase in ambient CO_2 concentration alleviates th signs of Na-deficiency but does not completely overcome them.

As the chl *a*/*b* ratios were unaffected by the ambient CO_2 concentration, it would appear tha an increased ambient CO_2 concentration alleviates the signs of Na-deficiency by by-passing th C_4 CO_2 concentrating system which is thought to require Na for its normal operation. This consistent with the suggestion of Johnston *et al.* (1984a) that the ambient **CO_2 concentration** change the intensity of the signs of Na-deficiency by altering the rates of diffusion of CO_2 either into o out of the bundle sheath cells.

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Free Amino Acid Concentrations in Leaves of Sodium-deficient C_4 Plants

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Abstract

Consistent changes in the free amino acid concentrations in response to sodium nutrition were observed in mature leaves of the C_4 species *Amaranthus tricolor* and *Chloris gayana*. The amino acids alanine, γ-aminobutyric acid and glycine were present in greater and aspartate and arginine in lower concentrations in mature leaves of sodium-deficient than in normal plants of both species.

Introduction

Sodium has been shown to be an essential micronutrient for C_4 but not C_3 plants (Brownell and Crossland 1972). Nable and Brownell (1984) found that the concentration of alanine was greater in the leaves of sodium-deficient than normal C_4 plants. They suggested that this increased alanine concentration in sodium-deficient plants may be associated with a corresponding increase in the concentration of pyruvate resulting from a limitation in the conversion of pyruvate to phospho*enol*pyruvate in sodium-deficient plants.

The free amino acid composition of the leaves of sodium-deficient and normal C_4 plants was examined to determine if only the amino acids involved in the C_4 pathway, alanine and aspartate, were consistently affected by sodium nutrition.

Materials and Methods

The procedures for the germination and growth of *Amaranthus tricolor* L. (non-pigmented form) and *Chloris gayana* Kunth under conditions of low sodium have been described (Brownell 1979). The total sodium concentration of the complete culture solution as an impurity was approximately 0·08 μM. Normal plants were obtained by supplying sodium chloride to give a final concentration of 0·1 mM in appropriate cultures.

Plants were grown in either 2-litre or 3·5-litre culture vessels containing 8 and 40 plants each, respectively, in a naturally illuminated growth cabinet supplied continuously with filtered air to minimise sodium contamination. The maximum photon irradiance in the growth cabinet was approximately 2000 μmol m^{-2} s^{-1}. The age of plants at harvest was between 25 and 32 days.

Before extraction, plant cultures were illuminated by a Philips 500-W flood lamp for 60 min at a photon irradiance of 1700 μmol m^{-2} s^{-1} at plant height. Light was passed through a 10-cm water filter which acted as a heat absorber.

Wallace *et al.* (1984) found that the concentrations of some amino acids were affected by treatments such as reduced light, reduced temperature and mechanical damage, immediately prior to killing. To prevent these changes, mature leaf tissue was rapidly weighed with minimal manipulation and dropped into 40 ml of boiling 80% (v/v) ethanol and boiled for 10 min. The leaf material was homogenised with a mortar and pestle and approximately 0·5 g of acid washed sand. The homogenate was boiled for a further 10 min and then centrifuged at 10 000 *g* for 20 min. The supernatant was removed and taken to dryness using a jet of air. The extracts were resuspended in 20% (v/v) ethanol.

Samples were deproteinised by adding a known volume of extract to an equal volume of 2 M ammonium acetate (pH 5·3), containing an internal standard (1 mM α-aminobutyric acid) and then

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placed in a boiling water bath for 3 min. When cool, twice the volume of chloroform was added and the samples were immediately mixed. After 10 min, the samples were remixed. Following centrifugation at 3000 *g* for 10 min, the aqueous phase of the samples was removed and stored at −20°C. The concentrations of the free amino acids in the deproteinised samples were determined using the method of McEvoy-Bowe (1985). A calibration factor for each individual amino acid was determined to correct for differential ninhydrin colour development. The internal standard was used as the basis against which the concentrations of the other amino acids were calculated to correct for experimental variation.

As asparagine does not stain blue with ninhydrin, a calibration factor was not determined. However, the absorbance of the asparagine spot after ninhydrin treatment was routinely read with the other amino acids at both 410 and 455 nm. The values given for asparagine are arbitrary and not absolute.

There was no detectable hydrolysis of asparagine to aspartate or glutamine to glutamate during the extraction and assay procedure. Some loss of glutamine was incurred during the extraction procedure and the data were corrected accordingly.

As values obtained for aspartate, when present in larger concentrations, were variable, the enzymatic method of Hatch (1979) was followed to determine aspartate concentrations in *A. tricolor*.

Table 1. Effect of sodium nutrition on the free amino acid concentrations in mature leaves of *Amaranthus tricolor* and *Chloris gayana*

Each value is the mean of 13–19 replicates for *A. tricolor* and 5–8 replicates for *C. gayana*, with standard errors in parentheses. Significance of differences between adjacent values: n.s., not significant; * $P<0·05$; ** $P<0·01$; *** $P<0·001$

	Concentration [μmol (g fresh wt)$^{-1}$]					
	Amaranthus tricolor			*Chloris gayana*		
	Sodium-deficient (No addition)		Normal (0·1 mM NaCl)	Sodium-deficient (No addition)		Normal (0·1 mM NaCl)
Glu	12·71(0·92)	***	6·13(0·28)	6·00(0·47)	n.s.	5·55(0·18)
Ala	10·18(0·89)	***	3·50(0·53)	9·18(0·50)	***	6·16(0·22)
Gly	4·57(0·56)	***	0·97(0·07)	3·14(0·16)	**	2·36(0·29)
Asp	4·46(0·25)	**	6·92(0·35)	1·45(0·15)	**	2·81(0·24)
Ser	3·50(0·34)	***	1·68(0·13)	2·13(0·07)	n.s.	2·26(0·14)
Gln	2·51(0·21)	n.s.	2·29(0·29)	1·39(0·09)	**	1·91(0·14)
Thr	1·32(0·08)	*	0·87(0·05)	1·23(0·10)	*	1·61(0·11)
Cys[A]	1·18(0·08)	***	0·73(0·04)	0·68(0·04)	*	0·88(0·07)
γ-Abu	0·84(0·10)	**	0·50(0·04)	1·23(0·07)	**	0·82(0·09)
Val	0·74(0·04)	**	0·51(0·03)	0·91(0·06)	n.s.	0·85(0·07)
Lys	0·63(0·07)	n.s.	0·50(0·04)	0·36(0·05)	***	0·86(0·07)
Leu + Ile	0·49(0·03)	*	0·35(0·03)	0·74(0·05)	n.s.	0·68(0·05)
Arg	0·47(0·06)	*	0·71(0·08)	0·48(0·08)	**	0·75(0·03)
Phe	0·32(0·04)	n.s.	0·34(0·03)	0·28(0·03)	**	0·49(0·04)
Tyr	0·30(0·03)	n.s.	0·32(0·04)	0·32(0·02)	**	0·59(0·06)
Asn[B]	n.d.		n.d.	1·64(0·26)	*	2·57(0·14)
Total	44·22	***	26·32	31·16	n.s.	31·15

[A] Cys includes both cysteine and cystine. [B] Arbitrary units.

Results and Discussion

Significantly higher concentrations of glutamate, alanine, glycine, serine, threonine, cysteine plus cystine, γ-aminobutyric acid, valine and leucine plus isoleucine were found in leaves of sodium-deficient than normal plants of *A. tricolor* (Table 1). In *C. gayana*, the concentrations of alanine, glycine, and γ-aminobutyric acid were higher in leaves of sodium-deficient than normal plants (Table 1). Not all the amino acids increased by similar extents; for example in *A. tricolor* alanine, which constituted approximately

13% of the total free amino acid pool in normal plants, increased three times in concentration to be approximately 25% of the total in sodium-deficient plants. However, other amino acids such as valine and threonine increased in concentration by only about one-and-a-half times in sodium-deficient plants and constituted approximately the same proportion of the total free amino acid pool in both sodium-deficient and normal plants. In *C. gayana*, the concentrations of alanine, glycine, and γ-aminobutyric acid were

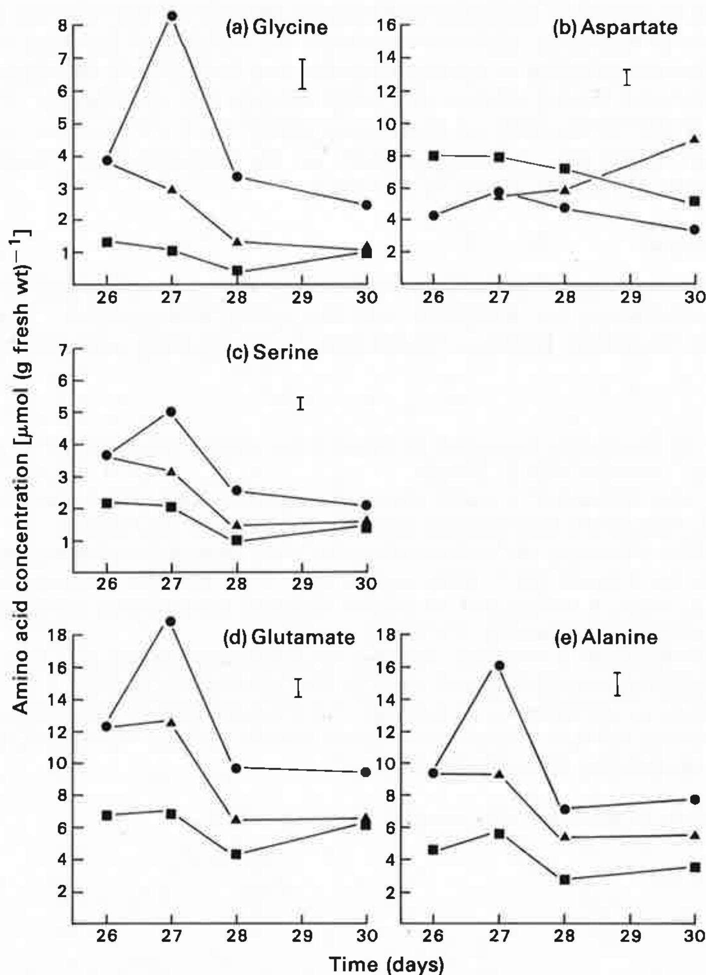

Fig. 1. Changes in concentration of amino acids in mature leaves of *A. tricolor* during recovery from sodium deficiency. ● No addition. ■ 0·1 mM NaCl, day 14. ▲ 0·1 mM NaCl, day 26. Bars indicate 2 × maximum s.e.m.

significantly higher in sodium-deficient plants even though the total concentration of free amino acids was unchanged by the sodium treatment.

In both species, aspartate was the only quantitatively important amino acid found in significantly lower concentrations in sodium-deficient than normal plants. However, the concentrations of arginine in both species, and glutamine, threonine, cysteine plus cystine, lysine, phenylalanine, tyrosine and asparagine in *C. gayana*, were also lower in sodium-deficient than normal plants, though these were present in relatively small quantities.

In *A. tricolor*, the pool sizes of the quantitatively important amino acids glycine, serine, glutamate and alanine decreased significantly in recovering plants within 1 day of receiving sodium (Fig. 1). Within 4 days, all but alanine had reached the concentrations observed in normal plants. The concentration of aspartate, however, increased slowly in recovering plants, becoming significantly greater than that of deficient plants within 4 days of receiving sodium. These changes preceded visual signs of recovery, indicating a relationship to sodium nutrition.

The finding of increased alanine and decreased aspartate concentrations in sodium-deficient plants is consistent with the suggestion by Nable and Brownell (1984) that increases in the concentration of alanine were due to a limitation in the regeneration of phospho*enol*pyruvate from pyruvate with which alanine is in equilibrium. If there was a limitation in the production of phospho*enol*pyruvate from pyruvate in sodium-deficient plants, aspartate, an early product of C_4 photosynthesis, would also be expected to decrease, as was found in this study.

Acknowledgments

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Publication 17

In vivo Chlorophyll *a* Fluorescence in Sodium-deficient C_4 Plants

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Abstract

The fluorescence ratio $(F_P - F_I)/F_I$ gave a consistent value when leaf tissue weights greater than 0·1 g were used and was lower in sodium-deficient than normal C_4 plants. Of the group I elements only sodium, irrespective of the salt supplied to deficient cultures, increased the fluorescence ratio to that observed in normal plants. The concentration of sodium required to increase the fluorescence ratio corresponded to the concentration of sodium required to bring about a growth response in *Kochia childsii*. The application of sodium, either directly onto the leaves or via the culture solution, brought about a rapid and parallel increase in the chlorophyll *a/b* ratio and the fluorescence ratio in *K. childsii.*

Introduction

Induced chlorophyll *a* fluorescence has been widely used as a non-destructive photosynthetic probe. Changes in chlorophyll *a* fluorescence *in vivo* reflect underlying changes in pigment composition and the electron transport through photosystem II (Papageorgiou 1975). The amount of variable fluorescence as a proportion of the maximum fluorescence yield has been used as a measure of photosynthetic efficiency (Simpson and Robinson 1984).

Manganese deficiencies in plants have been shown to be associated with reduced chlorophyll *a/b* ratios (Anderson and Thorne 1968; Simpson and Robinson 1984), changes in chlorophyll *a* fluorescence (Itoh 1968; Heath and Hind 1970; Simpson and Robinson 1984), differences in thylakoid ultrastructure, and reduced photosynthetic efficiency (Simpson and Robinson 1984).

As sodium-deficient plants have been shown to have reduced chlorophyll *a/b* ratios (Johnston *et al.* 1984*a*) and differences in chloroplast ultrastructure have been observed (Boag 1981), it is therefore likely that sodium nutrition would affect chlorophyll *a* fluorescence. The effect of sodium nutrition on room-temperature chlorophyll *a* fluorescence characteristics of C_4 plants is examined in this paper.

Materials and Methods

Plant Material

Amaranthus tricolor L. (non-pigmented form), *Chloris gayana* Kunth, *Echinochloa utilis* Ohwi et Yabuno and *Kochia childsii* Hort. were used as representatives of each of the three types of C_4 plants described by Gutierrez *et al.* (1974) and Hatch *et al.* (1975). The representative C_3 species used were *Atriplex prostrata* Boucher ex DC., *Lycopersicum esculentum* Miller (tomato) and *Triticum aestivum* L. em. Thell.

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Growth Conditions

The procedures for the germination and growth of plants under conditions of low sodium have been described previously (Brownell 1979). The concentration of sodium as an impurity in the complete culture solution was approximately 0·08 μM. Normal plants were obtained by supplying appropriate cultures with NaCl to give a final concentration of 0·1 mM except where otherwise specified. Where the other group I elements were used, they were supplied as chlorides. The sodium as an impurity contributed by these salts gave sodium concentrations less than 0·02 μM in the final culture solution. In experiments where sodium was supplied directly to the leaves of sodium-deficient plants, a 0·2 M NaCl solution in 40% ethanol was liberally sprayed onto the leaves using a hand spray.

Plants were grown in 3·5-litre culture vessels containing 20 or 40 plants each or 2-litre vessels containing eight plants each, in a naturally illuminated growth cabinet supplied continuously with filtered air to minimise sodium contamination. The maximum photon irradiance was approximately 2000 μmol m^{-2} s^{-1}.

Harvesting Procedure

The age of plants at harvest was between 25 and 35 days. Dry weights were obtained after drying to constant weight at 90°C. The concentration of sodium in the leaves was determined as described by Johnston *et al.* (1984*b*).

Chlorophyll Fluorescence

Fluorescence terminology used in this paper is as follows: non-variable fluorescence, F_o; the peak of induced fluorescence, F_p; variable fluorescence, F_v ($F_v = F_p - F_o$); the fluorescence rise F_o plus the activation rise from F_o to I, F_I; Papageorgiou (1975).

In vivo chlorophyll *a* fluorescence was measured using a Hansatech LD-2 instrument coupled to a Linear Instruments model 585 recorder. As the response time of the recorder was slow in relation to the fast fluorescence kinetics response, the F_o value could not be determined. The first interruption in the response curve corresponds to I as designated by Papageorgiou (1975). The actinic light from a Schott Mainz fibre optic halogen lamp was passed through a Schott BG38-2 filter which gave a photon irradiance of approximately 180 μmol m^{-2} s^{-1} at the leaf surface. The filter on the photodiode detector was a Wratten 87C supplied with the Hansatech instrument. Light and dark periods were manipulated in order to maintain metabolite pools at relatively high levels (Sivak and Walker 1983) and to provide a consistent pretreatment of the leaves. Three consecutive measurements were made in a sequence similar to that described by Sivak and Walker (1983). Mature leaf tissue removed from the plants in the growth cabinet was rapidly transferred to the fluorescence chamber and the first measurement was made. After the 100-s exposure to light, which constituted the first measurement, the chamber was darkened for 40 s, re-illuminated for 100 s, redarkened for 2 min and then re-illuminated for 100 s, at the onset of which the next reading was taken. Consecutive readings did not differ significantly from one another. The mean of the three consecutive readings was regarded as a single sample. Each sample was taken from a different plant.

Leaf discs were cut to fit the 37-mm-diameter fluorescence chamber. Those species which have strap-shaped leaves were cut into sections and enough leaves were used to cover the floor of the chamber. Air was passed through the chamber at a constant rate. Measurements were carried out in an air-conditioned environment controlled at 23±2°C.

The light/dark cycle used for the C_4 plants gave inconsistent results with the C_3 plants, as often the first measurement lacked a definable variable fluorescence yield. The C_3 plants were dark-treated for a minimum of 10 min prior to a single measurement.

In the experiments where chlorophyll *a*/*b* ratios were also determined, the leaf tissue was removed from the fluorescence chamber and ground with 96% ethanol (v/v) in the presence of 0·1 g of $CaCO_3$ and acid-washed sand. The chlorophyll extraction was carried out at 4°C under low light. Chlorophyll was determined using the equations of Wintermans and De Mots (1965).

Statistical Treatment

Data were analysed using logarithmic curvefit, analysis of variance and *t*-test.

Results and Discussion

Room temperature fluorescence induction kinetics of sodium-deficient and normal leaves of *K. childsii* are shown in Fig. 1. An increase in F_I and a decrease in $F_p - F_I$ fluorescence is apparent in sodium-deficient leaves.

In a preliminary experiment, the relationship between the weight of leaf tissue in the fluorescence chamber and fluorescence parameters was examined. Logarithmic increases in $F_p - F_I$ and F_I fluorescence with increasing tissue weight from leaves of both sodium-deficient and normal plants were observed ($R^2 > 0 \cdot 90$) in *K. childsii* (Fig. 2). The F_I component was significantly higher ($P < 0 \cdot 05$) in sodium-deficient than in normal leaves at all tissue weights. When tissue weights greater than 0·05 g were used, the $F_p - F_I$ fluorescence component was significantly lower ($P < 0 \cdot 05$) from sodium-deficient than normal leaves. The fluorescence ratio $(F_p - F_I)/F_I$, remained constant over a wide range of leaf tissue weights and was significantly lower ($P < 0 \cdot 001$) from sodium-deficient than normal leaves of *K. childsii* (Fig. 2). This fluorescence ratio was subsequently used as a reliable measure of fluorescence.

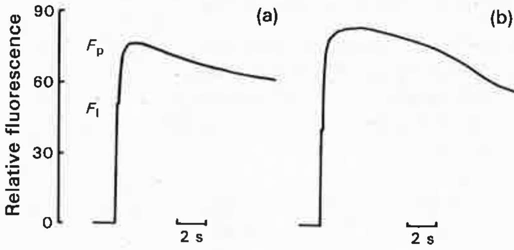

Fig. 1. Induction kinetics for chlorophyll *a* fluorescence from sodium-deficient (a) and normal leaves (b) of *K. childsii.*

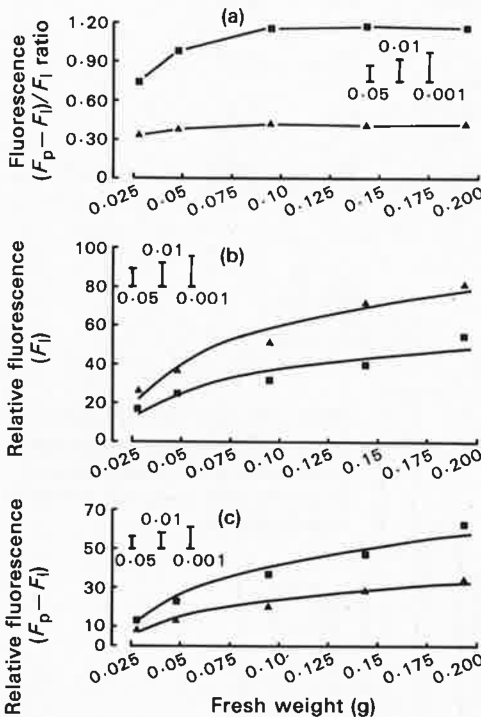

Fig. 2. Change in the relative fluorescence ratio (*a*), relative F_I fluorescence (*b*) and relative $F_p - F_I$ fluorescence (*c*) with increasing leaf weight of *K. childsii.* Bars show l.s.d. for the *P* levels indicated.
▲ No addition of sodium.
■ 0·1 mM NaCl.

A lower fluorescence ratio in sodium-deficient than normal plants was observed in each of the C_4 species (Table 1). The representative C_3 species showed no difference in

the fluorescence ratio when grown with or without added sodium. As sodium has been shown to be essential only for plants possessing the C_4 system (Brownell and Crossland 1972), and as sodium changed the fluorescence ratio only in the C_4 species, it appears that the reduction in fluorescence ratio could be linked to sodium nutrition.

Equivalent concentrations of sodium, supplied in cultures of sodium-deficient plants of *K. childsii* as the chloride, sulfate, nitrate, dihydrogen orthophosphate or bicarbonate

Table 1. Effect of sodium nutrition on fluorescence ratio $(F_p - F_I)/F_I$

Significance of differences between adjacent values: n.s., not significant; * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$. $n > 10$

	Fluorescence $(F_p - F_I)/F_I$ ratio No addition		0·1 mM NaCl
	C_4 species		
NADP-ME type			
Kochia childsii	0·49	***	1·08
Echinochloa utilis	0·67	***	0·81
PEP-CK type			
Chloris gayana	0·81	***	0·96
NAD-ME type			
Amaranthus tricolor	0·29	***	0·70
	C_3 species		
Atriplex prostrata	2·03	n.s.	2·41
Lycopersicum esculentum	2·10	n.s.	1·95
Triticum aestivum	1·73	n.s.	1·62

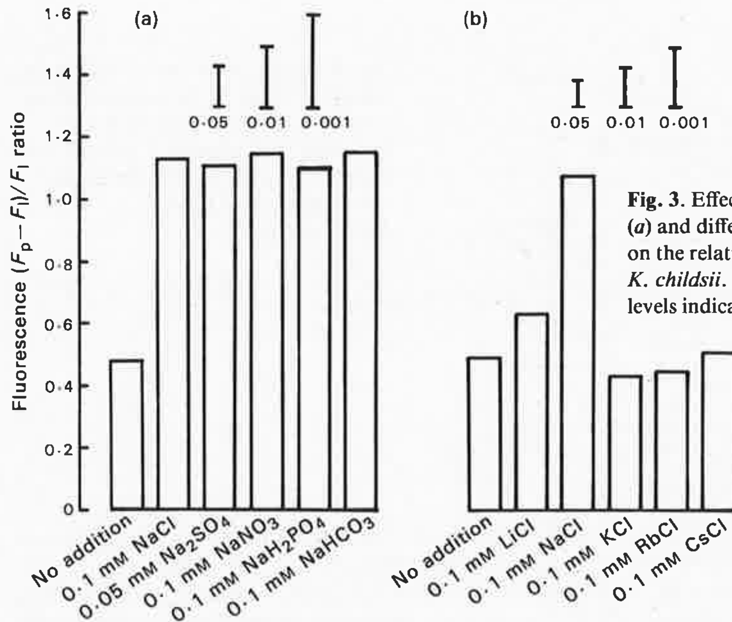

Fig. 3. Effect of different sodium salts (*a*) and different group I elements (*b*) on the relative fluorescence ratio of *K. childsii*. Bars show l.s.d. for the *P* levels indicated.

gave significant increases ($P < 0·001$) in the fluorescence ratio, compared to plants grown without added sodium (Fig. 3*a*).

Of the group I elements, supplied as the chlorides of lithium, sodium, potassium, rubidium and caesium to give a final concentration of 0·1 mM in cultures of sodium-deficient plants of *K. childsii*, only sodium markedly increased the fluorescence ratio

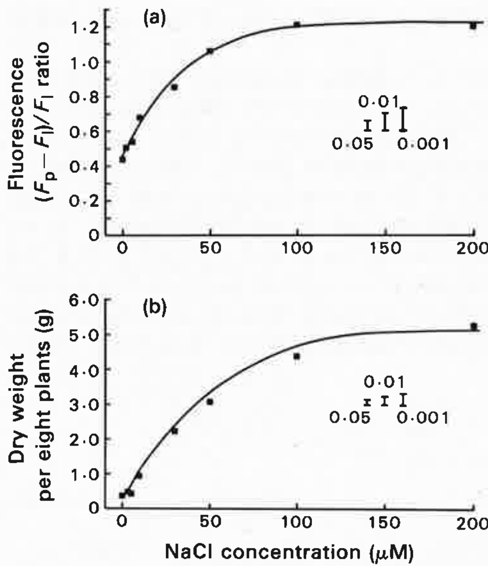

Fig. 4. Effect of sodium chloride concentration upon relative fluorescence ratio (a), and yield (b) of *K. childsii*. Bars show l.s.d. for the P levels indicated.

