Studies of crassulacean acid metabolism in *Mesembryanthemum crystallinum* and *Kalanchoe daigremontiana*

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A thesis presented for the degree of Doctor of Philosophy in the University of Leicester 1988.

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ACKNOWLEDGEMENTS

I am indebted to my supervisor, Dr. Bill Cockburn, for his encouragement and incisive guidance during my three years in the Botany Department at Leicester University (October 1984 - September 1987); and for his time and patience during the writing up which continued after taking up a post at Rothamsted Experimental Station in January 1988.

Thanks are also due to Dr. Dennis Ratcliffe for his help with the estimations of water and solute potential and the measurement of inorganic phosphate. In addition I am grateful to Sue Ogden for drawing the graphs.

Finally, my gratitude extends to Janet Why for her diligence and patience in typing the manuscript.

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ABBREVIATIONS

ABA	abscisic acid
ADP	adenosine diphosphate
ADPG	adenosine diphosphoglucose
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BES	N,N-bis[2-hydroxyethyl]-2-amino ethanesulphonic acid; 2-[bis(2-hydroxy ethyl)amino] ethanesulphonic acid
BSA	bovine serum albumin
CAM	crassulacean acid metabolism
cAMP	cyclic adenosine monophosphate
DHAP	dihydroxyacetone phosphate
DTT	dithiothreitol
EC	enzyme commission
EDTA	diaminoethanetetra acetic acid disodium salt
eq	equivalents
FBP	fructose 1,6 bisphosphate
F2,6BP	fructose 2,6 bisphosphate
F6P	fructose 6 phosphate
F.W.	fresh weight
G1P	glucose 1 phosphate
G6P	glucose 6 phosphate
G3P	glyceraldehyde 3 phosphate
GSH	reduced glutathione
Hepes	N-2-hydroxyethylpiperazine N-2-ethanesulphonic acid
HMDS	hexamethyl disilazane
HPLC	high performance liquid chromatography
Ma	malic acid
MES	(2-[N-morpholino]ethanesulphonic acid)
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ND	not detected
NS	not significant
OAA	oxaloacetic acid
Pa	Pascals
Pi	inorganic phosphate
PPi	pyrophosphate
PEG	polyethylene glycol
PEP	phosphoenol pyruvate
Pyr	pyruvate
1,3 PGA	1,3 phosphoglyceric acid
3 PGA	3 phosphoglyceric acid
RRT	relative retention time
RUBISCO	ribulose 1,5 bisphosphate carboxylase-oxygenase
St	starch
Sucrose P	sucrose 6 phosphate
TMCS	triemethylchlorosilane
Tris	(hydroxymethyl) aminomethane
TSC	total soluble carbohydrate
UDP	uridine diphosphate
UDPG	uridine diphosphoglucose
ψ	water potential
ψs	solute potential

INTRODUCTION

General Introduction to Photosynthesis and Crassulacean acid Metabolism

Photosynthesis is a process by which green plants use the electromagnetic energy of sunlight to synthesise carbohydrates and other cellular constituents from carbon dioxide and water.

$$CO_2 + 2H_2O \xrightarrow{\text{light}} (CH_2O) + O_2 + H_2O$$

Photosynthesis may be divided into two phases: a light phase, in which the electromagnetic energy of sunlight is trapped and converted into ATP and NADPH, and a synthetic phase, in which the ATP and NADPH generated by the light phase are used for biosynthetic carbon reduction.

All oxygenic organisms have a common pathway for the reduction of CO_2 to sugar phosphates. It is termed the C_3 pathway because the first product of carboxylation, 3, phosphoglyceric acid, (3PGA) contains three carbon atoms. It is also known as the Calvin-Benson cycle or the reductive pentose phosphate pathway. Ribulose 1,5 bisphosphate carboxylase-oxygenase (RUBISCO), (EC 4.1.1.39) is the carboxylating enzyme involved, possibly the most abundant protein in the world.

Although the C_3 cycle is the fundamental carboxylating mechanism,a number of plants have evolved adaptations in which CO_2 is first fixed by a supplementary pathway and then released in the cells in which the C_3 pathway operates. Two supplementary carboxylating pathways are known to occur in higher plants. These are the C_4 and crassulacean acid metabolism (CAM) pathways both of which are considered as forms of photosynthetic acid metabolism (Cockburn, 1985). The first products of CO_2 fixation in plants exhibiting these forms of photosynthesis are four carbon (C_4) acids. Phosphoenol

pyruvate carboxylase (PEP carboxylase), (EC 4.1.1.31) is the carboxylating enzyme of these supplementary pathways and the C_4 acids formed act as carbon carrying intermediates between this primary carboxylation and RUBISCO.

In C_4 photosynthesis the supplementary carboxylation pathway and the C_3 pathway are separated spatially in two cell types. The carboxylation catalysed by PEP carboxylase occurs in mesophyll cells. The second carboxylation involving the release of CO_2 from the C_4 acid and its refixation by RUBISCO occurs in bundle-sheath cells. The transport of CO_2 from the mesophyll cells to the bundle-sheath cells is facilitated by a radial leaf anatomy (Kranz anatomy), where the outer tissue layer consisting of mesophyll cells surrounds the inner tissue layer of bundle-sheath, or Kranz cells. The discovery of C_4 photosynthesis in sugar cane (Hatch and Slack, 1966) led to the speculation that the C_4 pathway was peculiar to tropical grasses, but it was later found in species of dicotyledons, *Amaranthus* (Amaranthaceae) and *Atriplex* (Chenopodiaceae) (Hatch and Kagawa, 1974).

In CAM tissue the two carboxylation reactions occur in the same cell, but are separated temporally, the C_3 pathway being dependent on ATP and NADPH occurs in the light, and the supplementary carboxylation pathway occurs at night.

The supplementary carboxylation pathways of C_4 and CAM photosynthesis both act to concentrate CO_2 at the site of RUBISCO activity in the light. This enhances the carboxylating activity of the enzyme and suppresses oxygenating photorespiratory activity which occurs in the presence of oxygen, releasing fixed CO_2. Enhanced carboxylating activity of RUBISCO relative to oxygenating activity may allow less investment of nitrogen in RUBISCO protein. In fully expanded leaves of the CAM plant *Kalanchoe pinnata* L. less than 20%

of total soluble leaf protein is invested in RUBISCO compared to 50% frequently seen in C_3 species (Winter, Foster, Schmitt and Edwards, 1982). The CO_2 concentrating mechanism means that the leaf requires access to atmospheric CO_2 for a shorter period of time than in C_3 plants. Stomatal opening can be reduced and less water is lost during the acquisition of atmospheric CO_2 than in the C_3 pathway. The CO_2 concentrating mechanism is particularly advantageous in hot, dry environments where the potentials for water loss and photorespiration which both increase with temperature are great.

In CAM plants this water-conserving feature is enhanced by the fact that the supplementary carboxylation reaction through open stomata occurs at night when the evaporative demand of the atmosphere is low. High internal CO_2 concentrations during the day - up to a hundred times ambient levels (Cockburn, Ting and Sternberg, 1979) - ensure that the stomata are kept shut when the evaporative demand of the atmosphere is greatest. Consequently, low ratios of water transpired to plant dry weight gain (transpiration ratio) are found in CAM plants (Winter, 1985). CAM values are typically below 100 compared to 500 for C_3 species and 300 for C_4 plants. Additionally the accumulation of malic acid at night and the resulting decrease in the water potential of the leaf tissue may aid water acquisition by the plant (Luttge, 1986). This diel fluctuation of organic acid ensures an inverse stomatal rhythm, a distinguishing feature of CAM vital in enabling these plants to conserve water and survive in arid conditions and one which leads to their distribution in natural environments being directly related to water availability (Ting, 1985).

The Romans may have been the first to notice the diel fluctuation of acid typical to CAM plants (Rowley, 1978). They noticed that the leaves of *Sedum acre* L. tasted less bitter towards the end of the day

than they did at the beginning of the day. De Saussure (1804) was the first to observe nocturnal CO_2 fixation by a CAM plant using *Opuntia* stem joints. Heyne (1813) like the Romans tasted leaves, and he explained the diel change in acid content in *Bryophyllum calycinum* L. in terms of O_2 uptake and release rather than fixation of CO_2. The notion that CO_2 fixation and organic acid metabolism were linked evaded scientists until the 1940s. Thomas (1949) and Thomas and Beevers (1949) observed a simultaneous uptake of CO_2 and O_2 in *Bryophyllum* plants in the dark. This together with the already known ability of the plant to accumulate acid and considerations of the newly discovered Wood and Werkman reaction proposing the carboxylation of pyruvate to form propionic acid (Wood and Werkman, 1938) led Thomas to suggest that CAM malate was synthesised from internal and external CO_2 by the carboxylation of an acceptor derived from starch and soluble sugar. This proposal led to more rapid progress in research work on CAM during the 1950s and 60s. The discovery of C_4 photosynthesis in the mid 1960s (Kortschak, Hartt and Burr, 1965; Hatch and Slack, 1966) and its similarity to CAM further stimulated research. Progress since then has been rapid and has highlighted the flexibility of the CAM mechanism.

Most CAM plants possess a number of distinguishing features. They fix CO_2 nocturnally and undergo a diel fluctuation of organic acid with a reciprocal diel fluctuation of storage carbohydrate. It is estimated that up to 20% of the plants organic material is causally linked to the production of PEP, the CO_2 acceptor (Black, Carnal and Kenyon, 1982). CAM plants tend to have large storage vacuoles in which molecules and ions are sequested. Up to 99% of the volume of *Kalanchoe daigremontiana* Hamet et Perrier protoplasts is taken up by the vacuole (Steudle, Smith and Luttge, 1980). CAM plants tend to have a high degree of succulence which is related to

the capacity of the vacuoles to accumulate malic acid during the dark (Winter, 1979a). The succulence of CAM plants together with the tendency to partition a lot of carbon into producing thick stems and thick leaves, when not reduced to spines, confers a low surface area to volume ratio assisting these plants to survive in conditions of low water availability. A low stomatal frequency common in CAM plants further reduces water loss (Kluge and Ting, 1978). The shallow roots of many CAM plants facilitate the quick uptake of surface water during short-lived periods of rainfall (Nobel and Sanderson, 1984).

The presence of CAM can be identified and the extent of its activity measured by observing gas exchange and acid levels on a diel basis. The degree of discrimination between the two isotopes of carbon can also identify the presence of CAM. PEP carboxylase discriminates less against ^{13}C than RUBISCO (Whelan, Sackett and Benedict, 1973; Deleens, Lerman, Nato and Moyse, 1974). Hence the ratio of $^{13}C/^{12}C$ ($\delta^{13}C$) is different between C_3, C_4 and CAM plants. C_3 and C_4 plants have $\delta^{13}C$ values that are fairly constant in the region of $-27°/\infty$ and $-11°/\infty$ respectively, but the value in CAM plants can vary from -14 to $-31°/\infty$ (Lerman, 1975), because in CAM plants the fixation of CO_2 from the atmosphere can be catalysed by PEP carboxylase and RUBISCO.

CAM has been found to occur in many species outside of the Crassulaceae family used in many of the early studies and after which CAM was named. It is a feature of species of the families Agavaceae, Aizoaceae, Apocynaceae, Asclepiadaceae, Bromeliaceae, Cactaceae, Celastraceae, Compositae, Crassulaceae, Cucurbitaceae, Didieriaceae, Ebenaceae, Euphorbiaceae, Flacourtiaceae, Geraniaceae, Labiatae, Liliaceae, Orchidaceae, Oxalidaceae, Piperaceae, Plantaginaceae, Portulacaceae, Rubiaceae, Sapindaceae, Vitaceae and members of the

non-flowering plant families Isoetaceae, Polypodiaceae and Welwitschiaceae.

These species inhibit a wide range of environments in addition to the deserts with which CAM has been traditionally associated. In the humid tropics, epiphytic orchids and epiphytic bromeliads exhibit CAM. The number of epihytic CAM plants in these two families alone is presumed to be of the same order as the total number of terrestrial CAM species (Smith, 1984). These epiphytic plants possess CAM as an adaptation to the low water availability of the epiphytic habit. Some epiphytic orchids fix CO_2 astomatally through aerial roots (Cockburn, Goh and Avadhani, 1985).

Stylites andicola L., a member of the Pteridophyte Isoetaceae family is a terrestrial plant possessing no functional stomata and is dependent on soil-borne CO_2 (Keeley, Osmond and Raven, 1984). It performs CAM like the submerged aquatic plants of the same family. The discovery by Keeley (1981) of CAM in permanently submerged aquatic *Isoetes howellii* plants was seen as an adaptation to overcome the daytime carbon limitation of photosynthesis. There is also a possibility that increasing the CO_2:O_2 ratio at the site of RUBISCO will increase the amount of CO_2 (moles) fixed per mole of enzyme. A consequence of this is that a given photosynthetic rate per unit biomass can occur with a lower RUBISCO content, reducing the plant's nitrogen requirement (Richardson, Griffiths, Reed, Raven and Griffiths, 1984). The shoreweed *Littorella uniflora* Aschers (Plantaginaceae) was the first isoetid seed plant shown to possess a CAM-like pathway (Aulio and Salin, 1982; Keeley, 1982). It possesses two life forms differing from each other both in morphological and biochemical characteristics. The submerged form possesses wax-covered functionless stomata and exhibits CAM (Aulio, 1985). The terrestrial form which occupies moist micro-habitats above the water

level has functioning stomata and acquires CO_2 entirely by the C_3 pathway.

The Four Phases of CAM Activity

During a 24 hour day consisting of 12 hours light and 12 hours dark a plant performing CAM exhibits a gas exchange pattern that can be divided into four phases (Osmond, 1978; Winter, 1985) (Figure 1). The activity of each phase may be modified in response to environmental conditions. This flexibility enables CAM to be of adaptive value in a range of environments. The properties of the four phases also vary between species.

Phase I of CAM is characterised by nocturnal dark fixation of CO_2 by PEP carboxylase and vacuolar malic acid storage. The carboxylation of PEP yields oxaloacetic acid (OAA) (Figure 2). The reduction of OAA to malic acid is catalysed by NAD-malate dehydrogenase (EC 1.1.1.37) and may occur in the cytosol and mitochondrion. The flow of carbon from PEP to OAA is probably rapid and must be at least as rapid as the synthesis of malic acid as indicated by the low pool sizes of PEP (Cockburn and McAulay, 1977; Kenyon, Holaday and Black, 1981), and by the high potential activity of PEP carboxylase (Holtum and Winter, 1982).

Leaf sap concentrations of malic acid may exceed 150 mM by the end of the dark period (Winter, 1985). Sequestration of malic acid away from the cytoplasm has always been thought to be probable since a low cytoplasm pH would presumably be damaging to the cell (Kluge and Osmond, 1972). Direct evidence locating malic acid in the vacuole was only obtained when an effective technique for the isolation of vacuoles was developed (Buser and Matile, 1977). Kenyon, Kringstad and Black (1978) found a variation of malic acid levels in isolated vacuoloes of *Sedum telephium* L. consistent with levels of leaf-titratable acidity. The uptake of malic acid into the vacuole is driven by a magnesium-dependent ATPase closely associated

Figure 1. Relationships between rate of CO_2 fixation, malic acid content and stomatal resistance in a well-irrigated CAM plant throughout the night ([redacted]), and day, showing the four separate phases of gas exchange. From Osmond (1978).

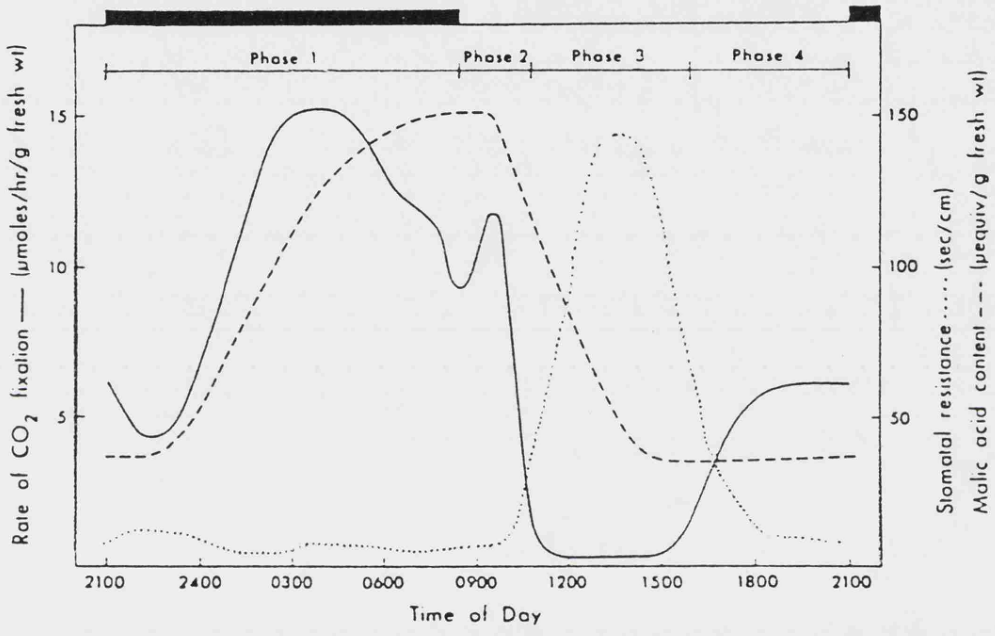
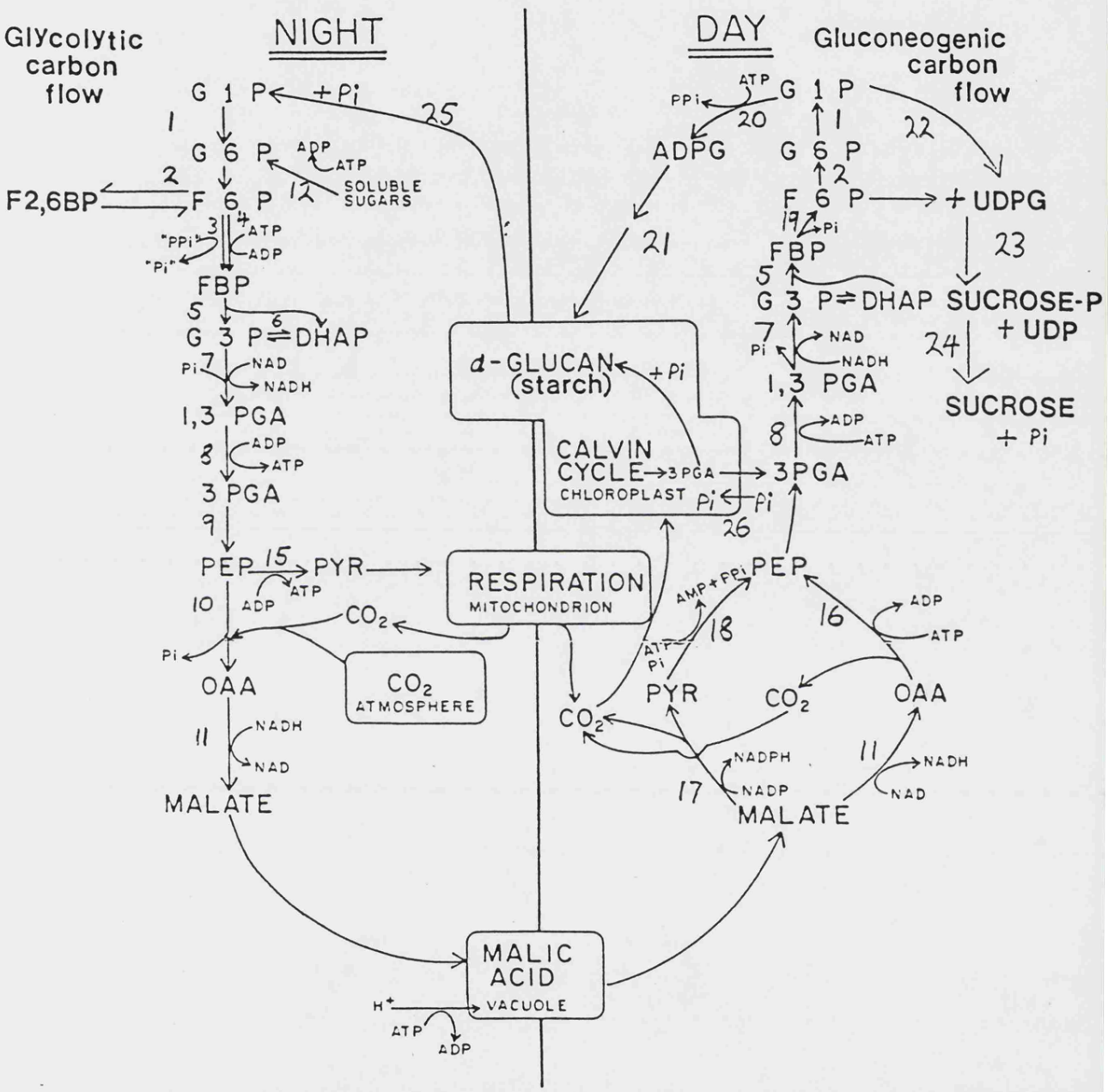

Phase 1
Phase 2
Phase 3
Phase 4
15
10
5
150
100
50
2100
2400
0300
0600
0900
1200
1500
1800
2100
Time of Day
Rate of CO_2 fixation —— (μmoles/hr/g fresh wt)
Stomatal resistance ····· (sec/cm)
Malic acid content – – – (μequiv/g fresh wt)

Figure 2. The carbohydrate metabolism of CAM. The location is in the cytosol except in the boxes. The enzymes are phosphoglucomutase (1) (EC 2.7.5.1), Phosphoglucose isomerase (2) (EC 5.3.1.9), pyrophosphate-dependent phosphofructokinase (3) (EC 2.7.1.90), ATP-dependent phosphofructokinase (4) (EC 2.7.1.11), aldolase (5) (EC 4.1.2.13), triose phosphate isomerase (6) (EC 5.3.1.1), glyceraldehyde 3 phosphate dehydrogenase (7) (EC 1.2.1.12), phosphoglycerate kinase (8) (EC 2.7.2.3), enolase (9) (EC 4.2.1.11), phosphoenol pyruvate carboxylase (10) (EC 4.1.1.31), malate dehydrogenase (11) (EC 1.1.1.37), hexokinase (12), (EC 2.7.1.2), fructose 6 phosphate dikinase (13) (EC 2.7.1.105), fructose 2,6 bisphosphatase (14) (EC 3.1.3.46), pyruvate kinase (15) (EC 2.7.1.40), phosphoenol pyruvate carboxykinase (16) (EC 4.1.1.31), NADP-malic enzyme (17) (EC 1.1.1.39), pyruvate Pi, dikinase (18) (EC 2.7.9.1), fructose 1,6 bisphosphatase (19) (EC 3.1.3.11), ADP-glucose pyrophosphorylase (20) (EC 2.7.7.27), starch synthase (21) (EC 2.4.1.21), UDPG-pyrophosphorylase (22) (EC 2.7.7.9), sucrose phosphate synthetase (23) (EC 2.4.1.14), sucrose phosphatase (24) (EC 3.1.3.24), α amylase (25) (EC 3.2.1.1), phosphorylase (25) (EC 2.4.1.1), and phosphate translocator (26).

Adapted from (Ting, 1987).


Glycolytic carbon flow
NIGHT
DAY
Gluconeogenic carbon flow
G 1 P
+Pi
25
1
G 6 P
ADP
ATP
2
F2,6BP
F 6 P
12
SOLUBLE SUGARS
"PPi"
"Pi"
3
4
ATP
ADP
FBP
5
G 3 P ⇌ DHAP
6
Pi
7
NAD
NADH
1,3 PGA
8
ADP
ATP
3 PGA
9
PEP
15
PYR
ATP
ADP
10
CO2
Pi
OAA
CO2 ATMOSPHERE
11
NADH
NAD
MALATE
α-GLUCAN (starch)
+Pi
CALVIN CYCLE
3 PGA
CHLOROPLAST
Pi
26
RESPIRATION
MITOCHONDRION
MALIC ACID
VACUOLE
H+
ATP
ADP
ADPG
ATP
PPi
20
21
G 1 P
1
G 6 P
2
F 6 P
+UDPG
19
Pi
FBP
23
5
G 3 P ⇌ DHAP
SUCROSE-P
+UDP
7
Pi
NAD
NADH
24
1,3 PGA
SUCROSE
+Pi
8
ADP
ATP
3PGA
22
PEP
AMP+PPi
ATP
Pi
18
16
ADP
ATP
PYR
CO2
OAA
CO2
NADPH
NADP
17
11
NADH
NAD
MALATE

with the vacuole (Luttge, Smith and Marigo, 1982). H^+ transport into the vacuole is active and magnesium and ATP-dependent (Jochem, 1986). The uptake of malate anions is passive via the proton-electrochemical gradient established by the ATPase (Smith, Marigo, Luttge and Ball, 1982). Two H^+ cations are pumped for every malate anion transported.

Synthesis of PEP, the CO_2 acceptor molecule must also occur during Phase 1. Early work stressed the function of starch as the precursor of PEP in CAM plants (Ransom and Thomas, 1960). However the diversification of this function to other carbohydrates in some species is apparent. In *Ananas comosus* L., hexoses consisting mainly of fructose contribute most of the carbon for PEP synthesis (Kenyon, Severson and Black, 1985). The breakdown of carbohydrate to provide PEP and the breakdown of carbohydrate to provide pyruvate for respiration are thought to occur independently utilising separate pools of carbohydrate (Deleens and Garnier-Dardart, 1977; Deleens, Garnier-Dardart and Queiroz, 1979). Starch granules are broken down to linear maltosaccharides by α-amylase (EC 3.2.1.1) and from linear maltosaccharides in the presence of inorganic phosphate (Pi) and phosphorylase (EC 2.4.1.1) to glucose 1 phosphate (Duffus, 1987) (Figure 2). Hexoses enter glycolysis via hexokinase (EC 2.7.1.2). Sucrose is not thought to be involved in the provision of carbon for the CO_2 acceptor (Pucher, Vickery, Abrahams and Leavenworth, 1949).

The degree of drought stress influences the amount of nocturnal CO_2 fixation. Increasing drought may delay the onset of nocturnal CO_2 fixation during Phase I and the stomata may remain shut for a significant part of the dark period. Under these conditions the recycling of respiratory CO_2 becomes important as the source for carbon flow (Szarek, Johnson and Ting, 1973; Holthe and Szarek, 1985). Increasing drought may climax in continuous stomatal closure and plants may exhibit CAM-idling. Day-night oscillations of malic

acid levels may be much reduced or entirely absent under these conditions. However, the capacity of PEP carboxylase remains high and the plants maintain their full capacity to metabolise CO_2 in the light and in the dark (Brulfert, Kluge, Guerrier and Queiroz, 1987). This prevents photo-oxidative inhibition of the photosystems which could result in irreversible injury. There is no net growth during CAM-idling, but metabolism is maintained in an active state so that the plant can respond quickly to small and short-lived amounts of precipitation.

Nocturnal acquisition of CO_2 from the atmosphere is favoured at cool temperatures between 5 and 20°C (Winter, Schroppel-Meier and Caldwell, 1986). At higher tissue temperatures there is a decline in net CO_2 uptake from the atmosphere but acid accumulation may increase up to 45°C due to more extensive recycling of respiratory CO_2 (Winter *et al.*, 1986).

Phase II commences at the beginning of the light period and is characterised in some species by a peak of CO_2 uptake, (Lange, Schulze, Kappen, Evenari and Buschbom, 1975, *Caralluma negevensis* L.; Nobel, 1976, *Agave deserti* L.; Winter and Tenhunen, 1982, *K. daigremontiana*; Acevedo, Badilla and Nobel, 1983, *Opuntia ficus-indica* L.). Fixation occurs via PEP carboxylase, and directly via RUBISCO with light dependent CO_2 assimilation. However, frequently in bromeliads such a peak is absent (Luttge, Stimmel, Smith and Griffiths, 1986), or there may be just a shoulder in the rapid decline of net CO_2 uptake in the first part of Phase II in other bromeliads (Luttge, 1987). The expression of Phase II gas exchange may depend on environmental conditions, and it has been found to decrease with increasing daytime tissue temperature (Nobel, 1976; Winter and Tenhunen, 1982). However, the details of such interactions are unclear and in bromeliads (Luttge, 1987) the early

morning peak of CO_2 uptake also occurs at high leaf temperatures after warm nights.

A passive efflux of malic acid from the vacuole into the cytoplasm commences during Phase II. The cause of the change from net accumulation to net remobilisation of malic acid is still an enigma (Luttge, 1987). It is likely to involve a mechanism of light activation (Nalborczyk, Lacroix and Hill, 1975), but endogenous elements are also implicated in CAM rhythmicity (Buchanan-Bollig, 1984). Regulation of the diel oscillations of malate levels could also be influenced by osmotic gradients between the vacuole and cytoplasm (Luttge, Kluge and Ball, 1975). The efflux of malic acid into the cytoplasm, suppresses the activity of PEP carboxylase (Kluge and Osmond, 1972). This effect of malate on PEP carboxylase activity is amplified by an additional mechanism through which PEP carboxylase becomes more susceptible to inhibition by malate during the light (Winter, 1982b). In experiments carried out on *Mesembryanthemum crystallinum* L. (Winter, 1981, 1982a) PEP carboxylase was more sensitive to inhibition by malate and exhibited a lower affinity for PEP during the light than during the dark. Similar properties of PEP carboxylase were found in other CAM species (von Willert, Brinckmann, Scheitler, Thomas and Treichel, 1979; Jones, Buchanan, Wilkins, Fewson and Malcolm, 1981).

Phase III of CAM is a period when behind closed stomata decarboxylation of malic acid follows its remobilisation from the vacuole. The CO_2 produced is refixed via RUBISCO and assimilated in the Calvin cycle. There are two mechanisms of malic acid decarboxylation (Figure 2). The single-step decarboxylation is catalysed by malic enzyme (EC 1.1.1.39) and can occur in the cytosol where NADP is the cofactor (Garnier-Dardart and Queiroz, 1974; Spalding, Schmitt, Ku and Edwards, 1979), or in the mitochondrion

where the cofactor is NAD (Dittrich, 1976; Rustin and Queiroz-Claret, 1985). Pyruvate and CO_2 are produced from these reactions. The two-step cytosolic mechanism involves the sequential action of NAD-malate dehydrogenase (EC 1.1.1.37) producing OAA which is then decarboxylated by PEP carboxykinase (EC 4.1.1.31) producing PEP and CO_2 (Daley, Ray, Vines and Black, 1977; Ku, Spalding and Edwards, 1980). The NADP and NAD malic enzymes involved in the single-step mechanism are present in all CAM species so far tested. However, their extractable activities vary (Dittrich, 1976) and one may distinguish between NAD malic enzyme species and NADP malic enzyme species. The occurrence of PEP carboxykinase involved in the two-step decarboxylation follows taxonomic grouping and only species of the Asclepiadaceae, Bromeliaceae and Euphorbiaceae show PEP carboxykinase activity. They are called PEP carboxykinase-type species and tend to have lower activities of the malic enzymes (Dittrich, Campbell and Black, 1973). Malic enzyme-type species lack PEP carboxykinase and presumably constitute the rest of CAM species. The light that promotes the decarboxylation of malate is absorbed by the same pigments that absorb the light that powers photosynthesis (Barrow and Cockburn, 1982). Very high internal CO_2 concentrations can build up in the leaves during this phase (Cockburn *et al.*, 1979). This may lead to some release of CO_2 even when stomatal conductance is low (Friemert, Kluge and Smith, 1986). The benefit of a high internal CO_2 concentration is seen in the rapid rates of photosynthesis that occur during this phase (Winter, 1985). A high temperature during this period will favour malic acid degradation (Wagner and Larcher, 1981).

Phase IV makes up the latter part of the light period and is characterised by a depletion of malic acid. Internal CO_2 levels fall and stomatal conductance increases as internal CO_2 fixation by

RUBISCO exceeds internal CO_2 production. There is direct fixation of CO_2 from the atmosphere via RUBISCO and PEP carboxylase (Ritz, Kluge and Veith, 1986). A low daytime temperature favours CO_2 uptake by PEP carboxylase during the light (Kluge, 1969).

In well-watered leaf-succulent CAM plants e.g. *K. daigremontiana* there may be considerable daytime atmospheric CO_2 acquisition (Kenyon *et al.*, 1981). This phenomenon is mainly absent from stem-succulent CAM plants (Cacti) (Winter, 1985). Daytime net CO_2 fixation is usually more sensitive to water stress than nocturnal CO_2 uptake (Winter, 1985), and Phase IV CO_2 uptake is eliminated first when water is scarce (Smith and Luttge, 1985).

Inducible CAM

The four phases of CAM activity and their modification by various parameters demonstrates the flexibility of CAM. This flexibility ensures that CAM is of adaptive value in a range of environmental conditions. Greater flexibility is exhibited by some plants which are able to attain the biochemical equipment necessary for CAM in response to an environmental stimulus. They change from C_3 photosynthesis to CAM in a short period of time relative to the total life cycle of the leaf. These plants are known as facultative CAM plants, inducible CAM plants or C_3-CAM intermediates (Lee and Griffiths, 1987).

CAM may be triggered by the length of the photoperiod (Queiroz, 1974). CAM is controlled in this way in *K. daigremontaina*, *K. blossfeldiana* L., *K. velutina* L. and *Aloe vera* L. In *K. blossfeldiana* short days favour CAM. In *K. daigremontiana* CAM is developed by a complex photoperiodic pattern in which short days are preceded by a number of long days in order to achieve a "juvenile phase", a pre-requisite for effective sensitivity to short days. In *K. blossfeldiana*, CAM induction resulted in an exponential increase in the capacity and activity of the enzymes of CAM and the connected pathways of glycolysis and amino acid synthesis (Queiroz and Brulfert, 1982). An increase in PEP carboxylase activity involved net synthesis of an enzyme form which appeared to be electrophoretically and immunologically different from that prevailing before the onset of the short-day treatment (Brulfert, Guerrier and Queiroz, 1982; Brulfert and Queiroz, 1982). The establishment of CAM in *K. blossfeldiana* is irreversible and accompanied by the induction of flowering. Leaves developing under long days acquire carbon predominantly by C_3 photosynthetic daytime

CO_2 fixation but ultimately also exhibit CAM when they exceed a certain age (Brulfert *et al.*, 1982). Although short-day treatment is much more effective than ageing in promoting CAM, it is quite obvious that the photoperiodic response of carbon metabolism in *K. blossfeldiana* is an example of the acceleration and amplification of physiological processes associated with leaf ontogeny (Winter, 1985).

A number of northern temperate species are facultative CAM plants, especially members of the Crassulaceae and genus *Sedum*. For example, *S. telephium* (Groenhof, Bryant and Etherington, 1986), *S. album* L. (Earnshaw, Carver and Lee, 1985), *S. acre* and *S. mite* L. (Kluge, 1977; Schuber and Kluge, 1981). *Sempervivum montanum* L. (Earnshaw *et al.*, 1985) and *Umbilicus rupestris* L. (Daniel, Woodward, Bryant and Etherington, 1985) also display facultative CAM. These plants tend to grow in rock crevices or in well-drained stony soils where periodic drought can develop rapidly and induce a change from C_3 to CAM which may be an important means of survival (Winter, 1985). Water stress may enhance latent CAM rather than induce CAM in *S. acre* and *S. mite* (Schuber and Kluge, 1981). *Portulaca oleraceae* L. is the only C_4 species known to express CAM characteristics (Koch and Kennedy, 1980). It relies on C_4 photosynthesis when well-watered and under long days, but exhibits CAM when subjected to drought stress under 8 hour photoperiods and day/night temperatures of 30/15°C.

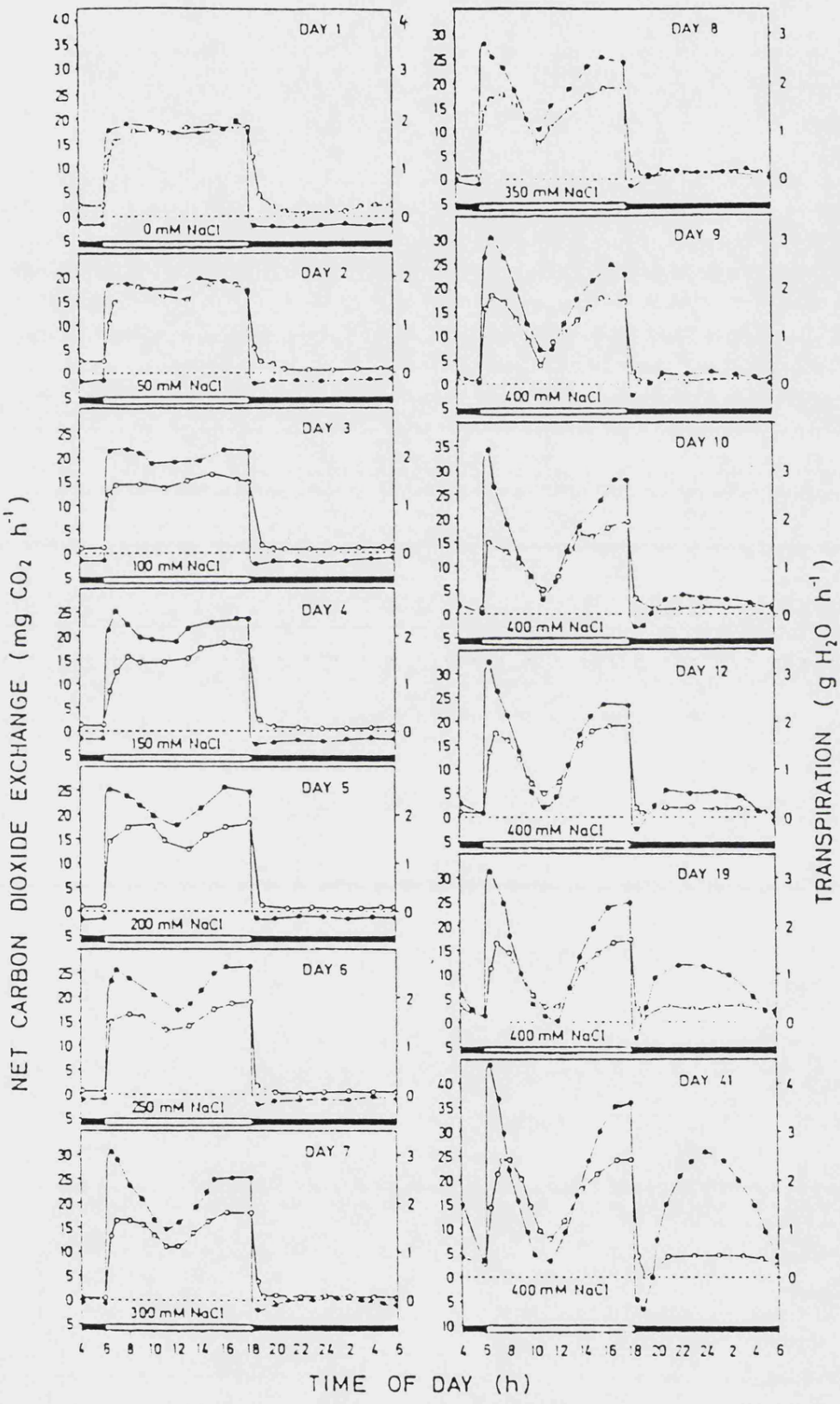
Mesembryanthemum crystallinum, an annual, halophic member of the Aizoaceae demonstrates facultative CAM. It is found predominantly in climates characterised by winter rain and warm dry summers (Winter, 1979a). There is a change from C_3 to CAM during its life cycle which is correlated with a change from the rainy to the dry season along the coast of the Mediterranean (Winter, Luttge, Winter and Troughton, 1978). This physiological flexibility means that during the first part of its life cycle, which corresponds with the wet season C_3

photosynthesis provides an effective means for carbon gain. Large leaves and high leaf:stem ratios during this period favour high rates of photosynthesis and growth allowing *M. crystallinum* to occupy its habitat often monophytically. The ability to perform CAM enables the plant to extend biomass production into the dry season. Water conservation is the overriding consideration during this time and new leaves are smaller and more succulent. The extended life cycle results in increased carbon gain and may be essential for the very high seed production. *M. nodiflorum* L. can also change its metabolism from C_3 photosynthesis to CAM. It has been observed at two sites in Israel (Winter and Troughton, 1978). At the drier site it changed to CAM earlier in its life cycle and CAM became more pronounced than at the wetter site.

Mesembryanthemum means "Midday flower" and describes the way its flowers only open during bright sunshine. Its specific name describes the crystal-like appearance of the leaves which is caused by bladder cells on the surface of the leaf which may have a volume of up to 2 µl (Luttge, Fischer and Steudle, 1978). They form reservoirs for salts and water and probably function as a buffering system, protecting the smaller mesophyll cells during water stress (Winter, 1979a).

The Aizoaceae have been extensively studied and described by Herre (1971). He subdivides the Aizoaceae into four subfamilies: Mesembryanthemoideae, of which the genus *Mesembryanthemum* is a member, Hymenogynoideae, Caryotophoroideae and Ruschioideae. The centre of dispersal of the Aizoid group is thought to be the frost-free region of South Africa (Volk, 1960). Many members of the family including *M. crystallinum* have spread world-wide from their South African origin and are now found in arid, hot environments as well as estuaries. Wild populations are found in Australia (Kloot, 1983), on

Figure 3. The course of net CO_2 exchange (-●-) and transpiration (-○-) of a *Mesembryanthemum crystallinum* plant (shoot) in response to a gradual increase in the NaCl content in the culture solution up to 400 mM. ═══ light, ▬▬▬ dark. Concerning net CO_2 exchange, positive values refer to net CO_2 uptake, negative values represent net CO_2 loss from the shoot (from Winter, 1975).


DAY 1
0 mM NaCl
DAY 2
50 mM NaCl
DAY 3
100 mM NaCl
DAY 4
150 mM NaCl
DAY 5
200 mM NaCl
DAY 6
250 mM NaCl
DAY 7
300 mM NaCl
DAY 8
350 mM NaCl
DAY 9
400 mM NaCl
DAY 10
400 mM NaCl
DAY 12
400 mM NaCl
DAY 19
400 mM NaCl
DAY 41
400 mM NaCl
NET CARBON DIOXIDE EXCHANGE (mg CO_2 h^{-1})
TRANSPIRATION (g H_2O h^{-1})
TIME OF DAY (h)

the Mediterranean coast (Winter *et al.*, 1978), the coastal desert of Southern Africa (von Willert, Brinckmann and Schulze, 1979), the coastal grassland of California (Vivrette and Muller, 1977) and along the Baja California peninsula, California (Wiggens, 1980).

