SEASONAL CHANGES IN PHOTOSYNTHESIS IN THE LICHEN *PELTIGERA RUFESCENS*.

By

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Abstract

The seasonal response of net photosynthesis to temperature, light and moisture has been examined in two populations of the lichen Peltigera rufescens.

The sub-arctic population had a temperature optimum of 35°C, and no seasonal changes in the photosynthetic response were observed.

In contrast, the gas exchange rate did change on a seasonal basis in the second population studied (from a temperate climatic zone). In this population, the temperature optimum for net photosynthesis shifted from 25°C in the winter to 35°C in the summer. This seasonal change can be induced in the laboratory by storing the lichen under appropriate temperature and photoperiod conditions. This change was shown, with the use of photosynthetic-illumination curves, to be restricted to alterations in the light saturated rates of net photosynthesis only, indicating that the acclimation process resulted from a change in the dark reactions of photosynthesis.

This hypothesis was further investigated with studies on an important rate controlling enzyme of the Calvin cycle, fructose-1,6-bisphosphatase. This enzyme was purified from summer and winter collections of the temperate population of P. rufescens and the total activity, thermal stability, substrate affinity and

activation energy was compared in these isolates. It was shown that the temperature response of the Km for this enzyme closely paralleled the net photosynthetic response and that the activation energy was lower im the summer form of the enzyme. These kinetic differences largely explain the seasonal changes in net photosynthetic capacity observed in the temperate population and support the enzymatic theory of acclimation suggested by the photosynthetic-illumination curves.

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List of Symbols

α	phtosynthetic efficiency
APC	allophycocyanin
AQE	apparent quantum efficiency
calib coef	calibration coeficient
CO_2ase	carboxylase
chl	chlorophyll
DTT	dithiothreitol
E_a	activation energy
Eqn.	equation
FBP	fructose-1,6-bisphosphate
F6P	fructose-6-phosphate
Fr-2,6-P	fructose-2,6-bisphosphate
G6PDH	glucose-6-phosphate dehydrogenase
IRGA	infra-red gas analyser
I_k	light intensity that saturates photosynthesis
$I_{k/2}$	light intensity that half-saturates photosynthesis
K_m	Michaelis constant, substrate affinity
n	number of observations, replicates
P	product
PAR	photosynthetically active radiation
PC	phycocyanin
PE	phycoerythrin

List of Symbols (continued)

PEP	phospho-enol pyruvate
PGI	phosphoglucose isomerase
PGM	phosphoglucomutase
P-I	photosynthetic-illumination
Pmax	maximum rate of net photosynthesis
PSU	photosynthetic unit
PS I,II	photosystem I,II
PVPP	polyvinylpolypyrrolidone
N/D	night/day
OD	optical density
RuBP	ribulose-1,5-bisphosphate
s	substrate concentration
$S_{0.5}$	half-saturating concentration
SBPase	sedoheptulose-1,7-bisphosphatase
SE	standard error of the mean
T	temperature
v	velocity of the reaction
V,Vmax	maximun velocity of the reaction
wt	weight
µE	$\mu E \cdot m^{-2} s^{-1}$

1. General Introduction

Photosynthesis is the major source of energy input into a living ecosystem. It is a two stage process whereby the energy associated with the visible light spectrum is first converted into chemical energy, and then stored as carbohydrates. The light driven reaction involves the oxidation of water to produce oxygen, ATP and NADPH. The ATP and NADPH are then used in the Calvin cycle to reduce carbon dioxide to carbohydrates (Salisbury and Ross 1978).

The process of photosynthesis is dependent on four environmental factors: light, CO_2, water and temperature. These factors can be very variable in the natural environment (both spatially and temporally) and plants show many adaptations to these changeable factors. Few studies on this photosynthetic plasticity were done prior to the mid 1960's. It was generally accepted that a plant which grew in a cold environment had a lower temperature optimum for net photosynthesis than a did plant which grew in a warmer environment (Berry and Bjorkman 1980). Thus arctic and alpine species usually have temperature optima several degrees lower than species from temperate and desert areas (Pearcy 1977; Mooney *et al.* 1978; Chapin and Oechel 1983; Addison and Bliss 1984).

In addition, it was known that the temperature and

light relationships of photosynthesis were greatly affected by the climatic growth history of the plant. If a plant was grown at either a high or low temperature, a stress response could be observed, where the gas exchange rate was reduced at all assay temperatures. However, in some species a shift in the temperature optimum for this process occurred, with or without concurrent changes in photosynthetic capacity (Mooney and West 1964). It was suggested that this change, or acclimation, of the temperature optimum could occur on a continual basis, enabling the plant to track temperature fluctuations in the field, thus maintaining a near maximal photosynthetic rate throughout the entire growing season (Larson and Kershaw 1975b: Kershaw 1977a,b: Berry and Bjorkman 1980).