(Fig. 3b). A marginal increase was also observed in the culture supplied with LiCl; however, it was still significantly less ($P < 0 \cdot 001$) than in the culture supplied with NaCl. These results indicate that the increase in fluorescence ratio is specifically attributable to sodium.

When plants of *K. childsii* were grown in increasing concentrations of sodium chloride, the fluorescence ratio increased sharply, reaching a plateau at approximately 100 μM NaCl (Fig. 4). The fluorescence ratio was similar to the yield in its response to sodium

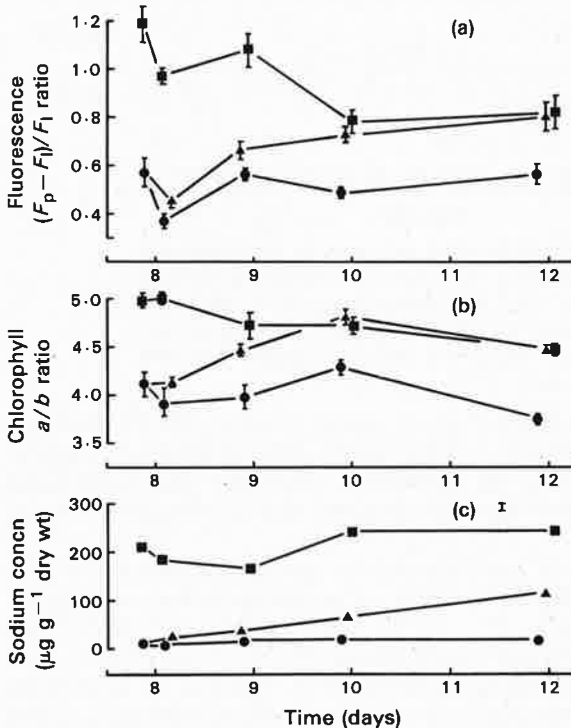

Fig. 5. Changes in relative fluorescence ratio (a), chlorophyll a/b ratio (b) and sodium concentration (c) in leaves of *K. childsii* during recovery from sodium deficiency, after the addition of sodium to the culture solution.
(a) Mean ± s.e.m., $n = 3$.
(b) Mean ± s.e.m., $n = 5$.
(c) Mean ± s.e.m., $n = 4$.
■ 0·1 mM NaCl., day 0.
▲ 0·1 mM NaCl, day 8.
● No addition.

concentration, suggesting a close relationship between the fluorescence ratio and sodium nutrition.

The introduction of sodium into the culture solution of sodium-deficient plants resulted in a small, significant increase in both the fluorescence ratio and chlorophyll *a/b* ratio within 24 h (Figs 5*a*, 5*b*). Within 2 days, the recovering plants had similar fluorescence ratios and chlorophyll *a/b* ratios to normal plants. The increases in fluorescence ratios and chlorophyll *a/b* ratios of the recovering plants may have been limited by the slow rate of sodium uptake from the culture solution (Fig. 5*c*). In an attempt to overcome this, leaves of sodium-deficient plants were sprayed with 0·2 M NaCl in 40% (v/v) ethanol. A significant increase ($P < 0{\cdot}05$) in both the fluorescence ratio and the chlorophyll *a/b* ratio was observed in leaves sprayed with the sodium solution within 4 h (Fig. 6). These rapid changes appear to be due to sodium, as the

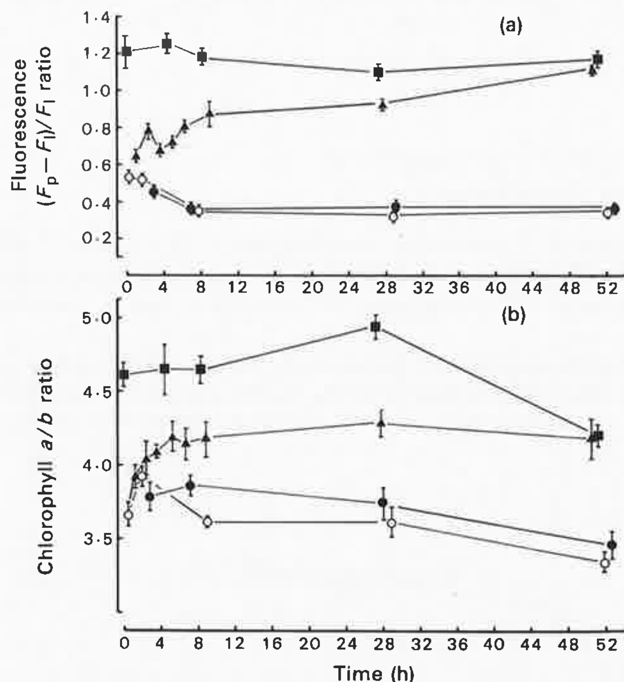

Fig. 6. Changes in the relative fluorescence ratio (*a*) and chlorophyll *a/b* ratio (*b*) during short-term recovery from sodium deficiency in leaves of *K. childsii*. (*a*) Mean ± s.e.m., $n = 3$. (*b*) Mean ± s.e.m., $n = 5$. ■ 0·1 mM NaCl supplied to culture solution 10 days prior to time 0. ▲ Leaves sprayed with 0·2 M NaCl in 40% ethanol at time 0. ● Leaves sprayed with 40% ethanol at time 0. ○ No treatment.

fluorescence ratio and the chlorophyll *a/b* ratio of plants sprayed with 40% ethanol alone did not differ significantly from those of the sodium-deficient plants throughout the experiment. The rapid increase in the fluorescence ratio and the concomitant increase in chlorophyll *a/b* ratio may represent an early step in the recovery from sodium deficiency.

Recent analysis of fast fluorescence kinetics using the system described by Norrish *et al.* (1983) enabled the determination of F_o values. A good relationship between the fluorescence ratio described in this paper $(F_p - F_I)/F_I$, and the F_v/F_o ratio ($R^2 > 0{\cdot}90$) was observed for both sodium-deficient and normal leaves. The F_v/F_o ratio may be used as a measure of photosynthetic efficiency as an increased F_o indicates a reduction in photon harvesting efficiency and a reduced F_v is related to reduced utilisation of the

absorbed light energy (Kriedemann *et al.* 1985). The lower $(F_p - F_I)/F_I$ ratio could therefore reflect reduced photosynthetic efficiency in sodium-deficient plants.

The decreased fluorescence ratios, and the correspondingly decreased chlorophyll *a/b* ratios in sodium-deficient compared to normal plants may be due to differences in the organisation and/or reduced numbers of light-harvesting and reaction centres in the mesophyll chloroplasts. This would be consistent with the suggestions of Johnston *et al.* (1984*a*) that sodium nutrition affects the energy production of mesophyll and/or bundle sheath cells and of Nable and Brownell (1984) that there is a limitation in the conversion of pyruvate to phospo*enol*pyruvate in the mesophyll cells of sodium-deficient plants. In the C_4 species *Chloris barbata* and *Eleusine indica*, Boag (1981) found differences in the organisation of chloroplasts from sodium-deficient and normal plants. Electron micrographs revealed fewer lamellae and distinctive areas of unappressed stromal lamellae in chloroplasts from sodium-deficient plants.

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PHOTOSYNTHETICA 22 (3): 459—461, 1988

BRIEF COMMUNICATION

Effect of Increased Ambient Oxygen Concentration on the Growth of Sodium-Deficient C_4 Plants*

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Abstract

Increased ambient O_2 concentration during the photoperiod significantly decreased the growth of sodium-deficient plants of *Amaranthus tricolor* L. and *Kochia childsii* hort., but sodium-sufficient plants were not affected.

Sodium is an essential micronutrient element for plants having the C_4 and CAM photosynthetic pathways but not those having the C_3 pathway (Brownell 1979). Sodium may be needed for the operation of the CO_2 concentrating mechanism in C_4 species thus maintaining the carboxylase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. In agreement with this, Na-deficiency is largely overcome by growing plants in atmospheres with elevated CO_2 concentrations (Johnston *et al.* 1984, 1986). If the concentration of CO_2 in the bundle-sheath is increased in Na-deficient plants growing in elevated concentrations of CO_2, it is likely that the concentration of oxygen in the bundle-sheath could also be maintained at high concentrations by growing plants in atmospheres with elevated concentrations of oxygen. Plants, in which the operation of the C_4 appendage is impaired by Na-deficiency, would then be expected to exhibit the Warburg effect (Forrester *et al.* 1966a) when grown in elevated ambient concentrations of oxygen. It was the aim of this work to study the effects of growing plants under increased concentrations of oxygen on dry mass of Na-deficient and Na-sufficient plants.

Amaranthus tricolor L. and *Kochia childsii hort.* were germinated and grown in 2000 cm^3 containers with 4 or 8 plants under low-Na conditions as described by Brownell (1979). The concentration of Na as an impurity was *ca.* 0.08 μM. Na-sufficient plants were grown in a final Na concentration of 0.1 mM. The containers were enclosed in 0.2 m^3 (200 l) naturally irradiated glass cabinets (maximum irradiance within 400—700 nm was *ca.* 2500 $\mu mol\ m^{-2}\ s^{-1}$). During the light period the growth cabinets were supplied with atmospheres containing 242 $cm^3\ (CO_2)\ m^{-3}$ and 21 % O_2 (control) or 42 % O_2 (high O_2); ambient air was supplied during the dark period. All cultures were aerated with normal air. Gas flow rates were [$cm^{-3}\ s^{-1}$] 16.67 for normal air, 7.80 for culture aeration, and 8.87 for CO_2-free air or oxygen. The age of plants at harvest was between 23 and 42 d. The samples were dried at 90 °C (to obtain dry mass). Results were analysed using analysis of variance and *t*-test. Log transformations were carried out on dry mass data to equalize the error variances.

The signs of sodium-deficiency were accentuated in Na-deficient plants grown in 42 % O_2 as compared with those grown in 21 % O_2. The Na-sufficient plants appeared relatively unaffected by the elevated O_2 concentration. The dry mass of both *K. childsii* and *A. tricolor* was reduced significantly ($P < 0.01$) in Na-deficient plants (Table 1). The dry mass of Na-sufficient plants was unaffected by the O_2 concentration. The difference between the responses of the Na-deficient and

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Table 1

The effect of sodium nutrition and oxygen concentration on growth of *Kochia childsii* and *Amaranthus tricolor*. Means are from two experiments. Plants were harvested on day 33 (*A*) or 26 (*B*) in experiment 1 and on day 42 (*A*) or 23 (*B*) in experiment 2. All values are the means of dry masses from a total of six (*A*) or eight (*B*) 2000 cm^3 cultures of 4 plants each. **: significant at $P < 0.02$, ***: significant at $P < 0.01$.

	Dry mass [g per plant]		
	21 % O_2		42 % O_2
(*A*) *K. childsii*			
No addition	0.060	**	0.037
	**		***
0.1 mM NaCl	0.178	n.s.	0.170
(*B*) *A. tricolor*			
No addition	0.016	***	0.008
	***		***
0.1 mM NaCl	0.052	n.s.	0.063

Na-sufficient C_4 plants was highlighted by the interaction of Na and O_2 which was significant ($P < 0.02$) in all experiments. Thus Na-deficient plants are more sensitive to ambient O_2 concentrations than Na-sufficient plants. The increased ambient O_2 concentration may lead to an increase of the O_2 concentration within the bundle-sheath cells. In Na-deficient plants, where the C_4 system may be impaired, the CO_2 concentration would be reduced and the subsequent increase in the O_2/CO_2 concentration ratio in the bundle-sheath could lead to increased activity of the photosynthetic carbon oxidation cycle. The observed significant reduction in dry mass production in Na-deficient plants with increased O_2 concentration supports this hypothesis. Increased concentrations of amino acid intermediates of the photorespiratory pathway, glycine and serine, in Na--deficient plants (Boag 1981, Grof *et al.* 1986) and the accumulation of ^{14}C label in these compounds reported by Boag (1981) also support this suggestion.

An alternative explanation requires that the C_4 pathway in both Na-deficient and Na-sufficient plants functions at a similar rate. Under normal conditions, the concentration of intermediate pools of CO_2 and HCO_3^- range from 0.6 to 2 mM in the bundle-sheath cells of C_4 plants (Hatch 1976, Hatch and Osmond 1976) creating a gradient between the bundle-sheath and the mesophyll cells. The reduction in yield observed in Na-deficient plants could be attributed to a possibly increased leakiness of the bundle-sheath to both CO_2 and O_2, thus negating the CO_2 concentrating effect of the C_4 appendage. If this were the case, the CO_2 and O_2 concentrations in the bundle-sheath would be predominantly governed by the concentration of the ambient gas. However, Boag (1981) found that the quantum yield in both Na-deficient and Na-sufficient plants was similar. This would not be so if the bundle-sheath was leakier in Na-deficient C_4 plants as additional energy would be required to carry out the CO_2 concentrating process in the bundle-sheath.

Previous work indicates that the response of plants to supra-atmospheric O_2 concentration and the effect on C_4 photosynthesis depends upon the species and the length of exposure (Forrester *et al.* 1966b, Gale and Tako 1976, Ku and Edwards 1980). Boag (1981) observed in three species of C_4 plants no significant change in photosynthesis in either Na-deficient or -sufficient plants when they were exposed to 2, 21 or 50 % O_2 for only 45 min. However, "prolonged" exposure to supra-atmospheric O_2 concentrations led to a decrease in the rate of CO_2 uptake in both Na-deficient

and Na-sufficient plants. Unlike C_3 plants which may equilibrate very rapidly, C_4 plants may take time to reach steady state photosynthesis (Gale and Tako 1976, Ku and Edwards 1980). Thus the implication by Boag (1981) that the C_4 pathway metabolism effectively suppresses photorespiration in Na-deficient plants may only be true in the short term.

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Effect of Sodium Nutrition on the Ultrastructure of Chloroplasts of C_4 Plants

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ABSTRACT

Mesophyll chloroplasts from sodium-deficient compared to normal plants of the C_4 species *Kochia childsii* and *Amaranthus tricolor* were found to have significantly less stacking in their grana. On the other hand, no marked difference of thylakoid arrangement between bundle sheath chloroplasts from sodium-deficient and normal plants of *A. tricolor* were observed.

Sodium is unique as a micronutrient for higher plants in that it is required only by plants possessing the C_4[1] CO_2-concentrating appendage (6, 7). The mesophyll cells are implicated as the site of the lesion since increased ambient CO_2 concentration alleviated the signs of sodium-deficiency without restoring the lower Chl *a/b* ratio, characteristic of sodium-deficiency, to that of normal plants (13–15).

Increased concentrations of alanine (9, 18) and pyruvate and decreased concentrations of PEP, OAA, malate, aspartate, and 3-PGA (M Johnston, CPL Grof, PF Brownell, unpublished observations) have been found in leaves of sodium-deficient compared to normal C_4 plants, suggesting a limitation in the conversion of pyruvate to PEP.

It is unlikely that this limitation is due to reduced activity of pyruvate orthophosphate dikinase, the enzyme catalyzing this reaction (4, 8). The transport of pyruvate into the mesophyll chloroplast and its subsequent conversion to PEP may be limited by the supply of ATP from photosynthetic phosphorylation. Decreased Chl *a/b* ratios (13), and fluorescence ratios (10) observed in sodium deficiency are consistent with dysfunction of the light reactions. These differences between sodium-deficient and normal plants suggest that the ultrastructure of mesophyll and/or bundle sheath chloroplasts may also be affected by sodium nutrition.

MATERIALS AND METHODS

Amaranthus tricolor L. (nonpigmented form) and *Kochia childsii* Hort. were used. The procedures for the germination and growth of plants under low sodium conditions have been described previously (5). The concentration of sodium as an impurity in the complete culture solution was approximately 0.08 μM. Normal plants were obtained by supplying appropriate cultures with NaCl to give a final concentration of 0.1 mM.

Leaves of *K. childsii* and *A. tricolor* were cut into thin sections in a fixative containing 3% glutaraldehyde in 0.05 M cacodylate (pH 7.0). The sections were rotated in a small volume of fixative at room temperature for 1 h, rinsed in 0.05 M cacodylate twice, and then left to stand in 0.05 M cacodylate overnight. The following day, the sections were postfixed in 1% OsO_4, in 0.05 M cacodylate for 1 h at room temperature. The OsO_4 was removed and replaced by distilled water. The leaf sections were then subjected to the following dehydration steps: 30% ethanol, 2 min; 50% ethanol, 2 min; 70% ethanol, 2 min; 80% ethanol, 5 min; 85% ethanol, 5 min; 90% ethanol, 5 min; 95% ethanol, 5 min; 100% ethanol, 10 min; 100% ethanol, 10 min; 100% ethanol (dehydrated), 10 min; 1:1, 100% ethanol: Spurr's resin, 2 h; 1:3, 100% ethanol: Spurr's resin, 3 h; 100% Spurr's resin, overnight; 100% Spurr's resin,

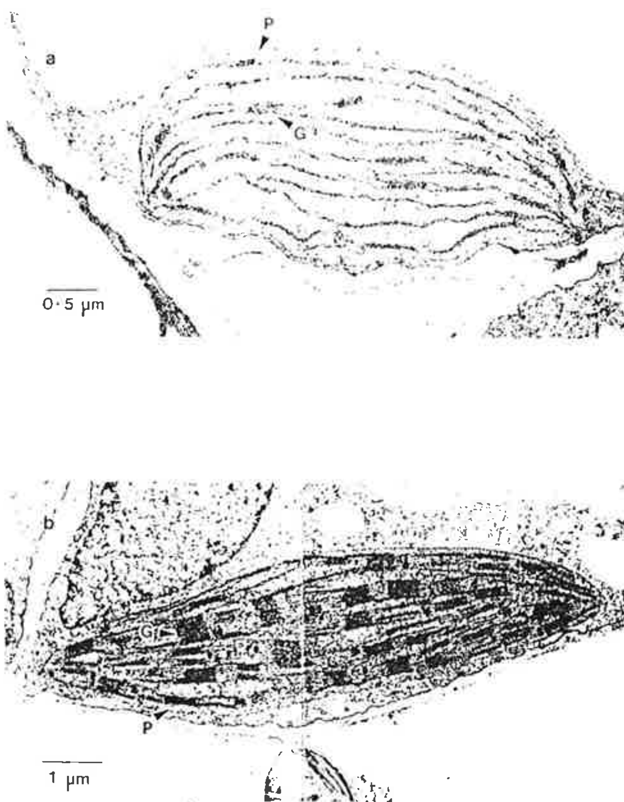

Figure 1. Ultrastructural features of mesophyll chloroplasts from a mature sodium-deficient (a) and a normal (b) leaf of *K. childsii* stained with uranyl acetate and lead citrate. G, grana; P, peripheral reticulum.

[1] Abbreviations: C_4, C_4 dicarboxylic photosynthetic pathway; PEP, phospho*enol*pyruvate; OAA, oxaloacetate; 3-PGA, 3-phosphoglycerate; LHC, light harvesting complex.

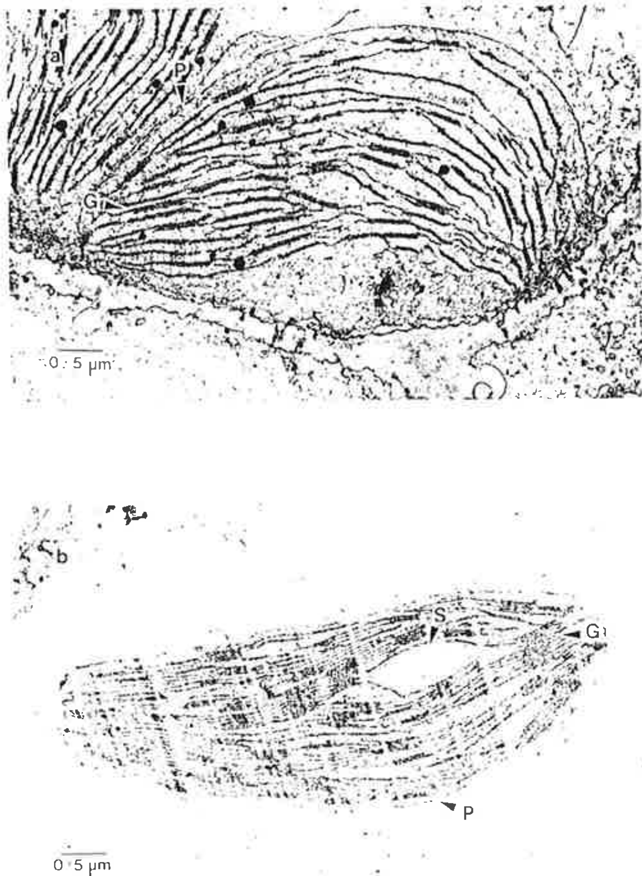

Figure 2. Ultrastructural features of mesophyll chloroplasts from a mature sodium-deficient (a) and a normal (b) leaf of *A. tricolor* stained with uranyl acetate and lead citrate. G, grana; P, peripheral reticulum; S, starch.

2 h. The sections were then transferred to embedding moulds containing 100% Spurr's resin and left overnight to cure at 60°C. Ultrathin sections (70–90 nm) were cut on an LKB Nova ultramicrotome using a diamond knife, stained with uranyl acetate (saturated in 50% acidified ethanol) for 6 min, and lead citrate (19) for 1 min, then examined using a JEOL FX2000 electron microscope.

Numerous sections were cut from a minimum of four leaves from three separate plants of each treatment. Once a particular section was photographed on account of its distinguishable detail within the chloroplast, a minimum of 5 µm was cut off the face of the block to ensure that the same chloroplast was not photographed again. Generally, the block was entirely refaced prior to more ultrathin sections being cut. Prints (10.15 × 12.70 cm) were made of each chloroplast. The thylakoids making up each granal stack were counted using a stereo dissecting microscope. The chloroplasts selected for counting were chosen on the basis of clarity to ensure a minimum of counting errors.

A chi-square analysis was carried out on the frequencies of granal stacks comprised of different numbers of thylakoids. Ten representative mesophyll and bundle sheath chloroplasts from both sodium-deficient and normal leaves of *K. childsii* and *A. tricolor* were examined.

RESULTS AND DISCUSSION

Mesophyll Chloroplasts

From Figures 1 and 2, it is evident that the amount of stacking in the grana of sodium-deficient mesophyll chloroplasts was markedly less than in the normal chloroplasts of both *Kochia childsii* and *Amaranthus tricolor*. This is supported by the statistical treatment of the data which show significant differences ($P < 0.01$, *K. childsii*; $P < 0.001$, *A.*

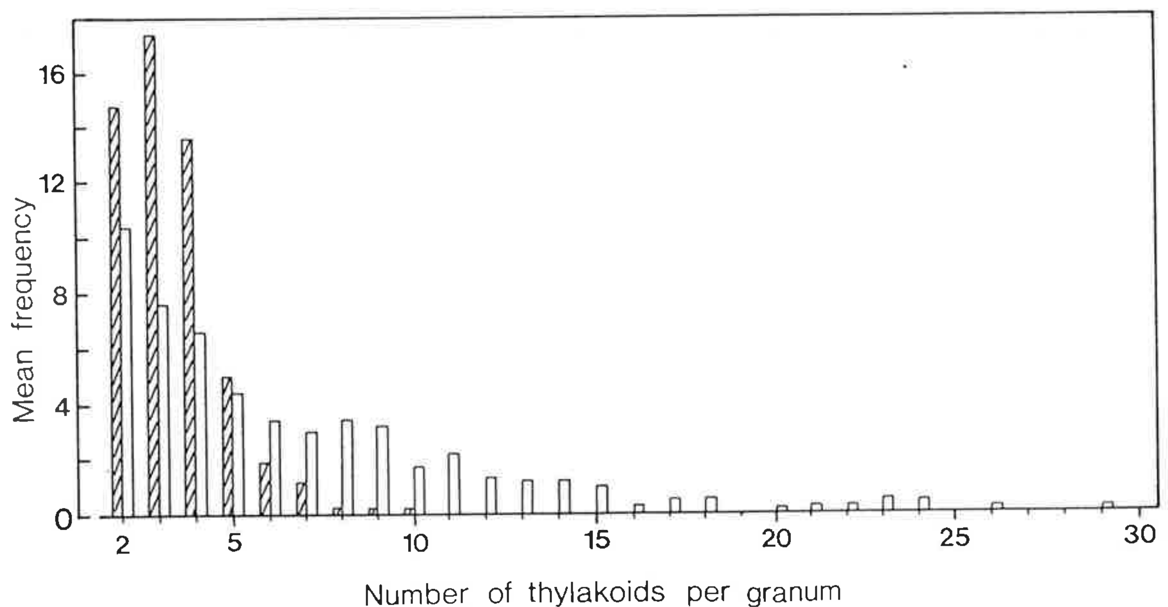

Figure 3. The mean frequency of grana containing 2 to 30 thylakoids per stack in mesophyll chloroplasts from mature sodium-deficient (hatched columns) and normal (open columns) leaves of *K. childsii*.