The induction of CAM in *M. crystallinum* in the laboratory can be performed by subjecting the plant to any treatment that reduces the water potential of the leaves. This is normally done by watering the plants with up to 500 mM NaCl (Winter and von Willert, 1972), or by exposing the roots to low temperature or oxygen deficiency (Winter, 1974). The shift from C_3 to CAM that accompanies water stress in *M. crystallinum* begins with a severe midday depression of net CO_2 uptake during the light period (Winter, 1975) (Figure 3). Nocturnal CO_2 fixation becomes more apparent (Winter, 1973b) and stomatal closure extends during the day until the four phases typical of CAM plants (Figure 3) can be distinguished (Luttge, 1987). The water use efficiency increases during this time (Winter and Luttge, 1976; Luttge and Smith, 1984). The induction of CAM is also accompanied by an increase in the activities of many enzymes (Winter, Foster, Edwards and Holtum, 1982; Holtum and Winter, 1982). These enzymes include PEP carboxylase responsible for the fixation of CO_2 from the atmosphere; mitochondrial NAD malic enzyme and cytosolic NADP malic enzyme responsible for the decarboxylation of malic acid; and pyruvate Pi dikinase (EC 2.7.9.1) responsible for the regeneration of PEP. The activities of many glycolytic enzymes also increase. These include enolase (EC 4.2.1.11), phosphoglyceromutase (EC 2.7.5.3), phosphoglycerate kinase (EC 2.7.2.3), NAD glyceraldehyde 3 phosphate dehydrogenase (EC 1.2.1.12) and glucose phosphate isomerase (EC 2.6.1.2). Activities of NAD-malate dehydrogenase, alanine and aspartate aminotransferases (EC 2.6.1.2 and EC 2.6.1.1 respectively) and NADP-glyceraldehyde 3 phosphate dehydrogenase (EC 1.2.1.13) also

increase. Determinations of the quantity of PEP carboxylase protein (Winter *et al.*, 1982b; Foster, Edwards and Winter, 1982) demonstrated that the increased enzyme activity is based on *de novo* protein synthesis. Ostrem, Olson, Schmitt and Bohnert (1987) showed that there was an increase in the level of translatable mRNA for this enzyme. Electrophoretic examination of the enzyme in CAM *M. crystallinum* showed it to have three protein bands. The deinduction of CAM resulted in the disappearance of one of these protein bands and a loss of enzyme activity (von Willert, Treichel, Kirst and Curdts, 1976). It would therefore appear that the increase in activity of the enzyme on induction is also the result of a change in the properties of the enzyme. An increased ability to transport malic acid into the vacuole also occurs when CAM is induced in *M. crystallinum* (Struve, Weber, Luttge, Ball and Smith, 1985), the ATPase responsible showing increased activity.

Inducible CAM plants like *M. crystallinum* provide ideal material for the study and comparison of C_3 and CAM photosynthesis since observations of both processes in the same species are free of interspecific variation and differences observed can be attributed to differences between the two forms of metabolism. However, the irrigation of *M. crystallinum* plants with NaCl solution to induce CAM also causes changes related to growth in saline conditions rather than changes directly associated with CAM.

Halophilism is a feature of the Aizoaceae (von Willert, Thomas, Lobin and Curdts, 1977). Halophytes have the capacity to complete their life cycles in environments of high salinity (>300 mM). In addition to a tolerance of salt, the growth of many halophytes is enhanced by salinity although appreciable growth takes place in its absence (Flowers, Troke and Yeo, 1977). Monocotyledonous halophytes largely restrict the entry of inorganic ions and use organic

compounds to maintain an adequate solute potential (Gorham, Hughes and Wyn Jones, 1980). Most other halophytes absorb large quantities of electrolytes particularly into their leaves and may be distinguished from mildly salt-tolerant glycophytes on the criterion that the latter exclude ions at the absorption sites in the roots (Greenway, 1973). The accumulation of ions in the vacuole away from metabolic processes that they could inhibit (Wyn Jones and Storey, 1981) allows osmotic adjustment in conditions of drought or salt-stress (Jones, Osmond and Turner, 1980), and allows the maintenance of internal positive turgor potentials necessary for growth (Hsaio, 1973). Metabolic events in halophytes and glycophytes do not differ markedly in their gross sensitivity to electrolytes (Wyn Jones and Pollard, 1982). Members of the Aizoaceae contain high concentrations of Na^+ and Cl^- ions even when they are at a low concentration in the soil (von Willert *et al.*, 1977). *M. crystallinum* plants grown at 400 mM NaCl had leaf sap concentrations of 500 mM Na^+ and 400 mM Cl^- (Demmig and Winter, 1986).

Compatible or non-toxic solutes are thought to accumulate in the cytoplasmic compartments of halophytes to counteract the osmotic perturbations caused by high vacuolar concentrations of inorganic ions (Wyn Jones, Storey, Leigh, Ahmad and Pollard, 1977). A number of possible compatible cytosolutes have been recognised in higher plants. These include glycinebetaine, proline, sucrose, sorbitol, prolinebetaine, alanine, dimethyl proprothetin and pinitol (Wyn Jones and Gorham, 1983). Designation of each of these compounds as a compatible solute is speculative (Greenway and Munns, 1980), and evidence that accumulation is of adaptive value and not a reflection of impaired metabolism is sparse. In sunflower proline only accumulates in response to severe stress (Lawlor and Fock, 1977) and is thought to be a result of protein breakdown serving little

adaptive function in conditions of severe water stress. Glycinebetaine (Pollard and Wyn Jones, 1979) and proline (Stewart and Lee, 1974) have been found to be compatible with metabolism in that they do not inhibit enzymes at high concentrations. Glycinebetaine has been found to be compatible with *in vitro* protein synthesis, polysome stability, chloroplast CO_2 fixation and coupled mitochondrial oxidative phosphorylation (Wyn Jones and Storey, 1981). Proline (Wyn Jones and Gorham, 1983) and glycinebetaine (Robinson and Jones, 1986) have also been found to accumulate to concentrations in cytoplasmic compartments high enough to be osmotically important. Elevated levels of proline in *M. crystallinum* in response to 400 mM NaCl (Heun, Gorham, Luttge and Wyn Jones, 1981; Demmig and Winter, 1986), and 500 mM NaCl (Ostrem, Vernon, Olson and Bohnert, 1987) have been found. Proline may function as a compatible solute in the cytoplasm (Ostrem *et al.*, 1987b), but the overall contribution of proline to the osmotic adjustment of *M. crystallinum* chloroplasts is thought to be small (Demmig and Winter, 1986).

The mechanism for the transformation of the physical stimulus of water stress into the biochemical response of CAM induction is not known. There is evidence that the shift from C_3 to CAM is the consequence of ontological development (Kluge and Ting, 1978). von Willert and Kramer (1972) in their observations of the ultrastructure of *M. crystallinum* leaves proposed that treatment with NaCl and hence CAM activity accelerated the normal ageing processes in the plant. NaCl-treated cells had the features of aged cells: empty vacuolised cytoplasm and reduced stromal thylakoids. In *M. crystallinum* PEP carboxylase was only active five weeks after germination (Ostrem *et al.*, 1987a), which suggests a certain degree of maturity is necessary before CAM can be induced. Ting and Rayder (1982) suggested that the stomata are the physiological receptors

that sense the environment and that the metabolic shifts in response to water stress come about because of stomatal closure, which is the first measurable physiological response to water stress. They found that artificial closure of the stomata of the inducible CAM plant *Portulacaria afra* L. with absciscic acid (ABA) caused organic acid decarboxylation during the light even when the plants were under good water status. Winter (1979b) found that in *M. crystallinum* stomatal closure which would change the access to external CO_2 could not initiate the expression of CAM and that the induction of CAM was not controlled by the availability of CO_2 in the light or dark. The effect of ABA on CAM may be more complex than simple stomatal closure. ABA is a maturation hormone and may induce CAM in *P. afra* by hastening maturation of the leaves. Furthermore, water stress which induces CAM in *P. afra* is associated with an increase in tissue ABA (Raschke, 1975). The production of ethylene in response to water stress in C_3 species (Wright, 1974) has not been investigated in inducible CAM species. It is possible that the complex process of CAM induction is elicited by a hormonal trigger. In addition, the localisation and concentration of ions near certain metabolic sites which may occur during the early stages of water stress might initiate CAM either as a result of the presence of the ions themselves or as a result of the lowered water potential that they could produce (von Willert *et al.*, 1977).

Carbohydrate Partitioning

The transition from C_3 to CAM photosynthesis involves many metabolic changes. These are likely to include an alteration in the distribution of assimilate within the plant.

There are distinct interspecific differences in carbohydrate partitioning in C_3 plants (Huber, 1983; Foyer and Spencer, 1986). One might expect these differences to be reflected in the mechanisms controlling the accumulation and partitioning of carbohydrate: namely the distribution of carbon between starch and sucrose, the principal end-products of photosynthesis.

In the chloroplast the fixation of CO_2 results in the formation of triose phosphate, 5/6 of which has to remain in the calvin cycle for the regeneration of the CO_2 acceptor, ribulose 1,5 bisphosphate. The remaining 1/6 is available for synthesis of starch in the chloroplast or the synthesis of sucrose in the cytoplasm. The partition of carbon between these two pools is determined by the flux of triose phosphate or 3PGA across the chloroplast envelope which can be regarded as the ultimate barrier between source and sink (Herold, 1980).

Flux of carbon from the chloroplast is facilitated by a carrier protein called the phosphate translocator (Heldt, 1976; Walker, 1976; Heber and Walker, 1979). It mediates the exchange of dihydroxyacetonephosphate (DHAP) and 3PGA with inorganic phosphate (Pi) between the chloroplast and cytoplasm. The exchange is strict and stoichiometric and consequently the level of total Pi in the chloroplast whether esterified or free remains constant. The alkaline pH of the stroma during the light ensures that DHAP is exchanged with Pi at this time. The return of stromal pH to neutrality in the dark enables 3PGA to be exchanged for Pi at night

(Macdonald and Buchanan, 1987). This is because a high pH renders 3PGA trivalent and immobile but does not change DHAP which remains divalent and freely transportable during illumination. The rate of operation of the Pi translocator is dependent on the concentration of the substances it transports (Flugge and Heldt, 1984). It is known that CAM plants possess a Pi translocator mechanism (Spalding and Edwards, 1980).

The availability of Pi regulates the activity of the Pi translocator and affects carbohydrate partitioning and the rate of photosynthesis. Isolated chloroplasts demand a carefully controlled concentration of Pi if photosynthesis is to occur (Cockburn, Baldry and Walker, 1967). Too much Pi causes a large flux of carbon from the chloroplast and not enough is retained to regenerate ribulose 1,5 bisphosphate. Too little Pi restricts the synthesis of the phosphorylated intermediates of the Calvin cycle.

The effect of Pi on the partitioning of assimilate into carbohydrate has been demonstrated by feeding Pi-sequestering compounds to leaf-discs (Chen-she, Lewis and Walker, 1975; Herold, Lewis and Walker, 1976; Herold, McGee and Lewis, 1980). Mannose, 2-deoxyglucose and glucosamine are phosphorylated in the presence of Pi and are not readily metabolised. The reduction of Pi availability produced causes an increase in the proportion of carbon retained in the chloroplast for starch synthesis in C_3 tissue. Plants grown in Pi-deficient conditions frequently have a high starch content (Herold and Lewis, 1977). The low availability of Pi leads to a high 3PGA:Pi ratio in the chloroplast, which stimulates the activity of ADP-glucose pyrophosphorylase (EC 2.7.7.27) (Preiss, Ghosh and Wittkop, 1967; Preiss, 1982), the major regulatory enzyme in starch synthesis (Preiss, Robinson, Spilatro and McNamara, 1985). At the same time, degradation of starch by phosphorylase (EC 2.4.1.1) is

impeded by low Pi. Starch synthesis releases Pi relieving to some extent the Pi deficiency. A stimulation of starch synthesis by low Pi occurs in the same way in C_4 plants. However, the net effect of low Pi on starch synthesis is less stimulatory and a decrease in the total amount of starch often occurs (Herold *et al.*, 1976). This could be due to a reduced rate of generation of PEP, the CO_2 acceptor, due to a lowered activity of pyruvate Pi dikinase (Huber and Edwards, 1975). A rise in ADP levels caused by low Pi could reduce CO_2 fixation by lowering the activity of PEP carboxylase (Danner and Ting, 1967).

High levels of Pi promote a flux of carbon from the chloroplast towards the sucrose biosynthetic pathway in the cytoplasm, a process analogous to gluconeogenesis (Cseke, Balogh, Wong, Buchanan, Stitt, Herzog and Heldt, 1984). Plants grown in conditions of enriched Pi show a decreased starch to sucrose ratio (Herold, 1984). An excessive flux of carbon from the chloroplast depletes levels of stromal intermediates and hence inhibits the regeneration of ribulose 1,5 bisphosphate.

The withdrawal of carbon from the Calvin cycle and the synthesis of sucrose are adjusted in such a way in C_3 plants that the withdrawal of triose phosphate does not exceed the limit set by the Calvin cycle and that from this surplus as much as possible is utilised (Heldt and Stitt, 1987). The rate of sucrose synthesis determines the partition of carbon between the chloroplast and the cytoplasm and hence influences the rate of photosynthesis.

Candidates for regulatory sites in the sucrose biosynthetic pathway are those processes which are displaced from equilibrium, possessing a large negative free energy change. On these grounds, the major control points in the pathway of sucrose synthesis are the reactions catalysed by cytosolic fructose 1,6 bisphosphatase (EC

3.1.3.11) and sucrose phosphate synthetase (EC 2.3.1.14).

The first step in the synthesis of sucrose is the conversion of triose phosphate exported from the chloroplast to fructose 1,6 bisphosphate catalysed by aldolase (EC 4.1.2.13) (Figure 2). The interconversion of fructose 1,6 bisphosphate and fructose 6 phosphate is catalysed by fructose 1,6 bisphosphatase. This catalysis is essentially irreversible in favour of sucrose synthesis (Black, Mustardy, Sung, Kormanik, Xu and Paz, 1987). The ATP-dependent phosphofructokinase (EC 2.7.1.11) catalyses this step irreversibly in the glycolytic direction (Black *et al.*, 1987). The reaction is also catalysed by a pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90), which is reversible (Kombrink, Kruger and Beevers, 1984). The regulator substance, fructose 2,6 bisphosphate has a decisive function in the regulation of the interconversion of fructose 1,6 bisphosphate and fructose 6 phosphate (Cseke *et al.*, 1984). The presence of fructose 2,6 bisphosphate was first discovered in mammalian tissue as a stimulator of ATP-dependent phosphofructokinase (van Schaftingen, Hue and Hers, 1980). It has been found in plant tissue (Sabularse and Anderson, 1981) and at micromolar concentrations is a potent inhibitor of fructose 1,6 bisphosphatase (Cseke, Weeden, Buchanan and Uyeda, 1982; Stitt, Mieskes, Soling and Heldt, 1982). The plant ATP-dependent phosphofructokinase is insensitive to fructose 2,6 bisphosphate but at nanomolar concentrations fructose 2,6 bisphosphate is a potent activator of glycolytic carbon flow through the enzyme pyrophosphate-dependent phosphofructokinase (Cseke *et al.*, 1984). Thus fructose 2,6 bisphosphate inhibits the flow of carbon towards sucrose synthesis (gluconeogenesis) and promotes sucrose breakdown (glycolysis) (Hers, Hue and van Schaftingen, 1982). The significance of the pyrophosphate-dependent phosphofructokinase to the control of sucrose

synthesis remains unclear. Plants possessing higher extractable activities of pyrophosphate-dependent phosphofructokinase than respective ATP- dependent phosphofructokinase activities tend to be succulent and tend to exhibit CAM (Carnal and Black, 1983). Ap Rees, Green and Wilson (1985) suggest a pyrophosphate synthesising role for pyrophosphate-dependent phosphofructokinase.

Levels of fructose 2,6 bisphosphate are determined by the relative activities of the enzymes which synthesise and degrade it, fructose 6 phosphate dikinase (EC 2.7.1.105) and fructose 2,6 bisphosphatase (EC 3.1.3.46), respectively. The synthesis of fructose 2,6 bisphosphate catalysed by fructose 6 phosphate dikinase is stimulated by fructose 6 phosphate and Pi and is inhibited by 3PGA and DHAP, whereas the hydrolysis of fructose 2,6 bisphosphate catalysed by fructose 2,6 bisphosphatase is inhibited by fructose 6 phosphate and Pi (Cseke, Stitt, Balogh and Buchanan, 1983; Stitt, Cseke and Buchanan, 1984). The effectors DHAP, 3PGA and Pi, are transported between the chloroplast and cytoplasm via the Pi translocator (Flugge and Heldt, 1984), and hence transmit information to the cytoplasm regarding the metabolic conditions of the chloroplast. The response of fructose 2,6 bisphosphate levels to changes in metabolic conditions is very rapid because it has a rapid rate of turnover (Heldt and Stitt, 1987). Thus an increase in triose phosphate results in a lowering of the fructose 2,6 bisphosphate level and hence an increase in the affinity of the cytosolic fructose 1,6 bisphosphatase towards its substrate, fructose 1,6 bisphosphate. Owing to the equilibrium of the aldolase catalysing the conversion of triose phosphate to fructose 1,6 bisphosphate an increase in triose phosphate also results in an increase in the concentration of fructose 1,6 bisphosphate. The concurrence of an increase in substrate concentration and an increase in substrate affinity results

in a sigmoidal dependence of fructose 1,6 bisphosphatase activity on the triose phosphate level (Heldt and Stitt, 1987). For withdrawal of fixed carbon for sucrose synthesis a certain threshold level of triose phosphate has to be exceeded, ensuring that a minimal triose phosphate level required for an efficient functioning of the Calvin cycle is maintained. An increase in triose phosphate beyond this threshold results in a dramatic rise in fructose 1,6 bisphosphatase activity, making all surplus triose phosphate available for sucrose synthesis, and Pi is recycled for maximal photosynthesis.

Sucrose phosphate synthetase is a strongly regulated enzyme. This is clearly demonstrated from results of subcellular metabolite analysis in spinach leaves (Heldt and Stitt, 1987), where the cytosolic levels of the substrates fructose 6 phosphate and UDP glucose do not differ largely under conditions for maximal rates of sucrose synthesis in the light and minimal rates of sucrose synthesis (from starch) in the dark. Sucrose phosphate synthetase is allosterically activated by glucose 6 phosphate and inhibited by Pi (Doelhert and Huber, 1983). Two forms of sucrose phosphate synthetase with respect to their sensitivity to Pi have been identified (Foyer, 1987). The activator glucose 6 phosphate is in equilibrium with fructose 6 phosphate, the substrate for sucrose phosphate synthetase, via phosphoglucose isomerase (EC 5.3.1.9). A change in the concentration of the substrate fructose 6 phosphate results in a three-fold change in the concentration of the activator. Since the sum of Pi and phosphorylated intermediates in the cytosol may be regarded as about constant the increase in the activator glucose 6 phosphate will be paralleled by a decrease in the inhibitory Pi level and vice versa. The activity of sucrose phosphate synthetase reveals a sigmoidal dependence on the fructose 6 phosphate concentration (Heldt and Stitt, 1987), and it also appears

that with this enzyme the substrate level has to exceed a threshold value for sucrose phosphate synthesis to occur.

Sucrose phosphatase (EC 3.1.3.24) catalyses the conversion of sucrose phosphate synthesised by sucrose phosphate synthetase into sucrose and Pi. It is an irreversible reaction and is competitively inhibited by sucrose (Hawker and Smith, 1984). However, the importance of this inhibition may not be great since the accumulation of sucrose during the day is due to storage in the vacuole (Stitt, Gerhardt, Wilke and Heldt, 1987). Additionally the fact that sucrose phosphatase usually occurs in at least a 10-fold excess over sucrose phosphate synthetase has led to the suggestion that it is unlikely to play a role in the regulation of sucrose synthesis (Habron, Foyer and Walker, 1981; Preiss, 1982). However, the situation is unclear and in some systems sucrose phosphatase may have a regulatory role (Ap Rees, 1988).

Thus although sucrose itself may not cause direct feedback inhibition of the enzymes of sucrose synthesis the accumulaton of sucrose in the leaf causes a profound change in the metabolism of the cytosol and cytoplasm resulting in a pronounced modulation of sucrose phosphate synthetase activity. Moreover, the regulatory effect of hexose phosphates and Pi on sucrose phosphate synthetase and via the formation of fructose 2,6 bisphosphate on fructose 1,6 bisphosphatase activity make it possible that the activities of the two enzymnes of sucrose synthesis are coordinated. In principle, these mechanisms provide a way of adjusting sucrose synthesis to process the triose phosphate made available by photosynthesis without this impinging on the ability of the chloroplast to carry out photosynthesis.

In C_3 plants there is diel modification of enzymes controlling sucrose synthesis such that the partitioning between sucrose and starch is affected independently of a source-sink regulation of

sucrose synthesis (Huber, Kalt-Torres, Usuda and Bickett, 1987). This may be a means of ensuring that the starch deposits of the chloroplasts are filled during the day in order to meet the metabolic demands of the plant cell in the following night. The reason for the gradual decline of sucrose phosphate synthetase activity during the day remains to be elucidated, but the changes in the activities of the enzymes responsible for the turnover of fructose 2,6 bisphosphate leading to a gradual rise during the day may be due to cAMP dependent protein phosphorylation (Hers *et al.*, 1982).

The control of carbohydrate partitioning is a complex process. Key regulatory enzymes and Pi regulate chloroplastic and cytoplasmic events and the flow of information between these compartments. Sucrose synthesis is related to the rate of photosynthesis and adjusted to allow alterations in carbon partitioning (Stitt *et al.*, 1987). The accumulation of starch is less closely related to the rate of photosynthesis and is altered in a goal-directed manner dependent on the requirement for starch (Chatterton and Silvius, 1979).

In CAM plants there is an absolute requirement for the nocturnal CO_2 acceptor PEP, if CO_2 fixation at night is to occur. One can speculate that the accumulation of the precursor of PEP might take precedence over the accumulation of other carbohydrate. This possibility and the mechanisms that might facilitate this requirement are investigated. Use is made of the inducible CAM plant *M. crystallinum* which enables comparisons to be made of C_3 and CAM photosynthesis that are free of interspecific variation.

The work is divided into seven sections. Section 1 observes the components of C_3 and CAM *M. crystallinum* and *K. daigremontiana* leaves during a 12 hour photoperiod. Section 2 analyses the effect of photon fluence rate on carbohydrate partitioning and levels of malic

acid in C_3 and CAM *M. crystallinum* and *K. daigremontiana*. Section 3 studies the induction of CAM in *M. crystallinum*, and Section 4 the deinduction and reinduction of CAM in *M. crystallinum*. Section 5 investigates the growth and induction of CAM in *M. crystallinum* plants at different levels of exogenous Pi. Section 6 studies the effect of Pi and solute potential on carbohydrate partitioning and levels of malic acid in leaf-discs of C_3 and CAM *M. crystallinum* and *K. daigremontiana*. Section 7 investigates the significance of the accumulation of pinitol accompanying the induction of CAM in *M. crystallinum*.

MATERIALS AND METHODS

Plant Material

Kalanchoe daigremontiana plants were grown in peat-based compost in a greenhouse at 22°C±3°C (day) and 15°C±3°C (night) with supplementary mercury vapour lighting to give a 14 hour photoperiod. The plants were transferred to growth cabinets at temperatures of 25°C (day) and 16°C (night) with a 12 hour photoperiod from 9:30 to 21:30 one week before the commencement of experiments. Warm white fluorescent tubes and tungsten lamps gave a photon fluence rate of 250 µmol m^{-2} s^{-1} between 400 and 700 nm. All plants grown in growth cabinets were cultivated under these growth cabinet conditions. Photon fluence rates were measured throughout with a LI-COR LI-185 quantum radiometer.

Mesembryanthemum crystallinum plants were grown from seed for 5 weeks in wet, peat-based compost in a greenhouse at 25°C with supplementary mercury vapour lighting to give a 16 hour photoperiod. Plants were then transferred to growth cabinet conditions.

Induction and deinduction of CAM activity in *M. crystallinum*

Plants which were to be maintained in the C_3 form were kept wet with tap water. CAM was induced by watering plants which were at least 6 weeks old with 400 mM NaCl solution made up in tap water. The soil was allowed to dry out between successive treatments with salt solution. The deinduction of CAM plants was achieved by drenching the soil of each plant with tap water and from then on maintaining plants in this state. Reinduction was achieved by allowing the soil of deinduced plants to dry out and then watering with 400 mM NaCl.

Sampling and extraction of water-soluble carbohydrate, pinitol, acid including malic acid, and inorganic phosphate (Pi) from leaf tissue of C_3 and CAM *M. crystallinum* and *K. daigremontiana*

Samples consisting of leaf-discs (0.5 cm diameter, 0.01 g fresh weight) were cut from leaves which were the most recently fully expanded leaves in *M. crystallinum* and the third most recently fully expanded leaves in *K. daigremontiana*, unless otherwise stated. Care was taken to ensure that replicate sampled leaves were as similar to each other as possible, and were receiving identical environmental conditions. Replicate variation caused by the mutual shading of *K. daigremontiana* plants was overcome by spacing the plants far enough apart to prevent shading. The exact number of leaf-discs sampled is detailed for each experiment in the Experiments and Results section.

Tissue killing and extraction of water-soluble compounds was effected in boiling water, extraction taking 2 hours. Five leaf-discs were extracted per ml of boiling water. The extraction of water-soluble carbohydrate and the recovery of known amounts of added carbohydrate exceeded 95% during this period of extraction.

Gas chromatographic analysis of water-soluble carbohydrates and pinitol

In C_3 and CAM *M. crystallinum* these compounds were identified and quantified by the gas chromatographic analysis of their trimethyl silyl derivatives according to the method of Holligan and Drew (1971). Pinitol in *Lotus corniculatus* was also identified in this way. Sub-samples consisting of less than 1/5 of the original leaf-disc sample were freeze-dried in pear-shaped flasks and then

dissolved in 0.85 ml of pyridine. The trimethyl silyl derivatives were formed by the addition of 0.1 ml of hexamethyl-disilazane (HMDS) and 0.05 ml of triemethylchlorosilane (TMCS), the reaction taking 30 minutes at room temperature. 1 µl volumes of silylated plant extract were injected into the injector port of a Pye Unicam series 204 Chromatograph. The injector port temperature was 250°C. The column temperature rose from 140°C to 250°C at a rate of 8°C per minute during the analysis. The detector temperature was 350°C. The glass column was 4 mm in diameter and 5 feet long and contained silanised chromosorb WHP 100-120 mesh coated with a stationary phase of 2% SE 52 (Phase-Sep Ltd.). The flow rate of the nitrogen carrier gas was 40 ml per minute. The instrument was connected to a Pye Unicam CDP1 computing integrator.

The retention times of peaks produced by the gas chromatograph were expressed relative to the retention time of commercially prepared α glucose, as relative retention time (RRT) (Table 1). The peaks were identified by comparison of their RRTs with those of commercially prepared compounds (standards). Quantification was based on the size of peaks produced by known amounts of standard which were taken through the same procedure as the leaf-disc samples. In the absence of commercially prepared pinitol, amounts of this substance were expressed as inositol equivalents. A known amount of an internal standard, ribose, was added to samples prior to freeze drying to ensure that errors were not incurred on injection, or due to incomplete derivatisation, evaporation, or malfunction of the chromatograph. Sampling and analysis of tissue by gas chromatography produced a detection limit of 0.05 µmol g^{-1} F.W. for each carbohydrate.

Table 1. The relative retention times of compounds analysed by gas chromatography from leaf-disc extracts of C_3 and CAM *M. crystallinum*.

Compound	Relative retention time
Ribose	0.58
Fructose	0.84
Pinitol	0.86
α Glucose	1.00
β Glucose	1.17
Sorbitol	1.11
(*myo*)Inositol	1.34
Sucrose	2.24

Anthrone method of determination of total water-soluble carbohydrate

Water-soluble carbohydrate was measured as total soluble carbohydrate using the Anthrone method of van Handel (1968). Anthrone (9,10-dihydro-9-oxoanthracene) reacts with carbohydrates in concentrated H_2SO_4 to produce a blue-green colour. 0.1 ml of plant extract was added to 1 ml of Anthrone reagent containing 1.5 mg of Anthrone per ml of 75% H_2SO_4. After 20 minutes incubation at 40°C the absorbance was read at 620 nm. One absorbance unit was equivalent to 24 µg of glucose, fructose or sucrose, the calibration passing through the origin. The method was linear up to 2 absorbance units. The Anthrone method is non-specific and will react with a variety of sugars. The rate and extent of colour development has been found to vary for many sugars with similar structures (Whistler and Wolfrom, 1962). Use of the method was discontinued because the identity of the soluble carbohydrate content of C_3 and CAM *M. crystallinum* and *K. daigremontiana* had not been established at this stage, and hence the range of rates and extents of colour development were not known. However, quantification of total water-soluble carbohydrate by the Anthrone method and gas chromatography produced very similar estimates.

Enzymic method of determination of glucose, fructose and sucrose

The enzymic method of Jones, Outlaw and Lowry (1977) was used instead of the Anthrone method. 0.1 ml of plant extract was added to 0.9 ml of reagent comprising 50 mM imidazole/HCl buffer pH 6.9, 0.4 mM NADP, 1 mM ATP, 5 mM $MgCl_2$, 1 mM DTT and 0.02% (w/v) BSA. Glucose was determined by adding 1 unit per ml of hexokinase and 0.5 units

per ml of glucose 6 phosphate dehydrogenase. Fructose was determined by subsequently adding 1 unit per ml of phosphoglucose isomerase and sucrose was determined by additionally including 0.2 mg per ml of invertase. The production of NADPH was determined spectrophotometrically at 340 nm. Quantifications produced by this method were very similar to estimations by the Anthrone method and gas chromatography.

Estimation of starch

Levels of starch in leaf tissue were estimated after the extraction of water-soluble carbohydrate from the leaf-discs. After the leaf-discs were washed in distilled water to remove any remnants of water-soluble carbohydrate they were put into 1 ml of 0.2 M sodium acetate/acetic acid buffer pH 4.5 containing 0.1 ml (1.4 units) of amyloglucosidase. The starch was broken down to glucose during the incubation at 55°C for 12 hours. No further glucose was released after the addition of extra enzyme after the period of incubation. The glucose produced was measured either by the Anthrone method or by the enzymic method.

Extraction of glucose 6 phosphate, fructose 6 phosphate and fructose 1,6 bisphosphate from C_3 and CAM *M. crystallinum* leaf tissue

The method of Cockburn and McAulay (1977) was followed. At least 3 g fresh weight of *M. crystallinum* leaf material comprising halves of leaves with the mid-rib removed were ground to powder in liquid N_2. Glucose 6 phosphate, fructose 6 phosphate and fructose 1,6

bisphosphate were extracted in cold, 5% (v/v) perchloric acid (1 ml per g fresh weight of leaf tissue). After centrifugation at 20,000 g for 5 minutes the supernatant was neutralised to a pH of 7-8 with cold saturated K_2CO_3. 5 mg per ml of noritol activated charcoal and 10% (w/v) polyvinylpyrrolidone (average molecular weight 360 000) were then added to the supernatant which was then left to stand on ice for 2 hours. The pellet was discarded after centrifugation at 20,000 g for 10 minutes. Extractions were also carried out in the presence of added known amounts of glucose 6 phosphate, fructose 6 phosphate and fructose 1,6 bisphosphate. Recoveries were all in excess of 80%.

Estimation of glucose 6 phosphate and fructose 6 phosphate

0.6 ml of leaf extract were added to 0.4 ml of 0.2 M imidazole/HCl buffer pH 7.1 containing 0.3 mM NADP and 5 mM EDTA. Amounts of glucose 6 phosphate were estimated by the addition of glucose 6 phosphate dehydrogenase, and amounts of fructose 6 phosphate by the sequential addition of phosphoglucose isomerase. The production of NADPH was measured spectrophotometrically at 340 nm. The addition of known amounts of authentic glucose 6 phosphate and fructose 6 phosphate to the assay mix in the presence of leaf extract produced the correct absorbance change showing that there was no inhibition of the assay by the leaf extract.

Estimation of fructose 1,6 bisphosphate

0.8 ml of leaf extract were added to 0.15 ml of 0.66 M imidazole/HCl buffer pH 7.3 containing 0.05 ml of 4 mM NADH. The enzymes α glycerophosphate dehydrogenase (3 units), triose phosphate isomerase (1 unit), and aldolase (2 units) were added. The absorbance change at 340 nm due to the disappearance of NADH after the addition of aldolase was proportional to the level of fructose 1,6 bisphosphate. The addition of authentic fructose 1,6 bisphosphate to the assay mix in the presence of leaf extract produced the correct absorbance change, validating the assay method.

Extraction and estimation of leaf chlorophyll content

The method of Arnon (1949) was followed. The chlorophyll was extracted from leaf-discs of C_3 and CAM *M. crystallinum* by boiling in 80% ethanol until the discs were free of green pigment. Amounts of chlorophyll a and b were determined from the absorbance of the extracts at 663 nm and 645 nm.

Estimation of Inorganic Phosphate (Pi)

The ammonium vanadate-molybdate method of Kitson and Mellon (1944) was followed. It is based on the reaction of Pi with molybdate and vanadate in acid solution. A complex, molybdivanadophosphoric acid develops which has a very stable yellow colour proportional to the Pi concentration of the solution.

0.6 ml of leaf-disc extract were added to 0.3 ml of reagent and reacted at room temperature for 10 minutes. One litre of the reagent contained 300 ml 50% HNO_3, 300 ml 0.25% ammonium vanadate, 300 ml 5% ammonium molybdate and 100 ml distilled water. The absorbance at 360 nm was measured. The change in absorbance due to the presence of Pi was linear up to 2 absorbance units. One absorbance unit was equal to 0.113 µmols of Pi, the calibration passing through the origin.

Performance of the assay in the presence of considerable sorbitol relative to Pi - a concentration of sorbitol 400-fold greater than that of Pi - rendered the assay inoperable. Above this ratio of sorbitol to Pi the absorbance peak at 360 nm was completely removed and negative absorbance changes were recorded. At a ratio of sorbitol:Pi of 120 the correct absorption change relative to the amount of Pi present took place more slowly, taking 40 minutes to complete compared to 10 minutes in the absence of sorbitol. It is thought that sorbitol may sequester the Pi, inhibiting the reaction with the reagent.

Estimation of leaf tissue acid content

The acid content of leaf-disc extracts was measured by titrating the extract with 0.01 M NaOH. The amount of NaOH required to neutralise the extract was proportional to the acid content. Acid was expressed as equivalents (eq).

Estimation of malic acid

The method of Mollering (1974) was followed. 0.1 ml of leaf-disc extract was added to 0.5 ml of 0.6 M glycylglycine/0.1 M glutamic acid/NaOH buffer pH 10.0, containing 0.1 ml 47 mM NAD, 5 µl of 2 mg/ml glutamate-oxaloacetate transaminase, 5 µl of 5 mg/ml malic acid dehydrogenase and 1 ml distilled water. The absorbance change due to the production of NADH was measured at 340 nm.

Estimation of the water potential (ψ) of leaf-discs and solute potentials (ψs) of leaf sap and solutions of polyethylene glycol

Measurements of water potential and solute potential were made using a Wescor Inc. HR-33T Dew Point Microvoltmeter. Leaf-disc water potential was measured by placing leaf-discs into the sample chamber storage well. Leaf sap solute potential was measured by expressing the sap from leaf-discs onto filter paper of the same size and placing the filter paper into the sample chamber storage well. The solute potential of polyethylene glycol was measured by soaking filter paper in the polyethylene glycol solution and placing the filter paper into the sample chamber storage well.

Measurement of leaf reflectance and leaf transmittance

The reflectance and transmittance of light between 400 nm and 700 nm from the adaxial surface of *M. crystallinum* leaves was measured using an LI-COR LI-1800 portable spectroradiometer with an attached LI-COR 1800-12 integrating sphere. Residual moisture on the

surface of leaves was carefully removed before measurements were made.

Gas chromatographic analysis of ethylene

1 ml gas samples were injected into a Pye series 104 gas chromatograph, fitted with a Poropak Q column at 90°C. The detector oven temperature was maintained at 150°C. The carrier gas flow rate was 40 ml per minute. The detection limit of the apparatus was 0.5 ppm ethylene.

Sequestration and addition of Pi to leaf-discs of C_3 and CAM *M. crystallinum* and *K. daigremontiana*

Pi sequestration was carried out by incubating leaf-discs in 50 mM BES buffer pH 6.8, containing 10 mM mannose or 10 mM glucosamine.

Incubation of leaf-discs in the presence of Pi was carried out by incubating leaf-discs in 100 mM Pi buffer made up of 50 mM K_2HPO_4 and 50 mM KH_2PO_4.

Control discs for all treatments were incubated in 50 mM BES buffer pH 6.8. The solute potential of all solutions was -0.65±0.14 MPa.

Analysis of pinitol by High Performance Liquid Chromatography (HPLC)

Leaf tissue extracts of *L. corniculatus* and CAM *M. crystallinum*, freeze dried in the same way as samples for gas chromatographic

analysis, were reacted with 85% acetonitrite at room temperature for 30 minutes. The Pye Unicam HPLC apparatus consisted of an LC-XP gradient programmer, an LC-UV detector, a PU 4023 refraction index detector and an LC-XPD pump. Microlitre samples were injected onto a spherisorb S-5 amino column with 85% acetronitrite as the mobile phase.

Isolation of protoplasts from CAM *M. crystallinum*

The method of Winter, Foster, Edwards and Holtum (1982) was followed. About 50 g of leaf tissue were excised from the plants, the mid-ribs removed and the leaf tissue sliced up into small squares no larger than 2 mm^2. They were then washed in incubation medium consisting of 800 mM sorbitol, 20 mM MES/KOH buffer pH 5.2, 0.5 mM $CaCl_2$ and 1 mM DTT, and placed into 75 ml of fresh incubation medium with 0.5 g cellulase and 0.25 g of macerozyme. Following vacuum infiltration for a few minutes the leaf squares were incubated for about $1\frac{1}{2}$ hours at room temperature. They were then washed through miracloth with incubation medium to remove the protoplasts from the debris which was discarded. The protoplasts were concentrated by centrifugation at 200 g and 4°C. The debris from this protoplast pellet was removed by mixing it with an equal volume of 20% Ficoll in incubation medium, overlayed by 10 ml of 6% Ficoll in incubation medium, which was overlayed by 5 ml of 3% Ficoll in incubation medium overlayed by 2 ml of incubation medium. Clean protoplasts floated to the top after centrifugation at 200 g for 5 minutes.

Isolation of vacuoles from CAM *M. crystallinum*

A minimum volume of lysis medium consisting of 100 mM Hepes/KOH buffer pH 8, 1 mM DTT and a few drops of neutral red were added to the clean protoplasts. The mixture was stirred gently and protoplast lysis observed microscopically. When protoplast lysis was complete the lysate was kept on ice and mixed with an equal volume of 1 M sorbitol containing 20% Ficoll in lysis medium. This was overlayed with concentrations of Ficoll in lysis medium containing 0.8 M sorbitol: 10 ml of 3.75% Ficoll, overlayed by 5 ml of 2.5% Ficoll, overlayed by 3 ml of 1% Ficoll, finally overlayed by 2 ml of lysis medium containing 0.8 M sorbitol. The vacuoles floated to the upper layers after centrifugation at 200 g for 10 minutes.

Isolation of chloroplasts from CAM *M. crystallinum*

The method of Demmig and Winter (1983) was followed. Clean protoplasts were ruptured by 2 passes through a 25 gauge needle. The chloroplasts were pelletted by centrifugation at 300 g for 5 minutes and the supernatant discarded. The chloroplasts were suspended in chloroplast storage medium consisting of 0.75 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCl and 50 mM MES/KOH pH 6.7. The intactness of chloroplasts produced in this way varied between 90-98% (Demmig and Winter, 1983).

Protoplasts and vacuoles were counted using a haemocytometer. Pinitol levels in protoplasts, vacuoles and chloroplasts were determined by gas chromatography.

Extraction and assay of glucose 6 phosphate cycloaldolase (EC 5.5.1.4) from *M. crystallinum*

The method of extraction and assay was adapted from Loewus and Loewus (1971). About 40 g of *M. crystallinum* leaf tissue 10 days into the induction period was ground to powder in liquid N_2 and homogenised in 0.2 M Tris/HCl buffer pH 8, containing 1 mM GSH and 10% polyvinylpyrollidone (average molecular weight 360 000). Samples from this were taken to assay for glucose 6 phosphate cycloaldolase.

The assay for glucose 6 phosphate cycloaldolase consisted of adding the following reagents to the enzyme extract to give final concentrations of 40 mM Tris/HCl buffer pH 8, 1 mM NAD, 12 mM NH_4Cl and 6 mM glucose 6 phosphate. The mixture was incubated at 30°C for $2\frac{1}{2}$ hours. It was then killed by boiling, cooled and 0.1 mg ml^{-1} of alkaline phosphatase and 1 mg ml^{-1} of $MgCl_2$ added. The extract was then analysed for the presence of inositol by gas chromatography. C_3 tissue was analysed for the presence of glucose 6 phosphate cycloaldolase in the same way.

EXPERIMENTS AND RESULTS

1. Analysis of leaf metabolites, chlorophyll and leaf sap solute potential (ψs) during a 12 hour photoperiod in C_3 and CAM *Mesembryanthemum crystallinum* and *Kalanchoe daigremontiana*, and the effect of leaf age on levels of malic acid in C_3 and CAM *M. crystallinum*

1.1 Starch, total soluble carbohydrate and malic acid levels at the beginning and end of a 12 hour photoperiod in C_3 and CAM *M. crystallinum* and *K. daigremontiana*

Sixteen leaf-discs from 4 plants, 4 discs being sampled from 1 leaf on each plant of C_3 and CAM *M. crystallinum* and *K. daigremontiana* were taken at 9:25 immediately prior to the start of the photoperiod and at 21:25, immediately prior to its end. There were 4 replicates each comprising 4 leaf-discs at each time. Starch and soluble carbohydrate were measured using the Anthrone technique.

Levels of starch and total soluble carbohydrate were higher in C_3 *M. crystallinum* than in CAM *M. crystallinum* and *K. daigremontiana*, the difference being most marked between C_3 and CAM *M. crystallinum* (Table 2). However, the factor increase in levels of carbohydrate during the 12 hour photoperiod was greater in the two CAM plants, with a factor increase for total carbohydrate of 4.7 in CAM *M. crystallinum* and 2.7 in *K. daigremontiana*, compared to 1.8 in C_3 *M. crystallinum*. In CAM *M. crystallinum* particularly, and in *K. daigremontiana* starch made up most of this increase with factor increases of 6.1 and 3.3 respectively, compared to 1.9 in C_3 *M. crystallinum*. In *K. daigremontiana* total soluble carbohydrate contributed more to this increase in total carbohydrate than in CAM *M. crystallinum*, with a factor increase of 2.1 compared to 1.7 in CAM *M. crystallinum* and 1.5 in C_3 *M. crystallinum*. In CAM *M. crystallinum* levels of total soluble carbohydrate were very low. The

ratio of starch to total soluble carbohydrate rose during the photoperiod in all three plants, the rise being the most marked in CAM *M. crystallinum* with a ratio of 7.1 at the end of the photoperiod compared to 2.4 in C_3 *M. crystallinum* and 1.7 in *K. daigremontiana*.

Levels of malic acid in CAM *M. crystallinum* and *K. daigremontiana* were high at the start of the photoperiod and fell by a factor of 4.9 and 4.4. respectively during the photoperiod (Table 2). In C_3 *M. crystallinum* levels of malic acid remained low during the whole photoperiod.