Some form of temperature acclimation has been documented in such diverse plant groups as algae, mosses, lichens, grasses, woody shrubs and trees (Oechel 1976: Sheridan and Ulik 1976: Davis and Dawes 1981: Drew and Legid 1981: Chapin and Oechel 1983: Kershaw 1984). In addition, several species with C_4 and CAM metabolism also have the ability to acclimate (Nobel and Hartsock 1981: Badger et al. 1982: Monson et al 1983). However, several other reports suggest that the photosynthetic response of some species are genetically fixed and cannot adapt to differing environments (Teeri et al. 1977: Kershaw and MacFarlane 1982: Coxson and Kershaw 1983). Even within a species or large population,

ecotypic differences in the acclimation potential of the plants could be observed (Mooney and Shropshire 1967; Smith and Hadley 1974; Pearcy 1977; Chapin and Oechel 1983).

The ability of a plant to acclimate would provide it with a large competitive advantage, and may enable several species to maintain niche diversity in the field (Regehr and Bazzaz 1976; Monson et al. 1983). It has been hypothesized that populations which grow in a widely fluctuating environment should have a higher acclimation potential than plants growing in stable environments. This has been demonstrated in some cases (Pearcy 1977) but not in others (Mooney 1980). Recently, Coxson and Kershaw (1983, 1984) have modified this hypothesis. They suggest that a high acclimation potential will be a viable strategy in environments which have predictable, long term (seasonal) variations in climate. They show that Caloplaca trachyphylla adapts to the predictable chinook weather patterns in the Alberta winter by acclimating to low temperatures (Coxson and Kershaw 1984), while the adaptation to low light intensity in Peltigera praetextata (Kershaw and Webber 1984) is a response to the predictable seasonal changes in light intensity on the floor of a deciduous forest. In contrast, species which grow in a fairly constant environment are expected to be well adapted to that particular environment, but have a low potential to acclimate to other conditions (Kershaw and MacFarlane 1982). A species

which lives in a rapidly fluctuating environment should show a very broad, generalized response, again with little acclimation potential (Coxson and Kershaw 1983).

Superimposed on this possible environmental predictability is an evolutionary factor. Evidence has been presented by Pearcy (1977) and Mooney (1980) which suggests that a population which has been established in an area for a long time period will have a higher acclimation potential than a recently established population. Thus there are still a large number of uncertainties involved in predicting whether or not a plant will show acclimatory changes in its photosynthetic metabolic rates throughout the growing season.

In the past, most workers have used higher plants for studies of photosynthetic acclimation. However, there are several drawbacks associated with the use of vascular plants for such measurements. The presence of special regulation systems (stomata and cuticle) complicates the gas exchange measurements. The experimenter must control the humidity of the air in the system such that excess transpiration does not cause an increase in the water deficit of the leaf. This would result in stomatal closure, decreasing the movement of CO_2 into the leaf and inhibiting photosynthesis.

Even if the stomatal resistance is maintained at a low level, the high net photosynthetic capacity of the vascular plant rapidly removes CO_2 from the assay system, resulting in a CO_2 limitation of net photosynthesis. This

must be prevented with the use of elaborate flow systems which maintain the CO_2 concentration in a narrow range (Jarvis et al. 1971, and Section 2.1), but which can usually handle only one individual at a time. These one-plant-per-experiment systems impose a large time constraint on the experimental design, thus most of the studies are univariate in nature. The light response is assayed at only one assay temperature, while the temperature response is determined for only one light intensity.

Higher plants also undergo periodic episodes of flowering and seed set. These processes represent complicating factors in the study of seasonal photosynthetic capacity changes. If such a change is observed, it is difficult to determine if this was a physiological response to a change in environmental conditions, or a change in the metabolism in preparation for a flowering event (Kershaw 1985).

Several of these drawbacks can be eliminated by switching to a lichen system for such studies.

The lack of cuticle and stomata greatly simplifies the gas exchange measurements. The experimenter does not have to control for the possible changes in stomatal resistance to CO_2 diffusion which may occur when a plant leaf is placed inside a gas exchange cuvette. Slight modifications to the assay procedure (see Larson and Kershaw 1975a, Kershaw et al. 1983) are required to compensate for

the lichens poikilohydric nature, but a much simpler system is necessary than that required to control the stomatal resistance between and within experiments in higher plants.

The lack of a root system also simplifies work with lichens. The thallus can be easily removed from its substrate without causing undue stress to the system. The entire plant can therefore be placed in the assay cuvette, whereas higher plant studies are often restricted to a single leaf or leaf section. Although the root system does not directly affect photosynthetic acclimation response of a plant, the root/soil interface is an ecologically important interaction which is greatly reduced in a lichen (Kershaw 1985).