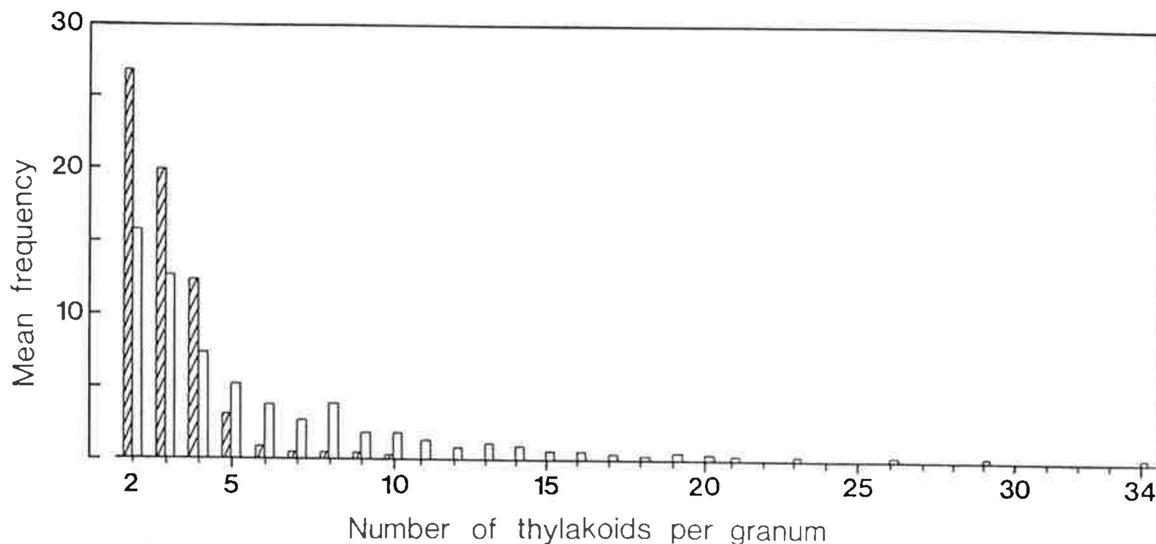

Figure 4. The mean frequency of grana containing 2 to 34 thylakoids per stack in mesophyll chloroplasts from mature sodium-deficient (hatched columns) and control (open columns) leaves of *A. tricolor*.

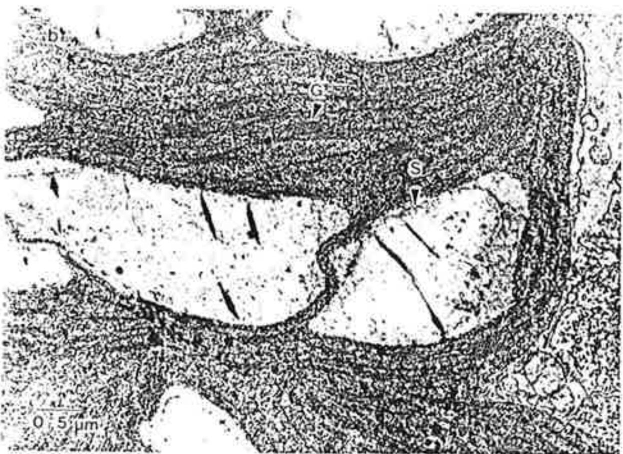

Figure 5. Ultrastructural features of bundle sheath chloroplasts from a mature sodium-deficient (a) and a normal (b) leaf of *A. tricolor* stained with uranyl acetate and lead citrate. G, grana; M, mitochondrion; S, starch.

tricolor) between sodium-deficient and normal chloroplasts in their arrangement of thylakoids into grana (Figs. 3 and 4).

Bundle Sheath Chloroplasts

No marked differences were observed between the thylakoid arrangement of bundle sheath chloroplasts from sodium-deficient and normal plants of *A. tricolor* (Fig. 5). Furthermore, the frequency of thylakoid stacking in the grana did not differ significantly (Fig. 6). The bundle sheath chloroplasts from both sodium-deficient and normal leaves of *K. childsii* were essentially agranal or paucigranal as has been previously reported in other NADP-ME types of C_4 plants (3).

Functional Importance of Granal Stacking

Weier and Benson (22) suggested that the accumulation of carotenoid pigments as a result of stacking may protect the Chl pigments from bleaching by light at high intensities. The concentration of photosynthetic pigments between the bilayer membranes could also facilitate energy transfer between molecules. Gunning and Steer (11) proposed that the greater stacking of grana may confer an advantage by providing a suitable environment for energy transfer between PSII and PSI. Kaplan and Arntzen (16) suggested that stacking has resulted in improved control of light harvesting and photochemical operations.

Murata (17) proposed a mechanism in intact algae which controls the relative proportions of light energy being directed to PSI and PSII due to structural rearrangements of the pigment antennae in response to a change in light conditions. This optimises the energy utilization.

The physical mechanisms which control granal stacking are described by Staehelin (20) and Barber (1). The physical changes responsible for the direction of the energy involve the phosphorylation/dephosphorylation of a mobile Chl *a*/*b* LHC (2, 21). This affects the membrane charge distribution and consequently the distance of the Chl *a*/*b* LHC from either the PSII-rich stacked or the PSI-rich unstacked regions (21). The decreased amount of granal stacking observed in sodium-

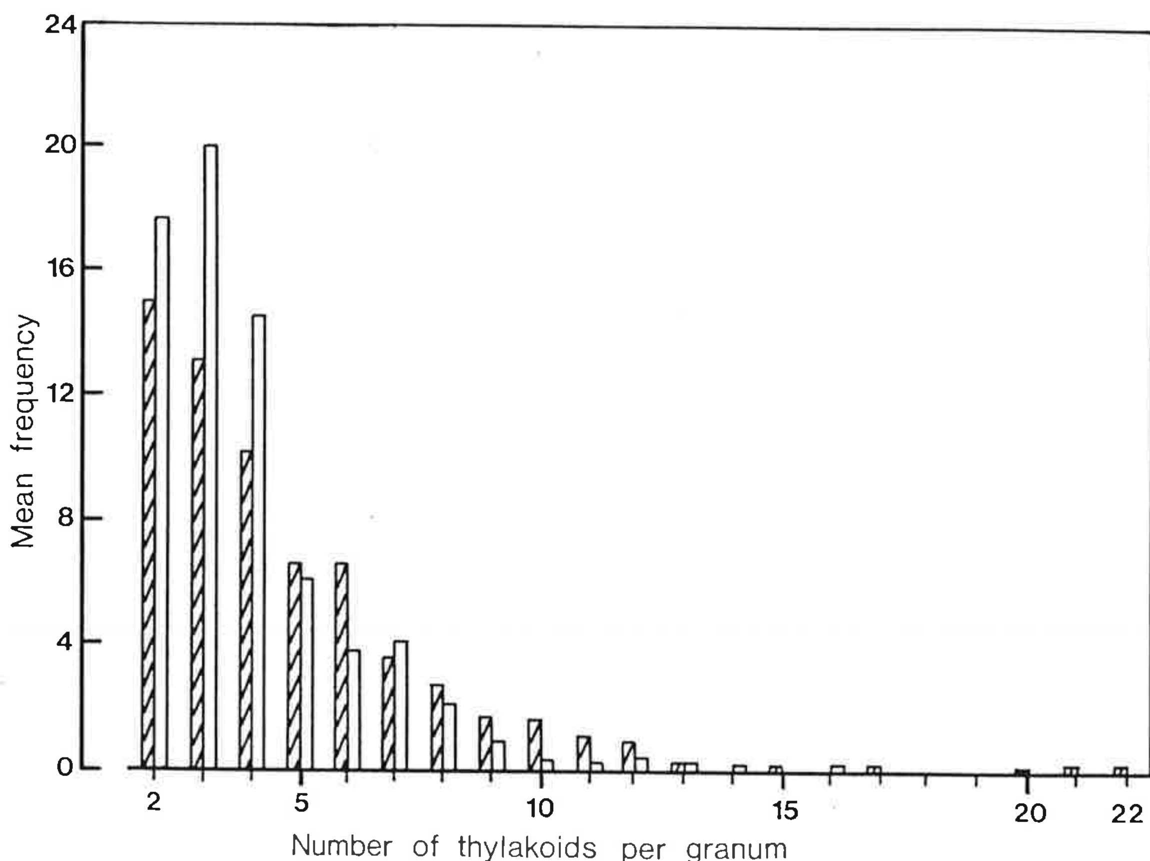

Figure 6. The mean frequency of grana containing 2 to 22 thylakoids per stack in bundle sheath chloroplasts from mature sodium-deficie (hatched columns) and control (open columns) leaves of *A. tricolor*.

deficient mesophyll chloroplasts suggests that a decreased PSII activity may be observed as energy would be directed toward PSI in these plants. It was found that PSII activity was markedly lower in mesophyll thylakoids extracted from sodium-deficient plants representing each of the groups of C_4 plants, *K. childsii* (NADP-ME type), *C. gayana* (PCK-type), and *A. tricolor* (NAD-ME type). PSI activity of mesophyll thylakoids was unaffected by sodium-deficiency in *K. childsii*; however, it was greater in sodium-deficient than control plants of *A. tricolor*. Bundle sheath thylakoids were relatively unaffected by sodium-deficiency (M Johnston, CPL Grof, PF Brownell, unpublished observations). Thus, structural differences in sodium-deficient chloroplasts may be responsible for observed differences in fluorescence (10) and electron transport rates (M Johnston, CPL Grof, PF Brownell, unpublished observations).

The possible reason why there is a difference in response to sodium between chloroplasts of C_3 plants and mesophyll chloroplasts of C_4 plants is that within the C_3 plant there is no physiological counterpart to the mesophyll chloroplasts of C_4 plants. These chloroplasts have the major specialized role of converting pyruvate to PEP whereas the chloroplasts of C_3 plants and bundle sheath cells of C_4 plants have the function of reducing CO_2 via the PCR cycle to photosynthates (12).

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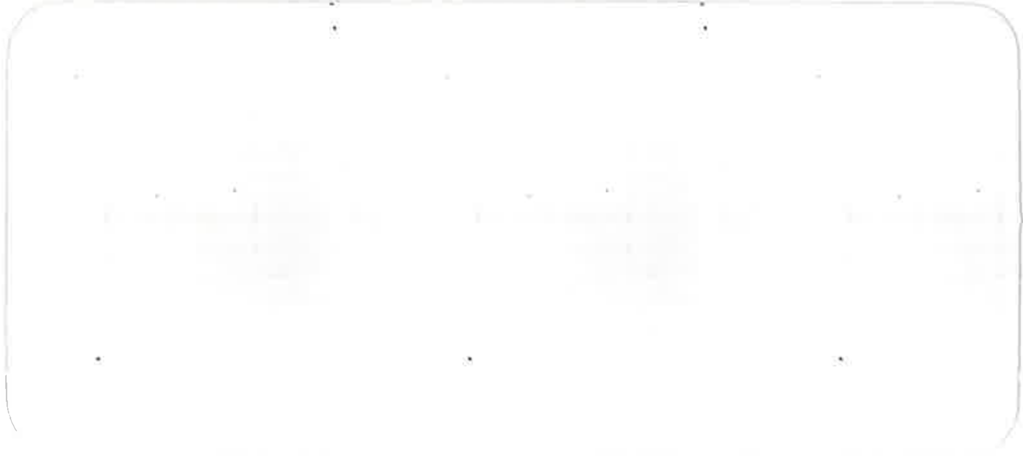

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Characterisation of Leaf Fluorescence of Sodium-deficient C_4 Plants: Kinetics of Emissions from Whole Leaves and Fluorescence Properties of Isolated Thylakoids

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Abstract

Examination of whole-leaf fluorescence kinetics by means of a second-degree polynomial function showed a decrease in the rate of the rise from F_d to F_p in sodium-deficient compared with normal leaves of the C_4 species *Kochia childsii* and *Amaranthus tricolor*. This suggests a decreased efficiency in light harvesting and/or utilisation in sodium-deficient plants.

Fluorescence ratios (F_v/F_o) of separated mesophyll and bundle sheath thylakoids were both lower from leaves of sodium-deficient compared with normal plants of *K. childsii, Chloris gayana, A. edulis* and *A. tricolor*.

Introduction

Sodium is an essential micronutrient for plants having the C_4 pathway but not for C_3 photosynthesis (Brownell and Crossland 1972). Sodium is required for the operation of the C_4 appendage in maintaining high concentrations of CO_2 in the bundle sheath cells, thus ensuring a high efficiency of the photosynthetic carbon reduction cycle (Johnston *et al.* 1984*a*, 1986). Increased levels of alanine (Nable and Brownell 1984; Grof *et al.* 1986*a*) and pyruvate (Johnston *et al.* 1988) and decreased concentrations of phospho*enol*pyruvate (Johnston *et al.* 1988) in sodium-deficient plants suggest a block in the regeneration of phospho*enol*pyruvate from pyruvate in the mesophyll chloroplasts.

Decreased chlorophyll *a*/*b* ratios (Johnston *et al.* 1984*b*), fluorescence yields (Grof *et al.* 1986*b*), PS II activity (Johnston *et al.* 1988) and changed ultrastructure of mesophyll chloroplasts from sodium-deficient leaves (Grof *et al.* 1989) suggest that a reduction in the energy available from the photosystems may limit the conversion of pyruvate to phospho*enol*pyruvate in sodium deficiency.

In an attempt to define the site(s) of the lesion in the chloroplasts of sodium-deficient plants, the effect of sodium nutrition on the kinetics of the whole-leaf fluorescence curve was examined by fitting a polynomial function to the part of the fluorescence emission corresponding to the designates F_d to F_p (Papageorgiou 1975). Such a fit would ensure greater precision, clarifying the comparison between treatments. This could indicate whether sodium deficiency affects the light-harvesting or energy-utilising systems in the chloroplast. Previous work of this type has been restricted to a determination of the maximal rate of induced fluorescence rise as a measure of chilling tolerance in maize populations (Hetherington *et al.* 1983). Papageorgiou (1975) considered that the rise in chlorophyll *a* fluorescence from F_d to F_p is synchronous with a decay in the rate of O_2 evolution. This reduction of O_2 evolution is thought to be brought about by a transient block of electron transport through PS I (Munday and Govindjee 1969), leading to an

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increase in the electron pool between PS I and PS II and ultimately resulting in a greater reduction of the PQ pool.

The relative height of F_p in relation to F_o is dependent primarily on excitation light irradiance and preconditioning in whole leaves. Increased irradiance results in increased ratios of variable to non-variable fluorescence (Krause and Weis 1984).

A further approach in attempting to define metabolic sites in which sodium is involved in C_4 plants, described in this paper, is to examine the effects of sodium nutrition on the fluorescence yields of mesophyll and bundle sheath thylakoids separated by differential blending.

Materials and Methods

The procedures for the germination and growth, under low-sodium conditions, of *Kochia childsii* Hort., *Amaranthus edulis* Speg., *Amaranthus tricolor* L. (non-pigmented form) and *Chloris gayana* Kunth, representing the three types of C_4 plants, have been described (Brownell 1979). The total sodium concentration of the complete culture solution was approximately 0·08 μM. Normal plants were obtained by supplying sodium chloride to give a final concentration of 0·1 mM in appropriate cultures. Plants were grown in either 2-L or 3·5-L culture vessels that contained 8 or 40 plants each and that were placed in a naturally illuminated growth cabinet supplied continuously with filtered air to minimise sodium contamination. The maximum irradiance in the growth cabinet was approximately 2000 μmol $m^{-2} s^{-1}$ (PAR). The age of plants at harvest was between 25 and 32 days.

In vivo chlorophyll *a* fluorescence was measured with a Hansatech LD-2 instrument. The actinic light source (Grof *et al.* 1986*b*) was activated by a strong solenoid-operated fish-eye shutter with an opening time of 1 ms. As the true F_o value is reached within 40–50 μs at room temperature (J. T. Wiskich, personal communication), it is unlikely that the true F_o values would have been obtained with the equipment used in this study. Therefore, the values designated F_o in this paper may not represent the absolute F_o values.

A Wratten 88A filter was used on the photodiode. Mature leaf tissue was removed from plants in the growth cabinet and placed in the fluorescence chamber as described by Grof *et al.* (1986*b*), and the measurement was made.

The fluorescence data were first stored in a fast data-acquisition system similar to that described by Norrish *et al.* (1983) and then transferred to a 640-kbyte IBM-compatible CCS computer. In collaboration with R. Rose (Physics Department, James Cook University of North Queensland), software was developed to display this data on the monitor screen and to allow the points in time corresponding to F_d and F_p to be selected.

Software was written to enable a polynomial function of the second degree to be fitted to the data points between the fluorescence parameters F_d and F_p. Tangents to this fitted line were calculated at selected points in time and the results plotted as the natural log of the tangent against time.

Particles and Thylakoids

'Mesophyll' and 'bundle sheath' thylakoids from sodium-deficient and normal plants were separated by differential blending as described by Johnston *et al.* (1989), who found little cross-contamination between mesophyll and bundle sheath cells.

The fast fluorescence characteristics were measured in a Hansatech chamber designed for liquid suspensions (model DW-2) and in a medium containing 100 mM sorbitol, 2 mM EDTA, 1 mM $MnCl_2$ and 50 mM HEPES (pH 7·6). The same filter and shutter mechanism described for the whole-leaf chamber were used. The irradiance within the chamber, 200–300 μmol $m^{-2} s^{-1}$, depending upon the chlorophyll content of the suspension, was measured with a Sharp blue-silicon photocell (SBC-255) calibrated against a Li-Cor model LI-188B light meter.

Results and Discussion

The rise from F_d to F_p was found to be steeper in the normal than the sodium-deficient leaves of *K. childsii* and *A. tricolor* (Figs 1 and 2), thus supporting the visual observation evident in Figs 3 and 4. If the slopes of these lines are generally equated with the rates of reduction of PQ, inferences can be drawn regarding the photosynthetic

efficiency of the normal compared to the sodium-deficient plants. The particular form of PQ reduced, viz. the bound (Q_a) or unbound (Q_b) pools, cannot be further resolved from this data. The F_d to F_p rise is apparently related to the decrease in O_2 evolution brought about by the lack of an electron acceptor in PS I. Krause and Weis (1984) suggest that the block in PS I is due to the lack of electron acceptors once the NADP pool has been depleted. In addition, the removal of electrons via the Mehler reaction is too slow relative to the size and rate of reduction of the PQ pool.

The height of F_p is dependent primarily upon the intensity of the actinic light source. It therefore follows that when the experimental conditions are equivalent for both treatments, a more rapid rate of reduction of PQ in the normal plants is a result of the increased light-harvesting or electron-transport efficiency of PS II.

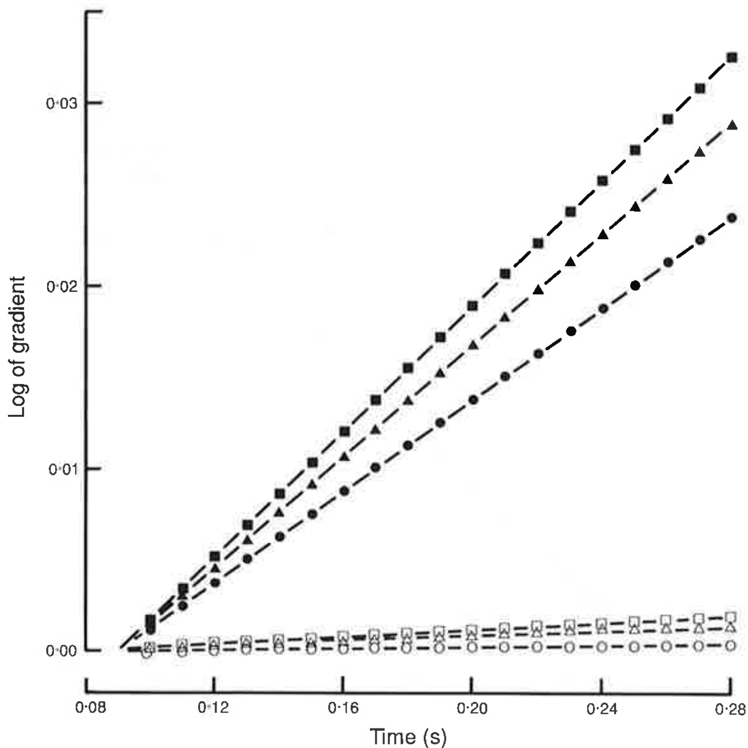

Fig. 1. An expression of the fluorescence rise from F_d to F_p of *Kochia childsii* determined by fitting a second-degree polynomial function. Open symbols, sodium-deficient leaves; solid symbols, normal leaves; each type of symbol represents a different leaf.

These data are consistent with the finding of Grof *et al.* (1986*b*, 1989) that a difference in ultrastructure between sodium-deficient and normal plants is evident in mesophyll chloroplasts and may be responsible for (1) the decrease in fluorescence ratios $(F_p - F_i)/F_i$ and F_v/F_o observed in whole leaves, and (2) a decrease in PS II activity determined *in vitro* (Johnston *et al.* 1989).

The F_v/F_o ratios measured for the separated mesophyll and bundle sheath thylakoids (Figs 5 and 6) are of the same order as those measured for whole leaves (Tables 1 and 2). The fluorescence ratio was significantly higher in both mesophyll and bundle sheath

thylakoids of normal than sodium-deficient plants in the representatives of each of the three subgroups of C_4 plants (Gutierrez *et al.* 1974; Hatch *et al.* 1975).

The F_v/F_o fluorescence ratio of mesophyll and bundle sheath thylakoids was found to decrease with increasing amounts of chlorophyll in the fluorescence chamber (Figs 7 and 8), possibly because of decreased irradiance with increasing chlorophyll concentrations. This is unlikely to have affected the results shown in Table 1 since similar concentrations of chlorophyll were used in comparing the fluorescence ratios of thylakoids extracted from sodium-deficient and normal leaves.

Furbank and Walker (1985) suggest that the mesophyll alone contributes to the whole-leaf fluorescence characteristics in C_4 species. Since *Zea mays*, an NADP-ME

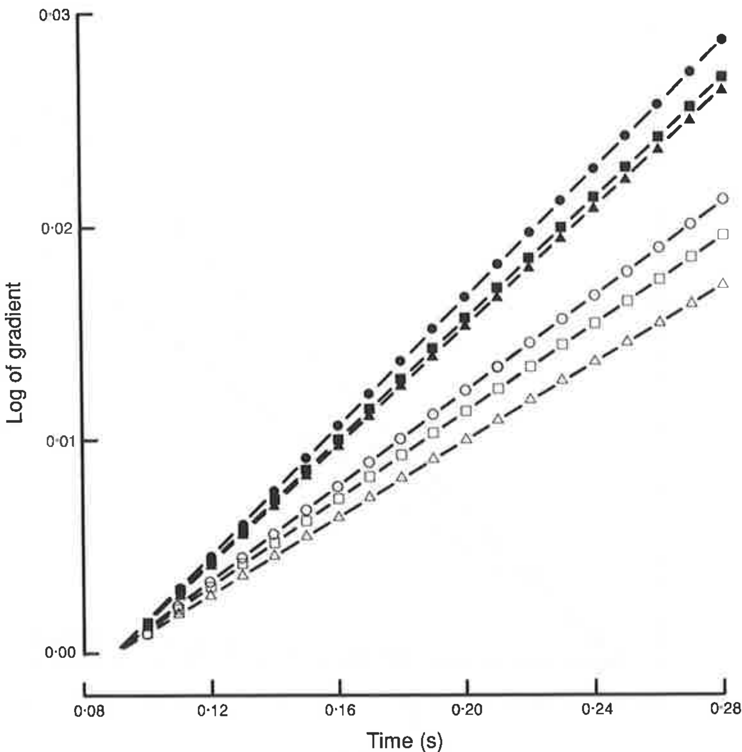

Fig. 2. An expression of the fluorescence rise from F_d to F_p of *Amaranthus tricolor* determined by fitting a second-degree polynomial function. Open symbols, sodium-deficient leaves; solid symbols, normal leaves; each type of symbol rpresents a different leaf.

type of C_4 plant, is deficient in PS II activity in the bundle sheath, and since representatives of the other two types of C_4 plants exhibit very similar fluorescence and CO_2 characteristics, the bundle sheath fluorescence in the PEP-CK and NAD-ME types may be shielded (Furbank and Walker 1985). Sufficient evidence demonstrating the low oxygen-evolving capacity of bundle sheath chloroplasts and thylakoids of maize and other NADP-ME types is documented (Woo *et al.* 1970; Anderson *et al.* 1972; Bazzaz and Govindjee 1973; Golbeck *et al.* 1981; Johnston *et al.* 1989). Golbeck *et al.* (1981) determined that the oxygen-evolving capacity of bundle sheath chloroplasts of *Z. mays*

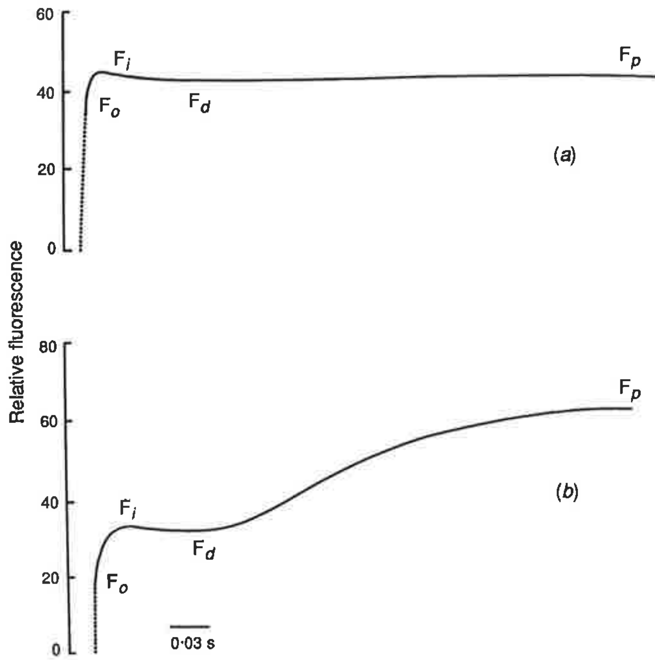

Fig. 3. Induction kinetics for chlorophyll *a* fluorescence from sodium-deficient (*a*) and normal (*b*) leaves of *Kochia childsii*, showing F_i, F_d and F_p transients.