1.2 A detailed analysis of leaf metabolites, chlorophyll and leaf sap solute potential during a 12 hour photoperiod in C_3 and CAM *M. crystallinum*

Twenty-seven leaf-discs from 9 plants, 3 discs being sampled from 1 leaf on each plant of C_3 and CAM *M. crystallinum* were taken at intervals of 2 hours between and including 9:30 and 21:30. There were 3 replicates, each comprising 9 leaf-discs. For the analysis of glucose 6 phosphate, fructose 6 phosphate and fructose 1,6 bisphosphate 2 replicates each comprising 3 g of 2 leaf-halves from 2 plants were sampled at 13:30 and 17:30. Starch was analysed by the enzymic method and soluble carbohydrate by gas chromatography.

A qualitative examination of total soluble carbohydrate revealed that it was composed of fructose, glucose, inositol, sorbitol and sucrose (Table 3). Levels of fructose and glucose were very low in CAM *M. crystallinum* in comparison to C_3 *M. crystallinum*. The maximum level of glucose was 1.3 µmol g^{-1} Fresh Weight (F.W.) in the CAM form compared to 22.7 µmol g^{-1} F.W. in the C_3 form. The maximum level of fructose was 0.7 µmol g^{-1} F.W. in the CAM form compared to 5.3 µmol g^{-1} F.W. in the C_3 form. This difference accounted for the large discrepancy in levels of total soluble carbohydrate between the two

forms of *M. crystallinum* (Table 2 and Figure 4). Levels of inositol were higher in the CAM form, maximal at 1.6 μmol g^{-1} F.W. compared to 0.5 μmol g^{-1} F.W. in the C_3 form. Sorbitol was maximal at 0.13 μmol g^{-1} F.W. in the CAM form and not detected (N.D.) in the C_3 form. Sucrose levels were slightly higher in the C_3 form, maximal at 2.4 μmol g^{-1} F.W. compared to a maximum value of 0.9 μmol g^{-1} F.W. in CAM *M. crystallinum*. The ratio of starch to total soluble carbohydrate (St:TSC) increased during the photoperiod from 2.0 at 9:30 to 7.1 at 21:30 in the CAM form compared to a smaller increase in the C_3 form from 1.9 to 2.4.

Amounts of glucose 6 phosphate (G6P) and fructose 6 phosphate (F6P) were much lower in CAM *M. crystallinum*. Maximum values were 0.007 μmol g^{-1} F.W. and 0.010 μmol g^{-1} F.W. respectively in CAM *M. crystallinum* and 0.028 μmol g^{-1} F.W. and 0.113 μmol g^{-1} F.W. respectively in C_3 *M. crystallinum*. Levels of fructose 1,6 bisphophate (FBP) were higher in CAM *M. crystallinum*, maximal at 0.011 μmol g^{-1} F.W. compared to a maximum value of 0.003 μmol g^{-1} F.W. in C_3 *M. crystallinum*.

Levels of Pi in CAM *M. crystallinum* were 2-fold higher than those in C_3 *M. crystallinum*, 15.9 μmol g^{-1} F.W. and 7.6 μmol g^{-1} F.W. respectively at 17:30.

Amounts of pinitol were negligible in C_3 *M. crystallinum*, at a maximum of 0.3 μmol g^{-1} F.W. compared to a maximum of 10.2 μmol g^{-1} F.W. in CAM *M. crystallinum*.

The solute potential (ψs) of leaf sap was more negative in CAM *M. crystallinum* than in C_3 *M. crystallinum* -3.8 MPa and -1.2 MPa respectively at 9:30. The values became less negative during the photoperiod, to -3.5 MPa in the CAM form and -1.0 MPa in the C_3 form.

Levels of chlorophyll were similar in the two forms of *M. crystallinum*: 0.41 mg g^{-1} F.W. in the CAM form and 0.50 mg g^{-1} F.W.

in the C_3 form. The ratio of chlorophyll a to b was higher in CAM *M. crystallinum* at 0.97 compared to 0.75 in the C_3 form.

1.3 The effect of leaf age on levels of malic acid in C_3 and CAM *M. crystallinum*

Twenty leaf-discs from 5 plants each of C_3 and CAM *M. crystallinum* were sampled at 9:25 and 15:00. One leaf was sampled per plant and 4 discs were taken from it. There were 4 replicates each consisting of 5 discs. 'Young' leaves were the most recently fully expanded leaves, and 'old' leaves the third most recently fully expanded leaf.

Levels of malic acid were higher and the amount of deacidification greater in older leaves in both C_3 and CAM *M. crystallinum* (Table 4). In old CAM leaves there was a decline of 7.9 μmol g^{-1} F.W. during the first six hours of the photoperiod from 11.4 μmol g^{-1} F.W. to 3.5 μmol g^{-1} F.W. In old C_3 leaves there was a decline of 3.3 μmol g^{-1} F.W. from 3.6 μmol g^{-1} F.W. to 0.3 μmol g^{-1} F.W. The amount of decline in malic acid in young CAM leaves was 4.1 μmol g^{-1} F.W. similar to that of old C_3 leaves, but the actual levels of malic acid in young CAM leaves was higher, 7.7 μmol g^{-1} F.W. at 9:30 and 3.6 μmol g^{-1} F.W. at 15:00. Levels of malic acid in young C_3 *M. crystallinum* leaves were low and rose by 1.4 μmol g^{-1} F.W. during the experimental period from 0.4 μmol g^{-1} F.W. to 1.8 μmol g^{-1} F.W.

1.1 Table 2. Starch (St), total soluble carbohydrate (TSC) and malic acid (Ma) levels at the beginning and end of the 12 hour photoperiod in *K. daigremontiana*, C_3 and CAM *M. crystallinum*.

K. daigremontiana

	At beginning of DAY		At beginning of NIGHT		µmol change during photoperiod	Factor increase or decrease
	µmol g^{-1} F.W.	Ratio of St:TSC	µmol g^{-1} F.W.	Ratio of St:TSC		
St	11.92± 5.61	1.1	39.25± 6.31	1.7	+27.3	+ 3.3
TSC	11.34± 2.45		23.73± 4.27		+12.4	+ 2.1
Ma	66.81±12.33		15.16± 3.15		-51.7	- 4.4
Total carbohydrate	23.3		63.0		+39.7	+ 2.7

CAM *M. crystallinum*

	At beginning of DAY		At beginning of NIGHT		µmol change during photoperiod	Factor increase or decrease
	µmol g^{-1} F.W.	Ratio of St:TSC	µmol g^{-1} F.W.	Ratio of St:TSC		
St	2.2 ± 0.9	2.0	13.5 ± 1.2	7.1	+11.3	+ 6.1
TSC	1.1 ± 0.4		1.9 ± 0.7		+ 0.8	+ 1.7
Ma	48.9 ± 0.2		9.9 ± 2.7		-39.0	- 4.9
Total carbohydrate	3.3		15.4		+12.1	+ 4.7

C_3 *M. crystallinum*

	At beginning of DAY		At beginning of NIGHT		µmol change during photoperiod	Factor increase or decrease
	µmol g^{-1} F.W.	Ratio of St:TSC	µmol g^{-1} F.W.	Ratio of St:TSC		
St	35.8 ± 1.9	1.9	69.2 ± 6.1	2.4	+33.4	+ 1.9
TSC	18.9 ± 1.6		29.0 ± 4.5		+10.1	+ 1.5
Ma	0.3 ± 0.1		4.1 ± 0.9		+ 3.8	+13.7
Total carbohydrate	54.7		98.2		+43.5	+ 1.8

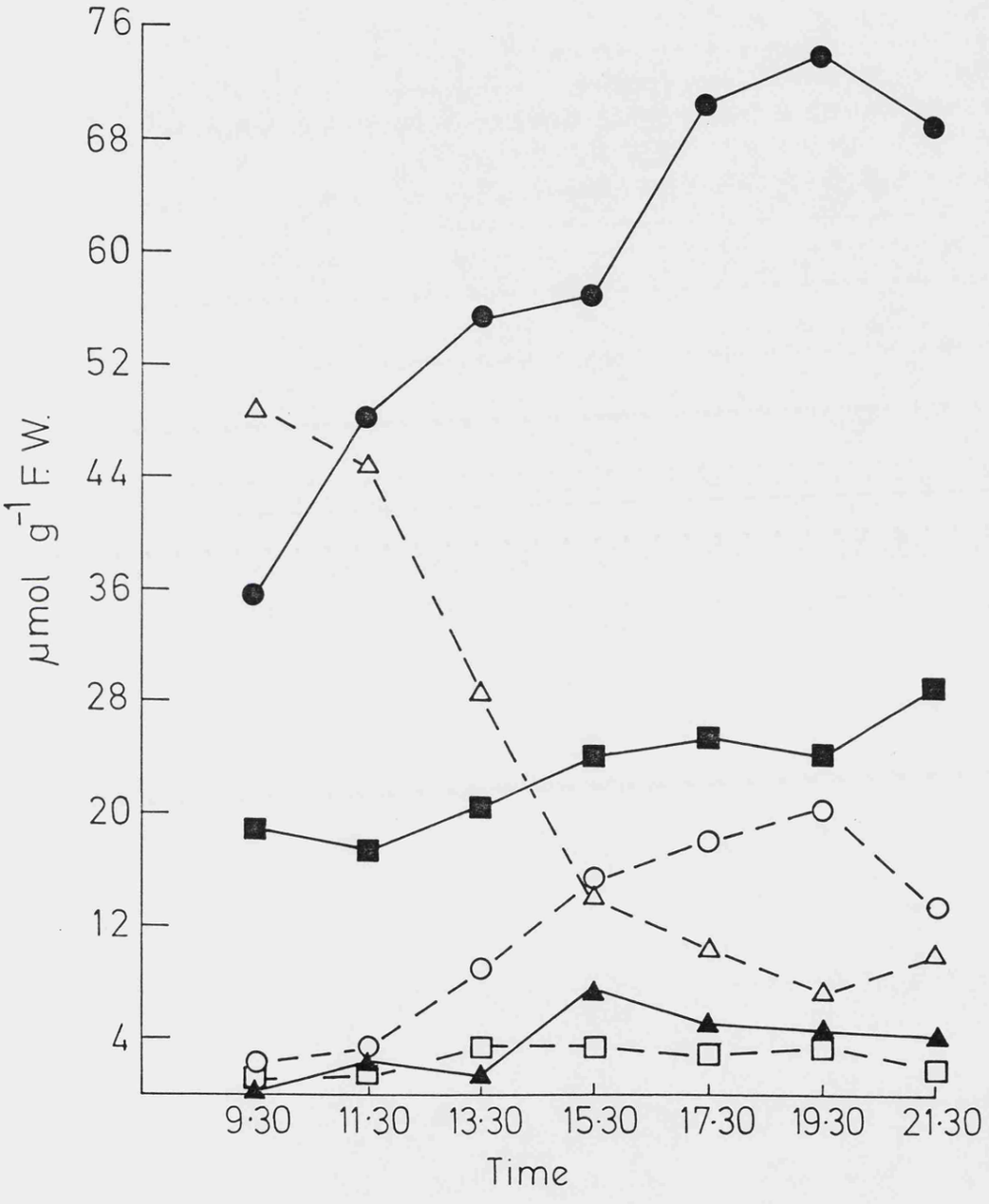
1.2 Table 3. Comparison of leaf metabolites, chlorophyll and leaf sap solute potential (ψs) during the 12 hour photoperiod in C_3 and CAM *M. crystallinum.*

µmol g^{-1} F.W.	9:30	11:30	13:30	15:30	17:30	19:30	21:30	9:30–21:30 µmol change	
Fructose	N.D.	N.D.	N.D.	0.7 ±0.2	0.2 ±0.1	0.7 ±0.2	N.D.	N.D.	
Glucose	0.5 ±0.2	0.4 ±0.1	1.3 ±0.8	1.1 ±0.5	0.9 ±0.4	0.8 ±0.2	1.3 ±0.5	+0.8	
Inositol	0.4 ±0.1	0.4 ±0.1	1.6 ±1.0	0.7 ±0.3	1.3 ±0.3	1.1 ±0.3	0.5 ±0.2	+0.1	
Sorbitol	0.13±0.04	0.09±0.01	0.09±0.01	0.10±0.01	0.13±0.01	0.12±0.03	0.10±0.004	−0.03	
Sucrose	0.1 ±0.02	0.7 ±0.05	0.4 ±0.14	0.9 ±0.44	0.4 ±0.14	0.6 ±0.12	N.D.	−0.1	
St:TSC	2.0	2.1	2.6	4.5	6.2	6.1	7.1		CAM
F6P			0.007±0.003		0.006±0.002				
G6P			0.010±0.004		0.010±0.002				
FBP			0.011±0.005		0.005±0.002				
Pi	14.1±0.2				15.9 ±0.4				
Inositol equivalents									
Pinitol	6.0 ±1.6	7.7 ±0.8	7.8 ±0.8	8.6 ±0.2	7.6 ±2.2	10.2 ±0.3	8.9 ±1.1	+2.9	
ψs leaf sap MPa	−3.8 ±0.4						−3.5 ±0.4		
mg g^{-1} F.W.									
Chlorophyll				0.41					
Ratio a:b				0.97					

µmol g^{-1} F.W.	9:30	11:30	13:30	15:30	17:30	19:30	21:30	9:30–21:30 µmol change	
Fructose	1.6 ±0.2	2.6 ±1.2	3.2 ±0.5	3.6 ±0.3	3.7 ±0.5	3.7 ±0.5	5.3 ±1.0	+3.7	
Glucose	16.7 ±0.2	14.2 ±5.6	15.4 ±6.5	19.4 ±3.2	20.5 ±3.4	17.5 ±6.9	22.7 ±3.0	+6.0	
Inositol	0.2 ±0.01	0.3 ±0.02	0.4 ±0.13	0.4 ±0.08	0.4 ±0.12	0.5 ±0.01	0.5 ±0.08	+0.3	
Sorbitol	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
Sucrose	0.4 ±0.2	0.5 ±0.3	1.2 ±0.7	0.7 ±0.1	0.9 ±0.1	2.4 ±0.7	0.5 ±0.4	+0.1	
St:TSC	1.9	2.7	2.8	2.4	2.8	3.1	2.4		C_3
F6P			0.028±0.010		0.016±0.006				
G6P			0.113±0.016		0.064±0.024				
FBP			0.003±0.002		0.003±0.001				
Pi	7.2 ±0.2				7.6 ±0.4				
Inositol equivalents									
Pinitol	6.3 ±0.20	0.1 ±0.05	0.1 ±0.06	0.3 ±0.10	0.3 ±0.15	0.2 ±0.11	0.2 ±0.09	−0.1	
ψs leaf sap MPa	−1.2 ±0.06						−1.0 ±0.04		
mg g^{-1} F.W.									
Chlorophyll				0.50					
Ratio a:b				0.75					

N.D. = not detected

1.2 Figure 4. Levels of starch (C_3—●— , CAM—○—), total soluble carbohydrate (C_3—■— , CAM—□—) and malic acid (C_3—▲— , CAM—△—) during a 12 hour photoperiod in C_3 and CAM *M. crystallinum*.


76
68
60
52
44
36
28
20
12
4
µmol g⁻¹ F.W.
9·30
11·30
13·30
15·30
17·30
19·30
21·30
Time

Standard errors for Figure 4 (μmol g^{-1} F.W.)

Time	9:30	11:30	13:30	15:30	17:30	19:30	21:30
CAM							
Starch	0.9	1.2	1.3	1.9	0.9	0.9	1.2
TSC	0.1	0.2	0.5	0.5	0.4	0.6	0.4
Malic acid	0.2	2.7	3.2	0.5	1.2	1.0	2.7
C_3							
Starch	1.9	5.6	1.8	2.6	2.4	2.8	6.1
TSC	3.7	3.3	4.1	4.0	4.4	4.6	4.7
Malic acid	0.1	1.4	0.7	2.9	3.1	2.8	2.6

1.3 Table 4. The effect of leaf age on malic acid levels (μmol g^{-1} F.W.) in C_3 and CAM *M. crystallinum*.

	9:30	15:00	Changes between 9:30 and 15:00
Young CAM	7.7 ± 1.6	3.6 ± 0.6	-4.1
Old CAM	11.4 ± 0.6	3.5 ± 2.1	-7.9
Young C_3	0.4 ± 0.2	1.8 ± 1.0	+1.4
Old C_3	3.6 ± 0.7	0.3 ± 0.2	-3.3

2. The effect of photon fluence rate on levels of carbohydrate and malic acid in C_3 and CAM *M. crystallinum* and *K. daigremontiana*

The different photon fluence rates were attained by placing the plants at different levels in the growth cabinet. The photon fluence rate of 1000 µmol m^{-2} s^{-1} was provided by a projector lamp.

Measurements of carbohydrate and malic acid at 9:30, immediately after the dark period (Tables 5, 6, 9, 10 and 11), which should ideally be the same for plants incubated at different photon fluence rates were not averaged to give one value because this would have given a less clear impression of the changes taking place at each photon fluence rate during the photoperiod. Differences at 9:30 are due to the inherent variability of the plant material.

2.1 The effect of photon fluence rate on levels of carbohydrate

Two plants each of C_3 and CAM *M. crystallinum* were incubated at the photon fluence rates of 0, 15, 30 and 1000 µmol m^{-2} s^{-1}. Six plants of each were incubated at 50 µmol m^{-2} s^{-1}, and 4 plants of each were incubated at 100 and 250 µmol m^{-2} s^{-1}. Replicate samples comprising 9 leaf-discs were taken from each plant at 4 times during the 12 hour photoperiod: 9:30, 13:30, 17:30 and 21:30. Two replicate samples were taken at each time at 0, 15, 30 and 1000 µmol m^{-2} s^{-1}. Six replicate samples were taken at 100 and 250 µmol m^{-2} s^{-1}. Levels of starch were measured by the enzymic method and soluble carbohydrate by gas chromatography.

Investigations on *K. daigremontiana* were carried out on excised leaves which were removed from the plants minutes prior to the start of the experiment. Two leaves excised from 2 plants were incubated at the photon fluence rates of 0, 50, 250 and 1000 µmol m^{-2} s^{-1}.

Four replicate samples consisting of 6 leaf-discs each were taken from both leaves at intervals of 2 hours between 9:30 and 17:30. Levels of starch and soluble carbohydrate were measured using the Anthrone method.

In CAM *M. crystallinum* there was a net accumulation of total soluble carbohydrate at all photon fluence rates above 0 μmol m^{-2} s^{-1} (Table 7). The amount of accumulation was low in comparison to C_3 *M. crystallinum* and *K. daigremontiana* (Tables 7 and 8), and reached a maximum of 1.2 μmol g^{-1} F.W. at 100 μmol m^{-2} s^{-1}. In C_3 *M. crystallinum* values for total soluble carbohydrate were more variable. At 0, 15, 250 and 1000 μmol m^{-2} s^{-1} there was net accumulation during the photoperiod, with a maximum of 22.6 μmol g^{-1} F.W. at 1000 μmol m^{-2} s^{-1} (Table 7). At 30, 50 and 100 μmol m^{-2} s^{-1} there was net consumption of total soluble carbohydrate. Sucrose accumulation rose with photon fluence rate up to 50 μmol m^{-2} s^{-1} in CAM *M. crystallinum*, where there was an accumulation of 0.9 μmol g^{-1} F.W. during the photoperiod (Table 6). In C_3 *M. crystallinum* accumulation of sucrose rose up to 1000 μmol m^{-2} s^{-1}, where there was an accumulation of 1.8 μmol g^{-1} F.W. during the photoperiod (Table 5). There was no correlation of fructose, glucose, inositol or pinitol levels with photon fluence rate.

In *K. daigremontiana* total soluble carbohydrate accumulated at all photon fluence rates above 0 μmol m^{-2} s^{-1}, and reached a maximum net accumulation value of 47.7 μmol g^{-1} F.W. at 1000 μmol m^{-2} s^{-1} (Table 8).

Starch accumulated at all photon fluence rates above 0 μmol m^{-2} s^{-1} in CAM *M. crystallinum* (Table 7 and Fig. 5). Accumulation was maximal at 250 μmol m^{-2} s^{-1} (15.0 μmol g^{-1} F.W.). In C_3 *M. crystallinum* starch consumption occurred at and below 50 μmol m^{-2} s^{-1}. At and above 100 μmol m^{-2} s^{-1} starch accumulation rose more

steeply than in CAM *M. crystallinum* and reached a maximum of 38.5 μmol g^{-1} F.W. at 1000 μmol m^{-2} s^{-1} (Fig. 5 and Table 7). In CAM *M. crystallinum* starch accumulation was always more or equal to, or consumption less than that of total soluble carbohydrate (St-TSC, Table 7). In C_3 *M. crystallinum* starch accumulation was only in excess of total soluble carbohydrate accumulation at and above 50 μmol m^{-2} s^{-1}.

In *K. daigremontiana* starch accumulation occurred at all photon fluence rates above 0 μmol m^{-2} s^{-1} (Table 8), and net accumulation reached a maximum value of 30.4 μmol g^{-1} F.W. at 250 μmol m^{-2} s^{-1}. At 0, 50 and 250 μmol m^{-2} s^{-1} starch accumulation was greater than total soluble carbohydrate accumulation (St-TSC, Table 8). At 1000 μmol m^{-2} s^{-1} total soluble carbohydrate accumulation exceeded starch accumulation.

Total carbohydrate (St+TSC) accumulated at every photon fluence rate above 0 μmol m^{-2} s^{-1} in CAM *M. crystallinum* (Table 7), reaching a peak of 16.1 μmol g^{-1} F.W. at 250 μmol m^{-2} s^{-1}. In C_3 *M. crystallinum* accumulation of total carbohydrate commenced at 100 μmol m^{-2} s^{-1}, rising with photon fluence rate up to 1000 μmol m^{-2} s^{-1} where 61.1 μmol g^{-1} F.W. accumulated during the photoperiod.

In *K. daigremontiana* total carbohydrate accumulation began at 50 μmol m^{-2} s^{-1}, rising up to 1000 μmol m^{-2} s^{-1} where 76.4 μmol g^{-1} F.W. accumulated (Table 8).

2.2 The effect of photon fluence rate on levels of malic acid.

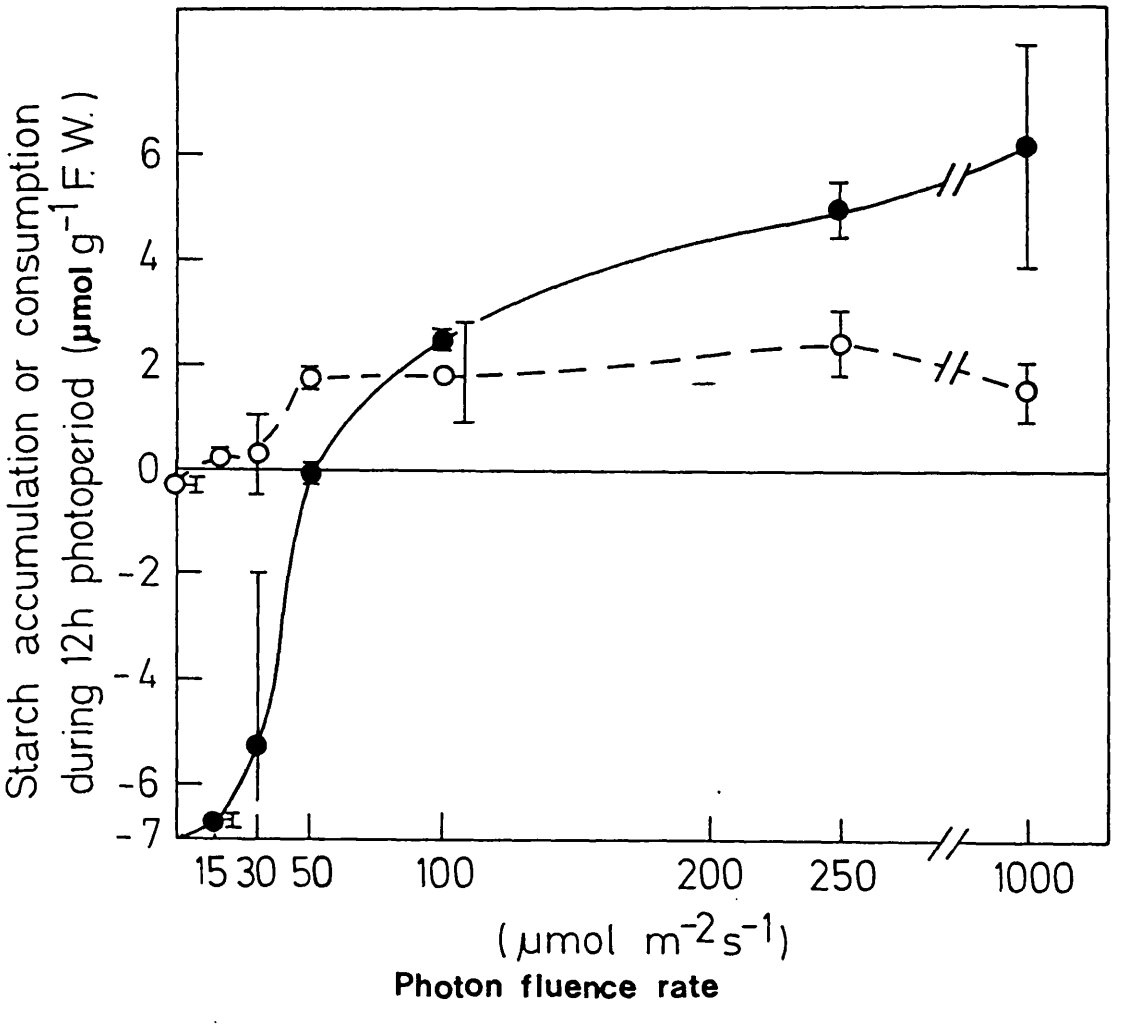
In C_3 and CAM *M. crystallinum* experimental conditions were as outlined in 2.1. In *K. daigremontiana* samples consisting of the same number of replicates and leaf-discs as 2.1 were sampled at intervals

of 2 hours between and including 10:00 and 16:00 at the photon fluence rates 0, 50, 250 and 1000 μmol m^{-2} s^{-1}.

In CAM *M. crystallinum* the consumption of malic acid during a 12 hour photoperiod rose with increasing photon fluence rate up to 250 μmol m^{-2} s^{-1} where 24.7 μmol g^{-1} F.W. were consumed (Table 10). Consumption of malic acid at 1000 μmol m^{-2} s^{-1} was 18.1 μmol g^{-1} F.W. The factor reduction in malic acid levels was greatest at 100 μmol m^{-2} s^{-1} (4.0).

In *K. daigremontiana* the actual consumption of acid and the factor reduction of acid levels rose up to the highest photon fluence rate, with values of 134 μeq and 7.4 respectively at 1000 μmol m^{-2} s^{-1} (Table 11).

2.1 Figure 5. Starch accumulation or consumption (C_3 —●— , CAM —○—) during a 12 hour photoperiod at different photon fluence rates in C_3 and CAM *M. crystallinum*.


Starch accumulation or consumption during 12h photoperiod (µmol g⁻¹ F. W.)
6
4
2
0
-2
-4
-6
-7
15 30 50 100 200 250 1000
(µmol m⁻² s⁻¹)
Photon fluence rate

2.1 Table 5. Levels of metabolites (μmol g^{-1} F.W.) during a 12 hour photoperiod at different photon fluence rates in C_3 *M. crystallinum*.

Photon fluence rate µmol m^{-2} s^{-1}	Time	Fructose	Glucose	Inositol	Sucrose	Starch	(inositol equivalents) pinitol
0	9:30	2.7±0.5	18.2± 3.1	0.3±0.1	0.3±0.1	122.3±42.0	1.8±0.3
	13:30	4.0±0.8	19.3± 3.5	0.4±0.2	0.3±0.1	119.0±34.9	2.0±0.4
	17:30	3.0±0.6	18.1± 3.0	0.3±0.1	0.4±0.1	117.5±46.7	1.6±0.3
	21:30	3.5±0.7	22.1± 5.9	0.4±0.1	0.6±0.2	116.1±32.1	1.8±0.5
15	9:30	3.8±0.8	14.9± 4.7	0.2±0.1	0.2±0.1	90.2± 5.2	1.2±0.5
	13:30	4.0±0.9	16.2± 4.9	0.3±0.1	0.2±0.1	63.7± 7.1	1.4±0.6
	17:30	4.3±1.0	19.2± 3.4	0.4±0.1	0.7±0.2	87.3±21.2	1.5±0.7
	21:30	5.8±1.2	15.2± 3.9	0.4±0.1	0.5±0.1	48.6± 5.2	1.6±0.6
30	9:30	3.6±0.7	21.8± 4.8	0.5±0.2	0.4±0.1	77.9±40.1	1.5±0.4
	13:30	0.9±0.7	17.8± 5.0	0.4±0.1	0.3±0.1	68.0±39.7	1.6±0.4
	17:30	3.4±1.0	21.1± 5.1	0.6±0.2	0.7±0.3	87.3±54.3	1.6±0.6
	21:30	3.2±0.9	18.9± 4.7	1.8±0.8	0.2±0.1	45.3±20.8	1.6±0.7
50	9:30	1.8±0.2	25.7± 7.6	0.6±0.1	0.8±0.3	66.0±12.6	1.0±0.3
	13:30	2.7±0.5	25.9± 6.4	0.6±0.1	0.9±0.2	62.2±14.0	0.9±0.3
	17:30	2.1±0.5	22.4± 6.8	0.6±0.2	0.9±0.1	59.1±11.7	1.1±0.6
	21:30	1.4±0.4	16.7± 7.1	0.5±0.2	1.1±0.2	62.7±17.0	1.1±0.4
100	9:30	1.1±0.4	17.3± 2.9	0.4±0.1	0.8±0.1	35.2± 5.5	0.4±0.1
	13:30	1.7±0.4	17.3± 3.6	0.4±0.1	1.1±0.5	48.3±15.8	0.4±0.1
	17:30	0.9±0.3	13.6± 4.3	0.5±0.2	1.7±0.8	54.1±10.1	0.5±0.2
	21:30	0.8±0.4	16.0± 6.8	0.6±0.1	1.2±0.2	63.0± 8.8	0.6±0.1
250	9:30	0.7±0.2	15.7± 4.8	0.5±0.2	0.4±0.1	32.0± 4.7	0.5±0.2
	13:30	2.2±0.6	18.7± 5.4	0.4±0.1	1.0±0.1	48.3±15.8	0.4±0.1
	17:30	2.1±0.5	16.2± 3.0	0.4±0.1	0.9±0.1	54.1±10.1	0.4±0.1
	21:30	1.3±0.3	18.2± 2.0	0.4±0.1	1.9±0.3	63.0± 8.8	0.4±0.1
1000	9:30	1.2±0.4	27.2±10.2	0.6±0.1	0.7±0.1	38.4± 1.3	0.6±0.1
	13:30	4.3±0.2	26.1± 8.5	0.6±0.1	1.3±0.1	65.5±23.5	0.6±0.1
	17:30	6.0±1.5	34.3±13.8	0.9±0.1	1.0±0.8	74.4±17.2	0.9±0.3
	21:30	4.7±0.5	30.3±12.2	0.9±0.1	2.5±0.6	76.9±11.5	0.9±0.2

2.1 Table 6. Levels of metabolites (μmol g^{-1} F.W.) during a 12 hour photoperiod at different photon fluence rates in CAM *M. crystallinum*.

Photon fluence rate μmol m^{-2} s^{-1}	Time	Fructose	Glucose	Inositol	Sucrose	Starch	(inositol equivalents) pinitol
0	9:30		0.3±0.10	0.2±0.04	0.2±0.05	2.9±1.5	9.9±1.2
	13:30		0.2±0.05	0.1±0.02	0.2±0.10	4.6±0.3	9.6±1.1
	17:30		0.1±0.03	0.1±0.02	0.2±0.08	2.8±0.9	10.5±1.2
	21:30		0.1±0.04	0.1±0.03	0.2±0.07	2.7±1.3	11.6±1.4
15	9:30		0.1±0.03	0.1±0.02	0.2±0.09	2.7±0.4	9.9±1.1
	13:30		0.7±0.20	0.2±0.06	0.5±0.2	2.6±0.4	11.1±1.3
	17:30		0.7±0.20	0.2±0.05	0.4±0.1	2.6±0.5	10.8±1.2
	21:30		0.6±0.10	0.2±0.06	0.5±0.2	3.6±0.2	10.7±1.2
30	9:30		0.1±0.04	0.2±0.07	0.5±0.2	3.8±2.3	9.5±1.1
	13:30		0.1±0.03	0.1±0.02	0.6±0.2	4.7±0.7	10.6±1.3
	17:30		0.1±0.03	0.1±0.01	0.6±0.2	4.1±1.8	11.3±1.4
	21:30		0.2±0.10	0.1±0.01	0.5±0.1	5.6±2.5	12.5±1.7
50	9:30		0.7±0.3	0.2±0.05	0.3±0.1	1.9±0.5	9.5±1.1
	13:30		0.4±0.1	0.2±0.03	0.6±0.1	5.2±0.6	9.7±1.0
	17:30		0.5±0.2	0.3±0.02	1.3±0.4	7.9±1.2	9.7±1.2
	21:30		0.7±0.3	0.3±0.08	1.2±0.4	12.7±1.8	10.8±1.4
100	9:30		0.1±0.05	0.3±0.01	0.3±0.1	3.1±0.6	9.5±1.0
	13:30		0.7±0.2	0.2±0.03	0.7±0.3	5.7±1.3	10.0±1.2
	17:30		0.8±0.3	0.3±0.03	0.5±0.3	9.3±0.8	7.1±1.4
	21:30		0.5±0.1	0.3±0.04	1.1±0.2	13.9±3.2	9.5±1.4
250	9:30		0.3±0.1	0.2±0.03	0.4±0.1	3.0±0.2	10.4±1.4
	13:30		0.7±0.2	0.3±0.03	0.5±0.1	8.0±1.7	9.5±1.1
	17:30		0.5±0.1	0.3±0.09	0.5±0.2	11.6±1.4	9.7±0.7
	21:30		0.6±0.2	0.4±0.05	1.0±0.4	18.0±2.3	10.9±1.2
1000	9:30		0.6±0.3	0.3±0.05	0.7±0.2	1.9±1.4	10.6±2.0
	13:30		0.9±0.6	0.2±0.10	1.7±0.4	8.0±2.8	10.5±2.8
	17:30		0.6±0.1	0.4±0.05	1.4±0.6	9.7±1.1	11.1±1.0
	21:30		0.5±0.2	0.4±0.10	1.6±0.7	12.1±4.6	12.1±1.4

2.1 Table 7. Starch (St) and total soluble carbohydrate (TSC) accumulation or consumption (μmol g^{-1} F.W.) during a 12 hour photoperiod at different photon fluence rates in C_3 and CAM *M. crystallinum*.

Photo fluence rate µmol m^{-2} s^{-1}	CAM				C_3			TSC
	St	TSC	St+TSC	St-TSC	St	TSC	St+TSC	St-TSC
0	- 0.2	-0.5	- 0.7	+ 0.3	- 6.1	+ 5.1	- 1	-11.2
15	+ 0.9	+0.9	+ 1.8	0	-41.5	+ 2.8	-38.7	-44.3
30	+ 1.8	+0.1	+ 1.9	+ 1.7	-32.6	-12.2	-44.8	-20.4
50	+10.8	+1.1	+11.9	+ 9.7	- 3.3	- 5.5	- 8.8	+ 2.2
100	+10.9	+1.2	+12.1	+ 9.7	+15.3	- 5.8	+ 9.5	+21.1
250	+15.0	+1.1	+16.1	+13.9	+30.9	+ 4.4	+35.3	+26.5
1000	+10.3	+0.8	+11.1	+ 9.4	+38.5	+22.6	+61.1	+15.9

2.1 Table 8. Starch (St) and total soluble carbohydrate (TSC) accumulation or consumpton (μmol g^{-1} F.W.) after the first 8 hours of a 12 hour photoperiod at different photon fluence rates in *K. daigremontiana*.

Photon fluence rate µmol m^{-2} s^{-1}	St	TSC	St+TSC	St-TSC
0	- 1.1	- 2.5	- 3.6	+ 1.4
50	+ 4.7	+ 4.1	+ 8.8	+ 0.6
250	+30.4	+23.0	+53.4	+ 7.4
1000	+28.7	+47.7	+76.4	-19.0

2.1 Table 9. Levels of starch (St) and total soluble carbohydrate (TSC) (μmol g^{-1} F.W.) during a 12 hour photoperiod at different photon fluence rates in *K. daigremontiana*.

Photon fluence rate µmol m^{-2} s^{-1}	Time	St	TSC
0	9:30	22.6±2.4	14.9±1.5
	11:30	22.5±3.1	11.5±1.8
	13:30	23.3±3.1	13.3±1.0
	15:30	21.5±3.2	17.1±3.5
	17:30	21.5±5.2	12.4±1.3
50	9:30	15.0±4.6	11.9±3.6
	11:30	11.9±2.7	12.6±0.1
	13:30	14.2±2.7	15.4±2.4
	15:30	18.0±1.7	15.0±1.7
	17:30	19.7±1.7	16.0±2.6
250	9:30	5.1±1.1	6.9±0.3
	11:30	8.4±1.1	11.8±0.4
	13:30	11.2±1.5	16.8±1.3
	15:30	32.6±0.6	32.1±0.7
	17:30	33.5±6.7	29.9±0.1
1000	9:30	9.5±2.2	7.4±0.4
	11:30	24.9±2.4	19.6±1.3
	13:30	36.7±1.0	40.6±2.5
	15:30	36.9±0.7	58.3±0.3
	17:30	38.2±0.6	55.1±3.8

2.2 Table 10. Malic acid consumption (μmol g^{-1} F.W.) during a 12 hour photoperiod at different photon fluence rates in CAM *M. crystallinum*.

	Photon fluence rate µmol m^{-2} s^{-1}						
	0	15	30	50	100	250	1000
Time 9:30	9.1±3.5	20.3±0.4	22.9±4.8	32.2±9.6	27.7±2.0	34.8±4.3	31.0± 2.3
13:30	4.1±1.1	17.6±6.1	16.4±3.5	27.7±5.3	21.1±3.9	22.8±2.4	25.9± 9.0
17:30	4.8±1.9	20.3±7.0	8.7±0.6	17.9±4.3	11.5±3.0	11.2±2.3	21.7±14.2
21:30	6.3±3.0	8.1±1.5	8.5±0.4	9.3±2.1	6.9±3.4	10.1±2.7	12.9± 8.1
µmol change during photoperiod	-2.8	-12.2	-14.4	-22.9	-20.8	-24.7	-18.1
Factor change during photoperiod	-1.4	- 2.5	- 2.7	- 3.5	- 4.0	- 3.4	- 2.4

2.2 Table 11. Acid consumption (μ equivalents (μeq) acid g^{-1} F.W.) during the first 6 hours of a 12 hour photoperiod at different photon fluence rates in *K. daigremontiana*.

	Photon fluence rate μmol m^{-2} s^{-1}			
	0	10	100	1000
Time 9:30	116±10	120±10	149± 1	155± 3
13:30	108±11	106± 4	100±11	47±11
17:30	106±15	64± 9	36± 6	22± 1
21:30	80± 5	70± 1	31± 1	21± 1
μeq change during photoperiod	-36	-50	-118	-134
Factor change during photoperiod	-1.5	-1.7	-4.8	-7.4

3. Measurement of the changes in levels of metabolites, dry weight and fresh weight, ethylene production and leaf reflectance and transmittance during the induction of CAM in *M. crystallinum*

Ten plants were maintained as C_3 plants and 10 were watered with 400 mM NaCl as outlined in Materials and Methods. For the measurement of carbohydrate, pinitol and Pi, 5 replicate samples each consisting of 5 leaf-discs were taken at 15:00. Starch was measured enzymically and soluble carbohydrate by gas chromatography. Samples for the measurement of malic acid and dry and fresh weight were taken at 9:25 and consisted of a single 50 leaf-disc sample. Samples for the measurement of glucose 6 phosphate, fructose 6 phosphate and fructose 1,6 bisphosphate were taken at 11:00 and consisted of 2 replicate samples of 3 g of leaf tissue from 2 leaf-halves.

The measurement of ethylene production was carried out on 6 C_3 and 6 inducing CAM *M. crystallinum* plants during the first 14 days of the induction period. The C_3 and inducing CAM plants were placed into 2 separate clear glass tanks each with a volume of 13.2 dm^3. Clear glass lids were sealed on to the tanks with high vacuum silicone grease, and were removed for 3 hours each day between 14:00 and 17:00. The tanks were kept under growth cabinet conditions. To allow gas samples to be taken from the tanks whilst sealed, tight-fitting suba-seal bungs were fitted into a hole in each lid. Gas samples were taken with a syringe just before 14:00 and analysed with a Pye series 104 gas chromatograph as outlined in Materials and Methods.

Leaf reflectance and transmittance were measured on the most recently fully expanded leaves attached to plants during the first 8 days of the induction period. Two C_3 leaves on different plants and 2 CAM leaves on different plants were sampled.

Levels of metabolites

Starch levels fell during the early part of the induction period in CAM *M. crystallinum* from 4 μmol g^{-1} F.W. at day 0 to 2.1 μmol g^{-1} F.W. at day 9 (Fig. 6). Starch levels then rose to 6.2 μmol g^{-1} F.W. by day 21. In C_3 *M. crystallinum* levels of starch rose steadily during the experimental period from 4 μmol g^{-1} F.W. at day 0 to 18 μmol g^{-1} F.W. at day 21.

Fructose and glucose contributed most to the difference in total soluble carbohydrate between C_3 and CAM *M. crystallinum* (Fig. 6 and Table 12). Fructose in CAM *M. crystallinum* was not detectable by day 7. In C_3 *M. crystallinum* fructose rose from 1.5 μmol g^{-1} F.W. at day 0 to 4.7 μmol g^{-1} F.W. at day 21. Levels of glucose in CAM *M. crystallinum* remained at or below the 1.8 μmol g^{-1} F.W. detected on day 0 except for a value of 3.1 μmol g^{-1} F.W. on day 2. In C_3 *M. crystallinum* glucose rose from 1.8 μmol g^{-1} F.W. at day 0 to 27 μmol g^{-1} F.W. at day 21. Sorbitol was present in CAM *M. crystallinum* from day 2 of the induction period at fairly steady levels between 0.1 and 1 μmol g^{-1} F.W. Sorbitol was present in C_3 *M. crystallinum* during the later half of the experimental period on days 14, 18, 21 and 36 between 0.1 and 0.6 μmol g^{-1} F.W. Inositol was found in CAM *M. crystallinum* throughout the induction period between 0.1 and 0.7 μmol g^{-1} F.W. In C_3 *M. crystallinum* inositol was undetectable on days 2 and 4, but was present at all other times during the experimental period between 0.1 and 0.8 μmol g^{-1} F.W. Sucrose levels were slightly higher in CAM *M. crystallinum* than in C_3 *M. crystallinum*, between 0.8 and 2.5 μmol g^{-1} F.W. and 0.5 and 2.0 μmol g^{-1} F.W. respectively. The ratio of starch to total soluble carbohydrate (St:TSC) was higher in CAM *M. crystallinum*, between 0.5 and 1.7 compared to between 0.3 and 1.3 in C_3 *M. crystallinum*.

Levels fell in CAM *M. crystallinum* during the induction period, but rose in C_3 *M. crystallinum*. Levels of glucose 6 phosphate and fructose 6 phosphate were higher in C_3 *M. crystallinum* than in CAM *M. crystallinum* from day 9 and day 7 respectively (Table 12). Levels of fructose 1,6 bisphosphate fell during the early part of the induction period up to day 9 in CAM *M. crystallinum* to become lower than levels in C_3 *M. crystallinum*. From day 14 until day 21 levels of fructose 1,6 bisphosphate were higher in CAM *M. crystallinum*.

Pinitol was present from day 4 in CAM *M. crystallinum* (Fig. 7 and Table 12), and then rose steadily to reach a peak of 10.5 µmol g^{-1} F.W. by day 21. If dry weight is about 2% of fresh weight (Fig. 10) then this level of pinitol represents 9.7% of dry weight. In C_3 *M. crystallinum* pinitol was not present until day 18, when it began to accumulate reaching 3.7 µmol g^{-1} F.W. by day 40.

Levels of Pi were higher in CAM *M. crystallinum* from day 3 of the induction period (Fig. 8). Pi rose from 3.3 µmol g^{-1} F.W. at day 0 to 15.4 µmol g^{-1} F.W. at day 21 in CAM *M. crystallinum* compared to 5.9 µmol g^{-1} F.W. by day 21 in the C_3 form.

In CAM *M. crystallinum* malic acid at 9:25, present from day 2 rose steeply to 21.1 µmol g^{-1} F.W. by day 21 (Fig. 9). C_3 *M. crystallinum* contained negligible malic acid for the entire experimental period.

Dry weight and Fresh weight

The dry weight of 50 leaf-discs was greater in CAM *M. crystallinum* until day 9 of the induction period, between 7.0 and 9.4 mg compared to between 4.8 and 9.4 mg in C_3 *M. crystallinum* (Table 13). After day 9 dry weights were higher in C_3 *M. crystallinum*, rising to 17.4 mg by day 36 compared to 14.0 mg in CAM *M. crystallinum*.

The fresh weights of 50 leaf-discs were similar in C_3 and CAM *M. crystallinum* until day 7 (Table 13). After this time the fresh weight rose to 810 mg by day 36 in CAM *M. crystallinum*, but remained more steady in C_3 *M. crystallinum* and was 610 mg at day 36.

These differences are reflected in the expression of dry weight as a percentage of fresh weight (Fig. 10). Until day 9 dry weight as a percentage of fresh weight was higher in CAM *M. crystallinum*. After day 9 it was higher in C_3 *M. crystallinum*.

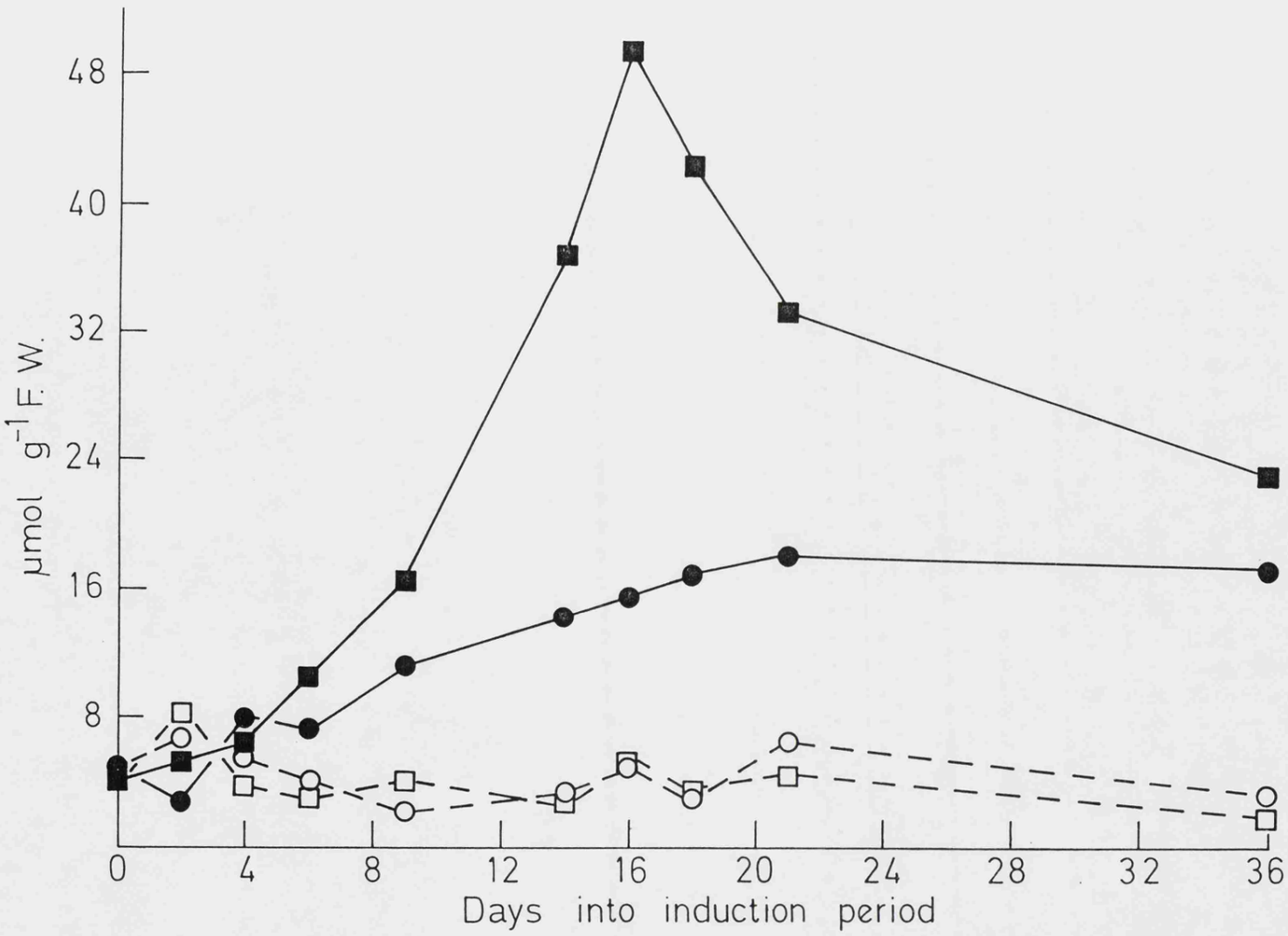
Ethylene

No emission of ethylene from tissue of C_3 and CAM *M. crystallinum* was detected during the whole experimental period.

Leaf reflectance and transmittance

Leaf reflectance and leaf transmittance remained unchanged during the first 8 days of the induction period (Figs 11 and 12).

3. Figure 6. Levels of starch (C_3 —●—, CAM —○—), and total soluble carbohydrate (C_3 —■—, CAM —□—) during the induction of CAM in *M. crystallinum*.


48
40
32
24
16
8
µmol g⁻¹ F. W.
0
4
8
12
16
20
24
28
32
36
Days into induction period

Standard errors for Figure 6 μmol g^{-1} F.W.

Days into induction period	Starch		Total soluble carbohydrate	
	C_3	CAM	C_3	CAM
0	0.6	0.6	0.5	0.5
2	0.7	1.3	0.7	1.3
4	2.0	1.1	1.2	0.8
6	2.2	0.9	2.4	0.7
9	2.0	0.4	4.8	0.9
14	2.8	0.6	7.2	0.8
16	2.7	0.5	10.6	0.8
18	2.3	0.4	9.9	0.9
21	2.2	0.4	8.8	1.1
36	2.9	0.2	5.1	1.0

3. Table 12. Levels of metabolites during the induction of CAM in *M. crystallinum*.

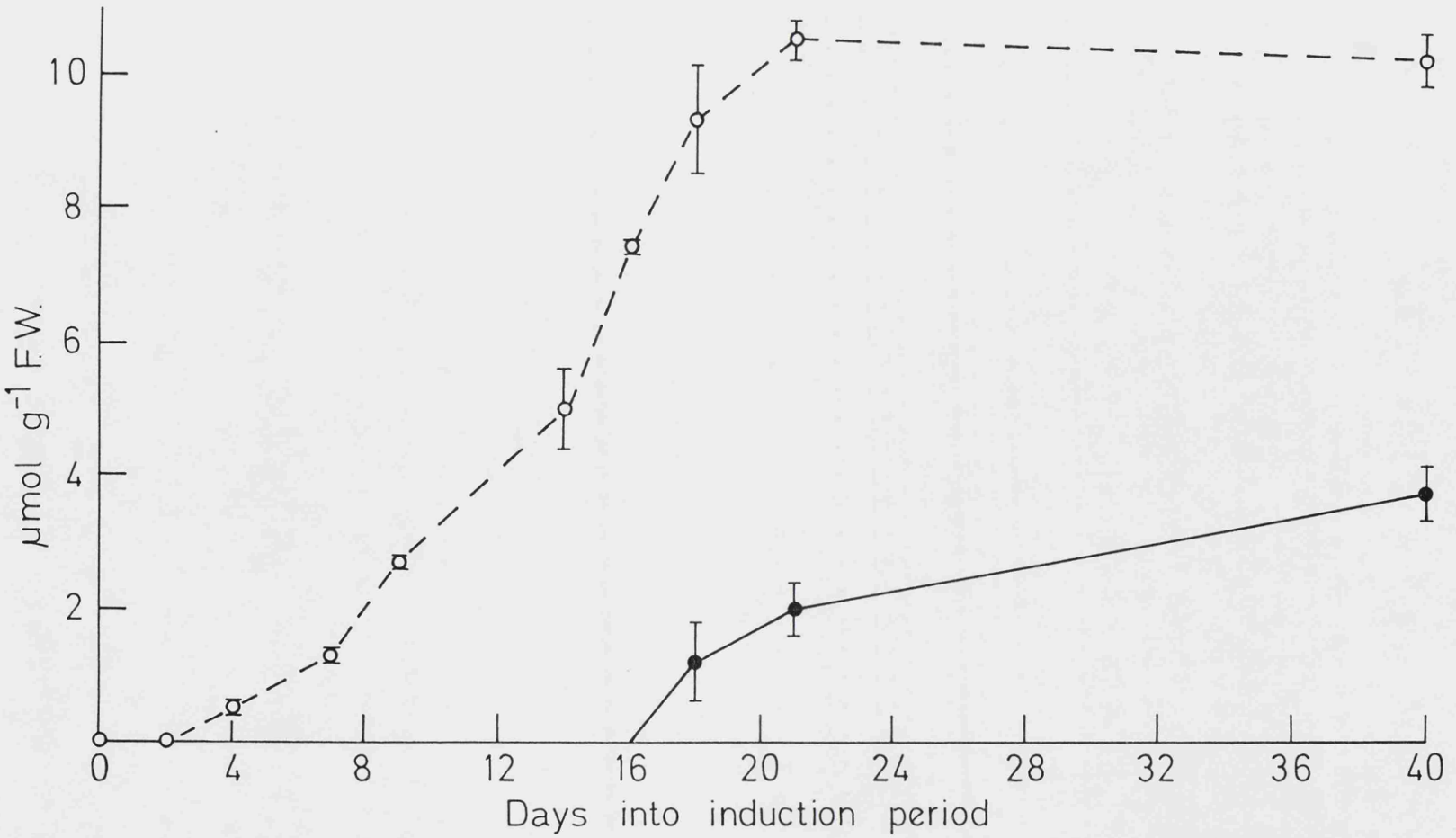
CAM

Days into induction period	μmol g^{-1} F.W.							nmol g^{-1} F.W.		
	Fructose	Glucose	Sorbitol	Inositol	Sucrose	Pinitol	St:TSC	G6P	F6P	F16BP
0	1.5±0.7	1.8±0.5	N.D.	0.1±0.1	0.8±0.3	N.D.	1.1	31.9±3.6	3.5±1.0	11.2±1.7
2	2.1±1.0	3.1±1.4	0.4±0.8	0.7±0.1	1.9±1.2	N.D.	0.8	61.3±4.7	1.9±0.6	7.4±0.8
4	0.1±0.1	1.1±0.3	0.5±1.0	0.7±0.1	1.5±0.1	0.4±0.1	1.5	3.2±5.2	0.4±0.2	0.5±0.3
7	N.D.	0.4±0.1	0.9±0.01	0.7±0.1	1.3±0.2	1.2±0.1	1.3	63.1±4.8	2.5±0.4	6.4±0.7
9	N.D.	0.9±0.1	1.0±0.14	0.4±0.1	1.8±0.1	2.8±0.1	0.5	30.1±2.1	5.0±0.9	0.8±0.4
14	N.D.	0.9±0.1	0.4±0.1	0.4±0.1	1.4±0.2	4.9±0.5	1.1	14.2±1.7	7.8±1.1	14.4±1.2
16	N.D.	1.1±0.3	1.0±0.1	0.5±0.1	2.5±0.3	7.3±0.1	1.0	16.0±1.8	5.7±0.8	14.9±1.3
18	N.D.	0.9±0.4	0.5±0.1	0.4±0.1	1.9±0.2	9.3±0.1	0.8	11.8±1.3	2.5±0.4	8.8±1.0
21	N.D.	1.8±0.5	0.6±0.1	0.4±0.1	1.5±0.1	10.5±0.3	1.5	10.3±1.1	2.5±0.3	12.8±1.4
36	N.D.	0.1±0.1	0.1±0.1	0.2±0.1	1.5±0.3	10.2±0.4	1.7	0.4±0.1	0.4±0.1	2.7±0.8

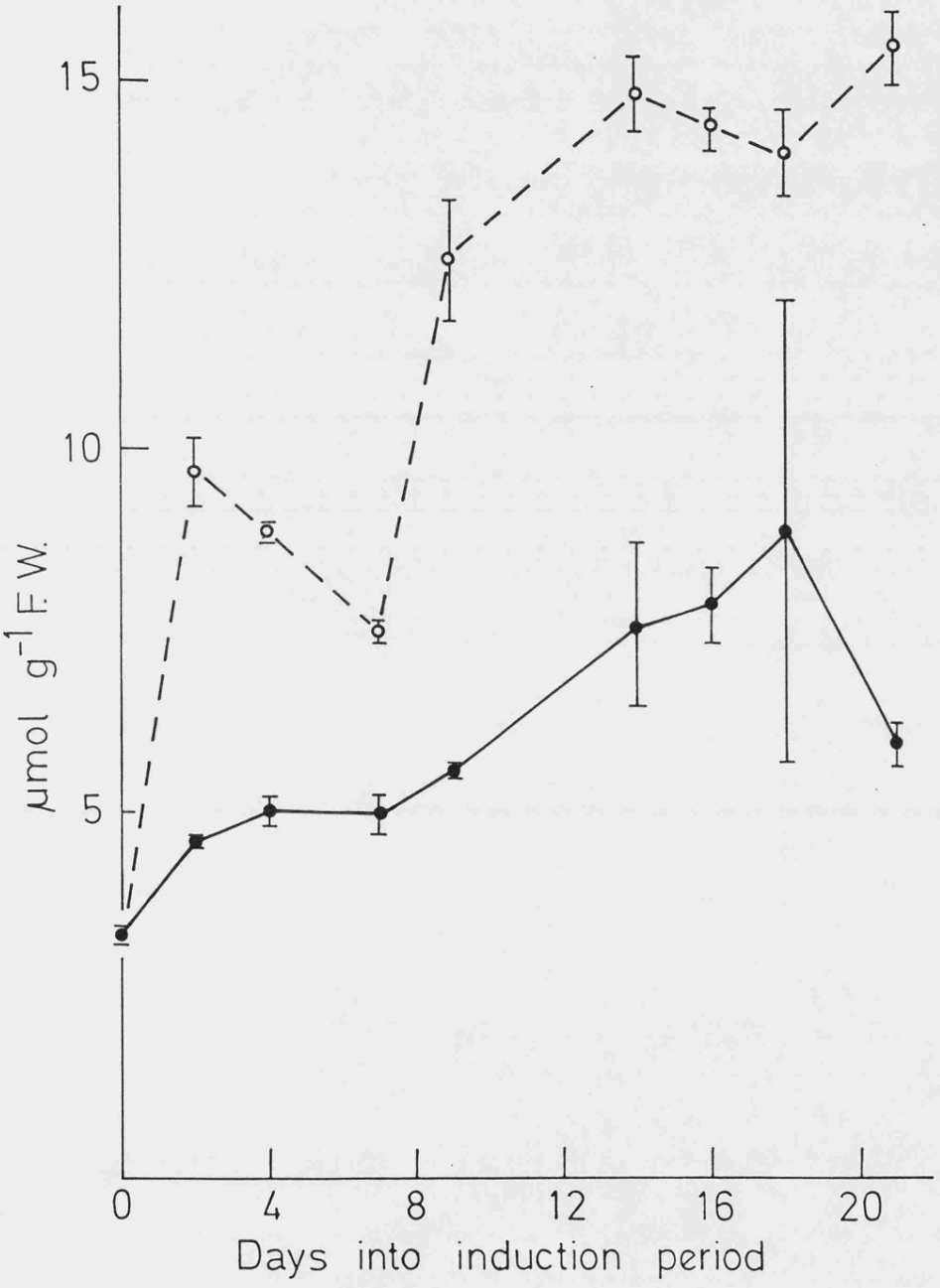
C_3

Days into induction period	μmol g^{-1} F.W.							nmol g^{-1} F.W.		
	Fructose	Glucose	Sorbitol	Inositol	Sucrose	Pinitol	St:TSC	G6P	F6P	F16BP
0	1.5±0.7	1.8±0.5	N.D.	0.1±0.1	0.8±0.3	N.D.	1.1	31.9±3.6	3.5±1.0	11.2±1.7
2	2.1±0.9	2.9±2.0	N.D.	N.D.	0.5±0.1	N.D.	0.5	52.1±3.9	18.8±2.8	22.1±2.2
4	1.5±0.2	4.3±0.4	N.D.	N.D.	0.6±0.1	N.D.	1.3	29.8±2.7	8.5±1.5	3.7±1.9
7	2.5±0.1	7.1±0.5	N.D.	0.2±0.1	0.7±0.1	N.D.	0.7	22.7±2.8	3.9±1.6	14.6±2.0
9	1.8±0.5	13.8±5.9	N.D.	0.2±0.1	0.7±0.1	N.D.	0.7	34.8±2.6	10.6±1.8	4.5±0.4
14	3.6±0.2	30.9±2.6	0.2±0.1	0.8±0.1	1.4±0.2	N.D.	0.4	57.4±2.6	19.9±2.1	2.7±0.3
16	9.7±0.8	37.4±5.3	N.D.	0.5±0.1	2.0±0.2	N.D.	0.3	69.9±3.2	14.9±2.0	10.1±1.1
18	3.0±0.5	36.6±7.0	0.5±0.1	0.7±0.2	1.3±0.2	0.9±0.4	0.4	59.6±3.1	11.0±1.9	2.7±0.5
21	4.7±0.5	27.0±4.5	0.1±0.1	0.5±0.1	1.0±0.3	1.9±0.3	0.5	84.4±5.2	4.3±1.1	4.8±0.9
36	1.6±0.5	19.8±5.0	0.6±0.1	0.3±0.1	0.7±0.2	3.7±0.3	0.7	38.7±2.7	6.4±1.0	9.8±1.2

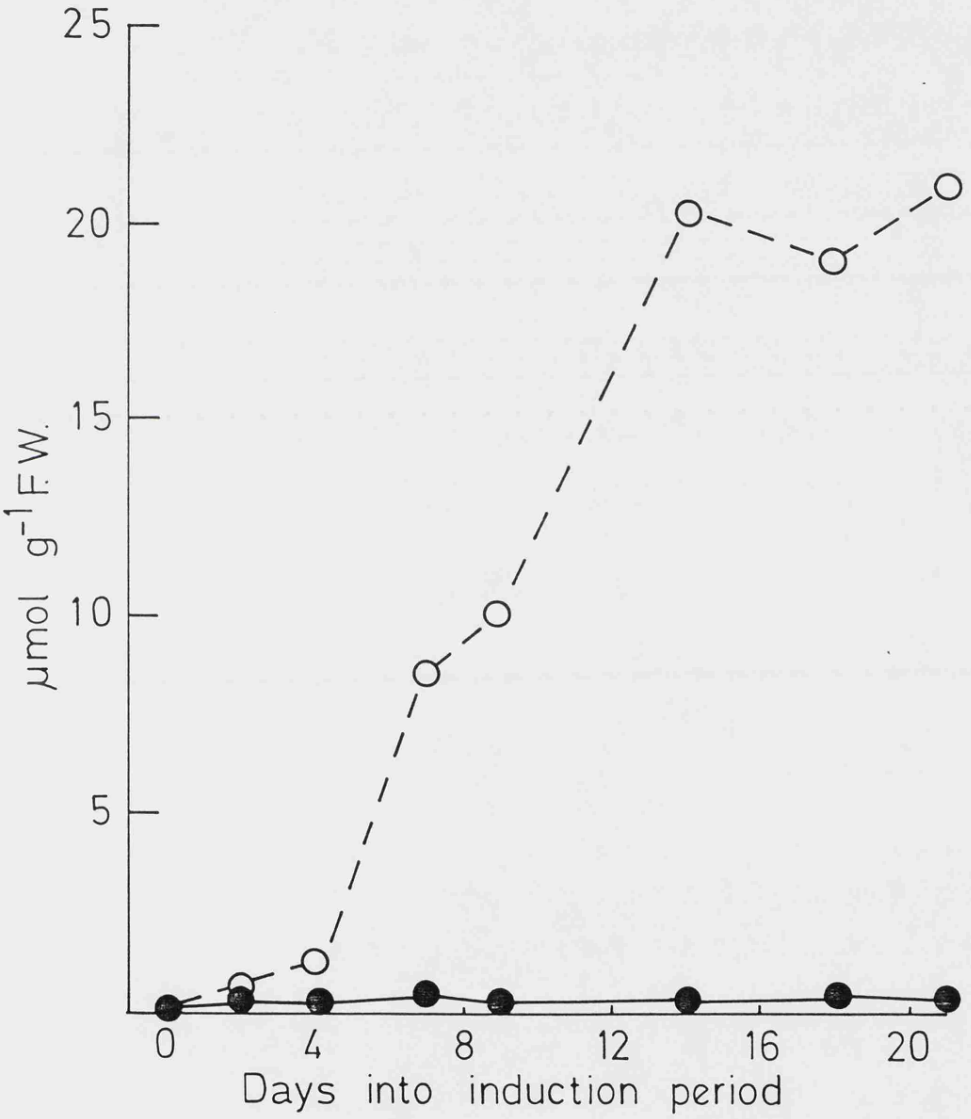
3. Figure 7. Levels of pinitol, inositol equivalents (C_3 —●— , CAM —○—) during the induction of CAM in *M. crystallinum*.


10
8
6
4
2
0
µmol g⁻¹ F.W.
0
4
8
12
16
20
24
28
32
36
40
Days into induction period

3. Figure 8. Levels of inorganic phosphate (C_3 —●— , CAM —○—) during the induction of CAM in *M. crystallinum*.


15
10
5
µmol g⁻¹ F.W.
0
4
8
12
16
20
Days into induction period

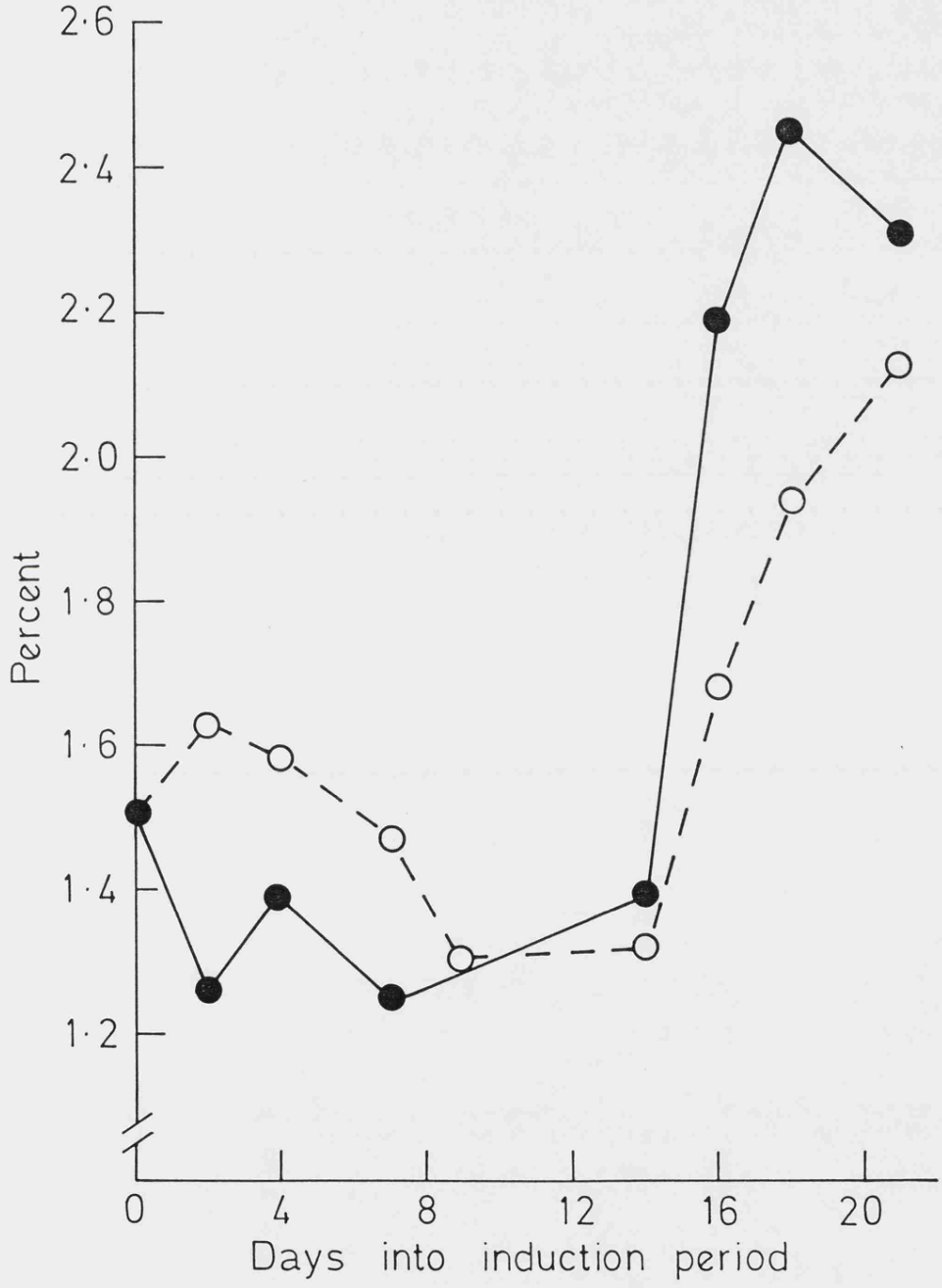
3. Figure 9. Levels of malic acid (C_3 —●— , CAM —○—) at 9:25 during the induction of CAM in *M. crystallinum*.


25
20
15
10
5
µmol g⁻¹F.W.
0
4
8
12
16
20
Days into induction period

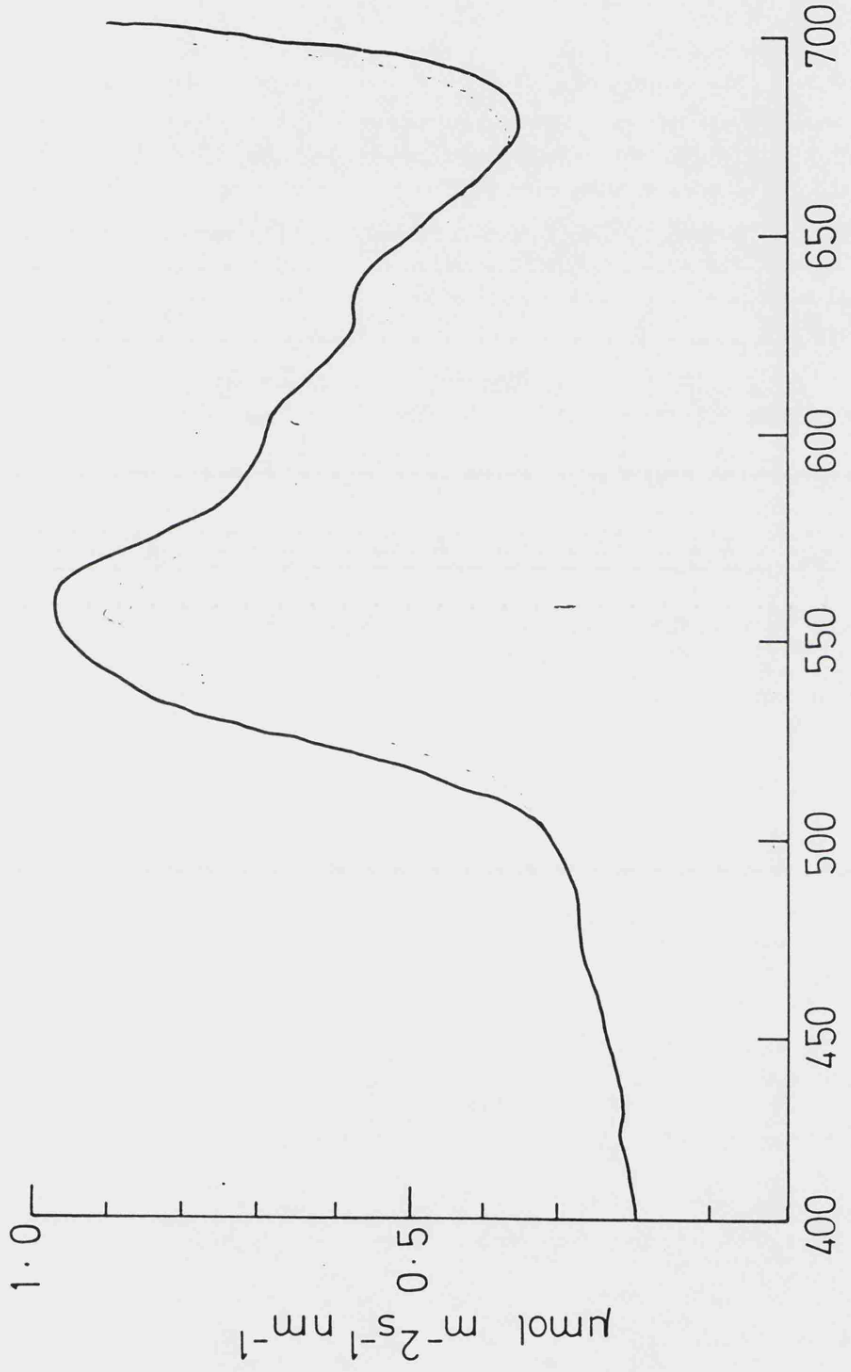
3. Table 13. Fresh weights and dry weights (mg) of 50 leaf-discs measured during the induction of CAM in *M. crystallinum*.

Days into induction period	Induction of CAM		C_3	
	Dry weight	Fresh weight	Dry weight	Fresh weight
0	9.4	618	9.4	618
2	8.0	490	6.3	500
4	7.6	480	6.8	490
7	7.2	490	4.8	380
9	7.0	540	6.4	500
14	4.5	340	7.8	560
16	10.4	620	11.6	530
18	12.2	630	12.5	510
21	14.7	690	9.8	424
36	14.0	810	17.4	610

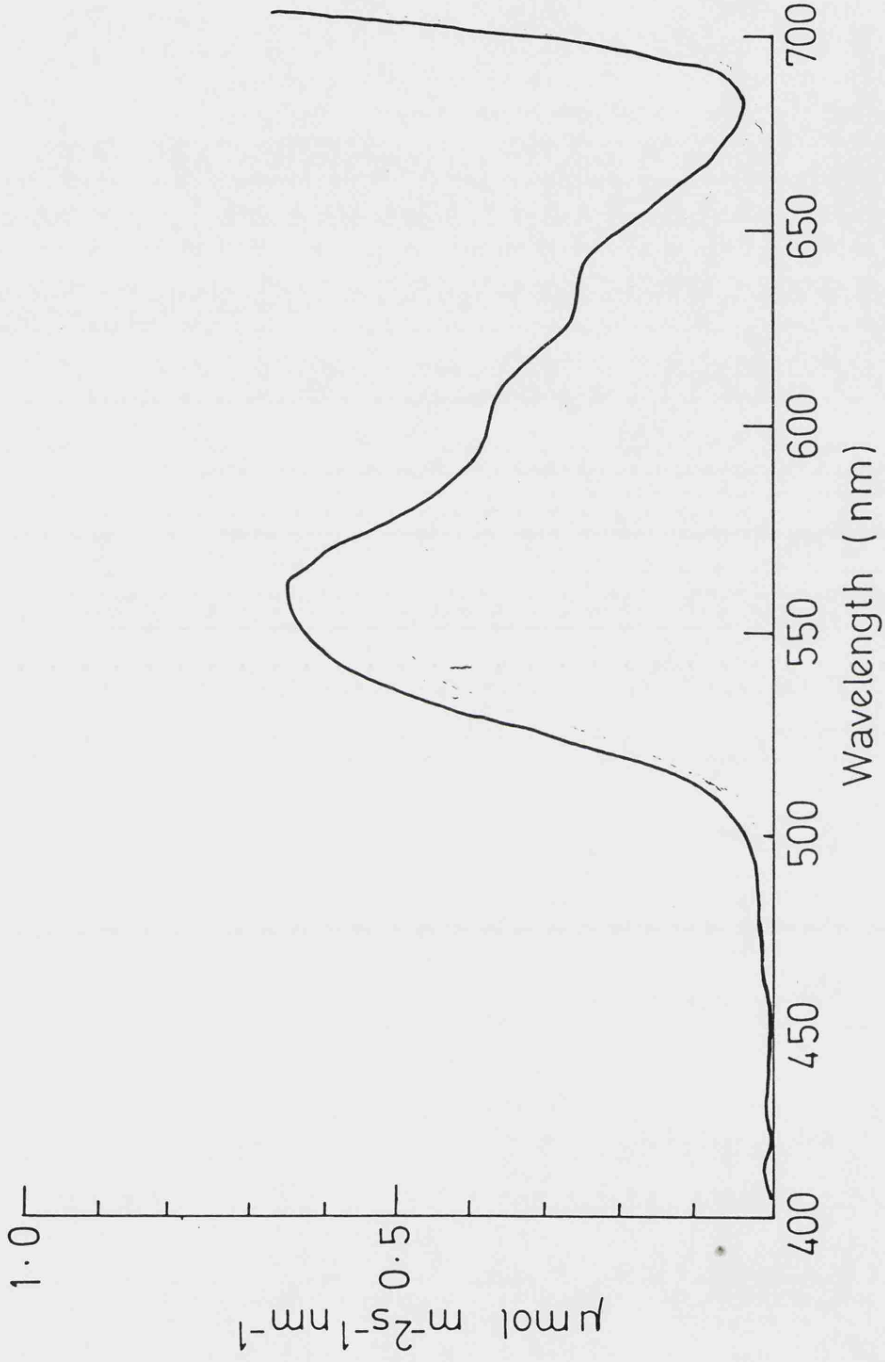
3. Figure 10. Dry weight as a percentage of fresh weight (C_3—●—, CAM——○——) during the induction of CAM in *M. crystallinum*.


2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
Percent
0
4
8
12
16
20
Days into induction period

3. Figure 11. Reflectance of C_3 and CAM *M. crystallinum* leaves during the first 8 days of the induction period.


1·0
0·5
μmol m⁻²s⁻¹ nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

3. Figure 12. Transmittance of C_3 and CAM *M. crystallinum* leaves during the first 8 days of the induction period.


1·0
0·5
μmol m⁻²s⁻¹nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

4. Measurement of the changes in levels of metabolites during the deinduction and reinduction of CAM; and of leaf reflectance and transmittance during the deinduction of CAM in *M. crystallinum*

Investigations were carried out on 30 *M. crystallinum* plants, 15 of which were induced into CAM. Forty days into the induction period 8 of these CAM *M. crystallinum* plants were deinduced. After 32 days of deinduction 4 of these 8 plants were reinduced. The method of deinduction and reinduction is outlined in the Materials and Methods section.

Samples for malic acid were taken at 9:25, and comprised 3 replicate samples of 5 leaf-discs each. Leaf-disc samples were also taken at 15:00 for malic acid and all the other metabolites. They consisted of 5 replicate samples of 5 leaf-discs each. Starch was measured enzymically and soluble carbohydrate by gas chromatography.

Measurements of leaf reflectance and leaf transmittance during the deinduction of CAM were carried out on leaves attached to the plants. Measurements of a "young" leaf, which was the most recently fully expanded leaf, were taken just before the start of deinduction and one day into the deinduction period when changes in optical properties were complete. Measurements of an "old" leaf, which was the third most recently fully expanded leaf, were taken just prior to deinduction and 2 and 3 days into the deinduction period when changes in optical properties were complete. Measurements of leaf reflectance and transmittance were also made on an excised leaf which was the equivalent to a "young" leaf. The leaf was immersed in a small volume of distilled water under growth cabinet conditions. Measurements were made just prior to the immersion in water and 1, 2, 5 and 7 days after immersion.

Levels of metabolites

Levels of starch in CAM *M. crystallinum* and deinducing *M. crystallinum* plants were very similar between day 0 and day 13 at between 8 and 11 μmol g^{-1} F.W. (Fig. 13). Between day 13 and day 37 levels of starch rose in the deinducing plants, stabilising at close to 15 μmol g^{-1} F.W. In CAM *M. crystallinum* levels of starch declined to 5.5 μmol g^{-1} F.W. by day 37.

Levels of glucose rose during deinduction from 0.04 μmol g^{-1} F.W. at day 0 to 0.48 μmol g^{-1} F.W. at day 27, compared to 0.13 μmol g^{-1} F.W. at day 27 in CAM *M. crystallinum* (Table 14). Sorbitol was detected intermittently in deinducing and CAM *M. crystallinum*, at a slightly higher level in the CAM form. Inositol was detected, except at day 0 at a slightly higher level in CAM *M. crystallinum*. Levels of sucrose fell slightly in deinducing plants from 0.33 μmol g^{-1} F.W. at day 0 to 0.25 μmol g^{-1} F.W. at day 27. Conversely, in CAM *M. crystallinum* sucrose rose from 0.33 μmol g^{-1} F.W. to 1.07 μmol g^{-1} F.W. at day 27. Levels of total soluble carbohydrate (TSC) rose in deinducing plants from 0.37 μmol g^{-1} F.W. at day 0 to 0.83 μmol g^{-1} F.W. by day 27. This increase was largely due to a rise in glucose levels. In CAM *M. crystallinum* the rise in total soluble carbohydrate levels from 0.37 μmol g^{-1} F.W. at day 0 to 1.28 μmol g^{-1} F.W. at day 27 was greater than in the deinducing plants and can be attributed to the rise in the level of sucrose. The ratio of starch to total soluble carbohydrate (St:TSC) was variable in CAM and deinducing *M. crystallinum* plants, but was consistently higher in the deinducing plants. At day 27 the ratio was 18.2 in the deinducing plants compared to 7.5 in CAM *M. crystallinum*.

Levels of pinitol fell slightly during the deinduction of CAM from 2 μmol g^{-1} F.W. at day 0 to between 1 and 1.7 μmol g^{-1} F.W. between day 3 and day 27 (Fig. 13). In CAM *M. crystallinum* pinitol

rose to 5.8 μmol g^{-1} F.W. by day 10, stabilising at this level for the rest of the period.

Deinduction of CAM caused a sharp decline in levels of Pi during the first 5 days of the deinduction period from 11.6 μmol g^{-1} F.W. at day 0 to 5.2 μmol g^{-1} F.W. at day 5 (Fig. 14). During the remainder of this period levels of Pi stabilised at between 6 and 8 μmol g^{-1} F.W. In CAM *M. crystallinum* levels of Pi were relatively steady between 11.6 and 14.3 μmol g^{-1} F.W. The reinduction of CAM in deinduced plants had little effect on levels of Pi.

Deinduction of CAM also caused a sharp drop in levels of malic acid measured at 9:25 (Fig. 15). Between day 0 and day 7 malic acid fell from 21.6 μmol g^{-1} F.W. to 7.1 μmol g^{-1} F.W. Between day 7 and the end of the deinduction period levels of malic acid measured at 9:25 fluctuated between 6.8 μmol g^{-1} F.W. and 15.2 μmol g^{-1} F.W. Levels of malic acid at 9:25 in CAM *M. crystallinum* remained high between 21.6 and 29.8 μmol g^{-1} F.W. during the first 25 days of the experimental period, but declined sharply after this time to 9.7 μmol g^{-1} F.W. by the end of the period. During the reinduction of CAM levels of malic acid measured at 9:25 rose from 13.8 μmol g^{-1} F.W. to 24.2 μmol g^{-1} F.W. at day 14 but declined sharply thereafter.

Deinduction of CAM produced little change in amounts of malic acid measured at 15:00 for the first 20 days of deinduction (Fig. 15). After this time levels fluctuated between 7.0 μmol g^{-1} F.W. and 16.4 μmol g^{-1} F.W. in the deinducing plants but declined gradually to 4.6 μmol g^{-1} F.W. by the end of the deinduction period in CAM *M. crystallinum*. The reinduction of CAM led to high and variable amounts of malic acid measured at 15:00. Levels were between 14.6 μmol g^{-1} F.W. and 19.9 μmol g^{-1} F.W., during the first 14 days of reinduction, but declined to 8.2 μmol g^{-1} F.W. by day 19.

The consumption of malic acid between 9:25 and 15:00 declined rapidly during deinduction from 16.9 μmol g^{-1} F.W. at day 0 to 0.5 μmol g^{-1} F.W. at day 7 (Fig. 16). On days 27 and 34 there was accumulation of malic acid between 9:25 and 15:00 - 0.8 μmol g^{-1} F.W. and 2.7 μmol g^{-1} F.W. respectively. In CAM *M. crystallinum* consumption of malic acid remained high until day 27 when there was a sharp reduction in consumption. The reinduction of CAM reintroduced higher levels of malic acid consumption.