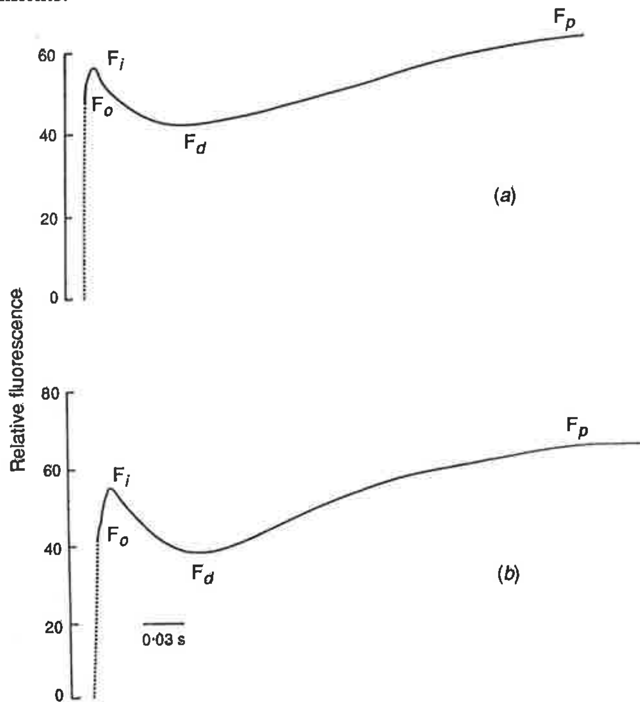

Fig. 4. Induction kinetics for chlorophyll *a* fluorescence from sodium-deficient (*a*) and normal (*b*) leaves of *Amaranthus tricolor*, showing F_i, F_d and F_p transients.

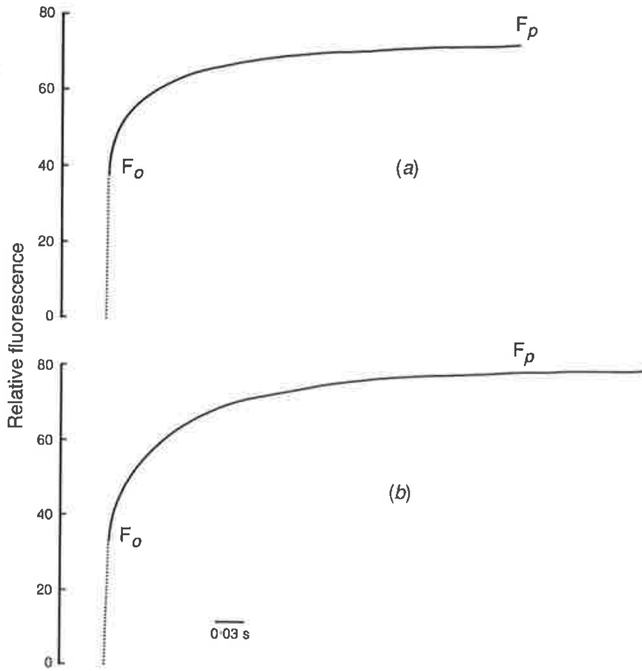

Fig. 5. Induction kinetics for chlorophyll *a* fluorescence of mesophyll thylakoids from leaves of sodium-deficient (*a*) and normal (*b*) *Amaranthus tricolor*.

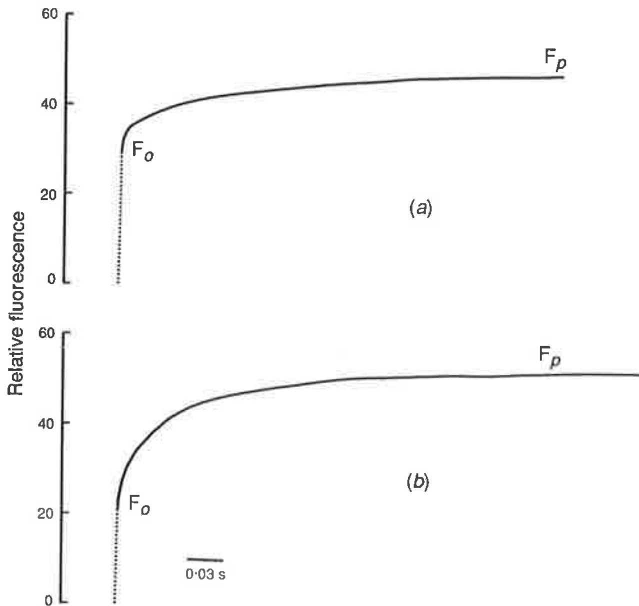

Fig. 6. Induction kinetics for chlorophyll *a* fluorescence of bundle sheath thylakoids from leaves of sodium-deficient (*a*) and normal (*b*) *amaranthus tricolor*.

was only 15–20% of the rates measured in mesophyll chloroplasts. Johnston *et al.* (1989) found that the bundle sheath thylakoids extracted from sodium-deficient plants of *K. childsii*, an NADP-ME type, were, when assayed under high or low light conditions, capable of 49% and 52% respectively of the rate of oxygen evolution of mesophyll thylakoids. Bundle sheath thylakoids extracted from normal plants of *K. childsii* and assayed under high or low light conditions evolved oxygen at rates of 33% and 20%, respectively of that observed in mesophyll thylakoids.

Table 1. Effect of sodium nutrition on F_v/F_o fluorescence ratio

Significance of difference between adjacent values: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $n > 3$

Plant species	F_v to F_o fluorescence ratio No addition		0·1 mM NaCl
NADP-ME type			
Kochia childsii	1·28	***	2·99
NAD-ME type			
Amaranthus tricolor	0·71	***	1·28
Amaranthus edulis	1·55	**	2·20

Table 2. Effect of sodium nutrition on F_v/F_o fluorescence ratio of separated mesophyll and bundle-sheath fractions

Significance of difference between adjacent values: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. Amounts of chlorophyll (mg) in the extract are given in parentheses

Plant species	F_v/F_o fluorescence ratio					
	Mesophyll			Bundle sheath		
	No addition		0·1 mM NaCl	No addition		0·1 mM NaCl
NADP-ME type						
Kochia childsii	1·264 (0·018)	***	2·345 (0·027)	1·412 (0·021)	***	2.307 (0·026)
NAD-ME TYPE						
Amaranthus tricolor	1·004 (0·008)	***	1·668 (0·006)	1·087 (0·005)	***	1·816 (0·005)
Amaranthus edulis	0·971 (0·013)	*	1·123 (0·012)	1·114 (0·009)	**	1·363 (0·009)
PEP-CK type						
Chloris gayana	1·075 (0·012)	***	1·336 (0·010)	0·978 (0·012)	*	1·149 (0·014)

Leegood *et al.* (1983) provided *in vitro* evidence of low activity of PS II as determined by fluorescence in mature bundle sheath cells of *Z. mays* and concluded that such activity is unlikely to be of importance *in vivo* regardless of the maximal rates that have been demonstrated with artificial electron donors and acceptors.

Although it is difficult to determine how much fluorescence is contributed to the whole leaf by either the mesophyll or the bundle sheath components, the fluorescence data suggest that both components are affected by sodium nutrition. Similarly, chloro-

phyll a/b ratios of both mesophyll and bundle sheath thylakoids are significantly higher in normal plants compared with those extracted from sodium-deficient plants (Johnston *et al.* 1989).

The decreased chlorophyll fluorescence ratios and the correspondingly decreased chlorophyll a/b ratios in organelles and whole leaves of sodium-deficient compared to

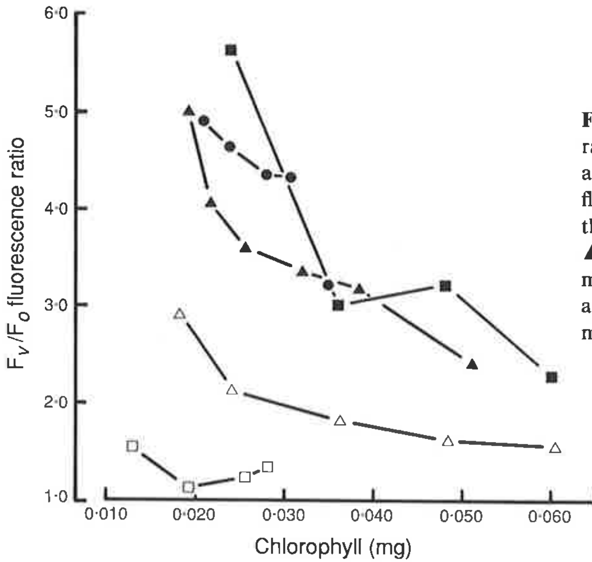

Fig. 7. Changes in the F_v/F_o fluorescence ratio of *Kochia childsii* with increasing amounts of chlorophyll in the fluorescence chamber. △ □, mesophyll thylakoids from sodium-deficient plants; ▲ ■ ●, mesophyll thylakoids from normal plants. Each symbol represents a separate extraction, and each value is the mean of two measurements.

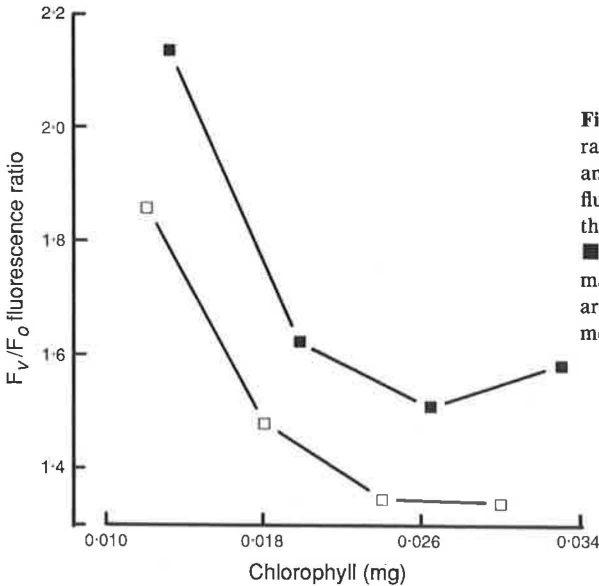

Fig. 8. Changes in the F_v/F_o fluorescence ratio of *Kochia childsii* with increasing amounts of chlorophyll in the fluorescence chamber. □, bundle sheath thylakoids from sodium-deficient plants; ■, bundle sheath thylakoids from normal plants. Each symbol represents a separate extraction, and each value is the mean of two measurements.

normal plants may be due to differences in the organisation and/or reduced number of light-harvesting and reaction centres in mesophyll and/or bundle sheath chloroplasts. This would be consistent with the suggestion of Johnston *et al.* (1984*b*) that sodium nutrition affects the energy production of mesophyll and/or bundle sheath cells, and the

suggestion of Nable and Brownell (1984) that there is a limitation in the conversion of pyruvate to phospho*enol*pyruvate in the mesophyll cells of sodium-deficient plants.

In the C_4 species *C. gayana* and *Eleusine indica*, electron micrographs have revealed fewer lamellae and distinctive areas of unappressed stromal lamellae in mesophyll chloroplasts from sodium-deficient compared to normal plants (Boag 1981). Grof *et al.* (1989) showed that mesophyll chloroplasts from sodium-deficient compared to normal plants of the C_4 species *K. childsii* and *A. tricolor* were found to have significantly less stacking in their grana. On the other hand, no marked differences of the thylakoid arrangement between bundle sheath chloroplasts from sodium-deficient and normal plants of *A. tricolor* were observed.

A recently described transport system unique to C_4 plants has been shown to have a specific sodium requirement (Ohnishi and Kanai 1987). This mechanism, located in the mesophyll chloroplast membrane, is responsible for the light-dependent uptake of pyruvate into the chloroplast. This transport system may represent a primary specific role for sodium. Alternatively, the data presented in this and other related papers (Johnston *et al.* 1984*b*, 1989; Grof *et al.* 1986*b*, 1989) would be consistent with sodium being involved in the production of the energy required for the conversion of pyruvate to phospho*enol*pyruvate in the mesophyll chloroplasts. However, it has been suggested by Boag *et al.* (1988) that the changes in the light-harvesting system in sodium-deficient plants (Johnston *et al.* 1984*b*, 1989; Grof *et al.* 1986*b*, 1989) may be a result of a limitation in pyruvate transport. Such a limitation would lower the energy normally utilised by mesophyll chloroplasts for the conversion of pyruvate to phospho*enol*pyruvate and increase the unutilised energy, which could damage the reaction centres. Thus, it is yet to be determined whether sodium is involved in the transport of pyruvate into the mesophyll chloroplasts and/or in the production of energy by the light reactions required for the conversion of pyruvate to phospho*enol*pyruvate.

Acknowledgments

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The Effect of Sodium Nutrition on the Pool Sizes of Intermediates of the C_4 Photosynthetic Pathway

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Abstract

Changes in C_4 pathway intermediates in response to sodium nutrition, consistent with the hypothesis that there is a limitation in the conversion of pyruvate to phospho*enol*pyruvate (PEP) in the mesophyll cells in sodium-deficiency, were observed in the C_4 species, *Kochia childsii, Chloris gayana, Amaranthus edulis, Amaranthus tricolor* and *Atriplex spongiosa*. In the C_3 species *Lycopersicon esculentum* (tomato), no differences were observed when grown with or without added sodium.

Of the group 1 elements, only sodium, irrespective of the salt supplied to deficient cultures, effected these changes in the C_4 species.

In the light, concentrations of aspartate, PEP and 3-phosphoglycerate (3-PGA) were lower and those of pyruvate and alanine were greater in sodium-deficient than normal plants. In the dark, concentrations of aspartate, pyruvate, alanine, PEP and 3-PGA were similar in sodium-deficient and normal plants.

In the C_4 species *Atriplex spongiosa*, the concentration of sodium required to bring about these changes corresponded to that required for growth responses.

Rapid increases in the concentrations of malate, PEP and 3-PGA and decreases in pyruvate and alanine were observed following the direct application of sodium to leaves.

Introduction

Sodium is unique as a micronutrient for higher plants in that it is required only by plants possessing the C_4 CO_2-concentrating appendage (Brownell and Crossland 1972, 1974). The mesophyll cells are implicated as the site of the lesion(s) since increased ambient CO_2 concentration alleviated the signs of sodium-deficiency without restoring the lower chlorophyll *a*/*b* ratio, characteristic of sodium-deficiency, to that of normal plants (Johnston *et al.* 1984*a*; 1984*b*; 1986).

Nable and Brownell (1984) found that the concentration of alanine was higher in sodium-deficient than in normal C_4 plants. They suggested that the increased concentration in sodium-deficient plants was due to a limitation in the conversion of pyruvate to PEP* in the mesophyll cells and that alanine and pyruvate were in equilibrium. If this were the case, it would be expected that the concentration of pyruvate and alanine would be greater and PEP, oxaloacetate and the C_4 compounds, malate and/or aspartate would be lower in sodium-deficient than in normal plants. This paper examines the effect of sodium nutrition on the levels of intermediates involved in C_4 photosynthesis.

Materials and Methods

The procedures for the germination and growth of *Kochia childsii* Hort., *Amaranthus edulis* Speg., *Amaranthus tricolor* L., *Chloris gayana* Kunth and *Atriplex spongiosa* F. Muell. and *Lycopersicon*

*Abbreviations used: ME, malic enzyme; PEP, phospho*enol*pyruvate; PEP-CK, PEP carboxykinase; 3-PGA, 3-phosphoglycerate.

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esculentum Mill. (tomato) under conditions of low sodium have been described (Brownell 1979). The total sodium concentration of the complete culture solution was approximately 0·08 μM. Normal plants were obtained by supplying sodium chloride to give a final concentration of 0·1 mM in appropriate culture solutions except where otherwise specified. Where the other group 1 elements were used, they were supplied as the chlorides when transplanting the seedlings to the culture vessels. The sodium as an impurity contributed by these salts gave sodium concentrations less than 0·02 μM in the final culture solution.

Plants were grown in either 2-litre or 3·5-litre culture vessels containing eight and 40 plants each, respectively, in a naturally illuminated growth cabinet supplied continuously with filtered air to minimise sodium contamination. The maximum irradiance in the growth cabinet was approximately 2000 μmol $m^{-2} s^{-1}$ (PAR). The age of plants at harvest was between 25 and 32 days.

Before extraction, plants were illuminated by a Philips 500-W flood lamp for 60 min at an irradiance of 1700 μmol $m^{-2} s^{-1}$ (PAR) at plant height. Light was passed through a 10 cm water filter which acted as a heat absorber. Alternatively, plants were exposed to an irradiance of approximately 2000 μmol $m^{-2} s^{-1}$ (PAR) in a naturally illuminated growth chamber.

Leaf tissue was extracted by a method modified from Hatch (1979). Approximately 1 g of mature leaf tissue was frozen with liquid nitrogen, ground in a mortar and pestle with approximately 0·4 g of acid-washed sand in a cold room at 0–4°C while allowing the liquid nitrogen to boil off. The powdered tissue was transferred to centrifuge tubes and 2·5 ml of 0·5 M HCl containing 0·2 mM EDTA was added. The contents were frozen with liquid nitrogen and later thawed and vacuum-infiltrated at 0°C. The mixture was shaken for 2 h to extract the soluble compounds then centrifuged at 10 000 *g* for 20 min. The extracts were neutralised by titrating 2 ml of the extract with 0·5 M KOH using phenolphthalein as an indicator. The ultraviolet absorbing material in the extracts and the indicator were removed by shaking with 50 mg of activated charcoal for 5 min at 0°C. The activated charcoal was removed by centrifugation at 10 000 *g* for 20 min at 0°C.

Oxaloacetate, pyruvate, PEP and 3-PGA were determined in sequence on the same sample using the method described by Hatch and Kagawa (1976). Malate was determined by the method of Gutmann and Wahlefeld (1974) and aspartate using the method of Hatch (1979). Alanine was measured according to the method of Nable and Brownell (1984).

Results and Discussion

The concentrations of the intermediates of the C_4 pathway, oxaloacetate, aspartate, malate, alanine, pyruvate and PEP and the product of the carboxylation in the photosynthetic carbon reduction cycle, 3-PGA, were determined in the leaves of sodium-deficient and normal plants of *Kochia childsii, Chloris gayana, Amaranthus edulis, Amaranthus tricolor* and *Atriplex spongiosa*, representing each of the three types of C_4 plants (Gutierrez *et al.* 1974; Hatch *et al.* 1975). Tomato was used as a control C_3-species.

The concentration of the product of the primary carboxylation via PEP carboxylase in C_4 plants, oxaloacetate, when detectable was lower in sodium-deficient than normal plants of *Chloris gayana, Amaranthus tricolor* and *Amaranthus edulis* (Table 1). As oxaloacetate is an unstable compound and is readily decarboxylated, it was not detectable in all experiments and no values were obtained for *Kochia childsii, Atriplex spongiosa* and the C_3 species, tomato.

In the PEP-CK and NAD-ME type C_4 plants, the oxaloacetate produced by the carboxylation of PEP in the mesophyll cells is aminated to form aspartate via aspartate aminotransferase (Hatch and Mau 1973; Hatch *et al.* 1975) and aspartate is transported to the bundle sheath cells. It was found that the aspartate concentration was lower in sodium-deficient than normal plants of *Chloris gayana* (PEP-CK type) and *Amaranthus tricolor, Amaranthus edulis* and *Atriplex spongiosa* (NAD-ME type) (Table 1). In NADP-ME type C_4 plants, aspartate is not directly involved in C_4 photosynthesis (Hatch *et al.* 1975). No difference was observed between the concentrations of aspartate in the leaves of sodium-deficient and normal plants of *Kochia childsii* (NADP-ME type) (Table 1).

Table 1. Effect of sodium nutrition on the leaf concentrations of intermediates of the C_4 pathway

Significance of difference between adjacent values: n.s., not significant; * $P<0\cdot05$; ** $P<0\cdot01$; *** $P<0\cdot001$. Numbers of replications are shown in parentheses. Concentrations are expressed as μmol g fresh weight^{-1}. n.d., not detectable; −Na, no addition; +Na, 0·1 mM NaCl

	Alanine			Pyruvate			PEP			OAA			Malate			Aspartate			3-PGA		
	−Na		+Na	−Na		+Na	−Na		+Na	−Na		+Na	−Na		+Na	−Na		+Na	−Na		+Na
C_4 species																					
NADP-ME type																					
Kochia childsii	18·36	*** (18)	9·88	3·95	*** (18)	1·41	0·52	*** (18)	1·82	nd.		n.d.	0·318	*** (18)	1·00	2·10	n.s. (18)	·24	0·21	*** (18)	0·46
PEP-CK type																					
Chloris gayana	32·47	*** (5)	15·88	1·81	** (5)	1·39	0·50	*** (5)	0·69	0·14	** (5)	0·25	25·77	* (6)	29·33	0·72	*** (6)	2·10	1·09	*** (5)	1·72
NAD-ME type																					
Amaranthus edulis	14·07	** (9)	6·4	2·53	** (9)	1·41	1·25	** (9)	1·63	0·06	* (3)	0·11	5·31	* (6)	8·40	1·21	** (3)	2·78	2·73	** (3)	3·80
Amaranthus tricolor	13·14	*** (28)	5·95	1·69	*** (26)	0·93	0·94	** (12)	2·33	0·07	— (1)	0·11	2·67	** (18)	4·82	1·63	** (18)	3·70	1·34	*** (12)	3·63
Atriplex spongiosa	7·29	*** (7)	3·96	1·24	** (6)	0·83	0·10	** (7)	0·36	n.d.		n.d.	2·79	*** (9)	6·94	1·17	*** (7)	1·84	0·10	*** (6)	0·24
C_3 species																					
Lycopersicon esculentum	2·49	n.s. (3)	2·58	0·11	n.s. (3)	0·12	0·17	n.s. (3)	0·18	n.d.		n.d.	11·27	n.s. (3)	11·28	1·94	n.s. (3)	1·91	1·45	n.s. (3)	1·46

In the NADP-ME type C_4 plants, oxaloacetate is reduced to malate via NADP malate dehydrogenase (Slack *et al.* 1969) and malate is transported to the bundle sheath cells. It was found that the concentration of malate was lower in sodium-deficient than normal plants of *Kochia childsii* (NADP-ME type) (Table 1). The concentration of malate was found to be lower in the leaves of sodium-deficient than normal plants of *Chloris gayana, Amaranthus tricolor, Amaranthus edulis* and *Atriplex spongiosa* even though it is unlikely to be directly involved in C_4 photosynthesis in these plants. These data suggest that the concentrations of the four-carbon intermediates of the C_4 pathway are lower in sodium-deficient than normal plants.

After the decarboxylation of the four-carbon compound in the bundle sheath cells of NADP-ME and NAD-ME type C_4 plants, pyruvate is produced and either pyruvate or alanine is transported back to the mesophyll cells (Hatch *et al.* 1975). The concentrations of pyruvate and alanine were found to be greater in the sodium-deficient than normal NADP-ME and the NAD-ME type plants examined (Table 1).

In the PEP-CK type plants there appear to be two routes for the transfer of carbon from bundle sheath to the mesophyll cells. In one route described by Hatch *et al.* (1975), PEP produced by the decarboxylation of oxaloacetate in the bundle sheath cells is converted to pyruvate. The pyruvate is then transaminated to alanine which is transferred to the mesophyll cells. Evidence for this scheme operating in *Chloris gayana* and *Panicum maximum* has been presented by Hatch and Kagawa (1976) and Hatch (1979). The other route, proposed by Rathnam and Edwards (1977), using *Eriochloa borumensis*, suggests that PEP is the major three-carbon compound accumulating during aspartate decarboxylation and that it may be returned directly to the mesophyll cells. A third possible route has been suggested in *Urochloa panicoides* by Burnell and Hatch (1988) in which pyruvate derived from the decarboxylation of malate by the mitochondrial NAD-malic enzyme in the bundle sheath is returned to the mesophyll.

In this study, the PEP-CK plant used was *Chloris gayana* in which Hatch (1979) showed by radiotracer analysis that the three-carbon compound transferred from the bundle sheath to the mesophyll cells was predominantly alanine. The concentrations of pyruvate and alanine were greater in sodium-deficient than normal plants of *Chloris gayana* (Table 1).

Alanine is transaminated in the mesophyll cells of C_4 plants and the pyruvate produced enters the mesophyll chloroplasts where it is converted to PEP (Hatch *et al.* 1975). PEP was found to be lower in sodium-deficient than normal C_4 plants (Table 1). These data suggest that the concentrations of the three-carbon compounds, pyruvate and alanine, returning from the bundle sheath to the mesophyll cells are greater in sodium-deficient than normal plants. On the other hand, the three-carbon acceptor of CO_2 in the primary carboxylation, PEP in C_4 plants, is lower in sodium-deficient than normal plants.

The concentration of 3-PGA, the first product of the photosynthetic carbon reduction cycle, was lower in sodium-deficient than normal C_4 plants (Table 1). This suggests that there is a limitation in the supply of substrate to the photosynthetic carbon reduction cycle in sodium-deficient C_4 plants. However, it is also possible that a decrease in the concentration of 3-PGA might occur in the mesophyll cells if it were in equilibrium with PEP.

There was no difference in the concentrations of aspartate, malate, pyruvate, alanine, PEP or 3-PGA in the leaves of the C_3 species, tomato, grown with or without added sodium (Table 1). As the concentrations of aspartate, malate, pyruvate, alanine, PEP and 3-PGA were found only to vary in response to sodium in C_4 plants which have been shown to require sodium as a micronutrient (Brownell and Crossland 1972), it is likely that these differences are related directly to sodium nutrition.