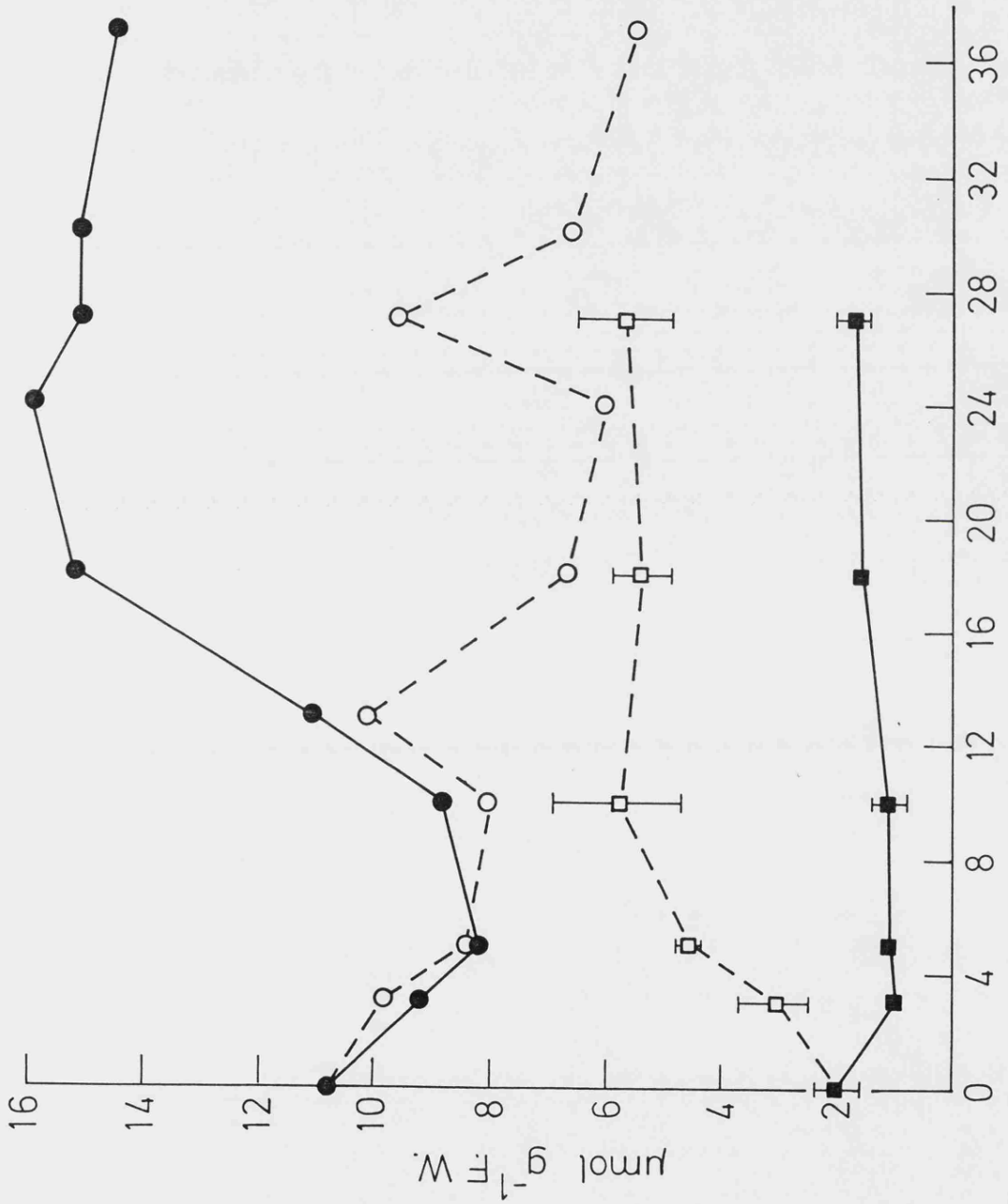
Leaf reflectance and transmittance

Leaf reflectance and leaf transmittance increased during the deinduction of CAM in attached *M. crystallinum* leaves (Figs 17, 18, 19 and 20). The increase was slightly greater in the old leaf, but took 3 days to complete compared to 1 day in the young leaf. Leaf reflectance and leaf transmittance were greater in young leaves.

Leaf reflectance and leaf transmittance also increased when an excised CAM *M. crystallinum* leaf was immersed in distilled water (Figs 21 and 22). The increase was greater than that caused by the deinduction of CAM in attached leaves and continued over a longer period. Leaf reflectance and transmittance were still increasing 7 days after the immersion of the leaf in water. The increase in leaf transmittance was greater than the increase in leaf reflectance.

The fresh weight of the excised leaf immersed in water increased linearly during the 7 days of immersion, from 3.1 g to 14.7 g (Fig. 23).

4. Figure 13. Levels of starch (deinducing —●— , CAM —○—) and pinitol (inositol equivalents) (deinducing —■— , CAM —□—) during the deinduction of CAM in *M. crystallinum*.


16
14
12
10
8
6
4
2
μmol g⁻¹ F.W.
0
4
8
12
16
20
24
28
32
36
Days into deinduction

Standard errors for Figure 13 μmol g^{-1} F.W.

	Starch		Pinitol	
	Deinducing	CAM	Deinducing	CAM
0	1.1	1.1	0.8	0.8
3	1.0	0.8	0.3	1.2
5	0.7	0.6	0.2	0.4
10	0.8	1.4	0.6	2.2
13	1.1	2.7		
18	2.6	1.6	0.2	1.0
24	3.1	2.9		
27	3.2	3.4	0.6	1.6
30	2.9	2.5		
37	3.0	2.3		

4.1 Table 14. Levels of metabolites (μmol g^{-1} F.W.) during the deinduction of CAM in *M. crystallinum*.

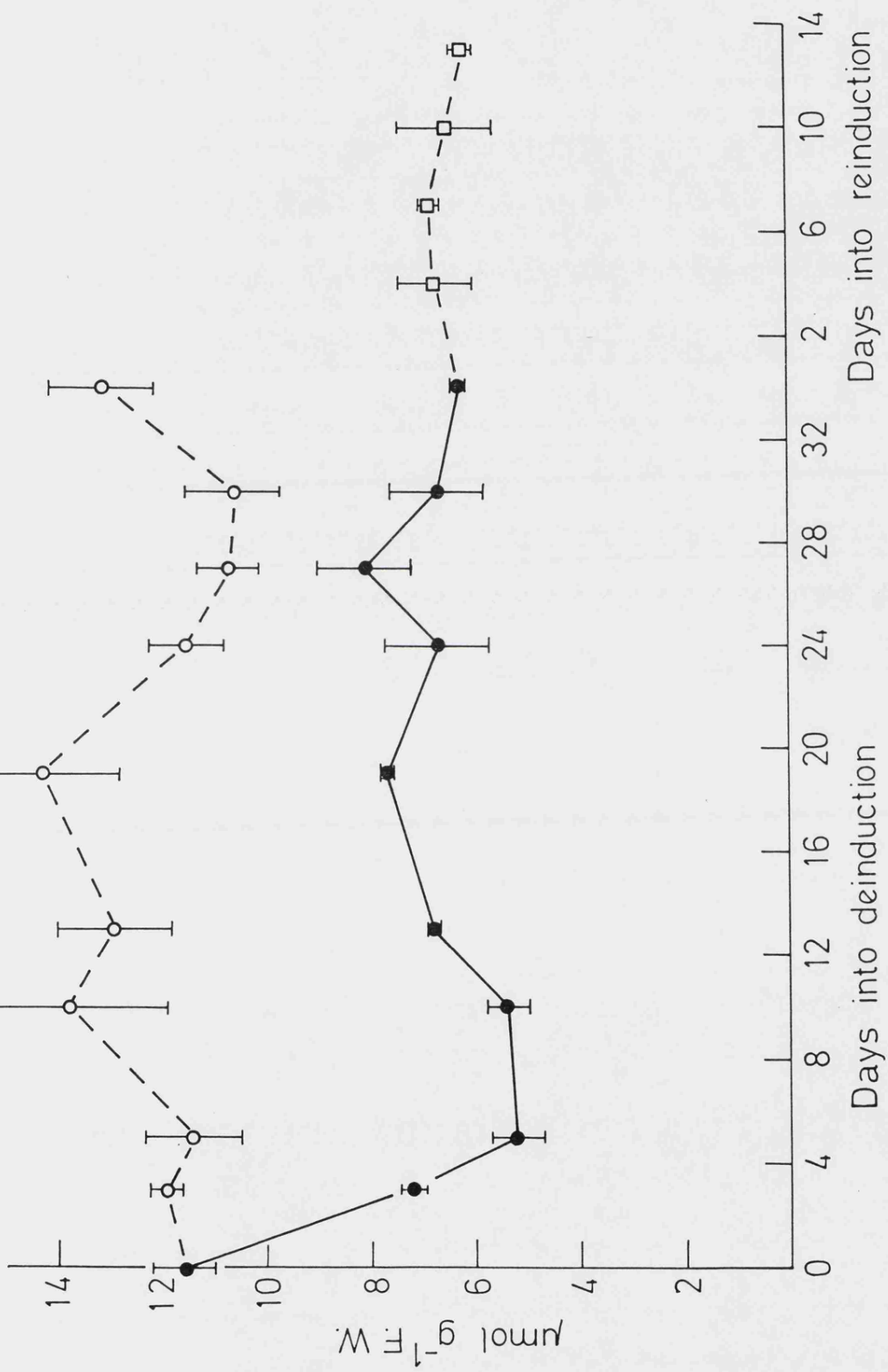
Deinduction of CAM

Days into deinduction	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC
0	0.04±0.01	N.D.	N.D.	0.33±0.19	0.37	29.2
3	0.06±0.02	N.D.	0.03±0.01	0.15±0.10	0.24	38.3
5	0.13±0.03	N.D.	0.06±0.02	0.18±0.09	0.37	22.2
10	0.13±0.02	0.08±0.01	0.08±0.01	0.17±0.12	0.46	19.1
18	0.37±0.05	0.08±0.01	0.04±0.01	0.25±0.04	0.74	20.5
27	0.48±0.23	0.06±0.01	0.04±0.01	0.25±0.08	0.83	18.2

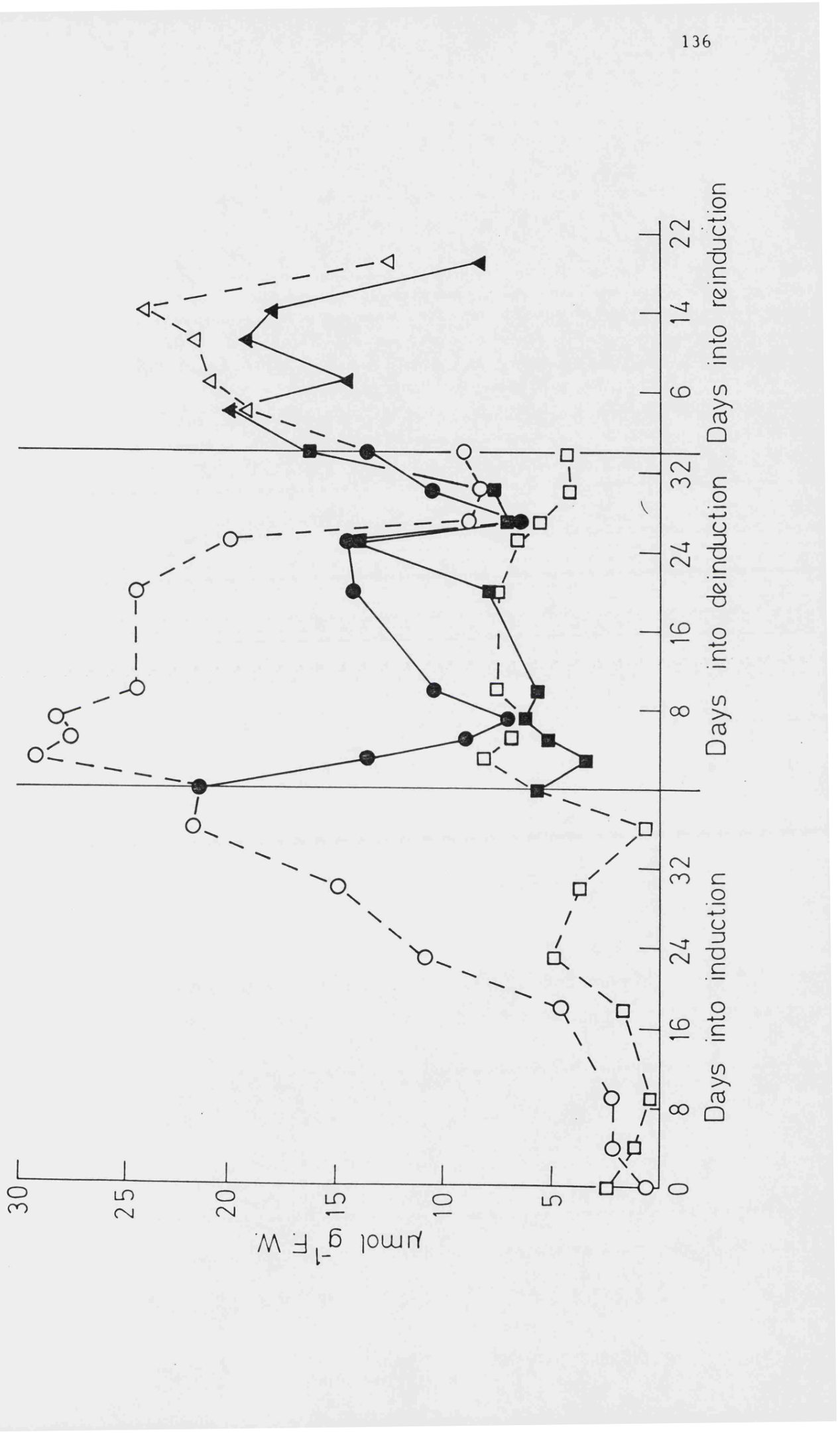
CAM Control

Days into deinduction	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC
0	0.04±0.01	N.D.	N.D.	0.33±0.19	0.37	29.2
3	0.18±0.03	N.D.	0.08±0.03	0.81±0.15	1.07	9.2
5	0.26±0.07	0.14±0.01	0.11±0.01	1.01±0.12	1.52	5.5
10	0.22±0.04	0.12±0.01	0.09±0.03	0.94±0.10	1.37	5.8
18	0.19±0.03	0.07±0.01	0.04±0.01	0.95±0.19	1.25	5.4
27	0.13±0.02	N.D.	0.08±0.02	1.07±0.23	1.28	7.5

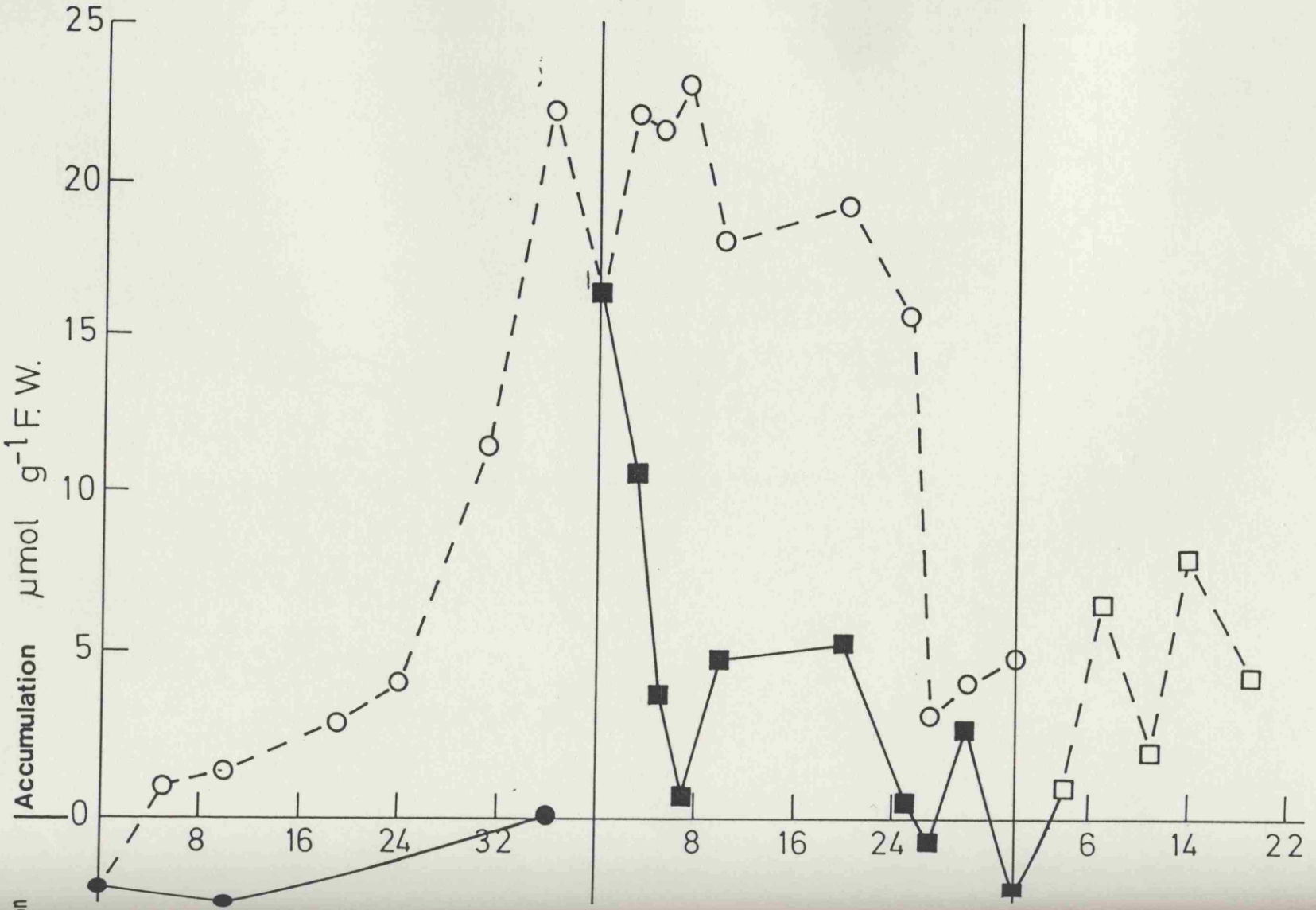
4. Figure 14. Levels of inorganic phosphate (deinducing —●— , CAM —○—) during the deinduction and reinduction (—□—) of CAM in *M. crystallinum*.


μmol g⁻¹ F.W.
14
12
10
8
6
4
2
0
4
8
12
16
20
24
28
32
2
6
10
14
Days into deinduction
Days into reinduction

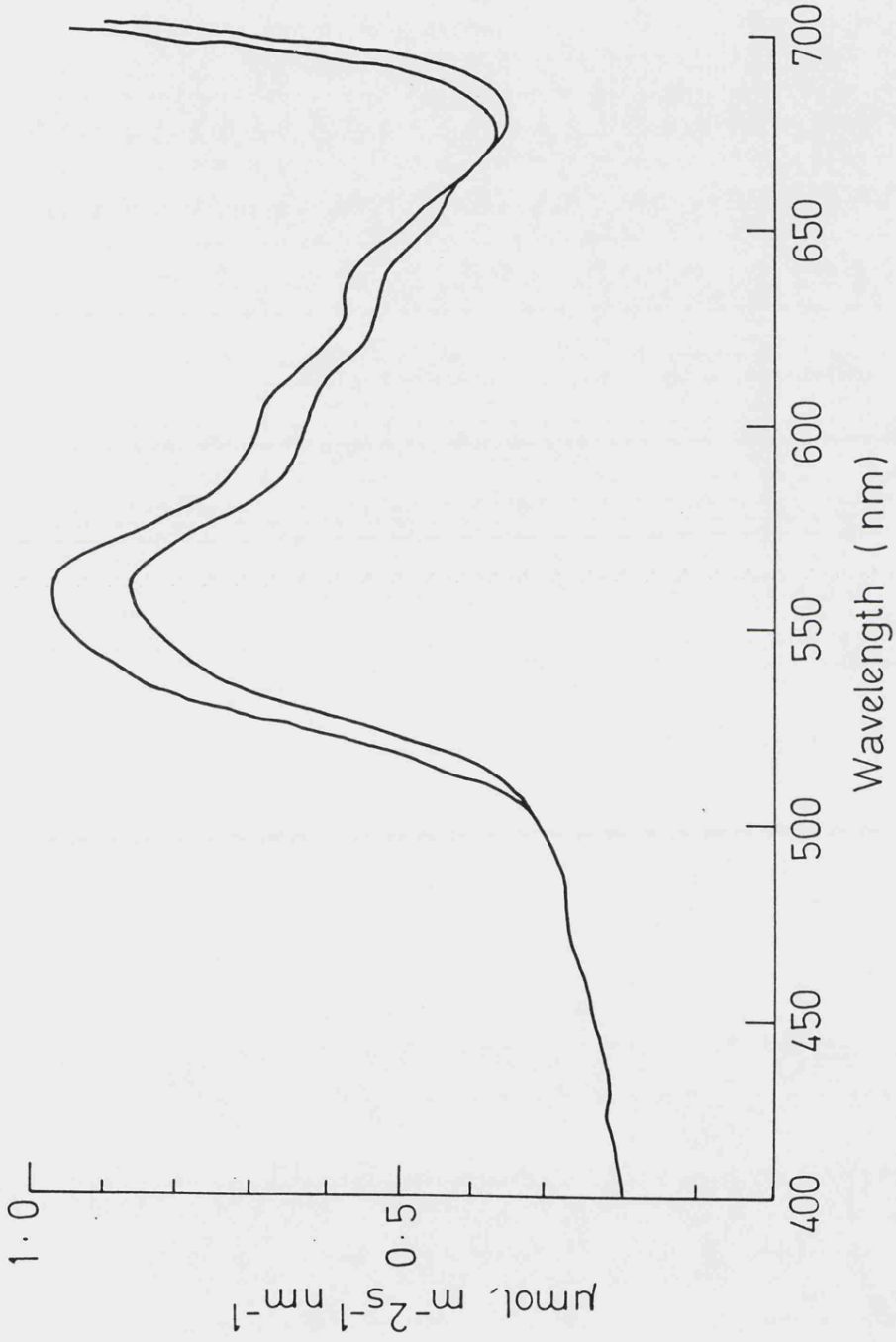
4. Figure 15. Levels of malic acid during the induction (9:25—○—, 15:00—□—), deinduction (deinducing 9:25—●— , 15:00 —■—) and reinduction (9:25—△—, 15:00—▲—) of CAM in *M. crystallinum*.


µmol g⁻¹ F.W.
30
25
20
15
10
5
0
8
16
24
32
Days into induction
8
16
24
32
Days into deinduction
6
14
22
Days into reinduction

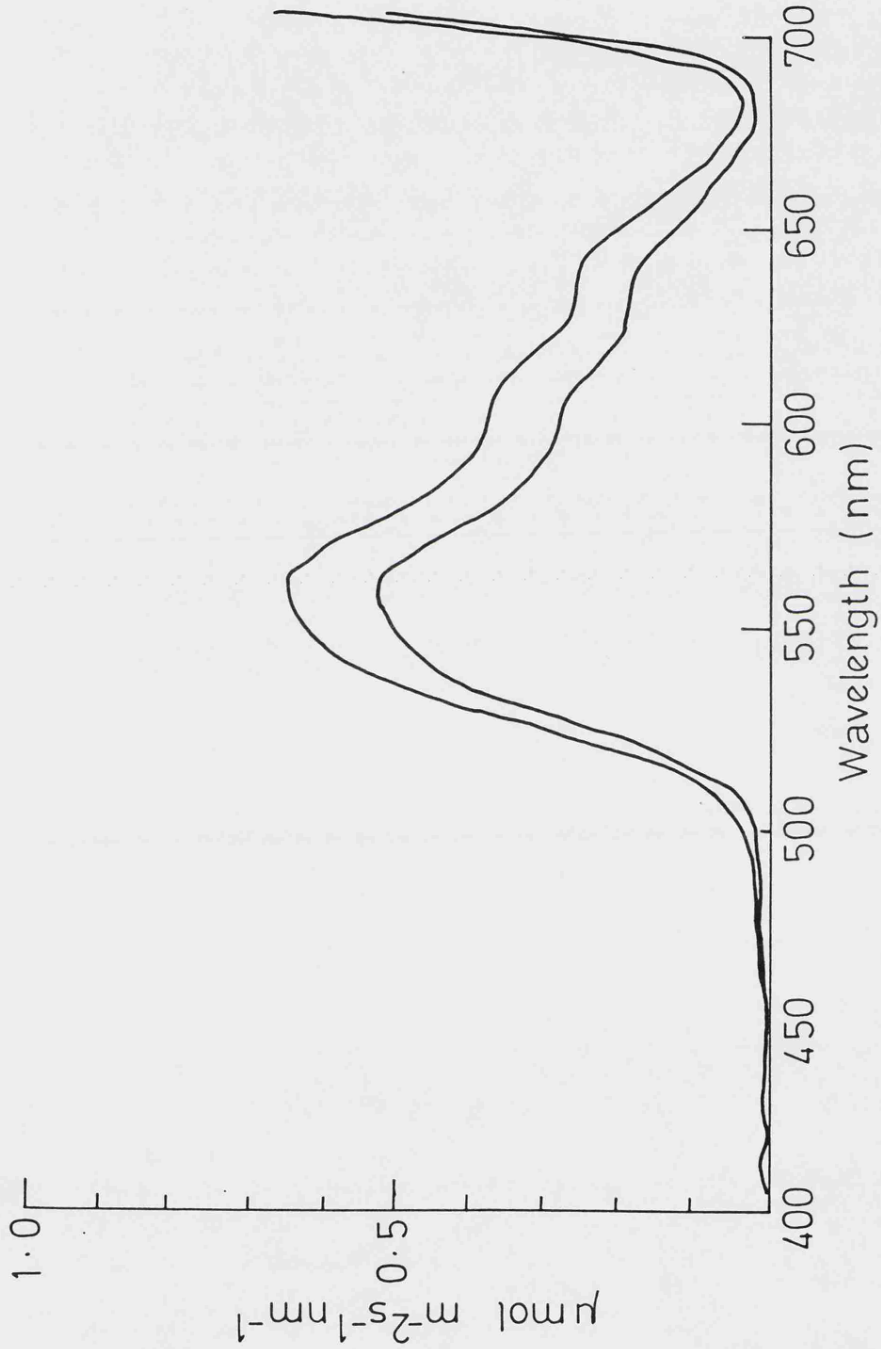
4. Figure 16. The consumption or accumulation of malic acid between 9:25 and 15:00 during the induction (C_3 —●— , CAM —○—), deinduction (deinducing—■— , CAM—○—) and reinduction (—□—) of CAM in *M. crystallinum*.


25
20
15
10
5
0
Accumulation µmol g⁻¹ F. W.
8
16
24
32
8
16
24
6
14
22
Days into induction
Days into deinduction
Days into reinduction

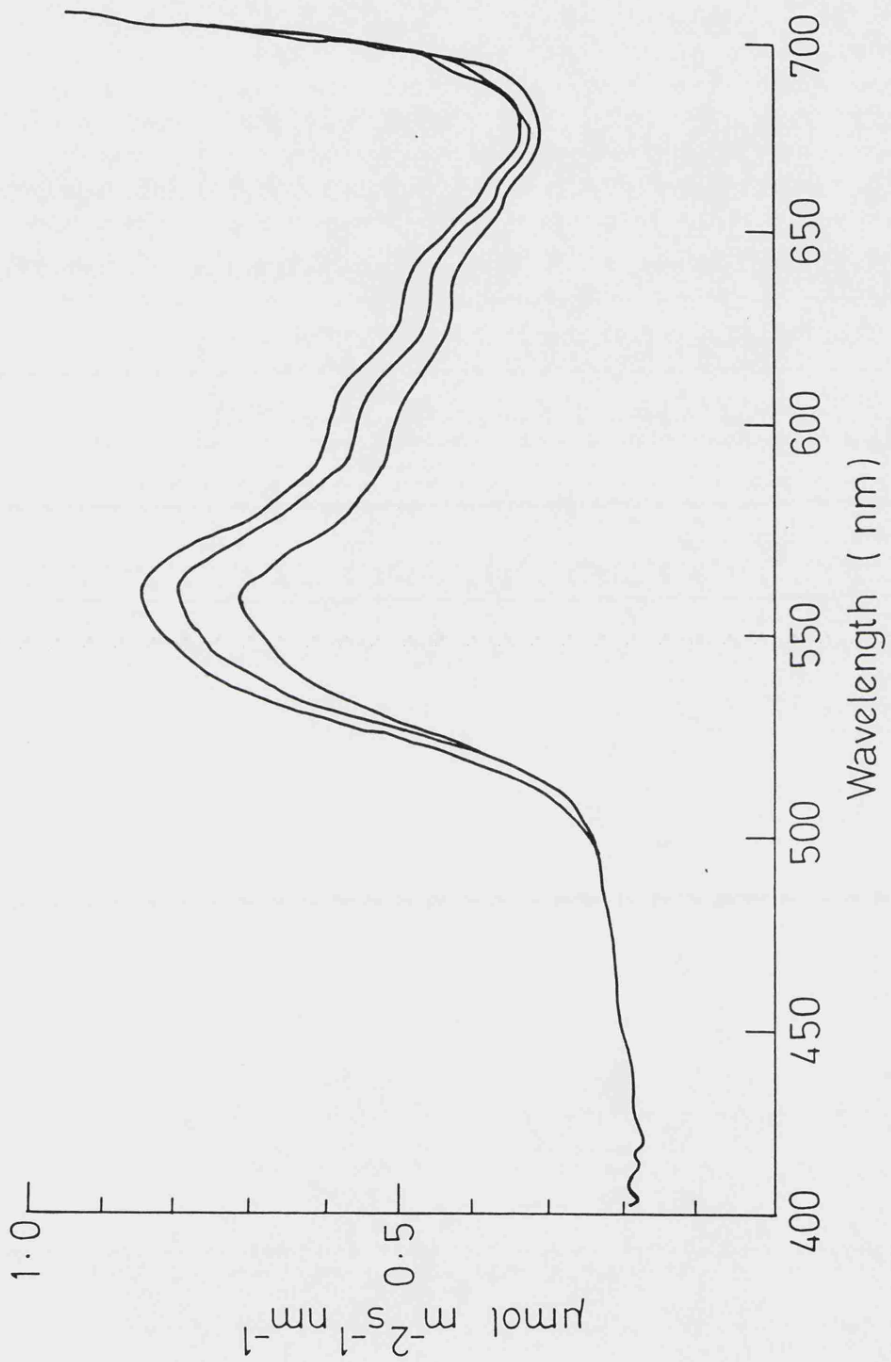
4. Figure 17. Reflectance of a young leaf before (lower line) and one day after (upper line) the commencement of deinduction in *M. crystallinum*.


1·0
0·5
μmol. m⁻²s⁻¹ nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

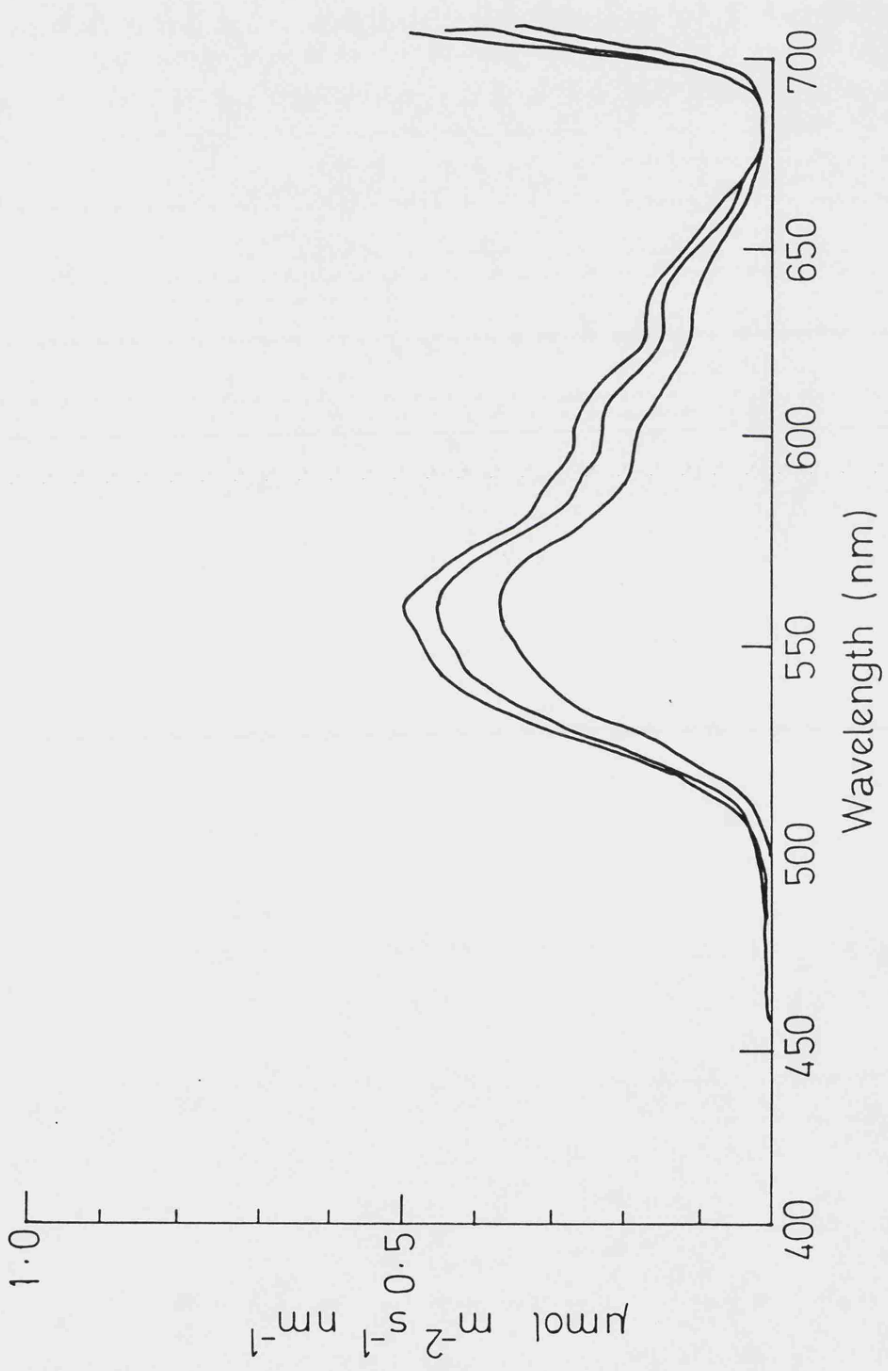
4. Figure 18. Transmittance of a young leaf before (lower line) and one day after (upper line) the commencement of deinduction in *M. crystallinum*.


1·0
0·5
μmol m⁻²s⁻¹nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

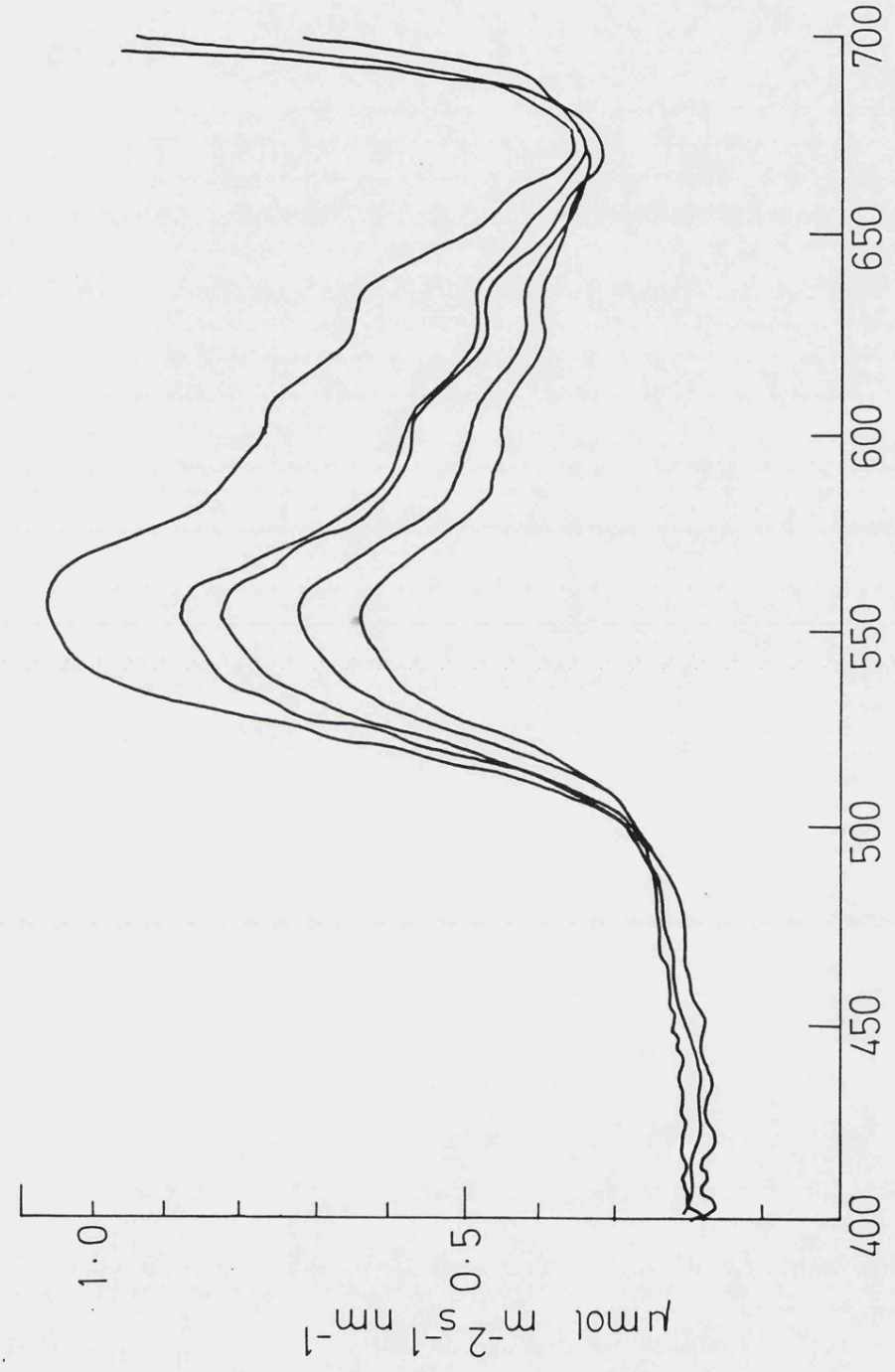
4. Figure 19. Reflectance of an old leaf before (lower line), two days after (middle line) and three days after (upper line) the commencement of deinduction in *M. crystallinum*.


1·0
0·5
μmol m⁻²s⁻¹nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

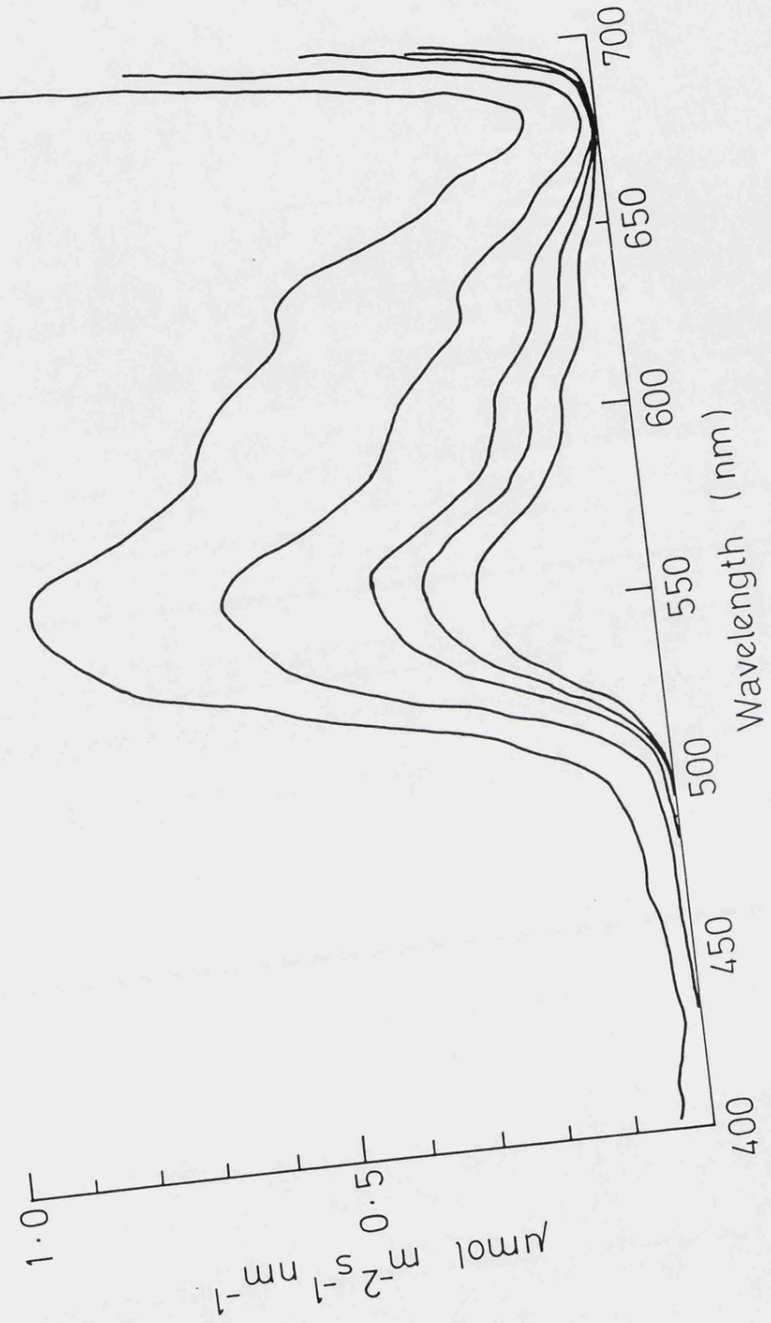
4. Figure 20. Transmittance of an old leaf before (lower line), two days after (middle line) and three days after (upper line) the commencement of deinduction in *M. crystallinum*.


1·0
0·5
μmol m⁻² s⁻¹ nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

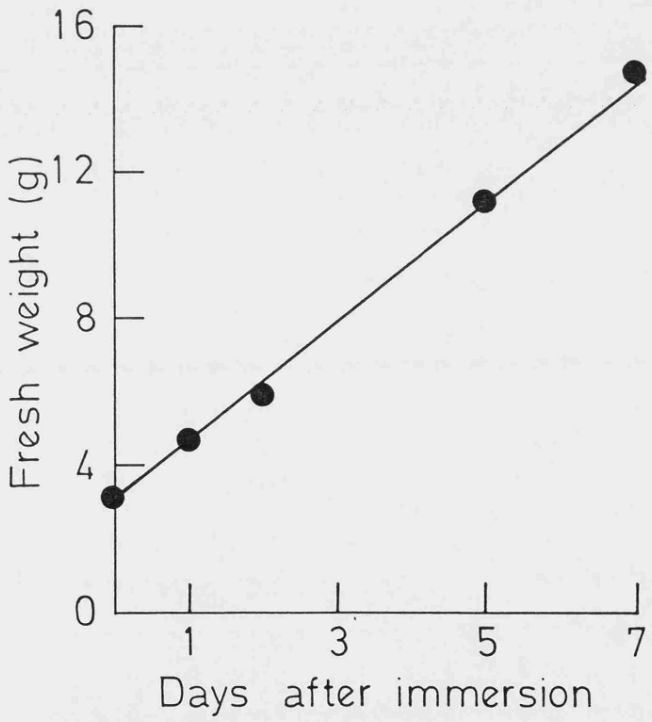
4. Figure 21. Reflectance of an excised CAM *M. crystallinum* leaf before (lower line), one day after (next line), two days after (middle line), five days after (next line) and seven days after (top line) its immersion in distilled water.


1·0
0·5
μmol m⁻² s⁻¹ nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

4. Figure 22. Transmittance of an excised CAM *M. crystallinum* leaf before (lower line), one day after (next line), two days after (middle line), five days after (next line) and seven days after (top line) its immersion in distilled water.


1·0
0·5
0
μmol m⁻² s⁻¹ nm⁻¹
400
450
500
550
600
650
700
Wavelength (nm)

4. Figure 23. The fresh weight of an excised CAM *M. crystallinum* leaf after its immersion in distilled water.


16
12
8
4
0
Fresh weight (g)
1
3
5
7
Days after immersion

5. Growth of and induction of CAM in *M. crystallinum* plants at different levels of exogenous Pi

M. crystallinum plants were grown from seed in "white silvaperl" under growth cabinet conditions. They were watered with nutrient medium (Table 15), containing 3 different concentrations of KH_2PO_4 - 0, 0.2 mM and 3 mM. Ten plants were grown at each level of Pi. When the plants were 8 weeks old 5 plants at each level of Pi were watered with nutrient medium supplemented with 400 mM NaCl to induce CAM.

Leaf-disc samples were taken before and throughout the induction period consisting of 3 replicate samples of 5 discs each. Sampling was carried out at 9:25 for malic acid and at 15:00 for malic acid and other leaf metabolites except Pi. Starch was analysed enzymically and soluble carbohydrate by gas chromatography. Leaf-disc samples for Pi measurement were taken 22 days after the start of CAM induction and consisted of 5 replicate samples of 5 leaf-discs each.

Measurement of dry weight and fresh weight was carried out 47 days after the start of CAM induction and consisted of the whole above ground biomass. Measurements were carried out on the most typical C_3 and CAM *M. crystallinum* plant in appearance at each level of Pi.

Observation of the appearance of plants was based on the subjective notion of green leaves being 'healthy' and yellowing leaves being less 'healthy'.

5. Table 15. Nutrient solution containing 3 different concentrations of KH_2PO_4.

Solution	Stock concentration	Volume (ml) per 20 litres nutrient solution made up with distilled water
KNO_3	1 M	120
$Ca(NO_3)_2$	1 M	80
$MgSO_4$	1 M	40
KH_2PO_4	1 M	0, 4 or 60
$MgCl_2$	1 M	80
Trace elements	(B, Mn, Zn, Cu, Mo)	20
NaFe-EDTA	(3.86 g 250 ml)	20

Trace elements	Quantity (mg) in 250 ml H_2O
H_3BO_3	715
$MnCl_2.4H_2O$	452
$ZnSO_4.7H_2O$	55
$CuSO_4.5H_2O$	20
$NaMoO_4.2H_2O$	7.25

From Edwards and Walker (1983).

Levels of metabolites

Levels of starch in CAM *M. crystallinum* were higher in plants grown at 0 Pi than in plants grown at 0.2 mM and 3 mM Pi where there was little difference between starch levels (Fig. 24). The difference between levels of starch in plants grown at 0 Pi and in plants grown at 0.2 mM and 3 mM Pi became less during the induction period up to day 13 where the difference was small. Between day 13 and day 29 the difference widened slightly.

In C_3 *M. crystallinum* levels of starch were also greater at 0 Pi than at 0.2 mM and 3 mM Pi, where the difference was small (Fig. 25). Starch levels were, however consistently higher at 0.2 mM Pi than 3 mM Pi, except at day 24. The difference between starch levels at 0 Pi and 0.2 mM and 3 mM Pi was greater than in the CAM *M. crystallinum* throughout the induction period. This was due to higher levels of starch in C_3 *M. crystallinum* than in CAM *M. crystallinum* at 0 Pi. The difference between starch levels at 0 Pi and 0.2 mM and 3 mM Pi in C_3 *M. crystallinum* diminished during the induction period.

The ratio of CAM starch to C_3 starch (starch CAM:C_3, Table 16, 17 and 18) became higher with the increasing Pi. It also rose during the induction period at 0 Pi and 0.2 mM Pi, but fluctuated between 0.97 and 1.44 at 3 mM Pi. At 0 Pi and 0.2 mM Pi the ratio was at or less than 1; at 3 mM Pi it was at or above 1, except on two occasions when it was 0.97.

Soluble carbohydrate with the exception of sorbitol was detected throughout the induction period in C_3 and CAM *M. crystallinum* plants grown at 0 Pi (Table 16). At 0.2 mM and 3 mM Pi soluble carbohydrates, particularly glucose, sorbitol and inositol were not detected in either C_3 or CAM *M. crystallinum* (Tables 17 and 18). At 0 Pi levels of soluble carbohydrate tended to fall during the induction period in both C_3 and CAM *M. crystallinum*. Levels of

glucose were slightly higher in C_3 *M. crystallinum* and levels of sucrose slightly higher in CAM *M. crystallinum*. There was little difference in levels of sorbitol, inositol and total soluble carbohydrate (TSC) between the C_3 and CAM forms at 0 Pi. The ratio of starch to total carbohydrate rose during the induction period in both C_3 and CAM forms grown at 0 Pi and was higher in C_3 *M. crystallinum*.

Sorbitol was not detected in C_3 *M. crystallinum* plants grown at 0.2 mM and 3 mM Pi, and inositol was only detected on one day - day 13 at 0.2 mM Pi (Tables 17 and 18). The patchy appearance of glucose in C_3 and CAM *M. crystallinum* at 0.2 mM and 3 mM Pi and of sorbitol and inositol in the CAM form makes comparisons of these sugars hard and also gives variable values for total soluble carbohydrate. Despite this, total soluble carbohydrate tended to be higher in CAM *M. crystallinum*. Sucrose levels were also quite variable but again tended to be higher in the CAM form. Values for the ratio of starch to total soluble carbohydrate (St:TSC) were very variable, but were higher in C_3 *M. crystallinum* except on day 13 at 0.2 mM Pi.

Levels of pinitol were higher in CAM *M. crystallinum* than in C_3 *M. crystallinum* at all 3 Pi levels after day 8 of the induction period (Fig. 26). Levels of pinitol in C_3 plants grown at 0.2 mM and 3 mM Pi were minimal at around 0.1 µmol g^{-1} F.W. up to day 24, rising to 1.0 µmol g^{-1} F.W. and 2.2 µmol g^{-1} F.W. respectively by day 35. Pinitol levels in C_3 plants grown at 0 Pi were consistently higher than in other C_3 *M. crystallinum* plants until after day 24 at between 1.1 and 2.5 µmol g^{-1} F.W. Levels declined to 0.4 µmol g^{-1} F.W. by day 35. Levles of pinitol in CAM *M. crystallinum* began to rise after day 3. The rise was quickest in plants grown at 0.2 mM Pi, reaching a maximum value of 8.8 µmol g^{-1} F.W. at day 18, declining to around 7 µmol g^{-1} F.W. thereafter. In CAM plants grown at 3 mM Pi the maximum

value was reached later at day 28, declining rapidly thereafter to reach 5.9 μmol g^{-1} F.W. at day 35. In CAM plants grown at 0 Pi levels of pinitol were close to 4 μmol g^{-1} F.W. from day 6.

Levels of malic acid in C_3 *M. crystallinum* at 9:25 and 15:00 became higher with decreasing Pi (Figs 27, 28 and 29). The consumption of malic acid between 9:25 and 15:00 increased and the frequency of accumulation decreased with decreasing Pi (Table 19). At 0 Pi levels of malic acid in C_3 *M. crystallinum* were higher than in inducing CAM *M. crystallinum* until day 21; and the consumption of malic acid was greater in C_3 *M. crystallinum* until day 18 (Fig. 27 and Table 19). In CAM *M. crystallinum* plants grown at 0.2 mM Pi levels of malic acid were higher than in C_3 *M. crystallinum* by day 3 (Fig. 28) and at 3 mM Pi by day 13 (Fig. 29). As in C_3 *M. crystallinum* levels of malic acid at 9:25 increased with decreasing Pi in CAM *M. crystallinum*. Levels of malic acid at 15:00 were highest at 0 Pi and were similar at 0.2 mM and 3 mM Pi. There was no correlation between the amount of malic acid consumption and level of Pi in CAM *M. crystallinum*. Levels of malic acid and the amount of consumption in C_3 *M. crystallinum* plants grown at 0 Pi were very similar to values for CAM *M. cyrstallinum* plants grown at 3 mM Pi.

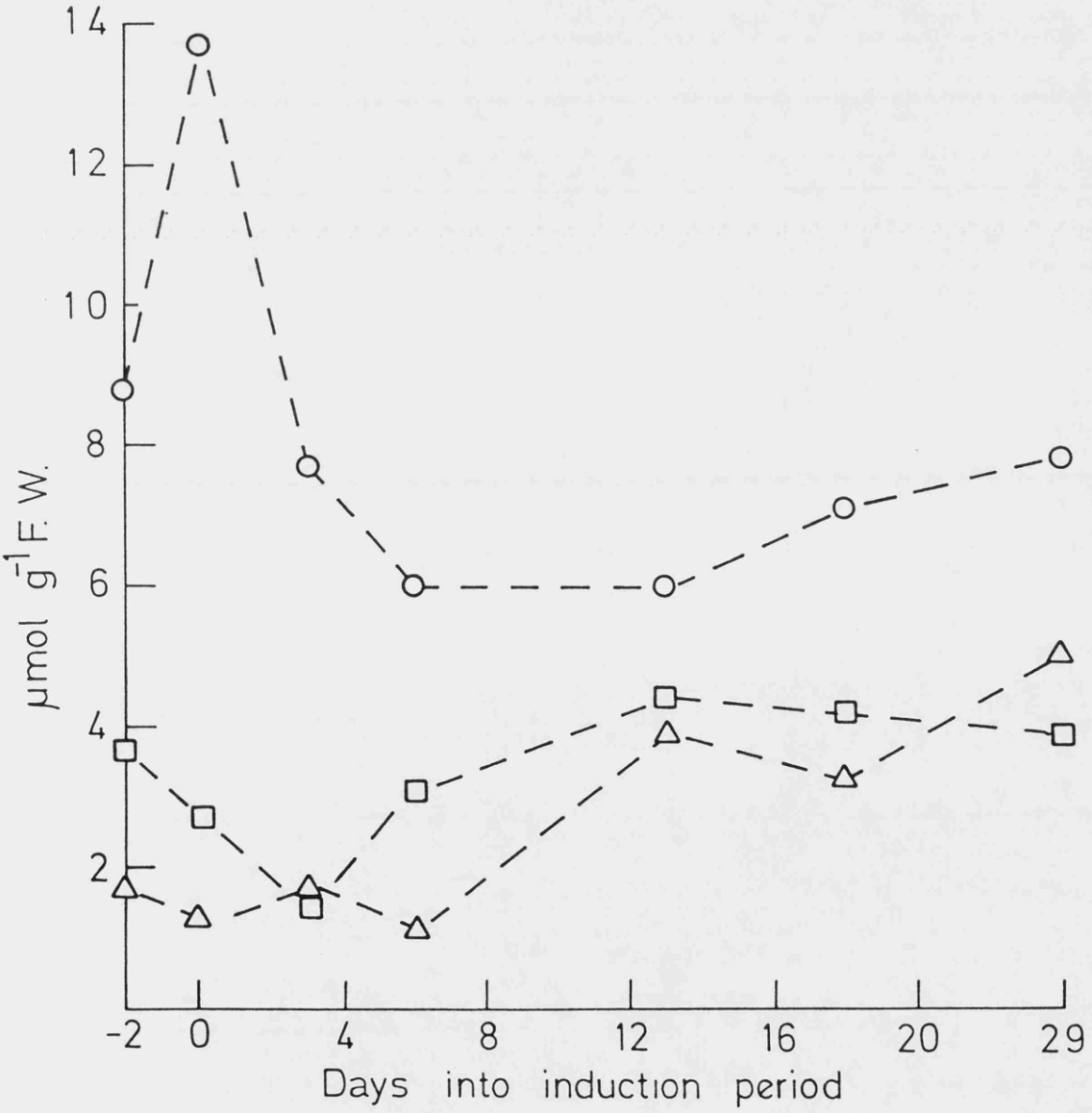
Levels of endogenous Pi were higher in CAM *M. crystallinum* than in C_3 *M. crystallinum* (Table 20). The difference between them increased as endogenous levels rose with increasing exogenous Pi.

Dry weights and fresh weights increased with increasing exogenous Pi in C_3 and CAM *M. crystallinum* (Table 21). Dry weights were similar in both forms of *M. crystallinum*, except at 0 Pi where it was 3-fold higher in CAM *M. crystallinum*. Fresh weight was also higher in CAM *M. crystallinum* at 0 Pi, but at 0.2 mM and 3 mM Pi fresh weights were about 4-fold higher in C_3 *M. crystallinum*. Dry weight as a percentage of fresh weight was fairly constant between 16.9 and

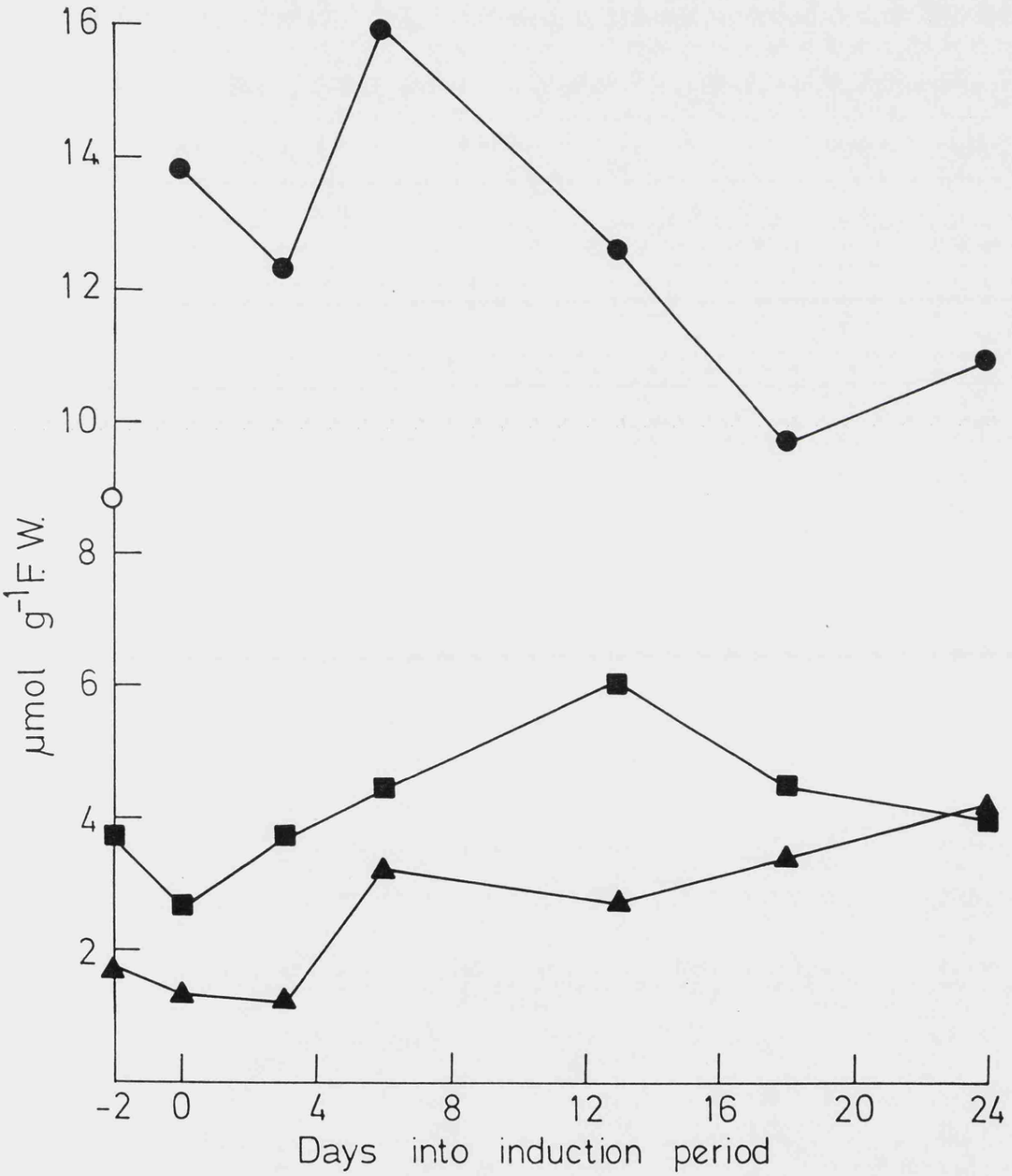
17.4 at different levels of Pi in CAM *M. crystallinum.* In C_3 *M. crystallinum* it decreased with increasing Pi from 8.2 at 0 Pi to 4.8 at 3 mM Pi.

There were marked differences in the appearance of C_3 and CAM *M. crystallinum* plants. CAM *M. crystallinum* plants appeared healthiest at 0 Pi possessing small dark green leaves with tinges of red at the petioles. At 3 mM Pi CAM *M. crystallinum* plants appeared unhealthy with yellowing leaves. This is in stark contrast to C_3 *M. crystallinum* plants grown at 3 mM Pi where they appeared the healthiest with many large green leaves. C_3 *M. crystallinum* plants grown at 0 Pi were similar in appearance to the CAM *M. crystallinum* plants grown at 0 Pi with the exception of red tinges on the leaves.

5. Figure 24. Levels of starch during the induction of CAM in *M. crystallinum* plants grown at 0 Pi (—○—), 0.2 mM Pi (—□—) and 3 mM Pi (—△—).


14
12
10
8
6
4
2
µmol g⁻¹F.W.
-2
0
4
8
12
16
20
29
Days into induction period

5. Figure 25. Levels of starch in C_3 *M. crystallinum* plants grown at 0 Pi (—●—), 0.2 mM Pi (—■—) and 3 mM Pi (—▲—).


16
14
12
10
8
6
4
2
µmol g⁻¹F.W.
-2
0
4
8
12
16
20
24
Days into induction period

5. Table 16. Levels of carbohydrate (μmol g^{-1} F.W.) during the induction of CAM in *M. crystallinum* grown at 0 Pi.

Induction of CAM

Days into induction period	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC	Starch CAM:C_3
-2	2.9±2.5	N.D.	0.5±0.45	0.9±0.5	4.3	2.0	1
0	4.6±1.1	N.D.	0.2±0.01	0.5±0.1	5.3	2.6	1
3	1.0±0.2	0.16±0.04	0.4±0.01	1.0±0.2	2.6	3.0	0.63
6	0.3±0.1	0.14±0.01	0.4±0.04	0.9±0.3	1.7	3.5	0.38
13	0.5±0.2	0.11±0.01	0.3±0.06	0.8±0.1	1.7	3.5	0.48
18	0.4±0.2	N.D.	0.4±0.20	0.7±0.1	1.5	4.7	0.73
24	0.3±0.1	N.D.	0.2±0.07	0.8±0.1	1.3	5.2	0.72

C_3 Control

Days into induction period	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC
-2	2.9±2.5	N.D.	0.5±0.45	0.9±0.5	4.3	2.0
0	4.6±1.1	N.D.	0.2±0.01	0.5±0.1	5.3	2.6
3	2.7±1.1	0.05±0.05	0.4±0.40	0.8±0.1	4.0	3.1
6	2.6±0.5	0.12±0.12	0.3±0.04	0.8±0.1	3.2	3.9
13	0.9±0.1	0.08±0.08	0.2±0.05	0.3±0.3	1.5	8.4
18	0.5±0.3	N.D.	0.2±0.02	0.6±0.4	1.3	8.8
24	1.0±0.3	N.D.	0.2±0.04	0.4±0.1	1.6	6.8

5. Table 17. Levels of carbohydrate (μmol g^{-1} F.W.) during the induction of CAM in *M. crystallinum* grown at 0.2 mM Pi.

Induction of CAM

Days into induction period	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC	Starch CAM:C_3
-2	N.D.	N.D.	N.D.	N.D.	N.D.	-	1
0	N.D.	N.D.	N.D.	0.2±0.1	0.2	13.5	1
3	N.D.	N.D.	N.D.	0.1±0.1	0.1	14.0	0.38
6	1.3±0.6	0.3±0.1	0.5±0.3	0.8±0.2	2.9	1.1	0.70
13	N.D.	N.D.	N.D.	0.7±0.2	0.7	6.3	0.73
18	1.1±0.1	N.D.	0.5±0.1	2.1±0.1	3.7	1.1	0.95
24	N.D.	N.D.	0.2±0.1	1.2±0.2	1.4	3.3	1.0

C_3 Control

Days into induction period	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC
-2	N.D.	N.D.	N.D.	N.D.	N.D.	-
0	N.D.	N.D.	N.D.	0.2±0.1	0.2	13.5
3	N.D.	N.D.	N.D.	N.D.	N.D.	-
6	0.2±0.1	N.D.	N.D.	0.1±0.1	0.3	14.7
13	1.1±0.5	N.D.	0.4±0.1	0.8±0.1	2.3	2.6
18	0.5±0.1	N.D.	N.D.	0.5±0.3	1.0	4.4
24	N.D.	N.D.	N.D.	N.D.	N.D.	-

5. Table 18. Levels of carbohydrate (μmol g^{-1} F.W.) during the induction of CAM in *M. crystallinum* grown at 3 mM Pi.

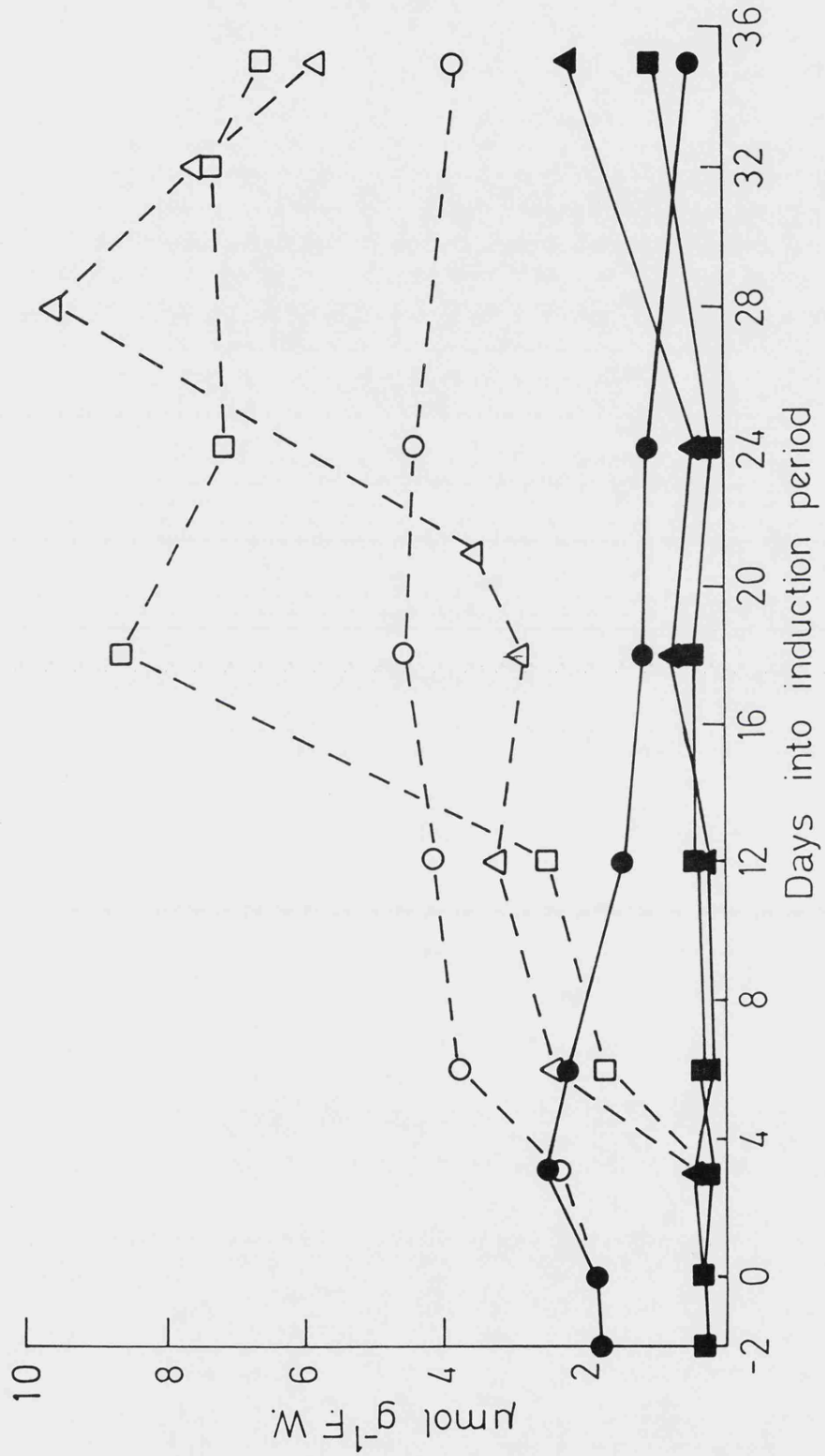
Induction of CAM

Days into induction period	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC	Starch CAM:C_3
-2	N.D.	N.D.	N.D.	0.2±0.03	0.2	8.5	1
0	N.D.	N.D.	N.D.	0.2±0.02	0.2	6.5	1
3	N.D.	N.D.	N.D.	0.3±0.06	0.3	5.7	1.42
6	0.1±0.01	N.D.	0.2±0.02	0.8±0.20	1.1	1.0	0.97
13	0.2±0.03	N.D.	0.2±0.01	1.4±0.15	1.8	2.2	1.44
18	0.2±0.01	N.D.	0.1±0.01	0.5±0.21	0.8	4.1	0.97
24	N.D.	N.D.	N.D.	N.D.	N.D.	-	1.22

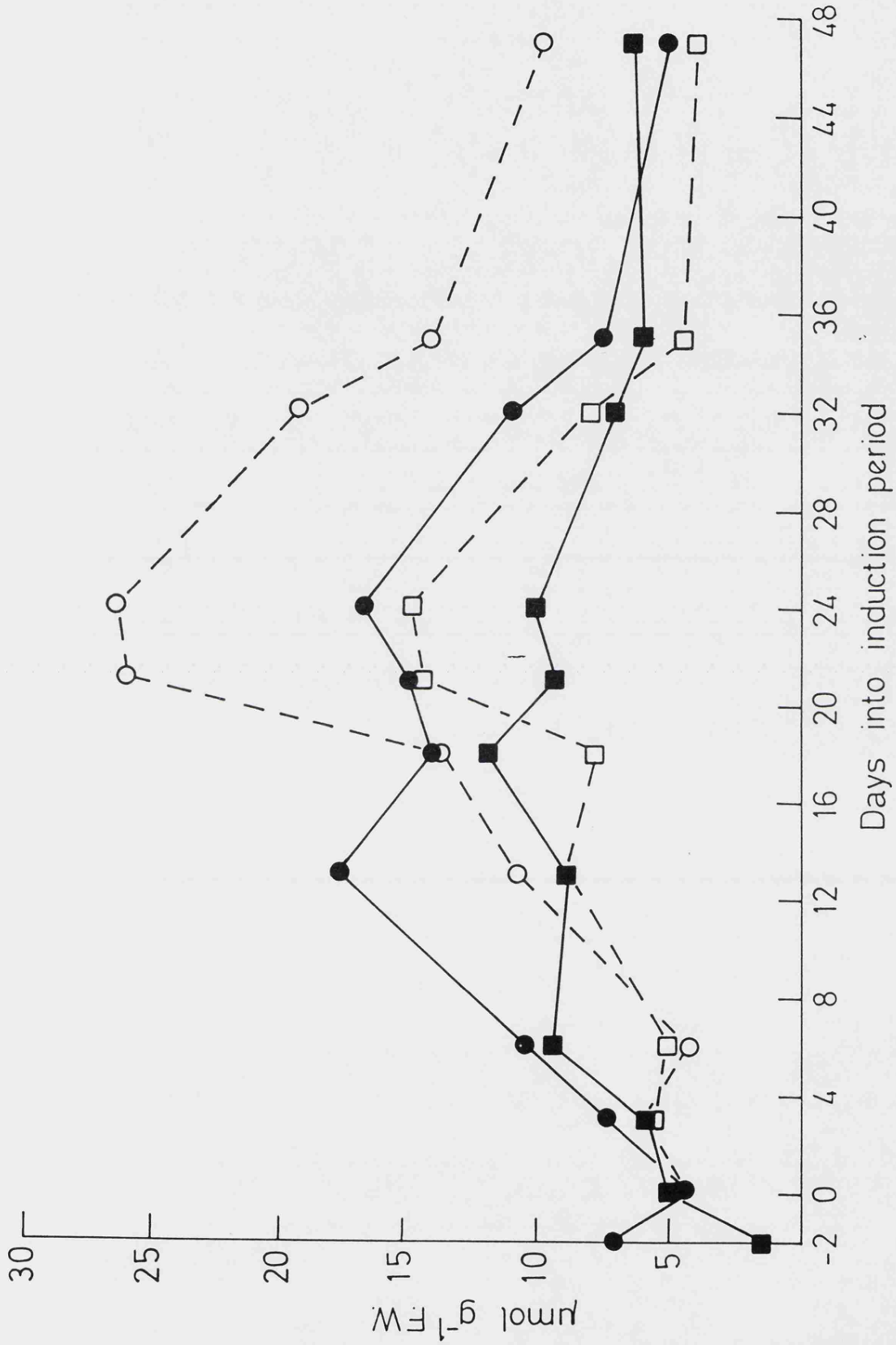
C_3 Control

Days into induction period	Glucose	Sorbitol	Inositol	Sucrose	TSC	St:TSC
-2	N.D.	N.D.	N.D.	0.2±0.03	0.2	8.5
0	N.D.	N.D.	N.D.	0.2±0.02	0.2	6.5
3	N.D.	N.D.	N.D.	0.2±0.03	0.2	6.0
6	0.4±0.1	N.D.	N.D.	0.3±0.02	0.7	4.6
13	N.D.	N.D.	N.D.	0.2±0.01	0.2	13.5
18	0.3±0.1	N.D.	N.D.	0.4±0.04	0.7	4.9
24	N.D.	N.D.	N.D.	0.3±0.03	0.3	13.7

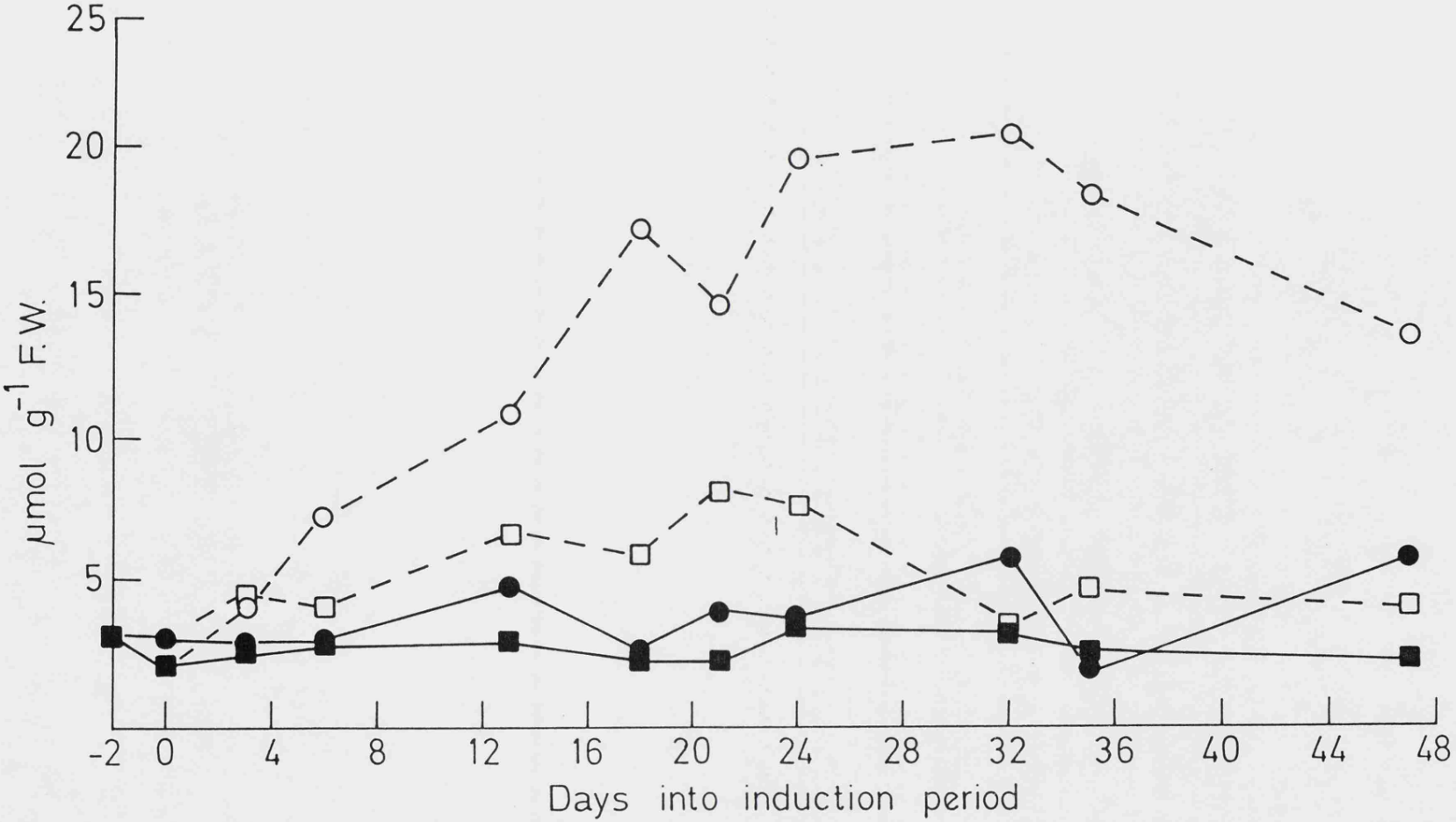
5. Figure 26. Levels of pinitol (inositol equivalents) during the induction of CAM in *M. crystallinum* plants grown at 0 Pi (C_3—●— , CAM—○—), 0.2 mM Pi (C_3—■— , CAM —□—) and 3 mM Pi (C_3—▲— , CAM—△—).


μmol g⁻¹ f.w.
10
8
6
4
2
-2
0
4
8
12
16
20
24
28
32
36
Days into induction period

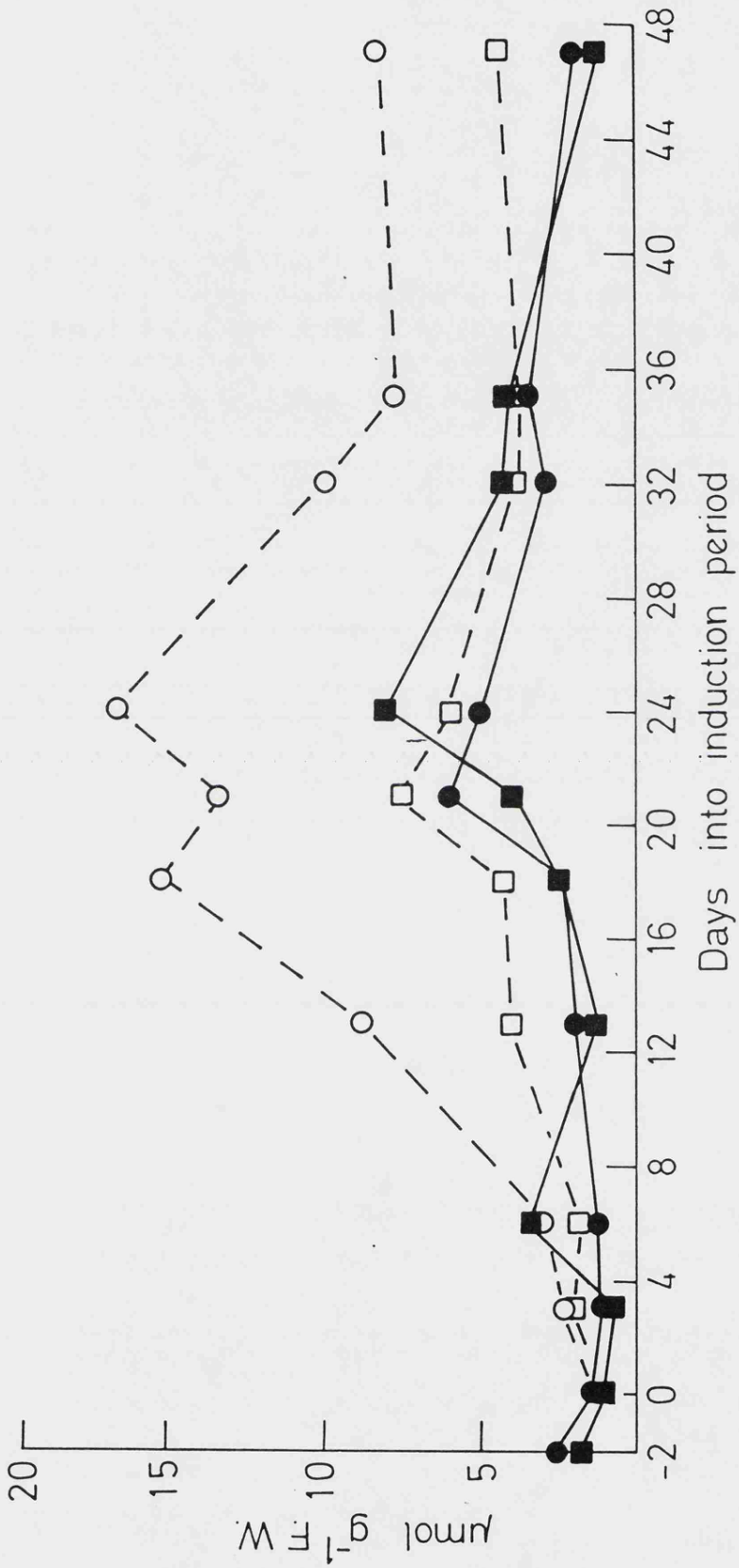
5. Figure 27. Levels of malic acid during the induction of CAM in *M. crystallinum* plants grown at 0 Pi, at 9:25 (C_3 —●—) CAM —○—) and 15:00 (C_3 —■— , CAM —□—).


30
25
20
15
10
5
μmol g⁻¹ FW
-2
0
4
8
12
16
20
24
28
32
36
40
44
48
Days into induction period

5. Figure 28. Levels of malic acid during the induction of CAM in *M. crystallinum* plants grown at 0.2 mM Pi at 9:25 (C_3 —●—, CAM—○—) and 15:00 (C_3 —■—, CAM—□—).


25
20
15
10
5
µmol g⁻¹ F.W.
-2
0
4
8
12
16
20
24
28
32
36
40
44
48
Days into induction period

5. Figure 29. Levels of malic acid during the induction of CAM in *M. crystallinum* plants grown at 3 mM Pi at 9:25 (C_3 —●—, CAM—○—) and 15:00 (C_3—■—, CAM—□—).


20
15
10
5
μmol g⁻¹ F W
-2
0
4
8
12
16
20
24
28
32
36
40
44
48
Days into induction period

Standard errors for Figures 24, 25, 26, 27, 28 and 29 (μmol g^{-1} F.W.)

Days into induction period	Starch						Pinitol						Malic acid											
	CAM			C_3			CAM			C_3			CAM						C_3					
													9:25	15:00	9:25	15:00	9:25	15:00	9:25	15:00	9:25	15:00	9:25	15:00
	0	0.2	3	0	0.2	3	0	0.2	3	0	0.2	3	0		0.2		3		0		0.2		3	
-2	1.8	3.5	0.3	1.8	3.5	0.3	0.8	0.1	0.1	0.8	0.1	0.1	0.8	0.5	0.4	0.4	0.3	0.1	0.8	0.5	0.4	0.4	0.3	0.1
0	0.8	0.7	0.1	0.8	0.7	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1.9	3.1	0.2	0.9	0.2	0.4	1.9	3.1	0.2	0.9	0.2	0.4
3	0.4	0.6	0.5	0.9	0.1	0.6	0.1	0.1	0.1	0.1	0.1	0.1	2.1	2.1	0.4	0.2	0.8	0.8	2.1	0.7	0.5	0.3	0.3	0.2
6	0.7	0.4	0.4	1.0	0.6	0.5	0.4	0.5	0.7	0.2	0.1	0.1	0.5	1.3	1.8	0.1	0.9	1.2	3.4	2.2	0.3	0.2	0.4	0.3
12	0.9	0.1	0.4	1.1	1.1	0.5	0.2	0.6	1.3	0.2	0.2	0.1	1.3	1.4	0.9	0.4	3.5	0.4	2.3	4.5	0.6	0.6	0.2	0.3
18	1.4	0.5	0.8	0.7	0.2	0.5	1.3	0.5	0.1	0.2	0.1	0.1	0.4	1.2	1.8	0.8	2.0	1.2	5.1	2.6	0.9	0.2	0.9	0.4
24	1.4	0.3	0.8	0.4	0.5	1.0	0.7	1.5	0.8	0.3	0.1	0.1	1.1	0.7	2.1	2.4	2.3	1.8	3.6	2.6	0.7	1.7	0.4	0.2
32													1.0	1.0	4.3	0.7	2.0	1.0	4.0	2.7	1.8	0.9	0.4	1.0
35							0.4	1.0	1.4	0.1	0.6	0.1	1.6	0.6	4.6	0.1	2.0	0.3	1.9	1.0	0.8	0.4	1.7	1.0
47													1.2	0.1	2.5	0.7	1.0	1.8	1.2	1.6	1.7	0.5	0.1	0.5

5. Table 19. Consumption (-) or accumulation (+) of malic acid (μmol g^{-1} F.W.) between 9:25 and 15:00 during the induction of CAM in *M. crystallinum* grown at 3 concentrations of Pi.

Days into induction period	0		0.2 mM		3 mM	
	CAM	C_3	CAM	C_3	CAM	C_3
-2	-0.76	-0.76	0	0	-0.10	-0.10
0	+0.08	+0.08	-0.14	-0.14	-0.05	-0.05
3	-0.07	-0.22	+0.06	-0.03	-0.03	-0.06
6	+0.11	-0.14	-0.41	+0.02	-0.18	+0.30
13	-0.24	-1.17	-0.56	-0.23	-0.64	-0.10
18	-0.77	-0.33	-1.54	-0.02	-1.47	+0.03
21	-1.51	-0.74	-0.86	-0.21	-0.78	-0.27
24	-1.50	-0.87	-1.61	-0.03	-1.44	+0.42
32	-1.48	-0.50	-2.28	-0.35	-0.82	+0.19
35	-1.28	-0.19	-1.84	+0.10	-0.52	+0.08
47	-0.52	-0.17	-1.31	-0.45	-0.52	-0.09

5. Table 20. Pi content of *M. crystallinum* 22 days into the induction period in plants grown at 3 concentrations of Pi.

Exogenous Pi (mM)	Pi µmol g^{-1} F.W. CAM	C_3
0	1.8 ± 0.5	1.4 ± 0.4
0.2	7.5 ± 0.1	6.1 ± 0.5
3.0	20.1 ± 4.2	9.9 ± 0.7

5. Table 21. Dry weights and fresh weights (g) of the whole above ground biomass of *M. crystallinum* 47 days into the induction period in plants grown at 3 concentrations of Pi.

Exogenous Pi (mM)	Dry weight		Fresh weight		Dry weight as a % of fresh weight	
	CAM	C_3	CAM	C_3	CAM	C_3
0	1.29	0.45	7.4	5.5	17.4	8.2
0.2	1.52	2.09	9.0	35.2	16.9	5.9
3.0	2.44	2.36	13.6	49.5	17.9	4.8

6. Effects of Pi and Pi-sequestration and solute potential on carbohydrate partitioning and levels of malic acid in leaf-discs of C_3 and CAM *M. crystallinum* and *K. daigremontiana*

6.1 Effects of Pi and Pi-sequestration on carbohydrate partitioning and levels of malic acid.

Effects were observed on C_3 and CAM *M. crystallinum* and *K. daigremontiana* leaf-discs incubated in petri dishes for 12 hours under daytime growth cabinet conditions and on separate discs incubated for 12 hours under nighttime growth cabinet conditions.