It would appear that, as there are decreased concentrations of PEP and the four-carbon compounds, oxaloacetate, aspartate and/or malate and 3-PGA but increased

Table 2. Effect of light/dark and sodium nutrition on the concentration of the intermediates of the C_4 pathway in *Amaranthus tricolor*

Significance of difference between adjacent values: n.s., not significant; * $P<0·05$; ** $P<0·01$; *** $P<0·001$. Numbers of replicates are shown in parentheses. Concentrations are expressed as μmol g fresh weight^{-1}. −Na, no addition; +Na, 0·1 mM NaCl. Oxaloacetate was not detected

	Alanine			Pyruvate			PEP			Malate			Aspartate			3-PGA		
	−Na		+Na	−Na		+Na	−Na		+Na	−Na		+Na	−Na		+Na	−Na		+Na
Light (80 min)	7·49	***	4·44	1·17	**	0·78	0·31	**	0·61	2·46	***	5·65	1·03	***	2·59	0·057	***	1·72
		(5)			(5)			(5)			(5)			(5)			(5)	
	**		n.s.	***		n.s.	n.s.		**	**		**	***		*	*		***
	(5)		(5)	(5)		(5)	(5)		(5)	(5)		(5)	(5)		(5)	(5)		(5)
Dark (60 min)	5·39	n.s.	4·79	0·63	n.s.	0·66	0·31	*	0·40	1·63	***	4·26	2·01	n.s.	2·17	0·32	**	0·62
		(5)			(5)			(5)			(5)			(5)			(5)	
	**		n.s.	*		n.s.	n.s.		**	n.s.		*	***		n.s.	n.s.		***
	(3)		(3)	(3)		(3)	(3)		(3)	(3)		(3)	(3)		(3)	(3)		(3)
Light (120 min)	3·63	n.s.	3·77	0·87	n.s.	0·65	0·29	***	0·59	2·07	***	3·57	0·87	***	2·41	0·43	***	1·30
		(3)			(3)			(3)			(3)			(3)			(3)	

concentrations of pyruvate and alanine in sodium-deficient plants, the conversion of pyruvate to PEP is limited in the mesophyll cells of sodium-deficient C_4 plants as suggested by Nable and Brownell (1984).

The data in Table 1 represent the total leaf pool sizes of the C_4 pathway intermediates and are not necessarily an indication of photosynthetically active pools. The effect of

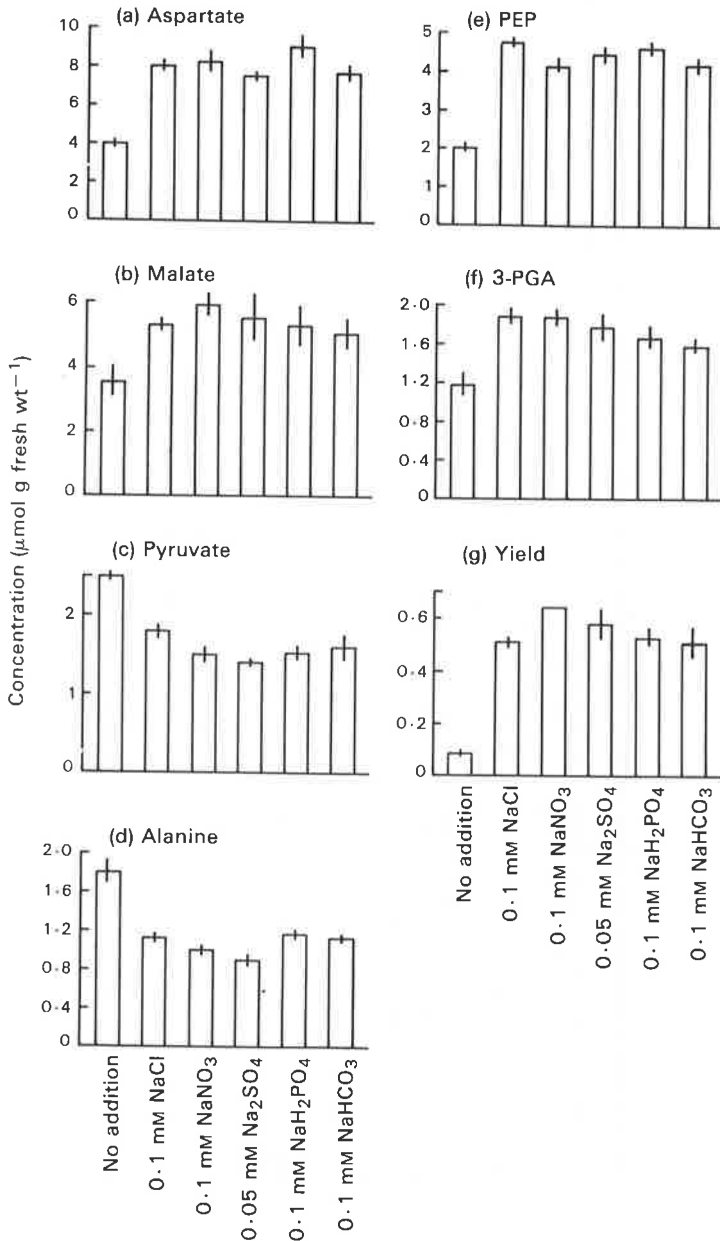

Fig. 1. Effect of different sodium salts on the concentrations of aspartate (*a*); malate (*b*); pyruvate (*c*); alanine (*d*); phospho*enol*pyruvate (*e*); 3-phosphoglycerate (*f*); and on yield (*g*) in *Amaranthus tricolor*. Mean ± s.e.m., $n = 4$.

light and dark on the pool sizes of the intermediates was examined (Table 2) in *Amaranthus tricolor* to determine if the pools were photosynthetically active. There was little effect of light and dark on the concentrations of aspartate, pyruvate and alanine in normal plants. The concentrations of PEP and 3-PGA decreased in the dark but were restored to their original levels within 120 min of being exposed to light in normal plants.

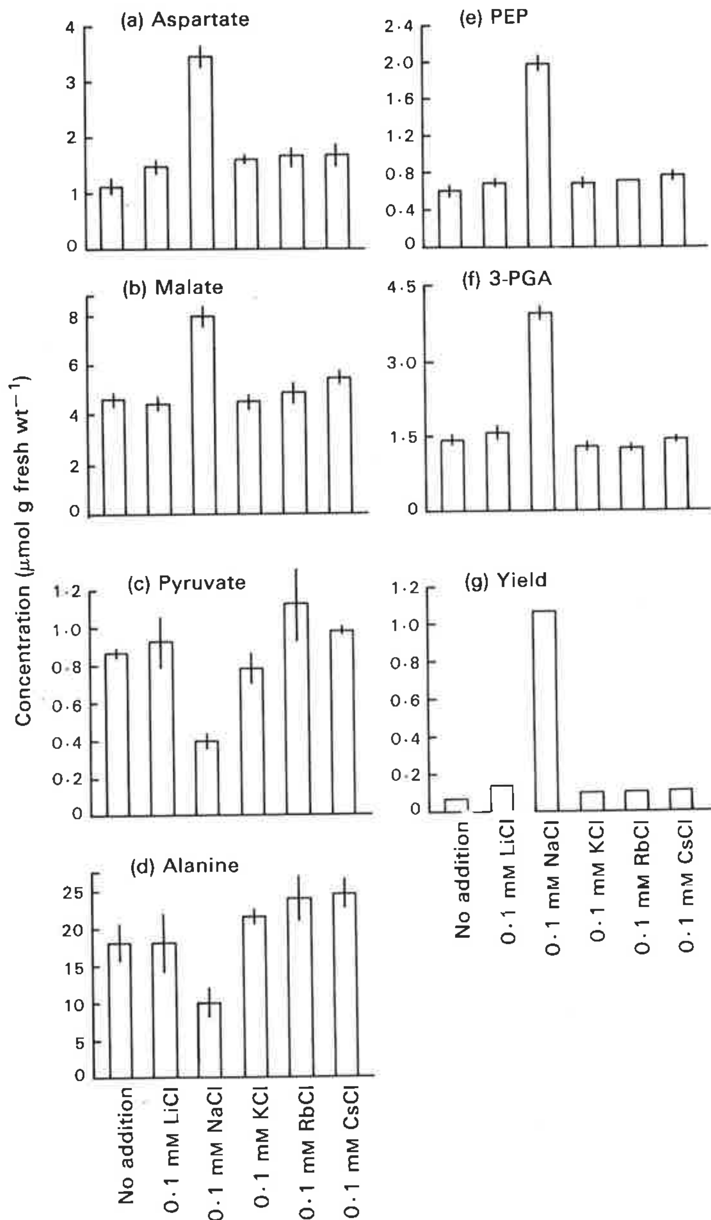

Fig. 2. Effect of group 1 elements on the concentrations of aspartate (*a*); malate (*b*); pyruvate (*c*); alanine (*d*); phospho*enol*pyruvate (*e*); 3-phosphoglycerate (*f*); and on yield (*g*) in *Amaranthus tricolor*. Mean ± s.e.m., $n = 3$.

In the light, the concentrations of aspartate, PEP and 3-PGA were lower and the concentrations of pyruvate and alanine were greater in sodium-deficient than normal plants. In the dark, the concentrations of aspartate, pyruvate, alanine and PEP in sodium-deficient and normal plants were similar. Generally, the concentrations of these compounds returned to approximately their original levels after reillumination in the light. In *Amaranthus tricolor* (NAD-ME type), malate is not directly involved in C_4 photosynthesis. As there were larger differences in the concentrations of aspartate, pyruvate, alanine, PEP and 3-PGA between sodium-deficient and normal plants of *Amaranthus tricolor* in the light than in the dark, it is likely that the differences are due to photosynthetically active pools.

Equivalent concentrations of sodium, supplied to cultures of sodium-deficient plants of *Amaranthus tricolor* as chloride, sulfate, nitrate, dihydrogen orthophosphate or bicarbonate, all produced similar increases in the concentrations of PEP, aspartate, 3-PGA and yield and decreases in the concentrations of pyruvate and alanine (Fig. 1). Of the group 1 elements supplied as LiCl, NaCl, KCl, RbCl and CsCl to give a final concentration of 0·1 mM to cultures of sodium-deficient plants of *Amaranthus tricolor*, only sodium significantly increased the concentrations of PEP, aspartate, 3-PGA and yield but decreased the concentrations of pyruvate and alanine (Fig. 2). These results demonstrate that the changes in the C_4 pathway intermediates are specifically due to sodium.

When plants of *Atriplex spongiosa* were grown in increasing concentrations of sodium chloride, the yield and chlorophyll concentrations reached a plateau between 10 and 50 μM sodium chloride (Fig. 3*a*, 3*b*). The concentrations of aspartate, malate, PEP and 3-PGA also increased to reach a plateau between 10 and 50 μM sodium chloride (Fig. 3*c*–3*f*) while concentrations of pyruvate and alanine decreased and levelled off between 10 and 50 μM sodium chloride (Fig. 3*g*, 3*h*). The changes in the concentrations of the C_4 intermediates and yield were brought about by similar concentrations of sodium. This suggests that these changes are intimately related to sodium nutrition.

The rate of recovery from sodium-deficiency is limited by the slow rate of sodium uptake from the culture solution (Grof *et al.* 1986). To overcome this, leaves of sodium-deficient *Kochia childsii* plants were sprayed with 0·2 M NaCl in 40% (v/v) ethanol and the concentrations of the intermediates of the C_4 pathway were monitored during recovery from sodium-deficiency. The concentrations of malate, PEP and 3-PGA increased to those of normal plants within 3–4 h of spraying with sodium chloride (Fig. 4). At the same time, the concentrations of pyruvate and alanine decreased rapidly during recovery from sodium-deficiency. The pyruvate and alanine concentrations of recovering plants were significantly lower ($P < 0.01$, *t*-test) than sodium-deficient plants within 3 and 9 h of receiving sodium chloride respectively and, within 24 h, the concentrations of both were similar to those of the normal plants (Fig. 4). These rapid changes in the concentrations of the intermediates of the C_4 pathway may represent an early step in the recovery from sodium-deficiency.

The diurnal changes in the concentrations of intermediates of the C_4 pathway can also be seen in Fig. 4. In normal plants, the concentrations of malate, PEP and 3-PGA increased rapidly during the early part of the light period and then decreased rapidly during the latter part of the light period. The concentrations of pyruvate and alanine in normal plants showed a similar trend but to a lesser extent than malate, 3-PGA and PEP. In sodium-deficient plants, pyruvate and alanine showed a marked increase during the light period whereas the concentrations of malate, 3-PGA and PEP showed relatively little diurnal change.

Nable and Brownell (1984) suggested that the regeneration of PEP is limited in the mesophyll chloroplasts of sodium-deficient C_4 plants. This could account for the observed differences in the concentrations of the intermediates of the C_4 pathway which

are related to sodium nutrition. The very rapid changes in the concentrations of the intermediates during recovery from sodium-deficiency would suggest that the changes are not developmental.

It is possible that the sodium may be needed for the normal action of the enzyme pyruvate orthophosphate dikinase, which catalyses the regeneration of PEP from pyruvate. Boag (1981) and Dorney (1985), however, showed that the *in vitro* activity of this enzyme was not affected by sodium nutrition. Even so, it is possible that sodium may be necessary for its *in vivo* activity.

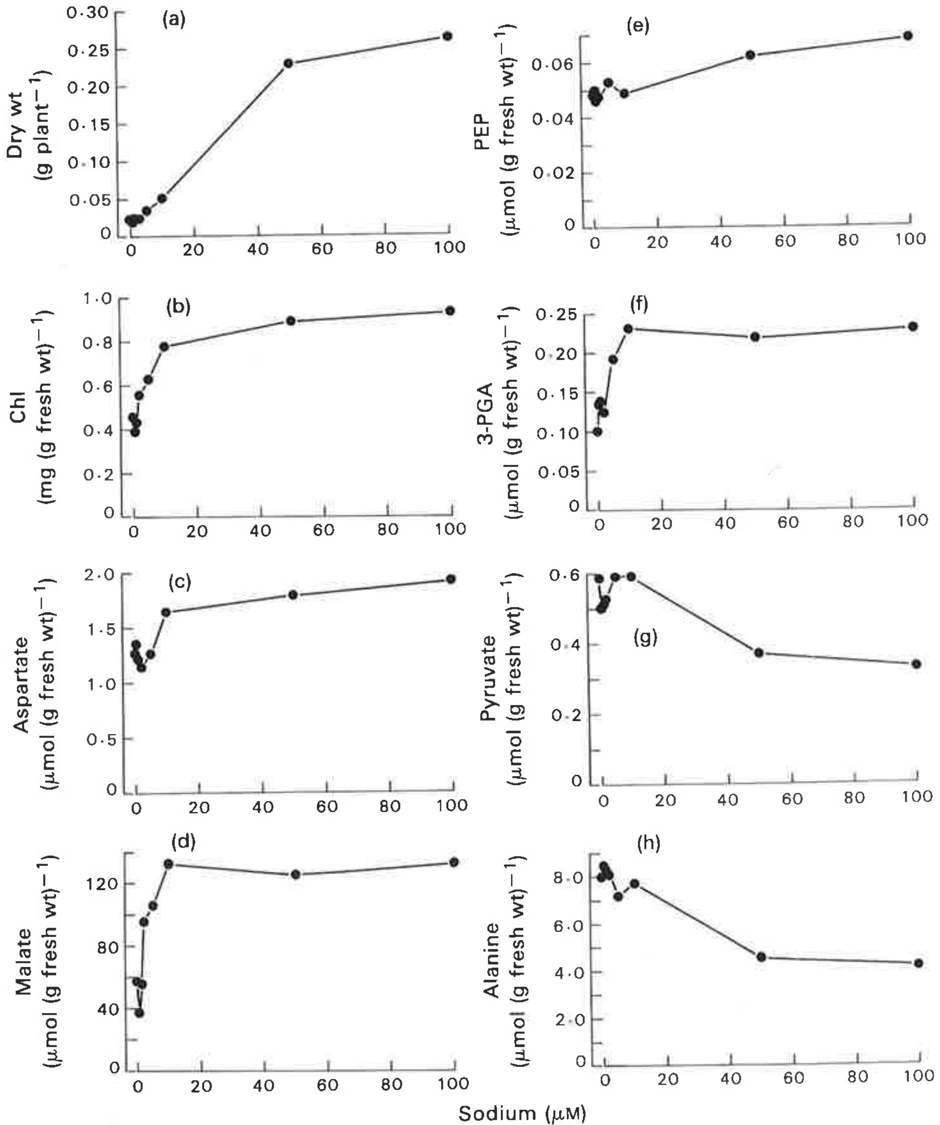

Fig. 3. Effect of sodium chloride concentrations on yield (*a*); chlorophyll (*b*); aspartate (*c*); malate (*d*); phospho*enol*pyruvate (*e*); 3-phosphoglycerate (*f*); pyruvate (*g*); and alanine (*h*); in *Atriplex spongiosa*. Mean ± s.e.m., $n = 3$.

Sodium might be required for the transport of intermediates such as pyruvate. Raghavendra and Das (1977, 1978) suggested that ATPases were involved in the active transport of metabolites and that sodium ions may be required for their activation. It has been calculated, however, from metabolite pool sizes that passive diffusion through the plasmodesmata could account for the movement of metabolites between the mesophyll and bundle sheath cells (Oleson 1975; Hatch and Osmond 1976). Huber and

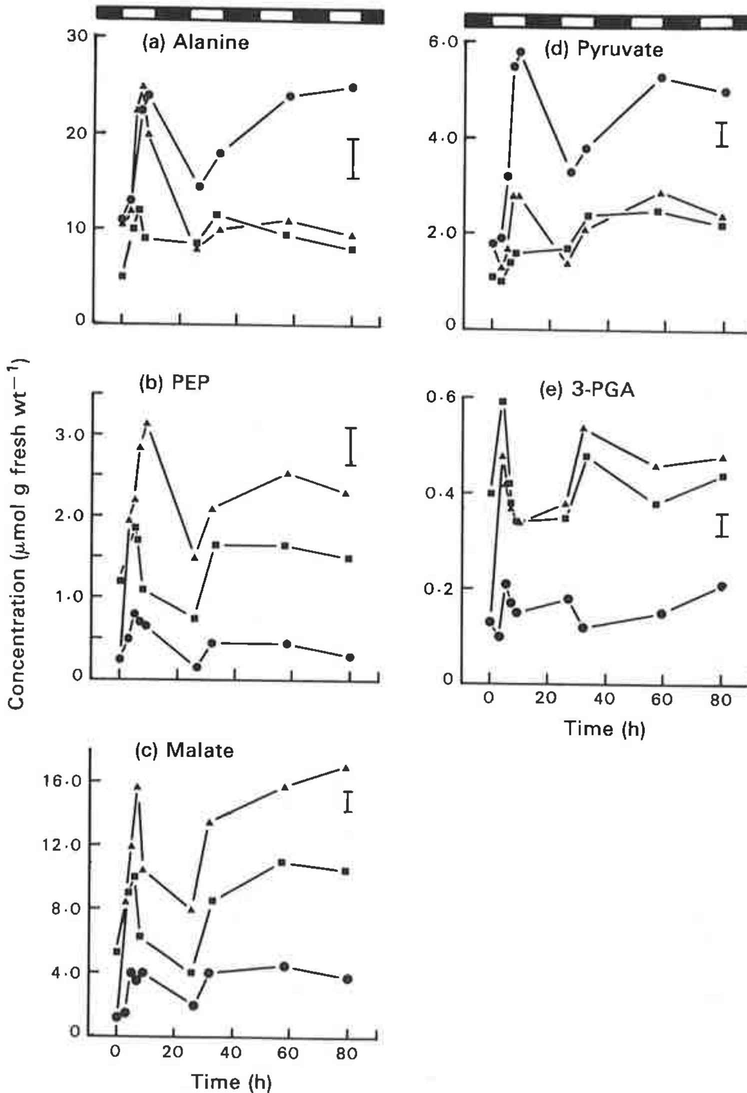

Fig. 4. Changes in concentrations of alanine (*a*); phospho*enol*pyruvate (*b*); malate (*c*); pyruvate (*d*); and 3-phosphoglycerate (*e*); in leaves of *Kochia childsii* during recovery from sodium-deficiency. Mean ± s.e.m., $n=3$. ■ 0·1 mM NaCl supplied to culture solution 15 days prior to time 0. ▲ Leaves sprayed with 0·2 M NaCl in 40% ethanol at time 0. ● No treatment.

Edwards (1977) reported a specific pyruvate transport into the mesophyll chloroplasts. An exciting development was the report by Ohnishi and Kanai (1987) who described a sodium requirement for the transport of pyruvate into the mesophyll chloroplast where it is converted to PEP, the CO_2 acceptor. The specific involvement of sodium in this process has been suggested as the primary function of sodium in the nutrition of C_4 plants (Boag *et al.* 1988).

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Chlorophyll *a/b* Ratios and Photosystem Activity of Mesophyll and Bundle Sheath Fractions from Sodium-deficient C_4 Plants

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Abstract

Significantly lower chlorophyll *a*/*b* ratios were observed in mesophyll thylakoids extracted from sodium-deficient than normal C_4 plants of *Amaranthus tricolor, Chloris gayana* and *Kochia childsii*. The chlorophyll *a*/*b* ratio of bundle sheath thylakoids was not generally as significantly affected by sodium nutrition as the mesophyll thylakoids.

The lower chlorophyll *a*/*b* ratio in mesophyll thylakoids of sodium-deficient plants was associated with a lower photosystem II activity. The effect of sodium nutrition on photosystem I activity in the mesophyll thylakoids was dependent upon the species. Although no effect of sodium nutrition on photosystem I was observed in mesophyll thylakoids of *K. childsii*, photosystem I activity was greater in sodium-deficient than normal plants in *A. tricolor*.

The photosystem II and I activities of the bundle sheath thylakoids were not affected by sodium nutrition in either *K. childsii* or *A. tricolor*.

Introduction

Sodium is unique as a micronutrient element for higher plants in that it is required only by plants possessing the C_4 appendage (Brownell 1979). Sodium is needed for the operation of the CO_2 concentrating mechanism in C_4 species, thereby maintaining the carboxylase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase and thus ensuring a high efficiency of the photosynthetic carbon reduction cycle (Johnston *et al.* 1984*a*, 1986).

Leaves of sodium-deficient C_4 plants have been shown to have increased concentrations of alanine (Nable and Brownell 1984; Grof *et al.* 1986*a*) and pyruvate (Johnston *et al.* 1988) and decreased concentration of PEP* (Johnston *et al.* 1988). This suggests a possible block in the regeneration of PEP from pyruvate in the mesophyll chloroplasts in sodium-deficient plants. The decreased chlorophyll *a*/*b* ratios (Johnston *et al.* 1984*b*), fluorescence yields (Grof *et al.* 1986*b*) and changed ultrastructure of chloroplasts from sodium-deficient leaves (Grof *et al.* 1989*a*) suggest that a reduction in the energy available from the photosystems may limit the conversion of pyruvate to PEP in sodium deficiency. It would therefore be expected that the effects of sodium nutrition on chlorophyll *a*/*b* ratios would be largely restricted to the mesophyll thylakoids from sodium-deficient plants.

*Abbreviations used: PS I, photosystem I; PS II, photosystem II; PEP, phospho*enol*pyruvate; BSA, bovine serum albumin; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; tricine, *N*-tris(hydroxymethyl)methylglycine; DMQ, 2,5-dimethyl-*p*-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron); MV, methyl viologen.

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In an attempt to define the site(s) of the lesion in the chloroplasts from sodium-deficient plants, the mesophyll and bundle-sheath cells were separated by differential blending and the effects of sodium nutrition on chlorophyll *a*/*b* ratios and the PS II and PS I activities of the thylakoids were examined.

Materials and Methods

The procedures for the germination and growth of *Kochia childsii* Hort., *Chloris gayana* Kunth and *Amaranthus tricolor* L. (non-pigmented form) have been described (Brownell 1979). The concentration of sodium as an impurity in the complete culture solution was approximately 0·08 μM. Normal plants were obtained by supplying appropriate cultures with NaCl to give a final concentration of 0·1 mM.

Separation of 'Mesophyll' and 'Bundle Sheath' Thylakoid Fractions

'Mesophyll' and 'bundle sheath' thylakoid fractions from sodium-deficient and normal plants were separated by differential blending. Approximately 10 g of leaves were washed twice with distilled H_2O, thinly sliced (<1 mm width) and blended for 6 s in a Sorvall Omni-mixer at 60% line voltage with 100 mL of extracting medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCl, 0·5 mM P_i, 2 mM isoascorbate, 50 mM MES and 0·4% (w/v) BSA (pH 6·2). The brei was filtered through two layers of Miracloth and the filtrate centrifuged at 10 000 *g* for 5 min, and the resulting pellet was designated the 'mesophyll' thylakoid fraction. The residue from the first blending was blended for a further 2 min, with a short pause after each 30 s interval, with 50 mL of extracting medium in a Virtis homogeniser at 60% line voltage and filtered through two layers of Miracloth. The filtrate was centrifuged at 10 000 *g* for 5 min and the resultant pellet designated the 'mesophyll plus bundle sheath' thylakoid fraction. The residue from the second blending was then ground in a mortar and pestle with 20 mL of extracting medium and approximately 0·5 g of acid-washed sand. The brei was filtered through two layers of Miracloth and centrifuged at 10 000 *g* for 5 min, and the pellet was designated the 'bundle sheath' thylakoid fraction.

To determine the purity of the 'mesophyll' and 'bundle sheath' fractions, PEP carboxylase and RuP_2 carboxylase were used as marker enzymes. The enzyme assays were carried out on the supernatant obtained after the centrifugation of each thylakoid-fraction pellet. The activity of the crude enzyme in each fraction, expressed as μmol NADH or bicarbonate fraction^{-1} min^{-1} (the absolute value of which varied with the weight of leaf tissue extracted), was totalled and the percentage of enzyme activity in each fraction calculated. Pellets of each fraction, containing intact and broken chloroplasts and cell debris, were washed in 20 mL of resuspending medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCl, 0·5 mM P_i, 50 mM HEPES and 0·4% (w/v) BSA (pH 6·7), then centrifuged at 10 000 *g* for 5 min and the pellet resuspended in 3–5 mL of resuspending medium. All operations were carried out at 0–5°C.

RuP_2 *Carboxylase Assay*

RuP_2 carboxylase activity was assayed with an isotopic method based on that of Lorimer *et al.* (1977). The assay medium contained 0·43 mL of buffer (100 mM tris-HCl, 5 mM DTT and 20 mM $MgCl_2$, pH 8·2), 0·01 mL of 60 mM ribose 5-phosphate, 0·01 mL of 350 mM ATP and 50 μL of 0·2 M $NaH^{14}CO_3$ of known specific activity (approximately 0·5 Ci mol^{-1}). After equilibration at 24°C, the reaction was initiated by the addition of 0·2 mL of the crude enzyme (activated by the addition of 10 mM $KHCO_3$ to the extracting medium). The reaction was terminated by the addition of 0·1 mL of 2 M formic acid. The assay measures Ru5*P* kinase, phosphoribuloseisomerase and RuP_2 carboxylase when ribose 5-phosphate is used in place of RuP_2 as the substrate. However, the enzymic activities catalysing the conversion of ribose 5-phosphate to RuP_2 are relatively high and are unlikely to be limiting in crude extracts (Lilley and Walker 1974). It appears, therefore, that the assay would be measuring the activity of RuP_2 carboxylase, the rate-limiting enzyme in the sequence. The RuP_2 carboxylase would have been in an activated state due to the presence of 10 mM $MgSO_4$ and 10 mM HCO_3^- in the reaction mixture prior to the assay.

After the reaction was terminated, the contents of the vials were air-dried overnight, then 5 mL of the scintillant Aquasol (New England Nuclear) was added. The vials were kept in the dark for 24 h after adding the scintillant in order to minimise chemiluminescence before counting on an LKB 1217 Rackbeta liquid scintillation counter.

PEP Carboxylase Assay

PEP carboxylase activity was assayed using the spectrophotometric method based on that of Hatch and Oliver (1978). The reaction medium contained 25 mM tricine-KOH (pH 8·3), 5 mM $MgCl_2$, 5 mM $KHCO_3$, 2·5 mM PEP, 1 mM glucose 6-phosphate, 2 mM NADH, 6 units of malate dehydrogenase and 30–200 μL of crude enzyme, all in a total volume of 3 mL. Activity was measured at 24°C by following, on a Varian series 634 spectrophotometer, the oxidation of NADH at 340 nm due to the reduction of oxaloacetate by malate dehydrogenase.

Determination of Chlorophyll in Extracts

Aliquots of extracts were centrifuged at 10 000 *g* for 10 min. The pellets were resuspended in 96% (v/v) ethanol. The homogenates were centrifuged at 10 000 *g* for 10 min and the pellets were re-extracted, if necessary, with 96% (v/v) ethanol until all colour was removed. Spectrophotometric measurements were made as described in Johnston *et al.* (1984*b*). Chlorophyll was determined using the equations of Wintermans and De Mots (1965).

Determination of PS I and II Activity of Isolated Thylakoids

The following modifications of the methods of Holloway *et al.* (1983) were used. Oxygen evolution or uptake was measured polarographically with a Clark-type oxygen electrode (Rank Brothers, Bottisham, U.K.) in a basic assay medium of 350 mM sorbitol, 25 mM HEPES, 2 mM $MgCl_2$, 1 mM KH_2PO_4 and 0·5 mM EDTA (pH 7·6). Thylakoid samples, obtained by osmotically shocking samples of fractions to rupture the envelope membranes of intact chloroplasts, were assayed at 25°C and illuminated by a Schött Mainz fibre-optic halogen lamp. The photon irradiance applied within the reaction suspensions was 400 $\mu mol\ m^{-2}\ s^{-1}$ (Griffiths *et al.* 1978). PS II activity was measured as the oxygen evolved during electron transport from H_2O to DMQ in 3 mL of the basic assay medium containing 0·13 mM DMQ, 4 mM ferricyanide and 20–30 μg of chlorophyll. PS I activity was measured as the O_2 uptake due to the electron transport from DCPIP to MV in 3 mL of the basic assay medium containing 4·5 mM isoascorbate, 0·1 mM DCPIP, 0·4 mM MV, 70 μM DCMU, 3·5 mM sodium azide and 10–20 μg of chlorophyll. In experiments where Triton X-100 was used, it was added to give a final concentration of 0·1% (v/v).

Statistical Treatment

The data were analysed with Student's *t* test.

Results and Discussion

The Separation of 'Mesophyll' and 'Bundle Sheath' Fractions

The data from Table 1 suggest that the differential blending method produced relatively pure 'mesophyll' and 'bundle sheath' fractions from *K. childsii* and *C. gayana*. More cross-contamination of the 'mesophyll' and 'bundle sheath' fractions in *A. tricolor* was evident (Table 1). The supernatants of the 'mesophyll plus bundle sheath' fractions from *K. childsii, C. gayana* and *A. tricolor* all contained high proportions of PEP carboxylase and RuP_2 carboxylase activities, verifying that these fractions were derived from a mixture of both cell types.

The Chlorophyll a/b Ratio of the Mesophyll and Bundle Sheath Thylakoids

The chlorophyll *a*/*b* ratios of the 'mesophyll' and 'bundle sheath' fractions from normal plants of the three types of C_4 species show similar trends to those observed by Ku *et al.* (1974) and Mayne *et al.* (1974). The NADP-ME type species, *K. childsii*, had a significantly higher ($P < 0·05$) chlorophyll *a*/*b* ratio in the 'bundle sheath' fraction than in the 'mesophyll' fraction (Fig. 1*a*). The NAD-ME type species, *A. tricolor*, had a significantly higher ($P < 0·01$) chlorophyll *a*/*b* ratio in the 'mesophyll' fraction than in the 'bundle sheath' fraction (Fig. 1*b*). The PEP-CK type species, *C. gayana*, had similar chlorophyll *a*/*b* ratios in both the 'mesophyll' and the 'bundle sheath' fractions

(Fig. 1*c*). The chlorophyll *a*/*b* ratios of the dimorphic fractions of the different types of C_4 plants have been related to their different energy requirements (Edwards *et al.* 1975).

The chlorophyll *a*/*b* ratios were significantly lower in the 'mesophyll' fraction from sodium-deficient plants than normal plants of *K. childsii, A. tricolor* and *C. gayana* (Fig. 1). The chlorophyll *a*/*b* ratios of the 'bundle sheath' fraction of *K. childsii* were unaffected by sodium nutrition. The chlorophyll *a*/*b* ratios were significantly lower in the 'bundle sheath' fractions from sodium-deficient plants than normal plants of *A. tricolor* and *C. gayana*. The effect of sodium nutrition on the chlorophyll *a*/*b* ratios was less marked in the bundle sheath cells compared with the mesophyll cells.

The lower chlorophyll *a*/*b* ratio in sodium-deficient than normal C_4 plants observed by Johnston *et al.* (1984*b*) appears to be predominantly due to a lower chlorophyll *a*/*b* ratio in the mesophyll cells.

Table 1. Efficiency of separation of 'mesophyll' and 'bundle sheath' fractions in *Kochia childsii, Chloris gayana* and *Amaranthus tricolor*

Enzyme assays were carried out on the supernatant obtained after centrifugation of each thylakoid-fraction pellet. Values (means ± s.e. from two experiments) represent activity of separate fractions expressed as a percentage of the total activity of PEP carboxylase or RuP_2 carboxylase in each species and treatment

Fraction	Distribution of marker enzymes (% of total activity)					
	K. childsii		*C. gayana*		*A. tricolor*	
	No addition	0·1 mM NaCl	No addition	0·1 mM NaCl	No addition	0·1 mM NaCl
	PEP carboxylase					
'Mesophyll'	47.00± 3.12	48.25±6.23	32.50±0.48	25.53±1.69	33.91±4.34	35.63±4.99
'Mesophyll plus bundle sheath'	50.20± 2.46	47.83±6.50	66.23±2.09	71.51±2.61	56.88±2.45	52.12±3.90
Bundle sheath'	2.78± 0.66	3.92±0.27	3.27±1.61	2.96±0.92	9.21±3.48	12.25±6.64
	RuP_2 carboxylase					
'Mesophyll'	2.17± 0.27	7.58±2.20	4.33±1.02	2.74±0.03	15.58±5.28	17.14±5.18
'Mesophyll plus bundle sheath'	41.13±11.10	42.39±2.84	51.68±7.63	44.30±7.36	48.11±7.96	51.45±7.13
'Bundle sheath'	56.70±11.38	50.03±0.63	43.99±6.61	52.96±7.38	36.21±9.37	31.54±1.60

PS I and II Activity

The electron transport from water to DMQ is an indication of PS II activity as DMQ accepts electrons from near Q in the electron transport from PS II to PS I (Trebst and Reimer 1976). In normal plants of *A. tricolor*, an NAD-ME type C_4 plant, the PS II activity was found to be significantly greater ($P < 0{\cdot}01$) in the 'mesophyll' than 'bundle sheath' fractions (Fig. 2*a*), and has been found in other NAD-ME type C_4 plants (Ku *et al.* 1974).

The electron transport from DCPIP, reduced with ascorbate, to MV is a measure of PS I activity. Mayne *et al.* (1974) found that PS I activity, measured as P700, was generally greater in the mesophyll than the bundle-sheath cells of NAD-ME type species. In normal plants of *A. tricolor*, PS I activity was found to be significantly greater ($P < 0{\cdot}01$) in bundle sheath rather than mesophyll thylakoids.

The activity of PS II was lower in the 'mesophyll' fraction of sodium-deficient than normal plants of *A. tricolor*. There was no significant difference, however, in the PS II

activity of the 'bundle sheath' fractions from sodium-deficient and normal plants. PS I activity, assayed in the presence of Triton X-100, was greater in the 'mesophyll' fraction from sodium-deficient than normal plants of *A. tricolor* (Fig. 2*b*). There was no significant difference in PS I activity of the 'bundle sheath' fractions from sodium-deficient and normal plants.

The PS II activity of normal plants of *K. childsii*, an NADP-ME type C_4 plant, was significantly greater ($P < 0 \cdot 01$) in the 'mesophyll' than 'bundle sheath' fraction

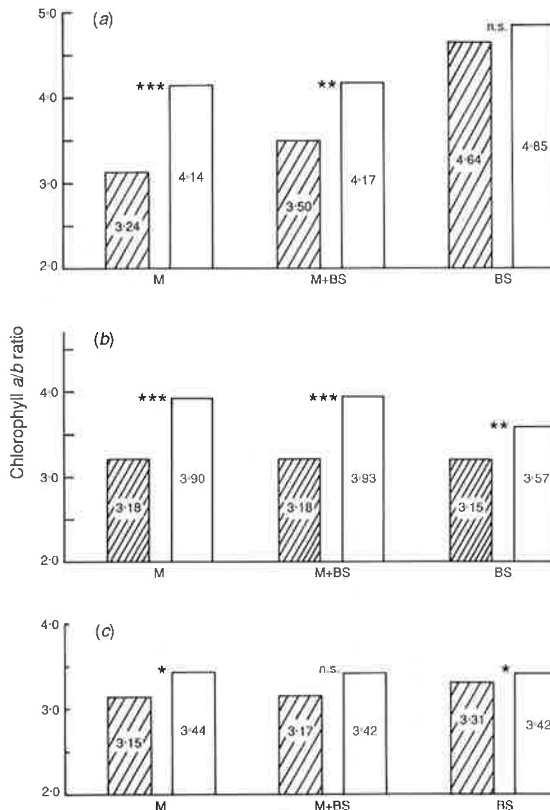

Fig. 1. Effect of NaCl on the chlorophyll *a*/*b* ratios of the 'mesophyll', 'mesophyll plus bundle sheath' (M + BS) and 'bundle sheath' (BS) fractions from leaves of sodium-deficient (hatched columns) and normal (open columns) plants of *Kochia childsii* (*a*), *Amaranthus tricolor* (*b*) and *Chloris gayana* (*c*). Significance of difference between values represented by adjacent columns: n.s., not significant; $^{*}P < 0 \cdot 05$; $^{**}P < 0 \cdot 01$; $^{***}P < 0 \cdot 001$.

(Fig. 3*a*). This is consistent with the findings that the bundle sheath chloroplasts of NADP-ME type C_4 plants are agranal, or have reduced grana, and are deficient in PS II (Anderson *et al.* 1971*a*,*b*; Andersen *et al.* 1972; Mayne *et al.* 1974). The PS II activity of the 'mesophyll' fraction of sodium-deficient plants of *K. childsii* was reduced compared to normal plants. Sodium nutrition, however, had no significant effect on the PS II activity of the 'bundle sheath' fractions or the PS I activity of either the 'mesophyll' or 'bundle sheath' fractions of *K. childsii* (Fig. 3*b*). Similar trends in the activities

of PS II and PS I in response to sodium nutrition were obtained when assays were carried out at photon irradiances exceeding saturation. The data indicate that there is less PS II activity in the mesophyll cells of sodium-deficient than in normal plants. The effect of sodium nutrition on the PS I activity varied with the different species. The PS I activity was greater in the 'mesophyll' fraction of sodium-deficient compared with normal plants of *A. tricolor*. There was no significant difference, however, in the PS I activity of either the 'mesophyll' or 'bundle sheath' fractions of *K. childsii* due to sodium nutrition.

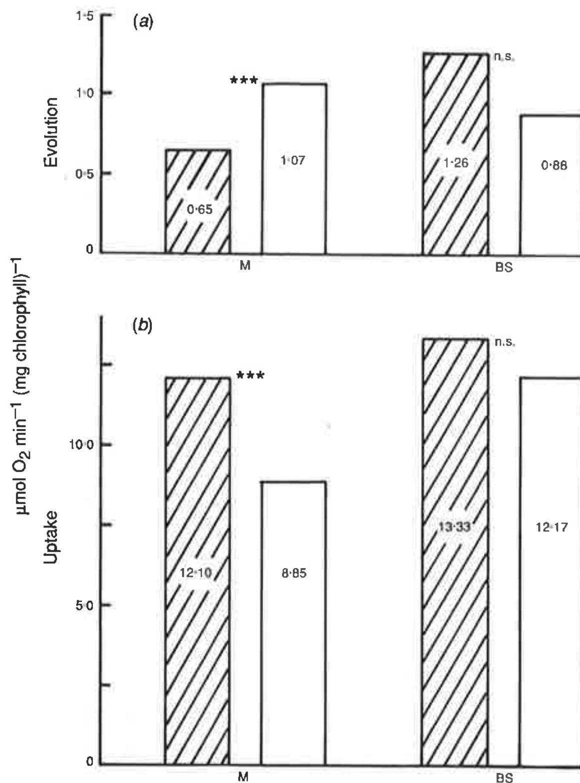

Fig. 2. Effect of NaCl on the activities of PS II (*a*) and PS I (*b*) of thylakoids from the 'mesophyll' (M) and 'bundle sheath' (BS) fractions of sodium-deficient (hatched columns) and normal (open columns) plants of *Amaranthus tricolor*. Significance of difference between values represented by adjacent columns: n.s., not significant $^{*}P<0\cdot05$; $^{**}P<0\cdot01$; $^{***}P<0\cdot001$.

According to a recent review on thylakoid structure (Thornber 1986), the chlorophyll protein of both PS I and PS II is composed of a core complex (reaction centre) and a light-harvesting complex. The chlorophyll *a*/*b* ratios are greater in the core complex than the light-harvesting complex in both PS I and PS II. The lower chlorophyll *a*/*b* ratios observed in sodium-deficient plants could imply an increased proportion of light-harvesting complexes relative to reaction centres. Simpson and Robinson (1984) reported that the reduction in chlorophyll *a*/*b* ratio in manganese-deficient plants was associated with a substantial loss of PS II reaction centres even though the light-

harvesting complex and PS I were unaffected. Similarly, a reduction in chlorophyll *a*/*b* ratio and PS II activity in sodium-deficient plants is reported in this work. Simpson and Robinson (1984) suggested that the manganese-deficient plants may be predisposed to photoinhibitory damage as there appear to be more light-harvesting complexes relative to reaction centres than in normal plants. Sodium deficiency in C_4 plants may also increase the chance of photoinhibitory damage.

Photoinhibition appears to directly affect photochemical reaction centres of PS II, reducing the electron transport of PS II while the electron transport of PS I is relatively

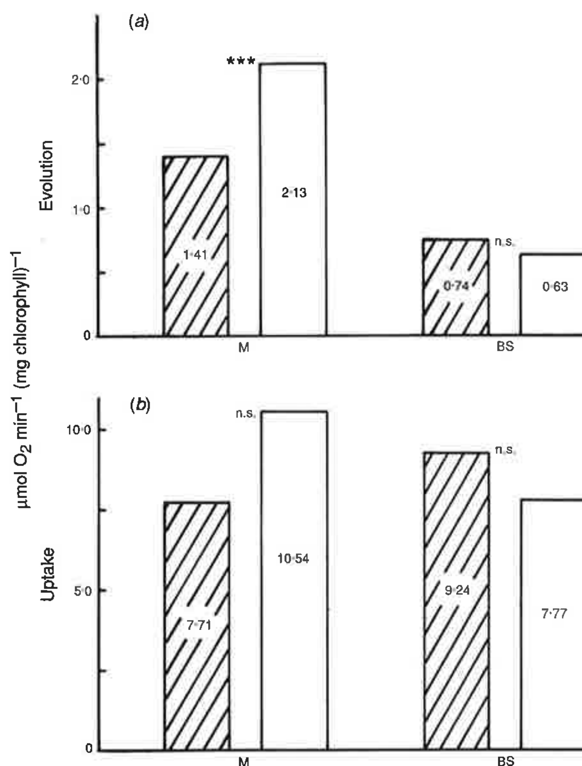

Fig. 3. Effect of NaCl on the activities of PS II (*a*) and PS I (*b*) of thylakoids from the 'mesophyll' (M) and 'bundle sheath' (BS) fractions of sodium-deficient (hatched columns) and normal (open columns) plants of *Kochia childsii*. Significance of difference between values represented by adjacent columns: n.s., not significant; $^{*}P<0\cdot05$; $^{**}P<0\cdot01$; $^{***}P<0\cdot001$.

unaffected (Critchley 1981, 1988; Powles 1984; Cleland 1988). Grof *et al.* (1989*b*) found that the F_V/F_0 fluorescence ratio of thylakoids was lower in the 'mesophyll' fractions of sodium-deficient than normal plants of *A. tricolor, K. childsii* and *C. gayana*. The lower F_V/F_0 ratio of thylakoids in the 'mesophyll' fractions of sodium-deficient than normal plants of *A. tricolor, K. childsii* and *C. gayana* (Grof *et al.* 1989*b*) is consistent with the suggestion that there is a substantial loss of PS II reaction centres, the source of fluorescence emissions, in sodium deficiency just as observed in manganese deficiency by Simpson and Robinson (1984).

The F_v/F_0 ratio of the 'bundle sheath' fraction in addition to that of the 'mesophyll' fraction has been found to be less in sodium-deficient than in normal plants (Grof *et al.* 1989*b*). It is difficult to reconcile the difference observed in the F_v/F_0 ratio between sodium-deficient and normal bundle sheath thylakoids and the apparent lack of difference in their granal arrangement (Grof *et al.* 1989*a*) and PS II activities. It is possible that this could be due to some cross-contamination between the 'mesophyll' and 'bundle sheath' fractions, particularly in *A. tricolor*. It could also be due to the sensitive fluorescence measurements responding to small changes brought about by sodium deficiency that are not detectable when measurements such as chlorophyll a/b ratio, granal counts and *in vitro* photosystem activity are made. However, the primary effects of sodium deficiency appear to be generally on mesophyll cells. This supports the suggestion by Nable and Brownell (1984) and Johnston *et al.* (1988) that there is a limitation in the conversion of pyruvate to PEP in sodium-deficient C_4 plants since the energy production for photosynthesis is altered in the mesophyll chloroplasts of sodium-deficient plants.

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Short Communication

The essentiality of sodium resolved ?

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Thirty years ago Brownell and Wood (1957) first reported a specific requirement for sodium by the higher plant, Atriplex vesicaria Heward ex Benth. Subsequently, sodium was mooted as an essential micronutrient element according to the rigidly defined criteria of essentiality proposed by Arnon and Stout (1939). Thus, plants grown in "sodium-free" culture (containing less than 0.07 mmol m^{-3} or 0.0016 ppm sodium as an impurity) showed characteristic deficiency symptoms - with yellowing of their leaves and development of white necrotic patches on leaf tips and margins. Indeed, plants died at an early stage thereby satisfying the first of the criteria, viz. "a deficiency of it makes it impossible for the plant to complete the vegetative or reproductive stage of its life-cycle". Of the Group I elements, only sodium brought about the recovery of sodium-deficient plants, therefore satisfying the second of the criteria, viz. "such deficiency is specific to the element in question, and can be prevented or corrected only by supplying this element".

However, the third of the criteria, viz. "the element is directly involved in the nutrition of the plant quite apart from its possible effects in correcting some unfavourable microbiological or chemical condition of the soil or other culture medium" was not wholly established (Brownell, 1965). In effect, this criterion necessitates the unequivocal demonstration of a specific role for sodium in the metabolism of the plant. By far the most exciting development in this field was the establishment of a correlation between the requirement for sodium and the possession of the C_4 photosynthetic pathway (Brownell and Crossland, 1972). It is in this context that we draw attention to recently published observations outlining a sodium-induced uptake of pryuvate into the mesophyll chloroplasts of C_4 plants (Ohnishi and Kanai, 1987a). This report explores the underlying mechanism of a unique metabolite translocator, located in the chloroplast envelope, which is responsible for the light-dependent uptake of pyruvate into these organelles (Flugge et al. 1985, Ohnishi and Kanai, 1982). Significantly, the properties of this translocator are quite different from those responsible for pyruvate transport in C_3 chloroplasts and bundle sheath chloroplasts of C_4 plants. Apparently, abrupt changes in the sodium concentration in the suspending medium of the chloroplasts in the dark induce the accumulation of pyruvate in the stroma, similar to the case of light-driven active uptake. Among various mono- and divalent cations the effect was specific to sodium with an apparent K_m for sodium in the range 2-5 mM. Presumably, a sodium gradient formed in the light may be the energy source for active pyruvate uptake into these chloroplasts.

C_4 photosynthesis itself involves the cooperative metabolism of two anatomically and biochemically distinct types of photosynthetic cells - termed mesophyll and bundle sheath cells. During C_4 photosynthesis atmospheric carbon dioxide is initially fixed by the carboxylation of phosphoenol-pyruvate in the mesophyll cells, producing four-carbon dicarboxylic acids - malate and aspartate (hence the term C_4 photosynthesis). These dicarboxylic acids are then transported to the bundle sheath cells where they are decarboxylated. The three-carbon compound formed during decarboxylation is then transported back to the mesophyll where it is used to regenerate PEP via pyruvate, Pi dikinase - an enzyme found in the mesophyll chloroplasts of all C_4 plants.

While the exact mechanism for pyruvate transport remains to be elucidated, we believe that this new result foreshadows the ultimate definition of a specific metabolic role for sodium - thus effectively satisfying the last criterion of essentiality.

This result sheds light on a range of physiological and biochemical attributes which characterise C_4 photosynthesis in sodium-deficient plants. Thus, the high concentrations of alanine in sodium-deficient leaves would indicate a specific lesion at the site of pyruvate to PEP conversion (Nable and Brownell, 1984) as alanine and pyruvate can be assumed to be in equilibrium (as catalysed by alanine aminotransferase). Whereas representative species from each of the three C_4 decarboxylation sub-groups have been shown to require sodium, it is significant that the PEP-carboxykinase types are generally less responsive to sodium limitations. Notably in this sub-group, PEP is the decarboxylation product which diminishes the overall flux of carbon via pyruvate to PEP conversion. A specific requirement for sodium, in terms of pyruvate transport, is consistent with this observation.

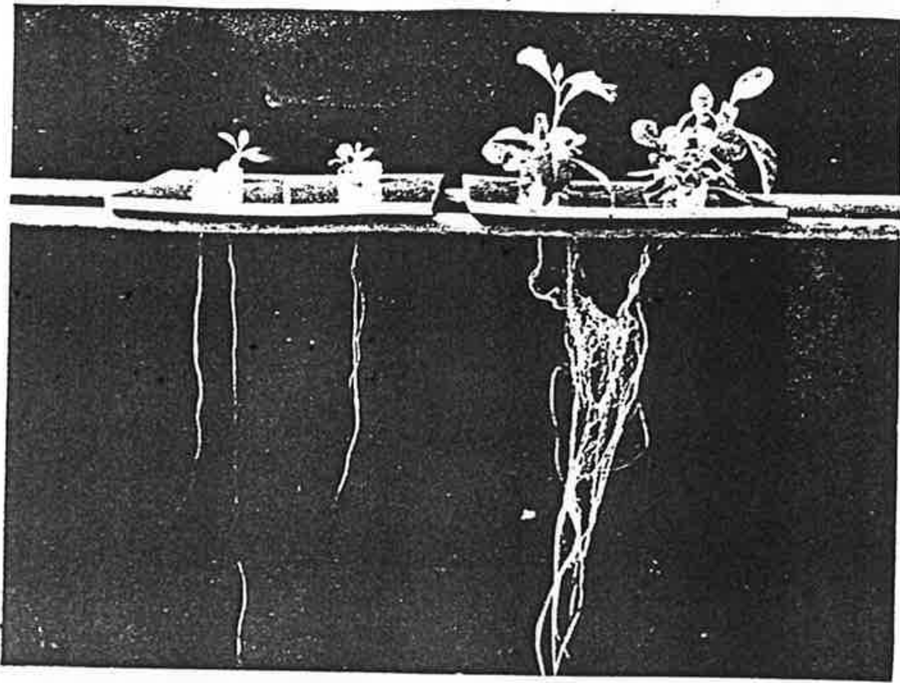

Plate 1. Comparisons between the top and root growth of Atriplex vesicaria Heward ex Benth grown in a basal culture solution which received no addition (left), and 0.60 meq/litre sodium sulphate (right).