Incubations of C_3 and CAM *M. crystallinum* tissue in the presence of Pi were carried out on 18 leaf-discs of each from 6 plants. For the analysis of carbohydrate and malic acid these were divided into 6 replicate samples of 3 discs each. In *K. daigremontiana* 24 leaf-discs from 4 plants were incubated in the presence of Pi. They were divided into 6 replicate samples of 4 discs each for carbohydrate and malic acid analysis.

Incubations and sampling of C_3 and CAM *M. crystallinum* leaf-discs in mannose and glucosamine (Pi-sequestration) were carried out in the same way as for incubations in the presence of Pi. *K. daigremontiana* leaf-disc incubations and sampling in mannose were also carried out in the same way. Eighteen leaf-discs from 6 *K. daigremontiana* plants were incubated in glucosamine. They were divided into 6 replicate samples of 3 leaf-discs each for carbohydrate and malic acid analysis.

The compositions of the incubation media are detailed in the Materials and Methods section. Starch and soluble carbohydrate were measured by the Anthrone method. The leaf-discs were rinsed first in

distilled water to remove the exogenous mannose and glucosamine. All incubations were carried out twice and instead of expressing data as the mean of the 2 incubations it was expressed as factor change during the 12 hours incubation. This gave a clearer representation of the effects of the different treatments.

Incubation of leaf-discs in mannose increased the accumulation of starch under daytime growth cabinet conditions in C_3 and CAM *M. crystallinum* and *K. daigremontiana* (Table 22). The increase in accumulation was most marked in *K. daigremontiana*, where there was a factor increase of 2.63 compared to 1.72 in the control. The factor increases in mannose-treated C_3 and CAM *M. crystallinum* leaf-discs were very similar at 1.09 and 1.05 respectively. The greater accumulation of starch in mannose treated leaf-discs under nighttime growth cabinet conditions was only statistically significant in C_3 *M. crystallinum* where there was a factor increase of 1.18 compared to a factor decrease of 1.10 in the control. Glucosamine had less effect on starch levels during the day and night. The only statistically significant effect of glucosamine on levels of starch was during the day in *K. daigremontiana* where starch increased by a factor of 2.82 compared to 2.36 in the controls. The effect of Pi on starch levels was the reverse of the effect of the Pi sequesters mannose and glucosamine. It caused a net consumption of starch during the day. The effect was again most marked in *K. daigremontiana* with a net consumption of 1.67 compared to 1.02 in the control. There was a difference between C_3 and CAM *M. crystallinum* with a net consumption of 1.33 in the CAM form compared to an accumulation in the control, and no statistically significant difference between treatment and control in the C_3 form. The effect of Pi at night was only statistically significant in the CAM form with a consumption of 1.47 compared to 1.10 in the control.

The effect of mannose, glucosamine and Pi on levels of total soluble carbohydrate was less uniform than the effects on starch levels. In CAM *M. crystallinum* mannose decreased the amount of total soluble carbohydrate consumption during the day to a factor decrease of 1.47 compared to 3.45 in the control. In C_3 *M. crystallinum* the reverse effect was observed; consumption was by a factor of 2.27 compared to 1.54 in the control. At night the only statistically significant effect of mannose on total soluble carbohydrate levels was in CAM *M. crystallinum* where it caused a consumption of 2.33 compared to 4.00 in the control. Total soluble carbohydrate levels in *K. daigremontiana* did not respond to mannose day or night. The response to glucosamine during the day was only statistically significant in C_3 *M. crystallinum* where the effect produced was similar to that caused by mannose with a factor decrease of 2.08 compared to 1.09 in the control. The response to glucosamine at night was different in C_3 and CAM *M. crystallinum*. In the C_3 form glucosamine increased consumption to 1.54 compared to 1.18 in the control, and in the CAM form it decreased consumption to 1.69 compared to 2.22 in the control. As with mannose, total soluble carbohydrate levels in *K. daigremontiana* did not respond to glucosamine day or night. The effect of Pi on levels of total soluble carbohydrate was only statistically significant in *K. daigremontiana* during the day where there was less accumulation, with a factor increase of 2.77 compared to 3.27 in the control.

Mannose increased the consumption of malic acid during the day in CAM *M. crystallinum* to a factor of 25.00 compared to 5.26 in the control. In C_3 *M. crystallinum* accumulation of malic acid by a factor of 1.23 in the control was turned into consumption of 4.35 in the presence of mannose. Consumption at night in C_3 *M. crystallinum* was also promoted in the presence of mannose to a factor of 1.28

compared to accumulation by a factor of 1.17 in the control. There was no effect of mannose on malic acid levels at night in CAM *M. crystallinum*. Glucosamine had no effect on daytime or nighttime levels of malic acid in *K. daigremontiana* or CAM *M. crystallinum*. Incubation of leaf-discs in Pi decreased the consumption of malic acid during the day and night in CAM *M. crystallinum*. In *K. daigremontiana* Pi increased the accumulation of malic acid during the night.

6.2 Effects of solute potential on carbohydrate partitioning and levels of malic acid.

Solutions of polyethylene glycol of different concentrations were used to obtain solutions varying in solute potential. Incubations and samplings were carried out in the same way as for C_3 and CAM *M. crystallinum* leaf-discs in the presence of Pi and mannose and glucosamine, but only one incubation during the day and night was carried out.

The incubation of leaf-discs in solutions of different solute potential reduced the amount of starch accumulation during the day in C_3 and CAM *M. crystallinum* and *K. daigremontiana* in comparison to the intact plant controls (Table 23). The difference between leaf-disc incubations and intact plant controls was greatest in CAM *M. crystallinum* and smallest in C_3 *M. crystallinum*. There was little difference in levels of starch between the incubations at different solute potentials; all incubations showed either a small accumulation or a small consumption of starch during the day. The effect on starch levels at night was less uniform. In CAM *M. crystallinum* starch consumption was less in leaf-disc incubations than in the

intact plant control, the difference being especially marked at -0.5 MPa. In C_3 *M. crystallinum* and *K. daigremontiana* there was little difference between intact plant controls and leaf-disc incubations at -0.5 MPa. In *K. daigremontiana* at -1.0 MPa there was markedly less consumption of starch at night.

The incubation of leaf-discs in solutions of different solute potentials produced contrasting effects on total soluble carbohydrate. In *K. daigremontiana* and CAM *M. crystallinum* the difference between intact plant controls and leaf-discs during the day was lowest at the solute potentials closest to the water potentials of the intact plant controls. These were -3.0 MPa in CAM *M. crystallinum* where there was accumulation during the day by a factor of 1.22 compared to 1.37 in the control; and -1.0 MPa in *K. daigremontiana* where there was an accumulation by a factor of 1.01 compared to 1.17 in the control. There was consumption of total soluble carbohydrate at -0.5 MPa and -3.0 MPa in *K. daigremontiana* and consumption at -0.5 MPa in CAM *M. crystallinum*, but accumulation at -6.0 MPa. In C_3 *M. crystallinum* the difference between the control and leaf-disc incubations decreased with a more negative solute potential. At -0.5 MPa and -1.0 MPa there was consumption of total soluble carbohydrate, but at -6.0 MPa there was accumulation by a factor of 1.18 close to the control value of 1.22. At night there was greater consumption of total soluble carbohydrate in leaf-discs of *K. daigremontiana* and C_3 *M. crystallinum* than in the intact plant controls. In CAM *M. crystallinum* the amount of consumption was greater than the control at -0.5 MPa and -3.0 MPa, but consumption declined with a more negative solute potential to be less than the control at -6.0 MPa.

Malic acid consumption during the day in leaf-discs was lower than in intact plant controls and decreased with a more negative

solute potential in CAM *M. crystallinum*. In *K. daigremontiana* consumption was lower at -0.5 MPa and -1.0 MPa, but at -3.0 MPa consumption was greater than in the control. The accumulation of malic acid during the night in *K. daigremontiana* and the CAM *M. crystallinum* was less in leaf-discs than in intact plant controls. At -0.5 MPa there was consumption of malic acid. In *K. daigremontiana* there was accumulation at -1.0 MPa by a factor of 2.74 compared to 27.10 in the control. In CAM *M. crystallinum* there was accumulation of malic acid at -3.0 MPa and -6.0 MPa by a factor of 2.45 and 2.38 respectively compared to 7.23 in the control.

6.1 Table 22. The effect of sequestration and additon of Pi on levels of starch (St), total soluble carbohydrate (TSC) and malic acid (Ma) in *K. daigremontiana*, C_3 and CAM *M. crystallinum*.

	Sequestration of Pi						Addition of Pi			
	Mannose				Glucosamine					
	Factor change during 12 h				Factor change during 12 h				Factor change during 12 h			
	DAY	P	NIGHT	P	DAY	P	NIGHT	P	DAY	P	NIGHT	P
K. daigremontiana												
St control	+1.72		+1.10		+2.36		−1.56		−1.02		−1.11	
St treatment	+2.63	0.02	+1.32	NS	+2.82	0.01	−1.56	NS	−1.67	0.05	−1.01	NS
TSC control	+2.18		−1.79		+2.56		−1.26		+3.27		+1.07	
TSC treatment	+1.92	NS	−1.89	NS	+2.63	NS	−1.26	NS	+2.77	0.1	−1.04	NS
Ma control					−5.88		−1.03				+2.11	
Ma treatment					−6.25	NS	+1.12	NS			+2.73	0.01
CAM *M. crystallinum*												
St control	−1.03		−1.06		+1.83		−1.20		+1.06		−1.10	
St treatment	+1.05	0.05	+1.04	NS	+1.67	NS	−1.30	NS	−1.33	0.02	−1.47	0.1
TSC control	−3.45		−4.00		−1.49		−2.22		−2.44		−2.27	
TSC treatment	−1.47	0.001	−2.33	0.01	−1.33	NS	−1.69	0.1	−2.00	NS	−2.13	NS
Ma control	−5.26		−1.69		−50.00		−2.27		−8.33		−2.50	
Ma treatment	−25.00	0.01	−1.67	NS	−33.33	NS	−1.33	NS	−5.00	0.02	−1.04	0.05
C_3 *M. crystallinum*												
St control	+1.05		−1.10		+1.16		+1.33		+1.12		−1.0	
St treatment	+1.09	0.05	+1.18	0.001	+1.44	NS	+1.08	NS	−1.09	NS	+1.01	NS
TSC control	−1.54		−1.75		−1.09		−1.18		−1.64		−1.43	
TSC treatment	−2.27	0.02	−1.69	NS	−2.08	0.02	−1.54	0.02	−1.27	NS	−1.37	NS
Ma control	+1.23		+1.17									
Ma treatment	−4.35	0.001	−1.28	0.01								

6.2 Table 23. The effect of external solution solute potential (ψs) on starch (St), total soluble carbohydrate (TSC) and malic acid (Ma) levels in leaf-discs of *K. daigremontiana*, C_3 and CAM *M. crystallinum*.

	Factor change during 12 hours DAY			Factor change during 12 hours NIGHT		
	St	TSC	Ma	St	TSC	Ma
K. daigremontiana		ψ of leaf-disc = -0.8 MPa				
Intact plant control	+1.46	+1.17	-25.00	-1.47	-1.16	+27.10
ψs of solution -0.5	+1.03	-1.16	-5.56	-1.56	-1.27	-1.03
(MPa) -1.0	-1.15	+1.01	-4.17	-1.03	-1.45	+2.74
-3.0	+1.03	-4.17	-10.00			
CAM *M. crystallinum*		ψ of leaf-disc = -3.5 MPa				
Intact plant control	+1.87	+1.37	-7.14	-1.89	-1.37	+7.23
ψs of solution -0.5	-1.03	-3.45	-5.26	-1.06	-4.0	-1.67
(MPa) -3.0	+1.08	+1.22	-2.44	-1.67	-1.83	+2.45
-6.0	-1.11	+1.92	-2.27	-1.56	-1.15	+2.38
C_3 *M. crystallinum*		ψ of leaf-disc = -0.8 MPa				
Intact plant control	+1.05	+1.22		-1.05	-1.22	
ψs of solution -0.5	+1.05	-1.54		-1.10	-1.75	
(MPa) -1.0	-1.11	-1.01				
-6.0	-1.10	+1.18				

7. Investigations of pinitol in *M. crystallinum*

7.1 Identification of pinitol.

Leaf tissue extracts from CAM *M. crystallinum* and *Lotus corniculatus* were analysed and compared by gas chromatography and HPLC (see Materials and Methods section). Pinitol is known to constitute about 1% of total dry weight in *L. corniculatus* (Riggs and Strong, 1967).

CAM *M. crystallinum* and *L. corniculatus* leaf tissue extracts were passed down an Amberlite resin IR-120(H) cation exchange column and a Dowex 1-X8 anion exchange column. The charge-free extract that remained was examined by gas chromatography.

A leaf tissue extract of CAM *M. crystallinum* was demethylated according to the method of Drew (1978). 2.5 g fresh weight of CAM *M. crystallinum* leaf tissue were extracted in the same way as leaf-disc samples (see Materials and Methods) and freeze-dried. This was then reacted with 2.5 ml of hydriodic acid (HI) and boiled under reflux. Iodine was removed by sublimation under vacuum at 80°C. The demethylated extract was analysed by gas chromatography.

Comparison of the leaf tissue extracts from CAM *M. crystallinum* and *L. corniculatus* by gas chromatography revealed that the putative pinitol peak in CAM *M. crystallinum* had an identical relative retention time (RRT) to a peak in *L. corniculatus* that represented more than 1% of dry weight. The putative pinitol peak had a RRT of 0.86 (Table 1) and a retention time relative to inositol of 0.61. The peak could be separated from fructose which had a RRT of 0.84. Analysis by HPLC also gave identical retention times for the putative pinitol peaks in CAM *M. crystallinum* and *L. corniculatus*. These peaks were the largest peaks produced by both leaf tissue extracts by

both methods of analysis.

The removal of charged particles from the CAM *M. crystallinum* and *L. corniculatus* leaf extracts did not affect the putative pinitol peak when analysed by gas chromatography.

The demethylation of a CAM *M. crystallinum* leaf extract yielded 5.7 μmol g^{-1} F.W. of inositol from a starting level of 0.5 μmol g^{-1} F.W.. Pinitol, at a starting level of 9.2 μmol g^{-1} F.W. was undetectable at the end of the demethylation treatment.

7.2 Dark incubation of CAM *M. crystallinum*.

Three plants were incubated in total darkness at room temperature for 96 hours. Two replicate samples of 10 leaf-discs each were taken at 9:30 immediately prior to incubation and at 9:30, after 96 hours in the dark. Starch was analysed enzymically and soluble carbohydrate by gas chromatography.

After 96 hours of darkness glucose and inositol had fallen from 1.0 μmol g^{-1} F.W. and 0.2 μmol g^{-1} F.W. respectively to undetectable levels (Table 24). Sucrose levels fell from 0.6 μmol g^{-1} F.W. to 0.2 μmol g^{-1} F.W. The level of pinitol remained unchanged after 96 hours of darkness.

7.3 Effect of two desiccating treatments on levels of pinitol and acid in *M. crystallinum*.

A comparison was made of plants which were watered with 400 mM NaCl to induce CAM as in the Materials and Methods section and plants which had all water withheld from them and were allowed to dry out. Three 6 week old C_3 *M. crystallinum* plants were given each treatment. Three replicate samples of 10 leaf-discs each were taken at 14:00 from plants at each treatment on day 0 just prior to the commencement of the treatment and on day 20.

Levels of pinitol and acid at day 20 were considerably higher in *M. crystallinum* plants treated with 400 mM NaCl than in C_3 *M. crystallinum* plants and plants with water withheld (Table 25). In plants treated with 400 mM NaCl there were 9.2 µmol g^{-1} F.W. of pinitol and 81 µeq g^{-1} F.W. of acid in comparison to 0.5 µmol g^{-1} F.W. and 6 µeq g^{-1} F.W. respectively in C_3 *M. crystallinum* plants. Levels of pinitol and acid were a little higher in plants with water withheld than in C_3 *M. crystallinum* at 1.1 µmol g^{-1} F.W. and 13 µeq g^{-1} F.W. respectively.

7.4 Effect of an overnight chilling temperature on levels of pinitol in C_3 *M. crystallinum*.

Three C_3 *M. crystallinum* plants were transferred from growth cabinet conditions to 5°C during the 12 hour dark period for a period of 9 days. Three C_3 *M. crystallinum* plants were kept under growth cabinet conditions during this period. The plants were 6 weeks old. Three replicate leaf-disc samples of 5 leaf-discs each were taken at 15:00 at the end of the 9 day period. Starch levels were analysed enzymically and soluble carbohydrate by gas chromatography.

Levels of pinitol were unaffected by the 9 day period of overnight chilling of C_3 *M. crystallinum* plants (Table 26). Levels of fructose, glucose, sucrose and consequently, total soluble carbohydrate (TSC) were significantly higher in chilled plants than in non-chilled plants. Levels of glucose, sucrose and TSC were about twice as high and levels of fructose 5-times as high as non-chilled plants. Levels of starch, sorbitol and inositol were not affected by the treatment.

7.5 Location of pinitol in the CAM *M. crystallinum* cell.

Pinitol levels in preparations of protoplasts, vacuoles and chloroplasts (see Materials and Methods section) were measured by gas chromatography. Three preparations were made of each.

No pinitol was found to be present in preparations of vacuoles. Pinitol levels in chloroplast preparations accounted for 37%±5.2% of the pinitol in the protoplast preparations. The remaining 63%±5.2% of the pinitol is assumed to be located in the cytosol. Assuming 10 µmol pinitol g^{-1} F.W. (Table 12) and that dry weight is 2% of fresh weight (Figure 10), and consequently 98% of fresh weight is water the concentration of pinitol in leaf sap is about 10 mM. Estimations can also be made of the concentrations of pinitol in the chloroplastic and cytosolic compartments. Assuming a volume of about 33 µl mg^{-1} of chlorophyll for CAM *M. crystallinum* chloroplasts grown at 400 mM NaCl (Demmig and Winter, 1983), and chlorophyll at 0.5 mg g^{-1} F.W. (Table 3) the chloroplastic concentration of pinitol is about 230 mM. Assuming that the relative volume of the cytosol is in the order of a few per cent (Heun, Gorham, Luttge and Wyn Jones, 1981) and less than 10% if the vacuole takes up over 90% in CAM tissue (Raven, 1987), and that dry weight is about 2% of fresh weight (Figure 10), then the cytosolic concentration of pinitol is about 100 mM.

7.6 Extraction and assay of glucose 6 phosphate cycloadolase from *M. crystallinum*.

The enzyme was extracted and assayed from CAM *M. crystallinum* and C_3 *M. crystallinum* leaf tissue (see Materials and Methods). Presence of glucose 6 phosphate cycloaldolase activity was indicated by the formation of inositol from glucose 6 phosphate, the reaction catalysed by the enzyme.

In the presence of CAM *M. crystallinum* leaf extract 1.1 μmol g^{-1} F.W. of inositol were formed. In the presence of C_3 *M. crystallinum* leaf extract 0.2 μmol g^{-1} F.W. of inositol were formed. This indicates the presence of greater glucose 6 phosphate cycloaldolase activity in CAM *M. crystallinum*.

7.2 Table 24. Levels of metabolites in CAM *M. crystallinum* before and after 96 hours of darkness (μmol g^{-1} F.W.).

	Glucose	Sorbitol	Inositol	Sucrose	Pinitol
0	1.0±0.3	0.05±0.01	0.2±0.02	0.6±0.2	13.7±4.4
96 hrs	N.D.	0.09±0.03	N.D.	0.2±0.1	11.9±3.7

7.3 Table 25. Levels of pinitol (μmol g^{-1} F.W., inositol equivalents) and acid (μeq g^{-1} F.W.) in *M. crystallinum* plants treated with 400 mM NaCl to induce CAM and in plants with all water withheld.

	C_3		400 mM NaCl		Water withheld	
	Pinitol	acid	Pinitol	acid	Pinitol	acid
Day 0	N.D.	3±1	N.D.	3±1	N.D.	3±1
Day 20	0.5±0.1	6±2	9.2±0.6	81±9	1.1±0.2	13±3

7.4 Table 26. Levels of metabolites (μmol g^{-1} F.W.) in response to overnight chilling in C_3 *M. crystallinum*.

	Fructose	Glucose	Sorbitol	Inositol	Sucrose	TSC	Starch	Pinitol
Non-chilling	0.5±0.1	13.1±1.6	0.5±0.10	0.3±0.03	0.6±0.2	15.0	6.8±1.5	0.3±0.08
Chilling	2.5±0.4	25.0±4.0	0.2±0.01	0.2±0.01	1.1±0.1	29.0	6.1±0.2	0.4±0.10

DISCUSSION

Provision of phosphoenol pyruvate, the CO_2 acceptor

CAM plants exhibit a diel fluctuation of organic acid and a reciprocal diel fluctuation in the level of storage carbohydrate. This is observed clearly in *Kalanchoe daigremontiana* (Table 2) and CAM *Mesembryanthemum crystallinum* (Table 2 and Fig. 4). The large fluctuation in levels of carbohydrate in CAM plants is due to the provision of the CO_2 acceptor phosphoenol pyruvate (PEP). In CAM *M. crystallinum* and *K. daigremontiana* carbohydrate accumulated predominantly as starch during the day (Table 2). The accumulation of carbon mainly as chloroplastic or extrachloroplastic carbohydrate during the day in CAM plants relates to the substrate utilised for PEP synthesis (Fahrendorf, Holtum, Mukherjee and Latzko, 1987). In CAM *M. crystallinum* and *K. daigremontiana* the large turnover of starch indicates that it is the major storage carbohydrate utilised for PEP synthesis. However, in *K. daigremontiana* soluble carbohydrate also contributed appreciably to daytime carbohydrate accumulation (Table 2): starch accounted for two thirds of carbohydrate accumulation and soluble carbohydrate for the other third. Thus, in *K. daigremontiana* soluble carbohydrate may also contribute to PEP synthesis. This confirms the work of Sutton (1975) who also concluded that starch donated two thirds of the carbon required for PEP synthesis and that a turnover of soluble glucan provided the remainder of the necessary carbon. The CAM plants *Ananas comosus* and *Aloe arborescens* predominantly accumulate extrachloroplastic assimilate during the day (Fahrendorf *et al.*, 1987). In *A. comosus*, soluble carbohydrate exhibits a diel fluctuation similar to that of the starch pool of starch accumulating CAM plants (Kenyon, Severson and Black, 1985).

The provision of a precursor for PEP is clearly vital to CAM, since it is a prerequisite for dark CO_2 fixation. Under conditions of limiting assimilate supply, at low photon fluence rates, starch accumulated more readily in CAM *M. crystallinum* than in C_3 *M. crystallinum* (Fig. 5). This confirms the present hypothesis that under conditions of restricted assimilate supply the CAM plant will divert carbon to the sink with the highest priority - the precursor of PEP. This may be termed high priority starch accumulation. The capacity for starch synthesis at low photon fluence rates - as low as 15 μmol m^{-2} s^{-1} in starch-storing CAM *M. crystallinum* (Fig. 5) - is paralleled in starch-storing soybean where the rate of starch synthesis was little affected by low photon fluence rates (Silvius, Chatterton and Kremer, 1979).

Starch and sucrose are the only carbohydrates that correlate in any quantitative way with photon fluence rate in C_3 and CAM *M. crystallinum* (Fig. 5, Tables 5 and 6). This confirms their identity as end-products of photosynthesis. Levels of sucrose tended to be slightly higher in CAM *M. crystallinum* than in C_3 *M. crystallinum* (Table 12). The maintenance of fairly high levels of sucrose throughout the day in CAM plants has been seen as a balancing of sucrose synthesis and sucrose transport (Kenyon *et al.*, 1985) and does not necessarily imply higher rates of sucrose synthesis than in C_3 plants. Sucrose exhibits little diel fluctuation in CAM plants (Pucher, Vickery, Abrahams and Leavenworth, 1949) and may have little function in the provision of PEP. The ratio of starch to total soluble carbohydrate is used in preference to the ratio of starch to sucrose (e.g. Table 2), because in CAM the accumulation of soluble carbohydrates other than sucrose may be considerable.

Thus, C_3 and CAM plants differ with regard to the partitioning of carbohydrate. The synthesis of PEP in CAM *M. crystallinum* from

starch dictates that the accumulation of this carbohydrate takes precedence over the accumulation of other carbohydrate. What mechanisms may account for this difference in carbohydrate partitioning between C_3 and CAM *M. crystallinum*?

The control of carbohydrate partitioning

The control of carbohydrate partitioning is a complex process and involves the interaction between several key enzymes and effectors. These key enzymes include fructose 1,6 bisphosphatase, sucrose phosphate synthetase and possibly sucrose phosphatase which are involved in sucrose synthesis, and ADP glucose pyrophosphorylase which is involved in starch synthesis. The effectors are fructose 2,6 bisphosphate, 3 phosphoglyceric acid (3PGA), dihydroxyacetone phosphate (DHAP) and inorganic phosphate (Pi) which influence the activities of these key enzymes and the rate of flux of carbon between the chloroplast and cytoplasm via the Pi translocator.

The chloroplast envelope can be regarded as the ultimate barrier between starch and sucrose partitioning (Herold, 1980). Pi regulates the flow of carbon across this barrier and is accorded a central role in the control of assimilate partitioning (Walker and Sivak, 1986). This central role was confirmed by manipulating the availability of Pi to whole plants of C_3 and CAM *M. crystallinum* and to leaf-discs of C_3 and CAM *M. crystallinum* and *K. daigremontiana* (sections 5 and 6).

If the sequestration of Pi by mannose and glucosamine as mannose phosphate and glucosamine phosphate surrounding leaf-discs of C_3 and CAM *M. crystallinum* and *K. daigremontiana* led to lower concentrations of cytosolic Pi, then this lack of Pi would restrict the activity of the Pi translocator and hence the export of triose phosphate from the chloroplast. Retention of triose phosphate in the chloroplast would enhance starch synthesis there and could explain the higher levels of starch in leaf-discs exposed to this treatment (Table 22). The metabolism of the Pi-sequester, mannose, exhibited by some species (Herold, Lewis and Walker, 1976), and of glucosamine which would presumably have led to higher soluble carbohydrate levels was not

apparent. The decrease in starch levels in response to elevated Pi can be explained by enhanced export of triose phosphate from the chloroplast and hence a low availability of triose phosphate in the chloroplast for starch synthesis. A comparison of starch accumulation in C_3 and CAM leaf-discs shows that there is no difference between them in their response to depressed or elevated Pi levels. Soluble carbohydrate accumulation however, is affected differently in C_3 leaf-discs compared to CAM leaf-discs (Table 22). In C_3 *M. crystallinum* Pi sequestration depressed soluble carbohydrate relative to the control discs. This is the effect that might be expected through the Pi translocator with a lack of Pi decreasing triose phosphate export from the chloroplast, resulting in lower cytosolic soluble carbohydrate levels. In CAM *M. crystallinum* the opposite effect was produced by Pi sequestration. In CAM *M. crystallinum* however, in contrast to C_3 *M. crystallinum*, the difference between the water potential of the leaf-discs (-3.5 MPa, CAM; -0.8 MPa, C_3) and the solute potential of the incubation medium (-0.65 MPa) was large. It may be that this difference was overriding the influence of Pi sequestration. Hiller and Greenway (1968) found that increasingly more negative solute potentials of solutions surrounding *Chlorella pyrenoidosa*, more negative than the water potential of this alga, directly stimulated the synthesis of sucrose. This was certainly true in C_3 and CAM *M. crystallinum* (Table 23), but in conditions of Pi-sequestration the effect of the solute potential of the medium may be more complex. The elevated soluble carbohydrate levels in comparison to the control produced in the conditions of Pi sequestration in CAM *M. crystallinum* may not reflect the situation *in vivo*. The method of cutting leaf-discs was an inadequate one for observing the intricacies of carbohydrate metabolism; it involved disrupting tissue and prevented the communication of events within

the whole leaf and plant. The results obtained from incubating leaf-discs at various exogenous levels of Pi provide no evidence for a difference in the properties of the C_3 and CAM Pi translocators of C_3 and CAM *M. crystallinum* and *K. daigremontiana* or for a difference in their ability to control Pi concentrations within the cell.

Growing intact plants of C_3 and CAM *M. crystallinum* at different exogenous Pi levels enabled a comparision to be made of the effects of Pi nutrition on carbohydrate partitioning that was free from the disruptive effect of cutting leaf-discs, the effect of the solute potential of the incubation medium and enabled communication within the whole plant. There was little discernable effect of exogenous Pi on soluble carbohydrate levels in CAM *M. crystallinum* (Tables 16, 17 and 18). The effect of exogenous Pi on soluble carbohydrate levels in C_3 *M. crystallinum* was, however, the opposite one would predict from the known regulatory properties of the Pi translocator. In intact plants, in contrast to leaf-discs, there is the possibility of movement of assimilate in whole plants, and a high level of Pi in the leaves of C_3 *M. crystallinum* could have stimulated export of soluble carbohydrate to the growing sinks (Hopkinson, 1964; Thorne and Koller, 1974). This would stimulate growth and account for the lower levels of soluble carbohydrate in the most recently fully expanded source leaves at high exogenous Pi. Indeed, if one excludes pinitol (about 10% of dry weight) and the inorganic ion content of CAM *M. crystallinum* mainly due to NaCl (Heun, Gorham, Luttge and Wyn Jones, 1981), from dry weights (Table 21), then dry weight is much greater in C_3 *M. crystallinum*. This indicates greater growth in the C_3 form, which could be reflected in the lower amounts of soluble carbohydrate.

Clear differences were apparent in amounts of starch between C_3 and CAM *M. crystallinum* plants grown at different exogenous levels of

Pi (Figs 24 and 25). The reduction in starch levels in *M. crystallinum* plants grown at high exogenous Pi was much less in the CAM form than in the C_3 form (starch CAM:C_3, Tables 16, 17 and 18). This suggests that the starch metabolism of CAM *M. crystallinum* chloroplasts may be less perturbed by high exogenous Pi than the metabolism of C_3 *M. crystallinum* chloroplasts. This may be due either to differences in the properties of the Pi translocator or because the CAM form is able to control cytosolic Pi levels and exclude Pi from the site of the Pi translocator. Information from other sources suggests that the properties of the Pi translocator may differ. Chloroplasts isolated from CAM *M. crystallinum* required less Pi for optimum O_2 evolution than those isolated from C_3 *M. crystallinum* (Monson, Rumpho and Edwards, 1983). O_2 evolution was also less perturbed by a high external Pi concentration in CAM *M. crystallinum*. Additionally, chloroplasts of the CAM plant *Sedum praealtum* L. have been found to be insensitive to a high level of external Pi (Piazza, Smith and Gibbs, 1982). Differences in the properties of the Pi translocator between the types of photosynthesis could therefore contribute to the differences in carbohydrate partitioning between C_3 and CAM *M. crystallinum*. This could also explain the anomaly of higher levels of cellular Pi in CAM *M. crystallinum*, where there is more bias towards starch synthesis than in C_3 *M. crystallinum* (Fig. 8). However, since the precise location of Pi within the cell is unknown and compartmentation of Pi within the cell away from the Pi translocator could equally explain this anomaly, one must remain cautious.

It is possible that regulation of cytosolic Pi levels in *M. crystallinum* cells may control the activity of the Pi translocator. The level of free Pi within *M. crystallinum* leaf tissue rose during the induction of CAM (Fig. 8) and fell during the deinduction of CAM

(Fig. 14). During CAM induction the accumulation of Pi within the leaves must be due to the uptake of Pi from the soil since a higher level of Pi accumulated in plants grown at the highest concentration of exogenous Pi (Table 20). This agrees with work on soybean leaves where changes in Pi levels were governed principally by the availability of phosphorus in the root environment (Mondal, Brun and Brenner, 1978). However, there is no evidence from other sources that the rapid depression of leaf Pi levels during the deinduction of CAM (Fig. 14) is a result of export of Pi from the leaf tissue. Instead the rapid decline may be due to the sequestration of Pi in the cell. The phosphorylation and dephosphorylation of a Pi-sequestering compound may quickly control the level of free Pi. The mechanism could be similar to the phosphorylation and dephosphorylation by protein kinase of enzymes in plant tissue which is responsible for variations in their activities (Preiss, 1987). Sorbitol was present in CAM *M. crystallinum* leaf tissue, usually at a more elevated level than in C_3 *M. crystallinum* (Table 12), and the sequestration of Pi could be carried out by sorbitol or by a compound with similar Pi-sequestering properties. Phytin (phytic acid), a mixed potassium, magnesium and calcium salt of (*myo*)inositol hexaphosphoric acid is found widely in mature seeds as the primary phosphorus reserve (Lott, 1984). In *Pisum sativum* L. seeds phytin can release 50% or more of the Pi required for growth (Guardiola and Sutcliffe, 1971; Ferguson and Bollard, 1976). It is possible that free Pi levels in *M. crystallinum* leaves could be regulated by the controlled release and sequestration of Pi by a Pi-storing compound. This in turn could influence carbohydrate partitioning.

Free Pi concentrations may additionally be controlled by compartmentation within the cell. The long term Pi concentration of the cytosol is rigidly conserved at the expense of the vacuolar pool

(Rebeille, Bligny, Martin and Douce, 1983). However, short-term changes in the cytosolic pool size may occur due to the slowness of Pi transport across the tonoplast in comparison to the movement of Pi across other cellular membranes such as the plasmalemma (Rebeille, Bligny and Douce, 1982) and the chloroplast envelope (Heldt and Rapley, 1970). The triggering of phosphofructokinase activity in tomato fruit cells accompanying a respiratory burst might be due to increased Pi levels in the cytoplasm resulting from changes in the flux of Pi across the tonoplast and plasmalemma (Chalmers and Rowan, 1971; Woodrow and Rowan, 1979). Evidence for the control of carbohydrate partitioning by a regulated compartmentation of Pi within the cell comes from work on *Sedum* (Black, Carnal and Kenyon, 1982). *Sedum* vacuoles were found to contain a large vacuolar pool of Pi which moved in and out with time opposite to the direction of malate movement. Presumably, this would ensure a high cytosolic Pi concentration at dawn which would then decrease throughout the day. Such a mechanism operating in CAM *M. crystallinum* could explain the large rise in the ratio of starch to soluble carbohydrate (St:TSC, Table 3) during the day in comparison to C_3 *M. crystallinum*. There is a change in the vacuolar development of *M. crystallinum* cells when CAM is induced (von Willert and Kramer, 1972): vacuolar-like spaces formed under the chloroplasts between the plasmalemma and cell wall together with a large number of vesicles and membranes in the central vacuole, at least some of which were in connection with the cytoplasm and chloroplasts. Such a change could facilitate the compartmentation of Pi within cells of CAM *M. crystallinum*.

It is clear from Table 22 that the response of leaf-discs to Pi and lack of Pi is different between day and night. It may be that the properties of the mechanisms responsible for the control of cellular Pi levels change on a diel basis. Black *et al.* (1982) have

already provided evidence for diel fluctuations of Pi levels facilitated by compartmentation. The sequestration of Pi by a putative Pi-sequestering compound and the properties of the Pi translocator may also change on a diel basis.

However, the precise mechanism by which Pi influences carbohydrate partitioning *in vivo* will remain unclear until the exact location of Pi within the cell whether as free Pi or as phosphorylated compounds is known.

Contributions to differences in carbohydrate partitioning between C_3 and CAM *M. crystallinum* could also be attributed to a modulation of the properties of the key enzymes involved in carbohydrate metabolism. Evidence presented here suggests that the properties of the key enzyme fructose 1,6 bisphosphatase may differ between C_3 and CAM *M. crystallinum*. Measurements of its substrate, fructose 1,6 bisphosphate and of its product fructose 6 phosphate (Table 3 and 12), showed that levels of the former were higher in CAM *M. crystallinum* and that levels of the latter were higher in C_3 *M. crystallinum*. This may indicate that the flow of substrate through fructose 1,6 bisphosphatase which is towards sucrose synthesis and away from starch synthesis, was greater in C_3 *M. crystallinum*. This was later confirmed by an analysis of the properties of the enzyme extracted from *M. crystallinum* (Keiller, Paul and Cockburn, 1987). Fructose 1,6 bisphosphatase had a five-fold greater affinity for its substrate in C_3 *M. crystallinum* than in the CAM form and was less sensitive to inhibition by fructose 2,6 bisphosphate and more sensitive to stimulation by Pi. Thus a further mechanism that could contribute to differences in carbohydrate partitioning between C_3 and CAM *M. crystallinum* appears to involve this enzyme. Fructose 1,6 bisphosphatase in the C_4 plant *Zea mays* also has a lower substrate affinity especially in the presence of fructose 2,6 bisphosphate,

than the enzyme extracted from C_3 species (Stitt and Heldt, 1985). This allows the synthesis of sucrose in the mesophyll to be reconciled with the maintenance of high concentrations of triose phosphate needed to provide a large enough diffusion gradient to allow rapid movement of triose phosphate back into the bundle-sheath.

The close co-ordination of fructose 1,6 bisphosphatase and the other key enzyme of sucrose synthesis, sucrose phosphate synthetase, may mean that their activities change together during the induction of CAM. Different levels of sucrose phosphate synthetase activity are a source of interspecific differences in carbohydrate partitioning in C_3 plants (Huber, 1983). Additionally, an alteration of the properties of ADP glucose pyrophosphorylase, the key regulatory enzyme of starch synthesis, may occur during CAM induction facilitating preferential starch synthesis in CAM *M. crystallinum*. No evidence has been found that differences in levels of fructose 2,6 bisphosphate account for differences in carbohydrate partitioning between C_3 and CAM *M. crystallinum* (Keiller *et al.*, 1987). It may be that a difference in the sensitivity of enzymes to effectors like fructose 2,6 bisphosphate is more important than absolute changes in levels of effector. However, the interpretation of fructose 2,6 bisphosphate levels requires caution. One might expect fructose 2,6 bisphosphate levels to be higher in CAM *M. crystallinum* because of the resultant inhibition of fructose 1,6 bisphosphatase and suppression of carbon flow towards sucrose. But, there is a tendency for fructose 2,6 bisphosphate levels to be lower in CAM plants that store starch (Fahrendorf *et al.*, 1987). This is because starch formers produce high cytoplasmic 3PGA and triose phosphate levels during deacidification which causes the retention of assimilate in the chloroplast for starch synthesis, and which also inhibits

fructose 6 phosphate dikinase the enzyme responsible for fructose 2,6 bisphosphate synthesis.

Thus, evidence is presented that reveals differences in the properties of mechanisms regulating carbohydrate synthesis and partitioning between C_3 and CAM *M. crystallinum*. The involvement of Pi is almost certain, but whether Pi levels are regulated by compartmentation or sequestration and whether the properties of the Pi translocator change is less clear. Differences in the properties of fructose 1,6 bisphosphatase are implicated. The picture is far from complete and requires a systematic survey of the possibilities discussed.

Biomass Production

In conditions of moderate and high exogenous Pi (0.5 mM and 3 mM Pi) the dry weights of the above ground biomass in C_3 and CAM *M. crystallinum* were similar (Table 21). However, if one excludes pinitol (about 10% of dry weight) and inorganic ions (probably of the same order) then dry weights were higher in C_3 *M. crystallinum*. Additionally, the saturation of carbohydrate accumulation at low photon fluence rates in CAM *M. crystallinum* and the lack of saturation of accumulation in C_3 *M. crystallinum* (Fig. 5 and Table 7); and greater carbohydrate accumulation during a 12 hour photoperiod in C_3 *M. crystallinum* (Table 2) indicate greater productivity in the C_3 form. This then is in accordance with the image of CAM plants as being unproductive and slow growing (Winter, 1985). Demmig and Winter (1983) have already found that chloroplasts isolated from C_3 *M. crystallinum* have higher rates of photosynthesis than those isolated from CAM *M. crystallinum*.

If the preferential partitioning of carbon into starch in CAM *M. crystallinum* is facilitated by a low level of Pi in the vicinity of the chloroplast envelope, then this may also lead to a decreased rate of photosynthesis. A low availability of Pi may limit ATP synthesis (Walker, 1976) and low Pi will reduce the flow of carbon through the Calvin cycle, by reducing the amounts of Calvin cycle enzymes (Geiger, 1987), and the activation of ribulose bisphosphate carboxylase-oxygenase (RUBISCO) (Dietz and Foyer, 1986). Low Pi also inhibits photosynthetic electron transport (Geiger, 1987). Sucrose synthesis is related to the rate of photosynthesis (Stitt, Gerhardt, Wilke and Heldt, 1987), but starch synthesis is less closely related to the rate of photosynthesis and is altered in a goal-directed manner dependent on the requirement for starch (Chatterton and

Silvius, 1979). This may mean that the accumulation of starch in preference to soluble carbohydrate in CAM *M. crystallinum* and its more loose association with the rate of photosynthesis in itself accounts for lower productivity and that starch accumulation for PEP synthesis takes precedence over biomass production. If this is so, one might expect CAM plants that store carbon extrachloroplastically to be more productive than ones that store starch. The CAM plant *A. comosus* (pineapple) stores carbon extrachloroplastically and has been shown to sustain an annual productivity over 2 years of 32 tons of dry matter ha^{-1} $year^{-1}$ (Bartholomew and Kadzimin, 1977), which is similar to the annual production of many C_3 and C_4 crops (Osmond, Winter and Ziegler, 1982). However, a comprehensive survey of CAM plants needs to be carried out before any assertions can be made.

Other processes may also account for the low productivity of CAM *M. crystallinum*. The provision of the enzymes necessary for CAM during its induction and the energetically expensive movement of malic acid in the vacuole (Jochem, 1986) means that energy is diverted away from growth. Acquisition of carbon from the atmosphere may be limited during periods of more extreme drought stress not only by stomatal closure but also by a limitation imposed on electron transport and phosphorylation which are inhibited by leaf water deficits (Kaiser, 1987). Water stress also limits RUBISCO activity (O'Toole, Crookston, Treharne and Ozbun, 1976) and the activities of other enzymes of the Calvin cycle (Plaut, 1971). During less extreme conditions carbon acquisition may be limited by the finite capacity of the vacuole to store malic acid. One may conclude that water conservation and survival rather than productivity are the overriding features of CAM, and clearly one of the costs of occupying an arid habitat and adopting water conservation strategies is a fairly slow growth rate.

Carbohydrate accumulation and growth was further restricted in CAM *M. crystallinum* by the relatively low photon fluence rate of 250 μmol m^{-2} s^{-1} at which deacidification saturated (Table 10). This was different to the situation in *K. daigremontiana* where decarboxylation of malic acid was not saturated at 1000 μmol m^{-2} s^{-1} (Table 11). As a result, carbohydrate accumulation in *K. daigremontiana* was not restricted by the decarboxylation of malic acid. Barrow and Cockburn (1982) found that saturation of deacidification in *K. daigremontiana* occurred at just above 1000 μmol m^{-2} s^{-1}. One might expect carbohydrate accumulation to saturate at or below this point since the internal generation of CO_2 in CAM plants during the day should always be greater than CO_2 consumption if CO_2 levels are to remain high enough to keep the stomata shut.