Additionally, higher intercellular CO_2 partial pressure and more positive $\delta^{13}C$ values seen in sodium-deficient plants are again consistent with a limitation at the site of PEP formation in the mesophyll compartment (Boag, 1981). Lastly, species possessing Crassulacean Acid Metabolism (CAM) exhibit symptoms of sodium deficiency under growth conditions where greater carbon flux is through the dark fixation pathway (Brownell, 1979). Under such conditions the conversion of pyruvate to PEP is the first step in the gluconeogenic conservation of the decarboxylation product (pyruvate) as carbohydrate. If this were the case then perhaps chloroplasts of CAM plants possess a mechanism for pyruvate uptake akin to that described for C_4 mesophyll chloroplasts (Demmig and Winter, 1983).

Moreover, the lower chlorophyll a/b ratios (Johnston et al. 1984), altered chlorophast ultrastructural features and chlorophyll a fluorescence kinetics (Grof et al. 1986) symptomatic of sodium-deficiency might simply reflect the altered energy requirements for photosynthesis under conditions of sodium limitation. Especially since the conversion of pyruvate to PEP consumes two molecules of ATP per molecule of carbon dioxide fixed during C_4 photosynthesis.

Whatever the final outcome, all of the above seems to signal the end of an era in considering the role of sodium in higher plants. This most recent work (Ohnishi and Kanai, 1987) confirms suspicions of workers in the fields of sodium nutrition and C_4 photosynthesis, that a hitherto undescribed transport system unique to C_4 plants may have a sodium requirement. It these terms confirmation of sodium's essential micronutrient status seems imminent.

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HYBRIDIZATION STUDIES USING AUSTRALIAN C_4 ATRIPLEX SPECIES AND THE C_3 SPECIES A. PROSTRATA

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To elucidate the mechanisms of inheritance of the C_4 photosynthetic pathway, C_4 species of *Atriplex*, endemic to Australia, were crossed as female parents with the C_3 species *A. prostrata*, which is regarded as a relatively recent introduction to Australia. Seed set ranged from 0.9% to 73.1%, depending on the particular female parent involved and the duration of pollen application. Of 102 seeds in embryo culture, only two F_1 individuals survived to maturity. Their morphology, leaf anatomy, and fruit structure showed hybrid characteristics. Pollen grain viability was only 7.8% and 2.0%, respectively, in the two hybrids compared with pollen grain viability in excess of 95% in each parent. Of 1,160 fruits examined from the F_1 plants, only 12 contained full seeds, while 67 contained endosperm-deficient seeds. These data indicate a very distant relationship between these Australian C_4 species and *A. prostrata*. In order to investigate the genetic control of C_4 photosynthesis, it may be necessary to generate interspecific $C_4 \times C_3$ hybrids via protoplast fusion.

Introduction

Plant breeders interested in incorporating at least some of the characteristics of C_4 photosynthesis into agronomically important plants require an understanding of the genetic control of C_4 photosynthesis. For this reason, attention has focused on genetic studies within genera which include both C_4 and C_3 species: *Atriplex* is one such genus. The aim of the research reported here was to elucidate the genetic control of the C_4 pathway in *Atriplex*, by hybridization of C_4 with C_3 plants. It was anticipated that analysis of the F_1 and F_2 progeny with regard to segregation for characteristics of C_4 photosynthesis such as leaf anatomy and requirement for sodium, would clarify the patterns of inheritance of C_4 photosynthesis.

BJORKMAN et al. (1970), NOBS et al. (1970), and NOBS (1976) worked extensively on the production and analysis of hybrids between the C_4 species, *A. rosea* L. and the C_3 species, *A. triangularis* Willd. However, inadequate F_2 populations were obtained to analyze the inheritance of the C_4 pathway (BJORKMAN 1976). *Atriplex triangularis* Willd. which is now regarded as an illegitimate name for *A. prostrata* Boucher ex DC. (BASSETT et al. 1983) is a native of North America, Europe, and Asia, while *A. rosea* is of Eurasian origin. We determined whether crosses between C_4 *Atriplex* species endemic to Australia and *A. prostrata* yielded sufficiently large populations to elucidate the mechanisms controlling inheritance of C_4 photosynthesis.

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Material and methods

PLANTS

The C_3 species, *Atriplex prostrata* Boucher ex DC., was crossed as male parent with six different C_4 accessions. Seeds of the C_4 parents were supplied by Dr. M. NOBS, Carnegie Institution of Washington, Dr. B. G. THOMSON, Department of Primary Industries, Alice Springs, and Mr. W. E. MULHAM, CSIRO, Deniliquin. New accessions received at the James Cook University were assigned a T (Townsville) accession number to differentiate each seed source. This was necessary because the taxonomy of *Atriplex* was confused. Although this has been largely rectified (WILSON 1984), there still exist areas of taxonomic uncertainty. When necessary, specimens were sent to Dr. PAUL WILSON, W. A. Herbarium, Perth, for positive identification.

The six C_4 accessions used and their accession numbers were: *A. limbata* Benth. (T22); *A. eardleyae* Aellen (T21); *A. lindleyi* subsp. *inflata* (F. Muell.) Paul G. Wilson (T24); *A. lindleyi* Moq. subsp. *lindleyi* (T3); *A. lindleyi* subsp. *conduplicata* (F. Muell.) Paul G. Wilson (T4), and a natural hybrid of T3 × T4 (T26). Seed of *A. prostrata* Boucher ex DC. (= *A. hastata* auct.) (= *A. triangularis* Willdenow) was already held at James Cook University, so no accession number was assigned. The identity of this material was confirmed by Dr. P. M. TASCHEREAU, Dalhousie University, Halifax.

Plants were grown in vermiculite in the glasshouse and provided with a mineral solution: 4,000 μM $Ca(NO_3)_2$; 6,000 μM KNO_3; 1,000 μM KH_2PO_4; 1,000 μM $MgSO_4$; 1,000 μM $(NH_4)_2HPO_4$; 44.7 μM ferric ammonium ethylenetetraacetate; 23.1 μM H_3BO_3; 4.5 μM $MnCl_2$; 0.4 μM $ZnSO_4$; 0.15 μM $CuSO_4$, 0.05 μM Na_2MoO_4.

Crosses

Atriplex prostrata was always used as male parent because in this species pistillate and staminate flowers are small and too closely associated to permit effective emasculation. The C_4 species chosen as female parents were selected from a range of *Atriplex* species because their floral morphology was such that the female flowers could be isolated. In these species, male flowers occur in terminal glomerules and female flowers in distinct axillary clusters. All terminal flowers were removed prior to anthesis, and the resulting female branches were bagged. Plants were checked daily and any staminate buds removed. Pollen from *A. prostrata* was applied to female flowers for as long as the stigmas appeared receptive. As new female flowers were continually maturing on any one plant, pollen was applied for between 1–5 wk, depending on the availability of pollen. Branches were rebagged after each pollen application. After several weeks, when fruiting bracts had ripened, fruits were examined to determine seed formation.

To confirm that the pollination technique was an appropriate means of ensuring maximum seed set, *A. eardleyae* flowers were emasculated, and pollen from other *A. eardleyae* was applied for as long as the stigmas were receptive. Seven selfings were carried out.

Other emasculated plants were used to test for the occurrence of apomixis. "Female isolates" of four accessions, T21, T22, T24, and T26 were produced by the removal of male flowers, prior to anthesis. The female isolates were kept under bags to prevent pollination. When the fruiting bracts ripened, the fruits were examined for seeds.

Embryo culture

To obtain viable F_1 seedlings, embryos were excised under aseptic conditions and grown on 0.8% agar containing one-fifth strength mineral solution (described above) and 6 mM sucrose. Embryos excised from seed obtained by selfing each parent served as controls. After 7–10 d, the developing seedlings were transferred to the glasshouse and planted in vermiculite with their roots still embedded in the solid agar medium.

Analysis of F_1 plants

Whole-plant and floral morphology as well as leaf size, shape, and color of F_1 plants were analyzed. Leaf structure was examined by light microscopy, using leaf tissue which had been fixed in 2.5% glutaraldehyde in 0.025 M phosphate buffer, embedded in Spurr epoxy resin, cut 1–2 μm thick and stained with 1.0% toluidine blue in 1% borax.

Inflorescences were fixed in Carnoy's solution (6:3:1) for 48 h and stored refrigerated in 70% ethanol. Squashes, made in acetocarmine, were examined using phase contrast optics. Fresh pollen grains were stained in acid fuchsin in lactophenol, to determine their viability. Viable pollen grains stained deep pink while nonviable grains remained yellow. Morphology of mature fruits was examined and seed set was ascertained by dissection of the ripened fruits.

Results

The selfings of *Atriplex eardleyae* to assess the efficacy of the crossing technique yielded 49 seeds in 69 female flowers pollinated (71% seed set). Crosses made when pollen was plentiful yielded 48 seeds in 52 flowers (92.3% seed set), while crosses made when the pollen supply was poor resulted in only one seed in 17 flowers (6% seed set). These data indicate that, provided adequate pollen is available, poor seed set in crosses is attributable to incompatibility between the parental species rather than inappropriate pollination technique.

In the test for apomictic seed set, 331 female flowers were isolated. No seeds formed on any of the female isolates. Dissection of the mature fruiting bracts showed the dried remains of ovaries and styles but no seeds. The absence of apomictic seed formation indicated that seeds formed on pollinated female parents were hybrid.

Crosses

All parents were 2n = 18 chromosomes; seed set in crosses between six C_4 accessions of *Atriplex* and the C_3 species *A. prostrata* varied, depending on the female parent used (table 1). In *A. limbata* × *A. prostrata* only four of the 468 pollinated female flowers yielded seed (0.9%). By contrast, crosses with *A. eardleyae* yielded 30 seeds in 52 flowers pollinated (58%).

The remaining four accessions used as female parents belong to the Spongiocarpus section. Seed set ranged from 2.0% in one cross involving *A. lindleyi* (T3) to 73% in the cross with accession T26 (table 1). The differential seed set in the crosses involving T3 and T4 is attributed to the variation in the availability of pollen. When pollen was applied over 5 wk, seed set was in the order of 60%, whereas only 2.0% seed set was observed when the pollination period was restricted to 3 d.

All seeds were extremely small, less than one-quarter the size of selfed seed obtained from each parental species. Microscopic examination indicated that the seeds contained a small embryo and an extremely shrivelled endosperm. Thus, the embryos were excised and cultured in vitro. The only F_1 seeds not cultured in vitro were the first four obtained from *A. limbata* × *A. prostrata*. Three of the four seeds germinated readily, but the seedlings remained viable for only 5–6 d.

TABLE 1

SEED SET IN CROSSES BETWEEN SIX C_4 ACCESSIONS OF ATRIPLEX AND THE C_3 SPECIES ATRIPLEX PROSTRATA

C_4 species (female parent)	Accession number	Seed set/ number flowers pollinated	% seed set	Number of seeds embryo cultured
Section Semibaccatae:				
A. limbata	T22	4/468	.9	0
A. eardleyae	T21	30/52	57.7	14
Section Spongiocarpus:				
A. lindleyi:				
subsp. *inflata*	T24	13/21	61.9	13
subsp. *lindleyi*	T3	31/52[a]	59.6[a]	30
		1/49[b]	2.0[b]	
subsp. *conduplicata*	T4	37/64[a]	57.8[a]	31
		5/18[c]	27.8[c]	
T3 × T4 hybrid	T26	19/26	73.1	14

[a] Pollen applied daily for 5 wk.
[b] Pollen applied for only 3 d.
[c] Pollen applied daily for 12 d.

EMBRYO CULTURE

Of the 102 embryos aseptically excised and cultured in vitro only two survived to maturity; one involved *A. lindleyi* subsp. *inflata* and one subsp. *lindleyi* as female parent (table 1). By contrast, all 60 of the control embryos, excised from seeds of the appropriate parental accessions, developed into viable seedlings.

F_1 PLANTS

Pollen mother cells of all parental accessions yielded clear squash preparations, showing nine bivalents at metaphase I. From the two F_1 individuals, however, no countable squashes were obtained. Bivalents could not be clearly distinguished. Pollen grain viability in the two F_1 individuals was only 7.8% and 2.0%, respectively, while the parental accessions showed pollen viability of 95.2% and 98.7% (table 2).

The *A. lindleyi* subsp. *inflata* × *A. prostrata* hybrid was intermediate between the C_4 and the C_3 parent with regard to several characteristics, including height, floral morphology, and leaf color (table 3). As indicated by leaf tracings, leaf morphology of the F_1 was distinct from either parent as the leaves were sessile with entire margins (fig. 1). This and the presence of some leaf vesicles on the epidermis of the F_1, but far fewer than in the C_4 parent, were indicative of the hybridity of the F_1. The leaf anatomy substantiated that: the F_1 was quite distinct from either parent (fig. 2). The C_4 parent possessed Kranz anatomy with a conspicuous bundle sheath consisting of thick-walled cells surrounding the vascular tissue. A discrete layer of mesophyll cells enclosed the bundle sheath; there were one to two layers of spongy mesophyll cells between the palisade mesophyll and the epidermis. The leaf of the C_3 parent lacked a bundle sheath: the thin-walled spongy mesophyll cells which lay adjacent to the vascular tissue were not differentiated from spongy mesophyll cells found elsewhere in the leaf. The F_1 was clearly intermediate in leaf structure; the vascular bundle was sur-

TABLE 2

STAINABILITY OF POLLEN GRAINS IN PARENTS AND F_1 HYBRIDS FROM THE CROSS OF ACCESSIONS T3 AND T24 × ATRIPLEX PROSTRATA

Source of pollen	Ratio of stainable (pink) : nonstainable (yellow) pollen grains	% viability
A. lindleyi subsp. *lindleyi* (T3)	3353 : 103	97.0
F_1 of T3 × *A. prostrata*	289 : 3378	7.8
A. prostrata	3175 : 161	95.2
A. lindleyi subsp. *inflata* (T24)	5675 : 75	98.7
F_1 of T24 × *A. prostrata*	93 : 4557	2.0

TABLE 3

WHOLE-PLANT MORPHOLOGY, FLORAL MORPHOLOGY, LEAF SIZE AND COLOR IN ATRIPLEX LINDLEYI SUBSP. INFLATA, A. PROSTRATA, AND THE F_1 BETWEEN THEM

Character	*A. lindleyi* subsp. *inflata* (C_4)	*A. prostrata* (C_3)	F_1
Whole plant morphology . .	Erect, 35 cm tall; intricately branched	Variable, prostrate to erect plants 20–38 cm tall; diffusely branched	Semi-prostrate plant, 17 cm tall, branched profusely
Floral morphology	Female flowers in axillary clusters along full extent of stem; male flowers terminal in short glomerules 3–5 mm long	Terminal inflorescences 15–30 mm long contain male and female flowers; axils of lower branches bear 3–5 mm long inflorescences with numerous female and some male flowers	Axillary and terminal inflorescences very short (2–3 mm long) contain both male and female flowers
Leaf color, epidermal morphology	Leaves gray in color: the upper and lower epidermis covered with a layer of densely packed bladder-like vesicles	Leaves light green; no vesicles are present	Leaves light green with a distinct grayish tinge due to the presence of some vesicles but far fewer than in the C_4 parent
Leaf length (including petiole); shape	Leaves 15–25 mm long; obovate with dentate margins; leaf tapers into a narrowly cuneate petiole about 2 mm long	Leaves 25–32 mm long subtended by a petiole 5–9 mm long; triangular in shape with irregularly dentate margins	Leaves sessile, 12–20 mm long; margins entire

rounded by a readily discernible ring of bundle sheath cells. However, this sheath was much less developed than that of the C_4 parent; the thick walls that characterize the bundle sheath in *A. lindleyi* subsp. *inflata* were lacking in the F_1.

Fruit shape and structure are an important characteristic in differentiating *Atriplex* species. The fruits borne by the F_1 plant differed from those of each parent (fig. 3). Fruiting bracteoles of *A. lindleyi* subsp. *inflata* are spongy, subglobose, and pale brown, whereas the bracteoles of *A. prostrata* are thin and papery, vary from entire to dentate, are triangular to hastate, and are black at maturity. The bracteoles of the F_1 became black at maturity as did those of *A. prostrata* and tended toward the globose shape of subsp. *inflata* fruits. The fruits of the F_1 were not spongy and bore sculpturing and spines which were not present in either parent.

The low frequency of F_2 seed set by the F_1 plants was in accordance with the low pollen viability (table 2). In 460 fruits examined from the F_1 of *A. lindleyi* subsp. *inflata* × *A. prostrata,* only five full seeds and 32 endosperm-deficient seeds were formed. Similarly, 700 fruits from the second F_1 individual (subsp. *lindleyi* × *prostrata*) yielded only seven full seeds and 35 incomplete seeds.

Discussion

This is the first report of hybridization involving C_4 species of *Atriplex* endemic to Australia and a C_3 *Atriplex* species. The first artificial hybridization of $C_4 \times C_3$ *Atriplex* species involved two species naturalized in North America, *A. rosea* × *A. triangularis* (= *A. prostrata*) (BJORKMAN et al. 1970). The resulting F_1 seeds germinated readily and produced vigorous F_1 seedlings (NOBS et al. 1970). This was not our experience: the extremely poor viability of the F_1 seedlings and the very low

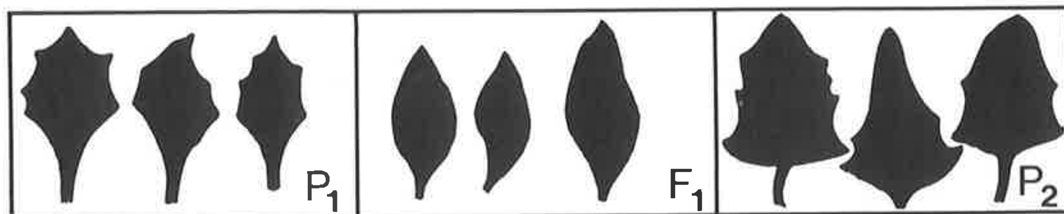

FIG. 1.—Leaf shape and size of *Atriplex lindleyi* subsp. *inflata* (P_1), *A. prostrata* (P_2), and their F_1 hybrid

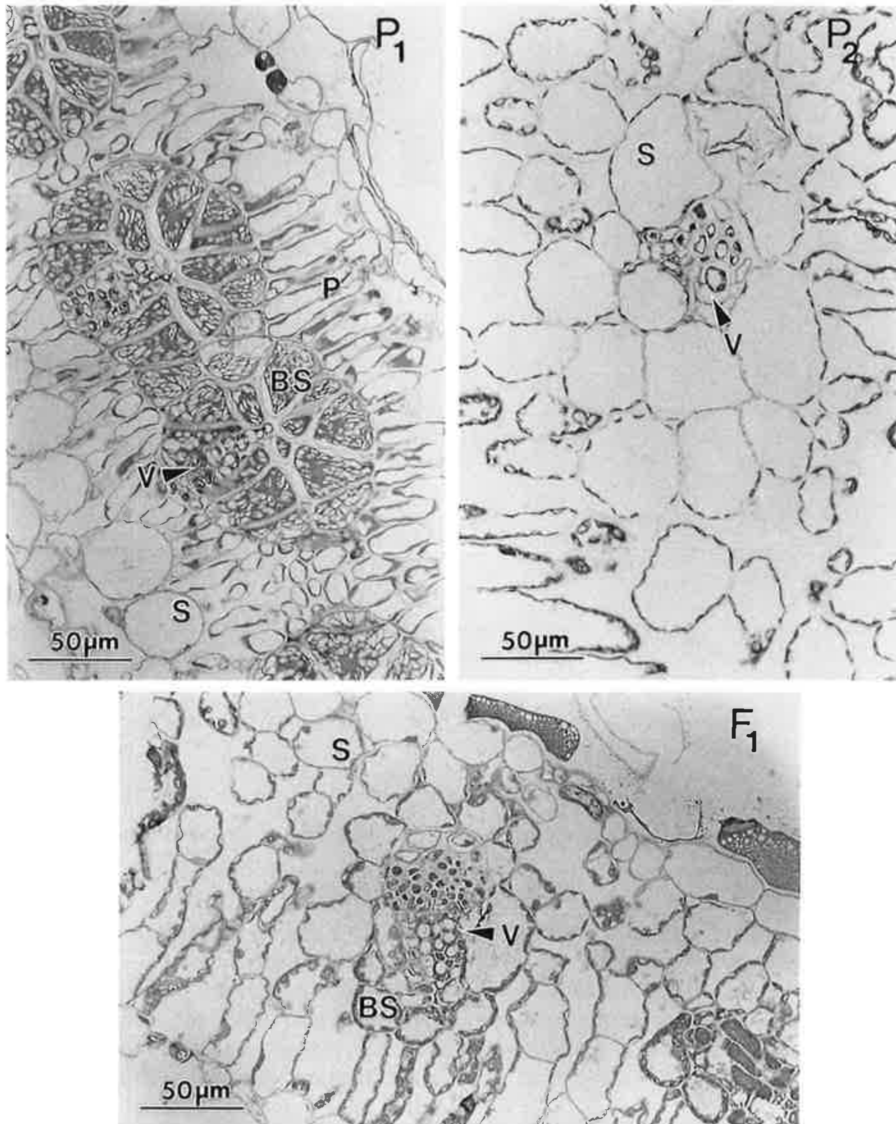

FIG. 2.—Light micrograph of cross sections of leaves of *Atriplex lindleyi* subsp. *inflata* (P_1), *A. prostrata* (P_2), and their F_1 hybrid. Vascular tissue (*V*) is enclosed in a bundle sheath (*BS*) in the C_4 parent and in the F_1, whereas in the C_3 parent undifferentiated spongy mesophyll (*S*) is associated with the vascular tissue. The bundle sheath in the C_4 parent is surrounded by a discrete layer of palisade mesophyll (*P*) cells.

frequency of F_2 seed set indicate that a strong compatibility barrier exists between the Australian C_4 species used and *A. prostrata*. The latter, a native of Europe, Asia, and North America (BASSETT et al. 1983; WILSON 1984), is probably a relatively recent introduction to Australia. Australian *Atriplex* species have been geographically isolated from those of Eurasia. The tropics of the East Indies are thought to have formed an effective barrier to gene flow (OSMOND et al. 1980). The only Australian species having possible affinity with European species is *A. australasica,* which is a C_3 plant (OSMOND 1974). However, there is some doubt as to its endemic status (WILSON 1984), and it is a tetraploid with 2n = 36 chromosomes (NOBS 1980): these two factors mitigated against its inclusion as male parent in this investigation.

Hybridization, both natural and artifical, has been well documented within the genus *Atriplex*. Evolutionary trends within the *A. prostrata* group in Scandinavia have been studied extensively (GUSTAFSSON 1973, 1976). In Britain, naturally occurring and artificial $C_3 \times C_3$ hybrids have been analyzed (TASCHEREAU 1986). The one intersectional $C_3 \times C_4$ cross attempted by TASCHEREAU (1986) was unsuccessful. These studies indicate that $C_3 \times C_3$ hybridization occurs readily in natural populations. Differing opinions have been expressed as regards $C_4 \times C_4$ hybridization: NOBS (1980) proposed that natural populations of diploid C_4 *Atri-*

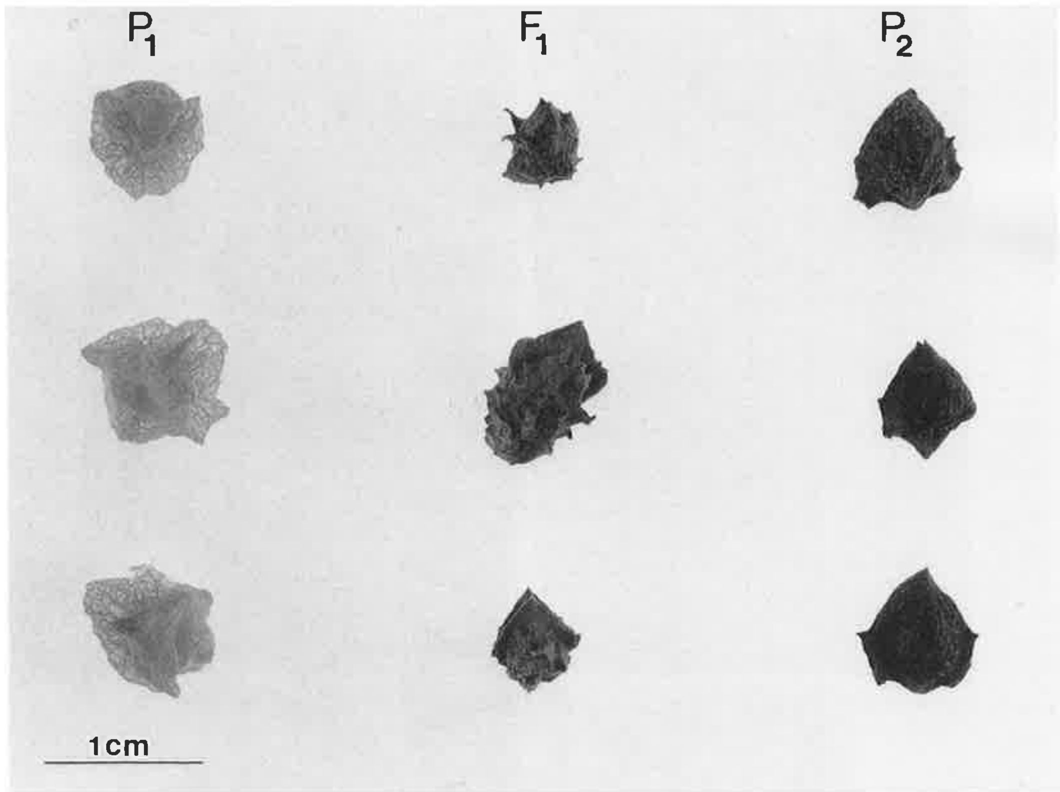

FIG. 3.—Fruit of *Atriplex lindleyi* subsp. *inflata* (P_1), *A. prostrata* (P_2), and their F_1 hybrid

plex species in Australia do not readily hybridize. However, WILSON (1984) suggested that crossing does occur, at least between subspecies of *A. lindleyi*. Spontaneous $C_4 \times C_3$ hybrids have not been observed, even between *A. rosea* and *A. triangularis*, whose distribution overlaps (OSMOND et al. 1980).

Ten percent of the *A. rosea* flowers pollinated by *A. prostrata* yielded F_1 seed (NOBS et al. 1970) while our crosses (table 1) gave much higher seed set. This suggests a closer affinity between the Australian C_4 species and *A. prostrata* than between *A. rosea* and *A. prostrata*. However, this may be a function of frequency and duration of artificial pollination. The fact that F_2 and subsequently F_3 and F_4 populations were obtained from the crosses to *A. rosea* (BJORKMAN et al. 1974), whereas we obtained very few F_2 seeds, indicates that *A. rosea* is more closely related genetically to *A. prostrata* than are any of the Australian C_4 species studied.

The two F_1 individuals which grew to maturity appeared to be hybrids, on the basis of their intermediate morphology, relative to the parents. The low viability of F_1 pollen and a seed set of only 12 full seeds in the 1,160 fruits analyzed from F_1 plants were also indicative of hybridity. It has been stated that low pollen fertility and low seed set may even occur in nonhybrid plants (BASSETT et al. 1983). We harvested many hundreds of fruit from parental plants which had been allowed to self. Those fruits rarely lacked seed, and pollen sampled from parental plants showed viability in excess of 95% (table 2).

Conventional hybridization has not resulted in the segregating diploid F_2 population which is required to analyze inheritance of C_4 photosynthesis in *Atriplex*. An alternative approach is now being undertaken: somatic hybridization of isolated protoplasts. Protoplasts have been isolated from C_4 and C_3 species of *Atriplex* (BIELIG and BROWNELL, unpublished). Somatic hybridization may overcome barriers to sexual incompatibility and result in larger F_1 and F_2 populations. Genomic incompatibility may, however, still be a barrier to interspecific hybridization.

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Increased Carbonic Anhydrase Activity in Leaves of Sodium-deficient C_4 Plants

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Abstract

The activitiy of carbonic anhydrase was determined in sodium-deficient and sodium-sufficient plants of *Kochia childsii* and *Amaranthus tricolor*. The enzyme extracted from leaves of sodium-deficient plants was found to be twice as active as that from leaves of control plants when expressed on a fresh weight or protein basis and three times as active on a chlorophyll basis. It was concluded that carbonic anhydrase activity was unlikely to limit the rate of photosynthesis in sodium-deficient C_4 plants.

Introduction

Species possessing the C_4 photosynthetic pathway and those undergoing crassulacean acid metabolism generally require sodium as a micronutrient (Brownell and Crossland 1972, 1974; Boag 1976). No requirement for sodium has been demonstrated in C_3 plants. It therefore appears likely that sodium might play a role within the C_4 pathway which is common to C_4 and CAM but not C_3 plants. *In vitro* assays of the two principal carboxylating enzymes in C_4 plants (Grof 1988) supported previous work by Brownell (1979) on phospho*enol*pyruvate (PEP) carboxylase in *Echinochloa utilis*, and Boag (1981) on PEP carboxylase and Rubisco in *Echinochloa crus-galli*, *Eleusine indica* and *Chloris barbata*. Numerous determinations carried out in these C_4 species indicate that the *in vitro* activities of the carboxylating enzymes extracted from sodium-deficient and control plants do not differ significantly. The other principal enzyme in the area of metabolism that appears to be affected by sodium nutrition, pyruvate orthophosphate dikinase, was assayed *in vitro* by Boag (1981) and Dorney (1985). They found no significant difference between the specific activities of this enzyme in extracts from sodium-deficient or control plants.

It has been shown that signs of sodium deficiency are alleviated in C_4 plants grown in atmospheres of elevated CO_2 concentrations whereas plants which received the sodium treatment did not respond to the high CO_2 concentrations (Johnston *et al.* 1984*a*). This suggested that sodium is required for the operation of the C_4 appendage in transporting CO_2 to the bundle sheath cells where it is reduced in the photosynthetic carbon reduction cycle to carbohydrate. It is unlikely that stomatal conductance of CO_2 limits photosynthesis in sodium deficiency as Boag (1981), working with species representing the three types of C_4 plants (Gutierrez *et al.* 1974; Hatch *et al.* 1975), showed stomatal physiology and function to be unaffected by sodium nutrition. He concluded that the internal CO_2 concentration would be unlikely to limit photosynthesis in sodium-deficiency at saturating irradiances. It also appeared possible that photosynthesis in sodium-deficient plants might be limited by having reduced activities of carbonic anhydrase in their leaves. This enzyme catalyses the interconversion of CO_2 and HCO_3^-, the latter being the substrate of PEP

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carboxylase in C_4 and CAM plants (Hatch and Osmond 1976). On the basis of observations on representatives of each of the three types of C_4 species (Gutierrez *et al.* 1974; Hatch *et al.* 1975), Hatch and Burnell (1990) concluded that the activity of this enzyme was only just sufficient to prevent the rate of conversion of CO_2 to HCO_3^- from limiting C_4 photosynthesis. Carbonic anhydrase is apparently restricted to the mesophyll cells of C_4 plants with the bundle sheath cells containing little or none of the enzyme (Burnell and Hatch 1988). This paper reports on experiments in which carbonic anhydrase activities measured in leaf extracts from sodium-deficient and control plants were compared on a fresh weight, protein and chlorophyll basis to determine if the activity of carbonic anhydrase might limit the rate of photosynthesis in sodium-deficient plants.

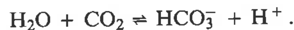
Materials and Methods

The procedures for the germination and growth of *Kochia childsii* Hort. and *Amaranthus tricolor* L. under conditions of low sodium have been described (Brownell 1979). The total sodium concentration of the complete culture solution was approximately 0·08 μM. Control plants were obtained by supplying sodium chloride to give a final concentration of 0·1 mM in appropriate culture solutions.

Plants were grown in 3·5 L vessels containing 40 plants each in a naturally illuminated growth cabinet supplied continuously with filtered air to minimise sodium contamination. The maximum irradiance in the growth cabinet was approximately 2000 $\mu mol\ m^{-2}\ s^{-1}$ (PAR). The age of plants at harvest was between 17 and 30 days.

Carbonic Anhydrase Assay

Carbonic anhydrase catalyses the following reaction:



$$H_2O + CO_2 \rightleftharpoons HCO_3^- + H^+ .$$

The activity of the enzyme was determined from the net rate of proton production (assumed to be equivalent to CO_2 hydration) during the reaction carried out under controlled conditions. The plant extract was prepared by grinding 0·5–0·8 g of fresh leaf material in 1 mL 50 mM HEPES-KOH (pH 8·2) containing 10 mM dithiothreitol. The grindate, after being filtered through Miracloth, was made up to a volume of 10 mL by the addition of the HEPES-KOH buffer.

Carbonic anhydrase activity was determined from the rate of hydration of CO_2 at 0°C by following the change of pH traced on a chart recorder according to a method similar to that of Hatch and Burnell (1990). The assay was carried out in a 0°C coldroom. A Metrohm pH meter was coupled to a recorder which was adjusted to give full-scale deflection with a decrease of pH from 8·3 to 7·1 units. Calibration was carried out by following the fall in pH from 8·3 to 7·3 units upon the sequential additions of 100 μL of 0·1 M H_2SO_4 to the assay medium which contained 5 mL 50 mM barbitone buffer (pH 8·3). It was found that there was a fall of 0·2 pH units per 100 μL of 0·1 M H_2SO_4. Therefore, 1 pH unit was equivalent to 0·1 mmol H^+ or 0·1 mmol CO_2 hydrated. The carbonic anhydrase activity was determined by the difference in the rates of decrease in pH in the assay medium containing 5 mL ice-cold CO_2-saturated water in the presence and absence of the 0·1 mL plant extract. The specific activities of carbonic anhydrase were expressed as mmol H^+s produced per minute on fresh weight, protein and chlorophyll bases. Protein concentrations were determined by the method of Lowry *et al.* (1951). Chlorophyll concentrations were estimated in ethanol extracts (Johnston *et al.* 1984*b*).

Results and Discussion

The activity of carbonic anhydrase, unlike that of the other enzymes previously studied in C_4 plants, was found to be markedly affected by sodium nutrition (Table 1). This enzyme was determined to be about twice as active in sodium-deficient as control plants when the activity was expressed on fresh weight and protein bases and three times as active when expressed on a chlorophyll basis. It now appears unlikely that the activity of carbonic anhydrase is a limiting factor in sodium-deficient plants. In addition, as stomatal physiology and function have been reported to be unaffected by sodium nutrition, it appears unlikely that internal CO_2 concentration is limiting photosynthesis (Boag 1981). The data reported

Table 1. Specific activities of carbonic anhydrase extracted from leaves of sodium-deficient and control C_4 plants

Significance of differences between adjacent values: n.s., not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n > 4$

Treatment	Specific activity of carbonic anhydrase[A]		
	Fresh weight basis [mmol CO_2 hydrated min^{-1} (g fresh wt)$^{-1}$]	Protein basis [mmol CO_2 hydrated min^{-1} (mg protein)$^{-1}$]	Chlorophyll basis [mmol CO_2 hydrated min^{-1} (mg Chl)$^{-1}$]
	Kochia childsii		
No addition	49·65	1·54	60·25
	***	***	***
0·1 mM NaCl	27·78	0·84	26·68
	Amaranthus tricolor		
No addition	40·37	2·22	45·75
	*	*	**
0·1 mM NaCl	16·37	0·51	10·53

[A] An average of at least two determinations each from three separate extracts.

here are consistent with the hypothesis of Nable and Brownell (1984) that the regeneration of the primary CO_2 acceptor, PEP, in mesophyll chloroplasts is limiting in sodium deficiency.

Increased levels of alanine and pyruvate and decreased levels of PEP, oxaloacetate, malate and aspartate in leaves of sodium-deficient C_4 plants (Nable and Brownell 1984; Grof *et al.* 1986*a*; Johnston *et al.* 1988) suggested a block in the conversion of pyruvate to PEP in mesophyll chloroplasts. The major steps in the process involve the transport of the pyruvate into the mesophyll chloroplast, its enzymatic conversion to PEP and the provision of energy required for the conversion. No effect of sodium nutrition on the activity of pyruvate phosphate dikinase, the enzyme that catalyses the conversion, has been observed (Boag 1981; Dorney 1985). However, a sodium-activated pyruvate transport system in mesophyll chloroplasts of *Panicum miliaceum*, a NAD-ME type of C_4 plant (Gutierrez *et al.* 1974; Hatch *et al.* 1975) has been reported by Ohnishi and Kanai (1987). Ohnishi *et al.* (1990) were able to demonstrate the requirement for sodium for the active uptake of pyruvate in *Urochloa panicoides* and *Panicum maximum* (phospho*enol*pyruvate carboxykinase-type C_4 plants) but not in *Zea mays* and *Sorghum bicolor* (NADP-ME type C_4 plants) (Gutierrez *et al.* 1974; Hatch *et al.* 1975). Sodium has been shown to be essential for *Echinochloa utilis* and *Kochia childsii* (Brownell and Crossland 1972) which are also NADP-ME type plants. It therefore appears that the activation of the pyruvate transport system may not be the sole function of sodium in the nutrition of C_4 plants.

There is evidence of damage to the light-harvesting system, the source of energy for the pyruvate transport and/or the regeneration of PEP in the mesophyll chloroplasts from sodium-deficient plants. The evidence includes lower chlorophyll *a*/*b* ratios (Johnston *et al.* 1984*b*, 1989), fluorescence yields (Grof *et al.* 1986*b*, 1989), and photosystem II activity (Johnston *et al.* 1989) and altered ultrastructure of the mesophyll chloroplasts (Grof *et al.* 1988).

It is of interest to note that increased activity of carbonic anhydrase has been reported in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* when grown under reduced CO_2 concentrations (Graham *et al.* 1970). Could these examples of increased activities of the enzyme represent a redeployment of the plants' protein resources in response to limited CO_2 availability to the Rubisco enzyme?

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The Function of Sodium as a Plant Micronutrient

Peter F. Brownell

Peter Brownell is a native of Australia, recently retired from the James Cook University of North Queensland in Townsville where, beginning in 1971, he taught plant physiology to students in a master of science course in tropical agriculture. As he tells us in this essay, he has long been interested in the role of sodium in plant nutrition and it was some of his early studies that convinced other plant physiologists that sodium is indeed an essential element—at least for some plants. Those plants turned out to be the ones that use C-4 photosynthesis (which we discuss in Chapter 11). Professor Brownell is now studying the biochemical steps in the process where sodium plays its role.

In 1954, when Professor J. G. Wood of the Botany Department at the University of Adelaide suggested a Ph.D. project to determine whether sodium and/or chlorine were essential micronutrients for plants, little did I think that I would still be working in this field with my students some 35 years later.

Sodium nas long been regarded as a possible *macro*nutrient. In 1945, Harmer and Benne arranged species into groups depending upon their responses to sodium with insufficient or sufficient supply of potassium. It was clear that a certain amount of potassium not replaceable by sodium was needed by all species, but there was no evidence for sodium being an essential element. Its main role was in its ability to substitute for some of the potassium needed for maximum growth.

The first suggestion that sodium might be needed as a *micro*nutrient element was probably made by Pfeffer in the late 19th century. Little attention was given to this possibility until the early 1950s, when Professor Wood suggested that sodium and/or chlorine could be essential for plants in very small amounts. At that time no experiments had been reported in which these elements had been carefully excluded from the plants' environment. Soon after the beginning of our study, Clarence Johnson and Perry Stout visited Adelaide with the news that their team at the University of California, Berkeley, had discovered chlorine to be an essential micronutrient for tomatoes. In the same year, also at Berkeley, Allen and Arnon found sodium to be an essential micronutrient element for the cyanobacterium *Anabaena cylindrica*. This was the first time that sodium had been shown to be essential for plant life. At this stage, we decided to invest all our efforts in determining whether higher plants might also require sodium.

Two developments greatly assisted the investigation. The first was the introduction of the flame photometer, followed soon after by Alan Walsh's invention of the atomic absorption spectrophotometer. This enabled us to measure sodium at low concentrations, accurately and rapidly. The second was the availability of plastic materials virtually free of sodium.

One species chosen for this work was *Atriplex vesicaria* (bladder salt-bush), which grows in the southern arid areas of Australia. Salt-bushes accumulate sodium and chlorine at high concentrations (up to 23 percent NaCl on a dry-weight basis) compared with other plants growing in similar habitats. The Australian salt-bushes also possess bundle sheath anatomy, which is now known to be a feature of C-4 plants [see Chapter 11 for description of C-4 plants]. We were very fortunate to have chosen a C-4 species, unknowingly, for our early work.

We took much care to exclude sodium from the environment of the plants. In early experiments, carried out in a conventional glass house, the amounts of sodium recovered in plant material plus that remaining in the culture solutions were several times greater than the amounts of sodium known to have been supplied as impurities of the water, culture media, and seeds. On the other hand, there was no detectable increase in sodium in later experiments conducted in a small greenhouse supplied continuously with air filtered to exclude dust that contained sodium. The water—which was distilled twice, the final stage in a silica still—had a sodium concentration of less than 0.0002 mg L^{-1}. Salts of culture solutions were generally purified by up to six recrystallizations in platinum or silica vessels. Some components of the culture solution were prepared from reagents redistilled in silica. For example, ammonium chloride was made up from silica-distilled ammonia and hydrochloric acid. The final concentration of sodium in a complete culture solution prepared from these salts was less than 0.0016 mg L^{-1}, which is only a little over a one-hundredth of the sodium which would have been derived from untreated analytical-reagent salts. When plants were grown under these conditions, they showed greatly reduced growth, chlorosis, and necrosis of the leaves. Plants receiving 1 mg L^{-1} sodium in their cultures, irrespective of the salt (the anion), showed normal growth. In some cases, growth of 45-day-old sodium-treated plants was over 20 times that of untreated plants. The response was specific to sodium, as no other group 1 elements were effective. It was very hard to leave such a project, and this is probably one reason why I am still working on it!

Two major questions arose from these results: What is the function of sodium in *Atriplex vesicaria*, and Do other plant species require sodium as a micronutrient element?

The functions of the essential transition elements, including iron, copper, zinc, manganese, and molybdenum, that can take part in oxidation/reduction reactions have generally been discovered incidentally during other metabolic studies. For example, from research on photosynthesis, a function for copper became obvious when it was shown to be a component of plastocyanin. Similarly, a function for molybdenum became apparent from studies of nitrogen metabolism; molybdenum was found to be a component of the nitrate reductase and nitrogenase enzymes. Our knowledge of the functions of other essential micronutrient elements—boron, chlorine, and sodium—has lagged behind because no direct effects on enzyme systems have been observed. Information of their functions had to be acquired by other means, often with little information from which to start.

We were puzzled when we attempted to answer the second question regarding whether or not there was a general requirement by higher plants for sodium. From the pattern observed with other essential elements, it was expected that sodium would be required by all higher plants. Surprisingly, of the 30 species examined, including halophytes, other chenopods, and other nonendemic species of *Atriplex*, only the 10 Australian species of *Atriplex* were found to require sodium. At this time, these differences in response could not be correlated with any obvious differences among the species studied. It still seemed possible that all higher plants might require sodium, but those plants that had grown normally without added sodium might require extremely small amounts compared with the Australian *Atriplex* species. However, after the discovery of the C-4 photosynthetic pathway by Hal Hatch and Roger Slack in 1966, it seemed likely that only plants having the C-4 pathway required sodium. Chris Crossland and I tested this possibility with C-4 plants from different families and found that they all responded to small amounts of sodium, as did the Australian species of *Atriplex*. We were excited at this discovery because it gave us a clue to the possible function of sodium in these plants. It suggested that sodium was required for the operation of the C-4 appendage in transporting CO_2 to the bundle sheath cells, where it is reduced to carbohydrates [as described in Chapter 11]. In support of this, we found that the signs of sodium deficiency were alleviated in plants grown in atmospheres with elevated CO_2 concentrations. However, plants that had received the sodium treatment showed little or no response to the high CO_2 treatments, which suggested that under conditions of sodium deficiency transport of CO_2 to the bundle sheath cells is decreased, thus limiting the rate of photosynthesis. When the atmospheric concentration of CO_2 in which the plants are grown is elevated to about 1,500 $\mu L\ L^{-1}$, CO_2 enters the bundle sheath cells by diffusion, thus bypassing the C-4 system.

Chris Crossland and I also found responses to sma amounts of sodium by a crassulacean-acid metabolisin (CAM) plant, *Bryophyllum tubiflorum*, when it was grown der certain conditions that encourage activity of the CAM pathway [see Chapter 11 for description of CAM]. There strong similarities in the photosynthetic metabolism of th plants with those of C-4 plants.

Ross Nable and Mark Johnston found high levels of nine and pyruvate and low levels of phosphoenolpyruva sodium-deficient C-4 plants. The pyruvate formed from C-3 compounds returning from the bundle sheath is con verted to phosphophenol pyruvate. The major steps in th process involve the transport of pyruvate into the mesop chloroplast, the enzymatic conversion of pyruvate to pho phoenolpyruvate within the stroma, and the provision of ergy required for the conversion reaction. No effect of so nutrition on the activity of pyruvate phosphate dikinase, enzyme catalyzing the conversion of pyruvate to phosph enolpyruvate, has been found.

Recently, Jun-ichi Ohnishi and Ryuzi Kanai discove a sodium-induced uptake of pyruvate into the mesophyll chloroplasts of *Panicum miliaceum*. This immediately su gested a role for sodium in C-4 plants. However, Mark Jo ston and Chris Grof have obtained evidence for damage the light-harvesting photosystem, the source of energy f ruvate transport and/or the regeneration of phosphoenol ruvate. In sodium deficiency, they found lower chlorophy ratios and lowered photosystem II activity, with altered u structure in the mesophyll chloroplasts. With the discove of the light- and sodium-activated membrane translocat system and of the damage to the energy-producing mac ery in the mesophyll chloroplasts, research on the actual function of sodium has reached an exciting phase. We st have a difficult question to answer: What is the primary fu tion of sodium? Is it needed to maintain the integrity of th light-harvesting and energy-transducing systems in the sophyll chloroplasts, or for transport of pyruvate into the sophyll chloroplasts? If the latter is the case, then damag the light-harvesting system observed in mesophyll chlor plasts of sodium-deficient plants could have been cause excess energy that normally would have been used to c vert pyruvate to phosphoenolpyruvate.

An intriguing feature of the hypothesis of sodium's i volvement in the transport of pyruvate across a membra is that a similar sodium-requiring system has been demo strated in species of cyanobacteria for the uptake of bica bonate ions. In 1967, Professor Don Nicholas and I found activity of nitrate reductase to be many times greater in s dium-deficient compared to normal cells of *Anabaena c drica*. We were unable to obtain a similar effect of sodium

(contir

The Function of Sodium *(continued)*

deficiency on nitrate reductase in C-4 plants. The effect of sodium nutrition on nitrate-reductase activity in *Anabaena* might have been a consequence of some earlier effects of the sodium treatment, perhaps those related to the transport of inorganic carbon.

It is unlikely that a lack of sodium will ever limit plant growth in nature. However, as sodium has an important role in C-4 photosynthesis, this continues to be a challenging and exciting project. It has been extremely rewarding to have been able to demonstrate the essentiality of another element, to show that its need is restricted to C-4 plants, and to have evidence that directs us to focus our attention on the metabolism of the mesophyll chloroplast. We have had our share of luck (which is necessary for some success in this kind of work), including the choice of a C-4 plant as our experimental material long before we knew what a C-4 plant was, and the timely discovery of this C-4 photosynthetic pathway during the project. Perhaps the greatest good fortune has been to have had excellent colleagues who have been generous with their help and interest. Another real asset has been having enthusiastic students who have shared the disappointments and periods of excitement that are part of such a project.

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percent silicon. The contents of most dicots are much lower than those of grasses or *E. arvense*.

As is so often the case in studies of essentiality, it is difficult to completely remove the element from the plants' environment to determine whether they can grow without it. But with silicon the problems are especially serious because it is present in glass and many nutrient salts, and it also exists as particulate SiO_2 in the atmosphere. In several species (rice, barley, sugarcane, tomato, and cucumber) the amount of silicon has been reduced enough to create deficiency symptoms (Miyake and Takahashi, 1985). In rice, for example, overall growth was retarded, transpiration was increased about 30 percent, and the older leaves died. In tomatoes, growth rates were lowered about 50 percent, new leaves of nearly mature plants were deformed, and many plants failed to set fruits. Werner and Roth (1983) and others indicated that silicon is generally an essential element. So far as we are aware, however, no dicot grown with limited silicon has failed to produce viable seeds, nor has any essential role for silicon in the plants been shown. Furthermore, soybean plants grown without silicon accumulated unusually high concentrations of phosphorus (Miyake and Takahashi, 1985), so it seems possible that symptoms of silicon deficiency sometimes represent phosphate toxicity. For certain algae (diatoms and some flagellate Chrysophyceae) that are surrounded by a silica-rich sheath, silicon is certainly essential.

Silicon exists in soil solutions as silicic acid, H_4SiO_4 [or $Si(OH)_4$], and is absorbed in this form. It accumulates largely as polymers of hydrated amorphous silica ($SiO_2 \cdot nH_2O$), most abundantly in walls of epidermal cells but also in primary and secondary walls of other cells of roots, stems, and leaves and in grass inflorescences (Kaufman et al., 1985; Sangster and Hodson, 1986). It also accumulates intracellularly in specialized epidermal cells called silica cells.

Various functions of silicon in plants have been suggested. When it accumulates in epidermal cell walls, it seems to cause less transpiration and fewer fungal infections. There is evidence that in xylem cells silicon provides rigidity and limits compression, such as that caused by bending in wind. In fact, it is well known that silicon-deficient cereal grain crops are more easily lodged (bent down by wind or rain) than those with adequate silicon. There are also claims that the silicates present in grass leaves and inflorescences reduce grazing (herbivory) by animals and insects. Prevention of lodging or herbivory would thus represent an ecological requirement for silicon rather than a physiological or biochemical requirement included in the two criteria for essentiality mentioned earlier.

When sheep and cattle eat grasses abundant in silica, they excrete most of it in the urine, but sometimes it forms kidney stones. Silica is also blamed for causing excessive wear on sheep's teeth, and it is implicated ir throat cancers of people from northern China and Irai who eat bracts of inflorescences of foxtail millet (*Setari italica*) and of *Phalaris minor*, respectively.

Cobalt is essential for many bacteria, including cy anobacteria (blue-green algae). It is required for nitro gen fixation by bacteria in root nodules on legume (Section 14.2). Figure 6-4 illustrates growth of soybean with and without cobalt and with only atmospheric ni trogen, which was fixed in root nodules. Cobalt conce