The rate of deacidification has been found to be irradiance-dependent (Kluge and Ting, 1978) and most evidence indicates that the saturation of CAM even at full sunlight does not occur (Nobel, 1982; Nobel and Hartsock, 1983). However, evidence supplied by Martin, Eades and Pitner (1986) shows that CAM in *Tillandsia usneoides* also saturates at photon fluence rates less than full sunlight. This demonstrated for the first time that a CAM plant did not require a high photon fluence rate and provided an explanation for the ability of *T. usneoides* to grow in the interior of tree canopies. *M. crystallinum* grows at high photon fluence rates in its natural environment (Winter, Luttge, Winter and Troughton, 1978). However, plants grown in growth cabinets received a maximum photon fluence rate of only 250 μmol m^{-2} s^{-1}. Growth under these conditions followed by a transfer to a higher photon fluence rate may require an acclimation period in CAM *M. crystallinum* to allow the adjustment of pigments and other components of the photosynthetic apparatus. Presumably this accumulation was not required by C_3 *M. crystallinum*

or *K. daigremontiana*. The induction of CAM in *M. crystallinum* resulted in a change in the ratio of chlorophyll a to b (Table 3). This is consistent with a rise in the ratio during drought stress in *Sedum sexangulare* (Tuba, 1984) which correlated with a higher proportion of pigment-proteins of photosystem I than pigment-proteins of photosystem II. Changes of photosynthetic pigments as an effect of drought can be considered to be an adaptive manifestation of the photosynthetic apparatus, similar to the adaptation of the pigment composition to different light conditions (Lichtenhaler, Kuhn, Prenzel, Buschmann and Meier, 1982). It may be associated with the saturation of photosynthesis and deacidification at 250 μmol m^{-2} s^{-1}.

Under conditions of zero exogenous Pi the dry weight and fresh weight of CAM *M. crystallinum* tissue were higher than those of C_3 *M. crystallinum* (Table 21). The accumulation of Na^+ and Cl^- ions led to the more negative water potential of CAM *M. crystallinum* leaf-discs (Table 23). This (Jones, Osmond and Turner, 1980), together with the accumulation of malic acid (Luttge, 1987), allows the osmotic acquisition of water and accounts for the greater succulence and fresh weight of CAM *M. crystallinum* under Pi-deficient conditions. Heun *et al.* (1981) found that the increase in ion levels of *M. crystallinum* leaf tissue when irrigated with salt water correlated with an increase in tissue dry weight. The accumulation of Na^+ and Cl^- ions in CAM *M. crystallinum* may account for some of the discrepancy in the dry weights of C_3 and CAM *M. crystallinum* tissue, but the difference was greater than could be accounted for by Na^+ and Cl^- uptake. Therefore, it appears that productivity in terms of dry matter gain is greater in CAM *M. crystallinum* than in C_3 *M. crystallinum* under conditions of Pi deficiency. It may be that these conditions are more compatible with the metabolism of CAM *M. crystallinum* and in particular with the preferential partitioning of

assimilate into starch. The tinges of red observed on the leaves of CAM *M. crystallinum* at zero Pi were due to the presence of betacyanins.

Dry weight as a percentage of fresh weight was high in the treatment where *M. crystallinum* plants were grown at different exogenous levels of Pi (Table 21). This may be because measurements were taken 47 days after the start of CAM induction by which time the plants possessed much lignified tissue. The higher values in CAM *M. crystallinum* were due to a higher dry weight at zero Pi than in C_3 *M. crystallinum*, but at 0.5 mM and 3 mM Pi this was due to lower fresh weights in CAM *M. crystallinum* due to the presence of tissue that was more desiccated.

Assuming that 1 mole of CO_2 is assimilated for every mole of malic acid decarboxylated, which is not necessarily so if the mitochondria are involved in the breakdown and subsequent CO_2 release from the remaining pyruvate, then the decarboxylation of 1.5 moles of malic acid should yield 1 mole of glucose or glucose equivalent. In *K. daigremontiana* the formation of 1 mole of glucose equivalent correlated with the decarboxylation of 1.3 moles of malic acid (Table 2). The slight discrepancy here may be due to the further release of CO_2 from pyruvate or due to fixation of CO_2 from the atmosphere during the day. In CAM *M. crystallinum* the formation of 1 mole of glucose equivalent correlated with the decarboxylation of 3.2 moles of malic acid. This is a shortfall in the amount of glucose equivalent produced. A loss of CO_2 because the stomata are not fully closed during the day is unlikely because the decarboxylation of malic acid in CAM *M. crystallinum* did not saturate at a photon fluence rate lower than that for carbohydrate accumulation (Tables 6, 7 and 10), and hence internal CO_2 levels are unlikely to have fallen to promote stomatal opening. The shortfall in the amount of glucose

equivalent produced in addition to the low productivity already associated with CAM could be due to the mode of CAM induction in *M. crystallinum* which may result in a further diversion of carbon away from growth processes.

The irrigation of *M. crystallinum* plants with salt water mediates the induction of CAM via an alteration in the water relations of the leaves. *M. crystallinum* is tolerant of high salinity because of the ability to accumulate and sequester high levels of Na^+ and Cl^- ions in the vacuole so that cellular metabolism is not inhibited (Wyn Jones and Storey, 1981). The irrigation of halophytes with salt water often stimulates growth (Greenway, 1968; Kaplan and Gale, 1972). However, a high concentration of NaCl (above 300 mM) may result in reduced growth (Wardlaw, 1967; Boyer, 1970).

The metabolic cost of growth in a highly saline environment may be great and may result in a considerable diversion of energy away from growth to maintenance processes (Penning de Vries, 1975) and reduction in productivity (Yeo, 1983). The metabolic cost is associated with the compartmentation of ions and the maintenance of membrane integrity (Rains, 1987). The higher levels of inositol observed in CAM *M. crystallinum* (Table 2) may be an indication of its release during membrane breakdown caused by water stress (D.W. Lawlor, Personal Communication). Additionally growth in saline conditions can result in a change in the pattern of dry weight allocation (Osmond *et al.*, 1982). A change in the shoot/root ratio is a common response to stress (Begg and Turner, 1976) and is assumed to be of adaptive value. However a decrease in the shoot/root ratio as often occurs under saline conditions represents a greater burden of heterotrophic tissue on the supply of photosynthates.

The osmotic pertubation caused by a high concentration of ions in the vacuole necessitates the accumulation of compatible solutes in

the cytoplasmic compartments. The synthesis of these osmotica diverts further energy away from growth towards maintenance processes. Evidence suggests that pinitol is a major compatible solute in *M. crystallinum* (Section 7). However the influence of putatively compatible solutes on integrated metabolic processes is unclear and could be a harmful one (Wyn Jones and Gorham, 1983). If NaCl leaks into the cytoplasmic compartments as it must do in its transit into the leaves growth will be further inhibitied by an NaCl-induced inhibition of PEP carboxylase (Von Willert, 1975b), of RUBISCO (Taleisnik, 1987), and possibly numerous other enzymes.

There is little doubt that the possession of CAM in *M. crystallinum* especially in a highly saline environment is detrimental to biomass production in comparison to well-watered C_3 *M. crystallinum* plants. However, the CAM strategy is able to maintain viable photosynthetic tissue throughout stress periods which other species survive only in the form of seeds or dormant reproductive structures. In *M. crystallinum* the induction of CAM allows net carbon gain and normal reproductive development to continue. The declining productivity associated with the switch to CAM is of little consequence and of no disadvantage in the prevailing conditions provided it remains sufficient for normal reproductive development to be completed. Indeed, in conditions of extreme aridity the productivity of CAM species outstrips that of C_3 or C_4 species. The possession of inducible CAM appears to be a distinct advantage in climates that fluctuate between drought and seasonal rainfall. It allows the plant to capitalise on the productivity of C_3 photosynthesis during periods of adequate moisture and also enables the plant to continue carbon gain through periods of drought via CAM photosynthesis. The merits of each mode of photosynthesis in its respective environment is emphasised by the rapid induction of CAM in

M. crystallinum during water stress and subsequent rapid deinduction of CAM upon the relief of water stress (Figs 9 and 15). The CAM plant *Agave deserti* switches to C_3-type gas exchange upon the relief of water stress (Hartsock and Nobel, 1976), even though it always possesses leaf structure and metabolic machinery associated with CAM. This emphasises the seeming advantage of the high productivity of C_3 photosynthesis during periods of adequate water supply. The retention of CAM leaf structure and metabolic machinery is an indication of the propitious nature of the CAM pathway in arid conditions. In the balance between productivity and survival CAM *M. crystallinum* plants are able to be both productive and survive by the integration of both modes of photosynthesis in a fluctuating environment. The productivity of the C_3 mode may be as necessary to the survival of inducible CAM plants as the endurability of the CAM mode.

Malic Acid, Pi and the Activity of CAM

A diagnostic expression of CAM activity is the accumulation of malic acid at night and its degradation during the day. The irrigation of *M. crystallinum* with 400 mM NaCl initiated nighttime malic acid synthesis (Fig. 9), and a diel fluctuation of malic acid (Figs 15 and 16). The cessation of salt treatment and the provision of a plentiful water supply dampened the ultimately abolished these oscillations (Figs 15 and 16). This induction of CAM is consistent with the increase in the amount and activity of PEP carboxylase protein paralleled by an increase in PEP carboxylase mRNA observed by Ostrem, Olson, Schmitt and Bohnert (1987). The increase in activity over a seven day induction period was 10-fold (Foster, Edwards and Winter, 1982). The deinduction of diel malic acid oscillations is consistent with a decline in PEP carboxylase activity, with a half-life of 2.5 days (Vernon, Ostrem, Schmitt and Bohnert, 1988). Levels of mRNA fell more rapidly with a 77% reduction over 2.5 days (Vernon *et al.*, 1988).

Thus, it appears that the establishment of diel malic acid rhythms is reversible. However, deinduction of CAM may not be simply a reversal of induction. The induction of nighttime malic acid accumulation took longer to establish than its deinduction (Figs 9 and 15). Nighttime malic acid accumulation during induction may be delayed whilst PEP carboxylase is being synthesised. However, during deinduction the more rapid fall in malic acid levels at 9:30 may be caused simply by declining PEP carboxylase activity. The higher levels of malic acid at 9:30 after deinduction compared to before induction may be due to PEP carboxylase remaining after deinduction. There was no evidence to suggest that these higher malic acid levels at 9:30 after deinduction were due to decreased deacidification

activity since malic acid levels at 15:00 were little different to those in induced plants (Fig. 15).

In addition to the induction of CAM by irrigation with 400 mM NaCl, CAM can also be induced in *M. crystallinum* by exposing the roots to low temperature or oxygen deficiency (Winter, 1974) and by irrigation with solutions of Na_2SO_4, KCl and K_2SO_4 (Winter, 1973a). PEP carboxylase protein, a major biochemical marker associated with CAM, can be induced with NaCl polyethylene glycol or drought (Bohnert, Ostrem, Cushman, Michalowski, Rickers, Meyer, deRocher, Vernon, Krueger, Vazquez-Moreno, Velten, Hoefner and Schmitt, 1988). This implies that regulation of the transition from C_3 to CAM is governed by a general mechanism responding to water stress and a more negative water potential in leaf tissue, and is not a specific response to salinity. However, CAM induction may be more specifically related to a lower solute potential caused by an increased concentration of ions due to uptake and cell dehydration. The observation that *M. crystallinum* plants irrigated with salt water following deinduction exhibited low levels of malic acid consumption (Fig. 16) was probably due to the hydrated nature of the soil following deinduction which prevented pronounced leaf dehydration. Von Willert, Treichel, Kirst and Curdts (1976b) found no increase in the activity of PEP carboxylase within 7 days of the reintroduction of 300 mM NaCl following the deinduction of CAM by saturating the soil with water.

The induction of CAM is conditioned by other factors. Leaf age affects nocturnal malic acid accumulation in *M. crystallinum* (Table 4). Older leaves of both the C_3 and CAM forms exhibited higher levels of malic acid at the start of a photoperiod and a greater diel fluctuation of malic acid. This trend is associated with an increase in PEP carboxylase activity in older leaves (Von Willert *et al.*,

1976b; Von Willert, Kirst, Treichel and Von Willert, 1976a). PEP carboxylase protein was present in young leaves (Von Willert *et al.*, 1976b), but young leaves lacked a third electrophoretic protein band with a high turnover rate which appeared crucial for activity. This phenomenon may be explained by a tendency for older leaves to wilt more readily (Winter, 1973b), and hence experience water stress more intensely than younger leaves. The translocation of water form old to young leaves during period of drought has been observed in cabbage (Catsky, 1962) and in *Kalanchoe daigremontiana* (Schafer and Luttge, 1987). This enables the continuing enlargement and growth of the young leaves (Schafer and Luttge, 1987). A concomitant of this may be greater CAM activity in older leaves. This theory is supported by the observation that artificial ageing induced by abscisic acid in *M. crystallinum* which incurred no water loss did not enhance CAM activity (Winter, 1975).

Ostrem *et al.* (1987a) have shown that the age of the *M. crystallinum* plant and not just the age of the tissue source within the plant influenced the induction of PEP carboxylase in response to salt stress. This apparent link between development and CAM induction in response to salt stress may be due to the influence of endogenous growth regulators. The elongation of axillary shoots may signal a change in the level of plant growth regulators (Phillips, 1975). The presence of ABA in leaf tissue is associated with maturation and senescence (Wareing and Phillips, 1981). Its accumulation in water-stressed leaves is also well-documented (Eamus, Fenton and Wilson, 1983), and water-stress induced CAM in *Portulacaria afra* is associated with an increase in tissue ABA (Raschke, 1975). Phytohormones such as ABA may have a direct effect on gene regulation (Theologis, 1986), and may be responsible for the induction of CAM in *M. crystallinum*. However, there is no evidence

that the application of ABA, indoleacetic acid, gibberellic acid or 5-benzyladenine to *M. crystallinum* leaves either induces CAM or inhibits CAM induction during salt stress (Bohnert *et al.*, 1988). Enhanced ethylene production is a response to water stress (Apelbaum and Yang, 1981). However, no evidence is presented here (Section 3) that ethylene is produced in response to water stress in *M. crystallinum* or that it might elicit the induction of CAM. The different lengths of time required for the induction of nighttime malic acid accumulation (Figs 9 and 15), taking 15 and over 32 days respectively for maximal malic acid accumulation may be due to slight differences in plant age on induction and hence differences in levels of endogenous plant growth regulators. Ostrem *et al.* (1987a) found no CAM induction when *M. crystallinum* plants were irrigated with NaCl solution during the fourth and fifth week after germination, but PEP carboxylase activity increased within 2 to 3 days when plants were salt stressed during the sixth week after germination. Pinitol and Pi associated with CAM activity in *M. crystallinum* also increased with increasing plant age in C_3 *M. crystallinum* (Figs 7 and 8). It could be argued that the induction of CAM in *M. crystallinum* by irrigation with NaCl is just an acceleration of the normal developmental process in this species. In its natural environment, the induction of CAM is associated with increasing plant age (Winter *et al.*, 1978). However, water availability decreases during the growing season, and the reversibility of CAM induction implies that water stress rather than plant development is the overriding initiator of CAM. Nevertheless, plant age and development may predispose *M. crystallinum* to CAM induction.

Von Willert, Thomas, Lobin and Curdts (1977) have proposed that an excess of anions like Cl^- already present in young leaves of *M. crystallinum* may restrict the accumulation of other anions like

malate. This theory receives some support from the finding that *M. crystallinum* plants, particularly the C_3 form, grown at high levels of Pi (PO_4^{3-}) contained less malate than those grown at low Pi (Figs 27, 28 and 29). Indeed the behaviour of the C_3 form at 0 Pi with no water stress treatment in terms of the diel malic acid fluctuation is CAM-like (Table 19). This is seemingly in contradiction with the induction of CAM activity in response to water stress and the observation that endogenous Pi levels correlate with CAM activity. The induction, deinduction and reinduction of CAM resulted in free cellular Pi levels proportional to the degree of diel malic acid fluctuation (Figs 8 and 9, and 14, 15 and 16). Indeed, the attempted reinduction of CAM which resulted in little increase in the level of diel malic acid fluctuation (Fig. 16), also resulted in little increase in amounts of Pi (Fig. 14). Yet, growth of *M. crystallinum* plants at 0 Pi which also resulted in low endogenous Pi (Table 20) correlated with nighttime malic acid accumulation and diel malic acid oscillations. The induction of CAM removed any difference in malic acid rhythms between the treatments, but overall levels of malic acid were still higher at 0 Pi (Figs 27, 28 and 29, and Table 19). Wong, Cowan and Farquhar (1985) have shown that plants grown at low Pi show a reduced stomatal conductance. It has been proposed that a reduced CO_2 supply in the light caused by a lower stomatal conductance brought about by water stress, rather than water stress itself could trigger the induction of CAM in *M. crystallinum* (Winter, 1979b). However, the growth of *M. crystallinum* plants under different CO_2 regimes showed this not to be the case (Winter, 1979b). Instead, the elevated malic acid levels in C_3 *M. crystallinum* plants grown at 0 Pi may demonstrate an additional role of malic acid. When there is a deficit of anions available in the soil malate may be synthesised to compensate for this anion deficiency within the cell. This function

is performed by malate in stomata, where it balances at least some of the K^+ transported into the guard cells during stomatal opening (Allaway, 1973; Travis and Mansfield, 1977). The behaviour of malate in this way suggests that normally this role would be performed by Pi. In the Aizoaceae at least, PO_4^{3-} may make a significant contribution to charge balancing within the cell (Von Willert *et al.*, 1977). In *Sedum* (Crassulaceae), Pi and malate move in opposite directions across the tonoplast (Black *et al.*, 1982).

Pi may also have an osmotic role. The involvement of Pi in osmoregulation has been suggested by Van Steveninck, Van Steveninck and Lauchli (1982). Its presence in the cytosol could provide significant osmotic adjustment to the presence of NaCl in the vacuole. The chloroplast envelope Pi-translocator in CAM *M. crystallinum* has been shown to be less sensitive to a high Pi concentration than that of the C_3 form (Monson *et al.*, 1983). The maintenance of a high cytosolic Pi concentration is therefore a possibility, affording osmotic stability without impairing metabolism.

This still does not, however, explain the greater diel fluctuations at 0 Pi in C_3 *M. crystallinum*. Owing to the possibility of the carbohydrate metabolism of C_3 *M. crystallinum* being more sensitive to perturbation by Pi than that of *M. crystallinum*, 0 Pi may induce sufficiently high starch levels to facilitate the provision of PEP and CAM activity. Low concentrations of Pi may, like plant development, predispose *M. crystallinum* to CAM. The high endogenous Pi levels associated with CAM activity may be compartmentalised away from carbohydrate metabolism and may be involved in water relations and ionic balancing. Von Willert (1975a) suggested that Pi may promote malate accumulation by a stimulation of the activity of PEP carboxylase. Pi-induced stimulation of PEP

carboxylase has been observed *in vitro* (Wong and Davies, 1973). It is also known that Pi may reverse the malate induced inhibition of PEP carboxylase (Von Willert, 1975b). It is tempting to suggest that the sequestration of Pi away from carbohydrate partitioning and its concentration in specific compartments due to uptake and tissue dehydration may be involved in the initiation of CAM activity.

In CAM *M. crystallinum* malate functions as a storage metabolite in which CO_2 is retained between the 2 temporally separated carboxylation reactions. Additionally, nocturnal malic acid accumulation drives significant osmotic acquisition of water (Luttge, 1986). An osmotic water flow accompanying malic acid accumulation is the basis of the model of Luttge, Kluge and Ball (1975) which proposes that malate levels in CAM are regulated by osmotic gradients. Results here on the effect of the solute potential of the incubation medium surrounding leaf-discs on malic acid levels (Table 23) confirm the effect of osmotic gradients on malic acid levels. Malic acid accumulation at night only occurred in tissue where the difference in solute potential of the medium and water potential of the leaf-discs was low. The model of Luttge *et al.* (1975) proposes that the change from a net malic acid influx into the vacuole to a net efflux is facilitated by an increase in turgor due to an osmotic water flow accompanying malic acid accumulation in the vacuoles. At a critical turgor pressure the properties of the tonoplast change in an "all or none" reaction so that passive loss of malic acid from the vacuoles becomes dominating. The hypothesis is supported by the property of membranes to respond to minimal pressure differences causing a change in thickness of a few Angstroms with the consequence of dramatic changes in membrane functions. The occurrence of a change between a net influx and a net efflux in a very narrow range of turgor pressure has been demonstrated in investigations of K^+

fluxes in *Valonia utricularis* (Zimmerman and Steudle, 1974). The model of Luttge *et al.* (1975) describes a mechanism for malic acid oscillation; it offers no explanation for the control or initiation of this phenomenon.

To sum up: malic acid and Pi appear to have multiple functions. Both may participate in water relations and ion balancing. Additionally, Pi may be involved in the initiation of CAM induction. An intruiging challenge lies in tracing the pathway through which the initial perception of water stress and the response and induction of CAM are linked.

Pinitol and Water Relations in *M. crystallinum*

During the induction of CAM in *M. crystallinum* a compound, not present before induction, began to accumulate to high levels (Table 12 and Fig. 7). By day 21 of the induction period there were 10.5 μmols g^{-1} F.W. of this compound, at least twice the level of combined soluble carbohydrate, and representing 9.7% of dry weight. During the deinduction of CAM the level of this compound although less than in CAM *M. crystallinum* remained higher than before CAM induction (Fig. 13).

Analysis of a leaf tissue extract of CAM *M. crystallinum* demonstrated that the compound was uncharged (Section 7). Observation of the peaks produced by gas chromatographic analysis of leaf extract showed that the compound had a relative retention time (RRT) very close to that of fructose. Very close elution times of fructose and pinitol have been noted by Riggs and Strong (1967). In CAM *M. crystallinum* the RRT of fructose was 0.84, and of the unknown compound 0.86 (Table 1). This compares with 0.85 for pinitol from *Pinus sylvestris*, separated using a 1% SE52 column (Drew, 1978). The retention time of the compound relative to (*myo*)inositol was 0.61 which compares with 0.64 obtained by Loewus (1966). On the basis of gas chromatography it was postulated that the compound was pinitol. Pinitol is known to be present in *Lotus corniculatus* leaf tissue (Riggs and Strong, 1967) and comparisons of the leaf tissue extracts of CAM *M. crystallinum* and *L. corniculatus* were made. Analysis of the extracts from both species by gas chromatography and HPLC revealed that the putative pinitol peak in CAM *M. crystallinum* had an identical RRT to one in *L. corniculatus*.

The demethylation of a CAM *M. crystallinum* leaf tissue extract resulted in the disappearance of pinitol and a higher level of

inositol (Section 7). This demonstrated that the compound could be a methylated form of inositol. The pathway of pinitol synthesis has been elucidated independently in Gymnosperms, the Leguminosae and Asclepiadaceae (Kindl and Hoffmann-Ostenhof, 1966). The reaction sequence was found to proceed by the methylation of inositol with subsequent epimerisation of the resulting sequoyitol to yield pinitol (Fig. 30). In *Simmondsia chinensis* (jojoba), inositol was found to be first converted to ononitol which was subsequently converted to pinitol (Dittrich and Korak, 1984). Inositol is synthesised via the cyclisation of glucose (Fig. 30). This pathway of inositol synthesis is widespread in plants (Kindl and Hoffmann-Ostenhof, 1964; Loewus and Loewus, 1971). The correlation between the fall in hexose levels during induction and depressed levels of hexose in CAM *M. crystallinum* compared to C_3 *M. crystallinum* (Table 12), is consistent with the glucose-utilising pathway of pinitol synthesis. The identity of pinitol in CAM *M. crystallinum* and its route of synthesis were further confirmed by the finding of greater inositol phosphate synthesising capacity in CAM *M. crystallinum* than in the C_3 form (Section 7). The greater activity of glucose 6 phosphate cycloaldolase may indicate that the synthesis of inositol phosphate is a key control point in the pathway of pinitol synthesis. However, one must remain cautious because glucose 6 phosphate cycloaldolase may simply be easier to extract from the CAM form. Possible identity as pinitol was made more plausible by its presence in another member of the Aizoaceae: *Tetragonia expansa* (Plouvier, 1954), and the observation that the identity of putative compatible solutes conforms to taxonomic grouping (Wyn Jones and Gorham, 1983).

Pinitol, 1 D-3-O-methyl-chiro-inositol (Fig. 31), is a member of the cyclitol group of compounds. These are relatively rare compounds in plants (Ford, 1982). D-pinitol is one of the most abundant

Figure 30. The pathway of pinitol synthesis (Kindl and Hoffman-Ostenhof, 1966; Dittrich and Korak, 1984).

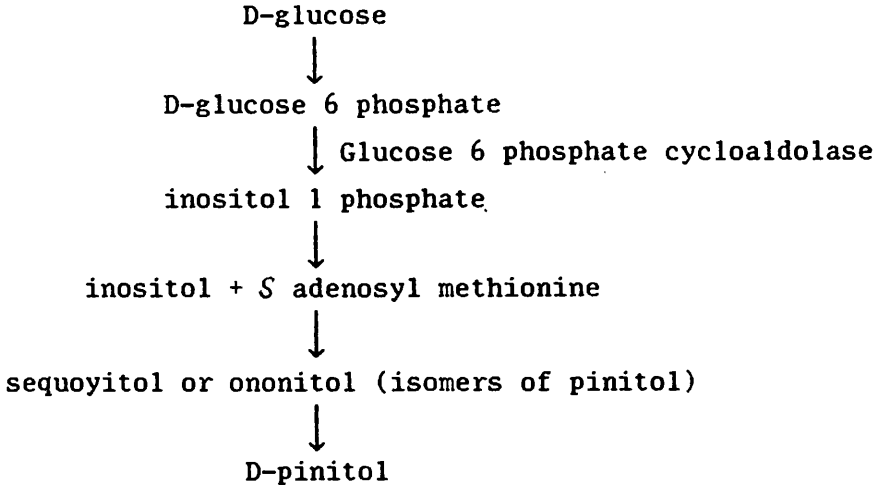
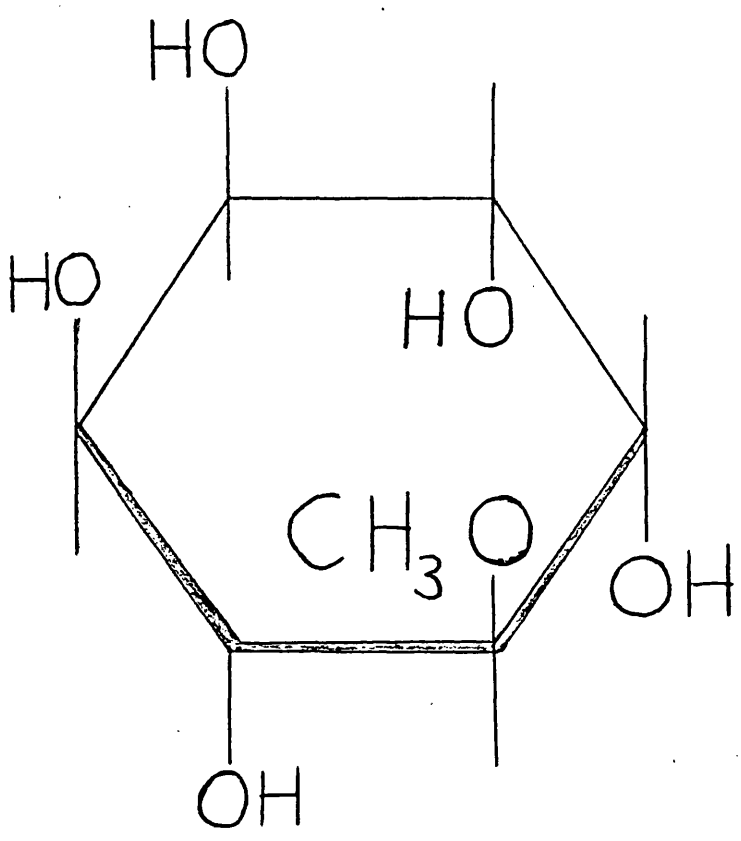

D-glucose
D-glucose 6 phosphate
Glucose 6 phosphate cycloaldolase
inositol 1 phosphate
inositol + S adenosyl methionine
sequoyitol or ononitol (isomers of pinitol)
D-pinitol

Figure 31. The structure of D-pinitol (1D-3-O-methyl-chiro-inositol)


HO
HO
HO
CH3O
OH
OH

cyclitols (Dittrich and Korak, 1984). Plouvier (1963) recorded pinitol in 6 families of Gymnosperms and 15 families of Angiosperms. Its isolation from many different types of plants, both woody and herbaceous and from plants of various geographical localities is seen as almost unique in chemical plant taxonomy (Plouvier, 1963). It is present in the CAM species *Hoya carnosa* (Asclepiadaceae) (Dittrich, Gietl and Kandler, 1971) and amongst species of the CAM family Euphorbiaceae (Yanagita, 1943).

There is a paucity of information on the physiological function of pinitol. There is no evidence to substantiate the proposal of Diamantoglou (1974) that pinitol serves as a storage carbohydrate: no information exists on the reactions that would form glucose 6 phosphate from pinitol. Indeed, the maintenance of pinitol levels during CAM deinduction (Fig. 13) and during dark starvation (Table 24) may indicate that pinitol is not easily metabolised, further refuting this hypothesis. Similar findings in response to dark starvation in *Trifolium repens* (Smith and Phillips, 1982) may indicate that pinitol is metabolically inert in plant tissue. The function of pinitol as a precursor of uronic acid or pentose residues in cell wall polysaccharides which contain O-methyl ethers or esters (Loewus, 1971; Karr, 1976) has not been confirmed. No pinitol accumulated in response to overnight chilling (Table 26). This indicates that pinitol accumulation is not a more general response to stress, but may be specific to water stress.

Pinitol and a number of other O-methyl inositols occur in many legumes (Smith and Phillips, 1980), and in *Macroptilium atropurpureum* (Ford and Wilson, 1981) and *Cajanus cajan* (Ford, 1982) pinitol increased markedly during drought. In the latter, pinitol increased from 1% to 5.3% of dry weight. In the salt-tolerant legumes *Sesbania aculeata* (Gorham, McDonnell and Wyn Jones, 1984) and *Sesbania*

bispinosa (Gorham, Tomar and Wyn Jones, 1988), pinitol accumulated when subjected to NaCl treatment. Pinitol also accumulated in *Honkenya peploides* (Caryophyllaceae) when irrigated with NaCl solution (Gorham, Hughes and Wyn Jones, 1981). Schobert (1977) suggested that the accumulation of low molecular weight carbohydrates (like pinitol) in plants under water stress may serve to increase the water-binding capacity of the cell walls. Wyn Jones (1981) and Ford (1982) have proposed that methyl inositols including pinitol could act as compatible solutes contributing to osmotic adjustment and hence plant survival during periods of water stress.

Halophytes absorb large quantities of ions into their leaves when exposed to high exogenous ion concentrations (Greenway, 1973). *M. crystallinum* plants grown at 400 mM NaCl had leaf sap concentrations of 500 mM Na^+ and 400 mM Cl^- (Demmig and Winter, 1986). This accumulation of ions in the vacuole away from metabolic processes that they could inhibit, allows water uptake and growth to continue in a soil environment with a low water potential (Jones *et al.*, 1980). Compatible solutes are thought to accumulate in the cytoplasmic compartments of halophytes to counteract the osmotic perturbations caused by a high vacuolar concentration of inorganic ions (Wyn Jones, Storey, Leigh, Ahmad and Pollard, 1977). Betaines and sugar compounds, putative compatible solutes in some species (Wyn Jones and Gorham, 1983), are considered to be of minor importance in the osmotic adjustment of *M. crystallinum* to 400 mM NaCl (Heun *et al.*, 1981). Proline levels have been found to increase five-fold in *M. crystallinum* plants irrigated with 500 mM NaCl (Ostrem, Vernon, Olson and Bohnert, 1987b). While proline may function as a compatible solute in the cytosol, it does not seem to have a major role in osmoregulation in the chloroplasts of *M. crystallinum* during salt-stress (Demmig and Winter, 1986). If pinitol is part of an

osmotic response in *M. crystallinum* to salt-stress, then it must be compartmentalised in the cytoplasm since amounts of pinitol measured in leaf tissue could only make a significant contribution to solute potential if concentrated in a relatively small volume. Spread evenly throughout the leaf, the concentration of pinitol would be about 10 mM. The accumulation of pinitol in CAM *M. crystallinum* is combined with low levels of fructose and glucose (Table 12). In C_3 *M. crystallinum* fructose and glucose levels are high, totalling 31.7 μmol g^{-1} F.W. 21 days into the induction period, compared to 10.5 μmol g^{-1} F.W. of pinitol in the CAM form (Table 12). If the replacement of glucose and fructose with pinitol fulfils an osmotic role then its compartmentation must be different to that of glucose and fructose in the C_3 form; otherwise a lower level of pinitol compared to glucose and fructose would provide less osmotic adjustment. Glucose was present in the vacuoles of C_3 *M. crystallinum* although quantification of it was not possible. Pinitol was absent from CAM *M. crystallinum* vacuoles and present in the chloroplasts at a probable concentration of about 230 mM, and in the cytosol at a probable concentration of about 100 mM (Section 7). At these concentrations pinitol could provide considerable osmotic adjustment to the accumulation of Na^+ and Cl^- in the vacuole. It could especially partake in the obvious osmotic adjustment observed in CAM *M. crystallinum* chloroplasts (Demmig and Winter, 1986), supplementing chloroplast levels of 160-230 mM Na^+ and 40-60 mM Cl^-. Chloroplastic osmotic adjustment enables maintenance of chloroplast volume in conditions of salt stress. The rate of photosynthesis is very sensitive to changes in chloroplast volume, being decreased both by shrinkage and swelling of the chloroplast (Robinson, 1985). A clear parallel exists between the accumulation of pinitol in *M. crystallinum* and the accumulation of glycinebetaine in salt-stressed

spinach (Robinson and Jones, 1986). Here chloroplastic glycinebetaine contributed 30-40% of the total leaf glycinebetaine comparable to the chloroplastic contribution of 37±5.2% of total leaf pinitol in CAM *M. crystallinum*.

Pinitol accumulated more readily in response to salt-stress than in response to the withholding of water from *M. crystallinum* plants (Table 25). This may be because treatment with salt causes more pronounced osmotic perturbation. Indeed, the level of malic acid in plants with all water withheld, was low (Table 25), perhaps indicating that the water stress was not great enough to induce appreciable CAM activity. A more lengthy drought period may have induced greater CAM activity and a higher level of pinitol. It is likely that pinitol is not causally related to CAM, but rather to the presence of high cellular Na^+ and Cl^- concentrations. The deinduction of CAM which would have resulted in the removal of most NaCl from the soil, but not immediately from the leaves, resulted in only a small decline in the level of pinitol in the leaves, although the difference in levels of pinitol between CAM and deinducing plants was quite high (Fig. 13). The increase in pinitol levels in plants already induced into CAM activity is probably due to renewed irrigation of these plants with 400 mM NaCl after a prolonged period of drought between successive salt waterings. It is very tempting therefore, to pronounce that pinitol functions as a compatible solute in *M. crystallinum*. However, affirmation of this requires the determination of its effects on metabolic processes to ensure that it is compatible, and to refute the notion that its production is the result of an impairment of metabolism or cell damage.

Other compounds may participate in osmotic adjustment in CAM *M. crystallinum*. Levels of sorbitol were found to be consistently higher in CAM *M. crystallinum* than in the C_3 form where it was often

absent (Tables 3 and 12). Polyols like sorbitol have often been implicated as compatible solutes (Ahmad, Larher and Stewart, 1979; Jefferies, Rudmik and Dillon, 1979). However, at the low levels found in CAM *M. crystallinum* it is unlikely that by itself sorbitol has any appreciable osmotic effect. Schobert (1977) proposed that an increase in polyol levels in water-stressed plants does not confer osmotic adjustment, but that polyols could replace water molecules by means of their water-like OH groups, thus participating in the structure of biopolymers in the cell cytoplasm. Levels of inositol and sucrose also tended to be higher in CAM *M. crystallinum*. These may provide a little osmotic adjustment, although the presence of higher levels of inositol may indicate membrane breakdown caused by water stress (D.W. Lawlor, personal communication). Higher levels of sucrose may be due to an NaCl-induced inhibition of acid invertase (Hawker, 1980). Levels of total soluble carbohydrate were also higher in leaf-discs incubated in solutes with a more negative solute potential (Table 23). It appears that the accumulation of soluble compounds could be a fundamental response to water-stess. By themselves these compounds may offer little osmotic adjustment. However, put together with proline and pinitol, osmotic adjustment in the cytosol may be substantial. Pinitol and Na^+ and Cl^- ions may make a significant contribution to osmotic adjustment in the chloroplasts of CAM *M. crystallinum*.

In halophytes, exposure to NaCl often results in cell expansion (Greenway, 1968; Kaplan and Gale, 1972). This is a consequence of increased water uptake brought about by a raised leaf ion content. The succulent nature of many halophytes is a means of diluting the high salt concentrations within the cells (Winter, 1979a). The decrease in the dry weight of 50 leaf-discs during CAM induction and the rise in fresh weight (Table 13), is a consequence of cell

expansion and greater leaf succulence. During the deinduction of CAM, leaves appeared brighter green. This could be due to cells which expanded during CAM induction filling with water. The increased availability of water during deinduction would facilitate this. The brighter green appearance of leaves correlated with the reflectance and transmittance of more green light (Figs 17, 18, 19 and 20). The extra water present could be responsible for this occurrence. The very large increase in the fresh weight of excised leaves of CAM *M. crystallinum* (Fig. 23) and the paralleled increase in leaf reflectance and transmittance (Figs 21 and 22) tends to confirm this. The quicker change in leaf reflectance and leaf transmittance of younger leaves than older leaves during deinduction (Figs 17, 18, 19 and 20), could be due to the greater plasticity of younger tissue. During the induction of CAM the change in leaf reflectance and transmittance over the first 8 days of induction was negligible (Figs 11 and 12). It could be that differences in water contents of the tissue were not yet great enough to cause a difference in leaf spectral properties.

Osmotic adjustment in addition to CAM is a response to the irrigation of *M. crystallinum* plants with 400 mM NaCl. Pinitol may function as a compatible solute particularly in chloroplasts of CAM *M. crystallinum*. There appears to be no causal link between pinitol and CAM.

Résumé

The irrigation of *M. crystallinum* plants with 400 mM NaCl results in fundamental changes in the physiology of the plant.

The induction of CAM by this treatment necessitates the provision of PEP, the substrate for the nighttime fixation of CO_2 from the atmosphere. This dictates that during the day the accumulation of starch, the precursor of PEP, takes precedence over the accumulation of other carbohydrate. Preferential synthesis of starch is reflected in the mechanisms controlling carbohydrate partitioning. The flow of assimilate towards sucrose and away from starch synthesis via the key enzyme fructose 1,6 bisphosphatase is less favoured in CAM *M. crystallinum* than in the C_3 form. Starch accumulation in intact plants of the CAM form is less perturbed by a high exogeneous Pi level than in C_3 *M. crystallinum*. This implies possible differences in the properties of the Pi translocator between the two forms and, or that the CAM form is able to compartment or sequester Pi within the cell. The other key enzyme of sucrose synthesis, sucrose phosphate synthetase, and the key enzyme of starch synthesis, ADP glucose pyrophosphorylase, are also likely to play a central role.

The preferential partitioning of carbohydrate into starch may in itself lower productivity in CAM *M. crystallinum*. Additionally, the adoption of the CAM strategy and the prevalance of an arid and saline environment ensure the diversion of resources away from biomass production. However, this loss of productivity is of no disadvantage in the prevailing conditions, since the endurability of the CAM form enables carbon gain to continue through periods of drought. The C_3 strategy enables the plant to capitalise on the productivity of this mode of photosynthesis during periods of more plentiful water availability. The ready induction and deinduction of CAM emphasises

the versatility of *M. crystallinum*, and the merits of each mode of photosynthesis in contrasting conditions. This ensures productivity and persistence in a fluctuating environment.

The induction of CAM is initiated by a reduction in the water potential of the leaves, and deinduction by a reversal of this process. It is unclear how this is translated into the induction of CAM. The concentration and compartmentation of ions like Pi within the cell due to a reduction in water content of the leaves and due to increased ion uptake may be influential. Endogenous plant growth regulators may have an involvement, in addition to plant development and leaf age.

The accumulation and compartmentation of Na^+ and Cl^- in the cell during the irrigation of plants with 400 mM NaCl facilitates water uptake. It also necessitates the proportionate accumulation and compartmentation of compatible solutes. The putative compatible solute, pinitol, may play a central role in the osmotic adjustment of chloroplasts in *M. crystallinum*. The accumulation of pinitol, though apparently not directly related to CAM may, like CAM be viewed as an aspect of the adaptation of the plant to a reduction in water availability.

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ABSTRACT

Studies of crassulacean acid metabolism in *Mesembryanthemum crystallinum* and *Kalanchoe daigremontiana*

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Studies of crassulacean acid metabolism (CAM), were undertaken in *Kalanchoe daigremontiana* and in the inducible CAM plant *Mesembryanthemum crystallinum*. Irrigation of *M. crystallinum* plants with 400 mM NaCl initiated CAM activity; CAM was deinduced by saturating the cultivation soil with water.

Diel fluctuations and amounts of malic acid in *M. crystallinum* correlated with levels of endogenous inorganic phosphate (Pi). However, growth of *M. crystallinum* plants at varying concentrations of exogenous Pi resulted in an inverse relationship between these components. Indeed, diel fluctuations of malic acid in C_3 *M. crystallinum* grown at 0 Pi were CAM-like. Cellular compartmentation of Pi and multi-functional roles of Pi and malic acid may explain these phenomena.

The response of carbohydrate levels in *M. crystallinum* to photon fluence rate revealed that in conditions of restricted assimilate supply the CAM form partitioned carbohydrate preferentially as starch. Investigations of the control of this partitioning implicated differences in the properties of cytosolic fructose 1,6 bisphosphatase between C_3 and CAM *M. crystallinum*. Growth of *M. crystallinum* at varying exogenous Pi concentrations demonstrated less perturbation of starch accumulation at high Pi in the CAM form. The involvement of fructose 1,6 bisphosphatase and Pi in carbohydrate partitioning is discussed.

Dry matter data, carbohydrate levels and saturation of carbohydrate accumulation and malic acid decarboxylation at 250 μmol m^{-2} s^{-1} in CAM *M. crystallinum* indicated low productivity. Preferential partitioning of carbohydrate into starch, the adoption of CAM and growth in a saline environment may contribute to this.

CAM induction in *M. crystallinum* was accompanied by the accumulation of pinitol. Preparations of protoplasts, vacuoles and chloroplasts showed pinitol to be chloroplastic at a probable concentration of about 230 mM and cytosolic at about 100 mM. No pinitol was detected in vacuoles. Pinitol, a putative compatible solute, may contribute to osmotic adjustment particularly in chloroplasts in response to the cellular accumulation of NaCl. There was no indication of a causal link between pinitol and CAM.