The low photosynthetic capacity of a lichen, and a lower dependence of this rate on the CO_2 concentration in the assay system has allowed Larson and Kershaw (1975a) to design an experimental system which can assay 15 to 20 individuals simultaneously. This system enables a much more rigorous experimental design, where the secondary and tertiary interactions between light, temperature, thallus moisture content and season can be determined.

This low net photosynthetic capacity is a biproduct of the unique association which forms the lichen. The thallus represents a symbiotic relationship between a fungus and a green algae or cyanobacterium. As the majority of the biomass is fungal, the net photosynthetic rate per unit of

dry weight is low. Also it is often assumed that the measured rate of respiration is primarily fungal in origin, and the algal respiration is thus insignificant. Gas exchange measurements provide an estimate of net photosynthesis, which is a balance between the CO_2 fixed by photosynthesis and the CO_2 produced from mitochondrial respiration and photorespiration. Mitochondrial respiration in plant leaves and algae may be strongly affected by the light environment (Sharp et al. 1984), complicating studies of interactions between light and net photosynthesis. The relative insignificance of algal mitochondrial respiration to the total respiration rate in lichens results in a measurement that is not affected by light intensity (Coxson et. al. 1982), thus simplifying the interactions.

While the above factors indicate how a lichen system simplifies gas exchanged measurements, other aspects of their physiology also make them good candidates for the study of photosynthetic acclimation. Lichens are long lived, evergreen species which are active year round. The plant will experience several changes in the environmental parameters on a regular and predictable basis. The lichen therefore is exposed to a wider environmental gradient on a seasonal basis to which it must adapt to than the light and temperature regime experienced by a short lived annual or deciduous perennial.

During the past decade, Kershaw and coworkers have

observed several adaptations in the photosynthetic capacity of lichens to differences in the light, temperature and moisture environment. Some of the adaptations represent a fixed response to a specific environment, while others exhibit a large plastic response.

For example, populations of Cladonia stellaris growing on the crest and valleys of a ridge system (Kershaw 1975) and Parmelia caperata and P. sulcata growing at different heights in a tree (Harris 1971) show a fixed adaptive response to the differences in moisture availability in their respective microenvironments.

The adaptations to light intensity in Cladonia stellaris and C. rangiferina are somewhat more plastic (Kershaw et al. 1983; MacFarlane et al. 1983). These species live in a patchy light environment which ranges from shaded regions to completely exposed areas. The species show large morphological variation to this light gradient, ranging from a short, stubby sun population with a low net photosynthetic capacity, to a tall, finely branched shade morphology capable of high CO_2 fixation rates. This represents a long term adaptation to light intensity, possibly due to an increased algal density. No short term adaptations to seasonal changes in the canopy density were observed (Kershaw et al. 1983; MacFarlane et al. 1983) indicating the response was genetically fixed in the population.

Amongst the many lichen species that have been

studied, the genus Peltigera has shown some of the most plastic photosynthetic responses. The temperature optimum for net photosynthesis in P. praetextata and P. polydactyla can adapt to both naturally occurring and laboratory controlled changes in temperature (Kershaw 1977a,b). P. praetextata can also alter its photosynthetic efficiency in response to changes in the density of the canopy (Kershaw and Webber 1984) while P. scabrosa can be experimentally acclimated to different light intensities (Kershaw and MacFarlane 1980). Two populations of P. aphthosa, one growing in a closed canopy spruce forest and the other in a recently burned area exhibit a fixed adaptive response to differences in thermal stress and moisture content in these environments (Kershaw and MacFarlane 1980; MacFarlane and Kershaw 1980).

The Peltigera species described above are primarily from woodland environments. Peltigera rufescens is a species with a distribution restricted to exposed habitats (Section 3.1). Unlike other Peltigera's, this species experiences little seasonal fluctuation in the light environment, but larger variations in the thermal regime. These thermal differences suggest that, if a seasonal pattern in net photosynthetic capacity was observed, it may be different than the light adaptations documented for the other species.

The following chapters represent a study of the net photosynthetic response to light, temperature and thallus moisture content in Peltigera rufescens. Two populations of

this species were studied, from environments representative of sub-arctic (Churchill, Manitoba) and temperate (Muskoka, Ontario) regions. Incorporated into the experimental design were studies of population adaptation to these widely contrasting geographic environments and a seasonal adaptation within a geographic location. The thesis has been divided into two parts. The first section characterizes and describes the seasonal changes in net photosynthetic capacity which occur in the two populations. Studies on this naturally occurring capacity change are supported by a study of the laboratory induced acclimation. The second part of the thesis investigates a hypothesis concerning the biochemical and physiological basis for the seasonal acclimation pattern observed in the Muskoka population.

Part I: The Gas Exchange Studies

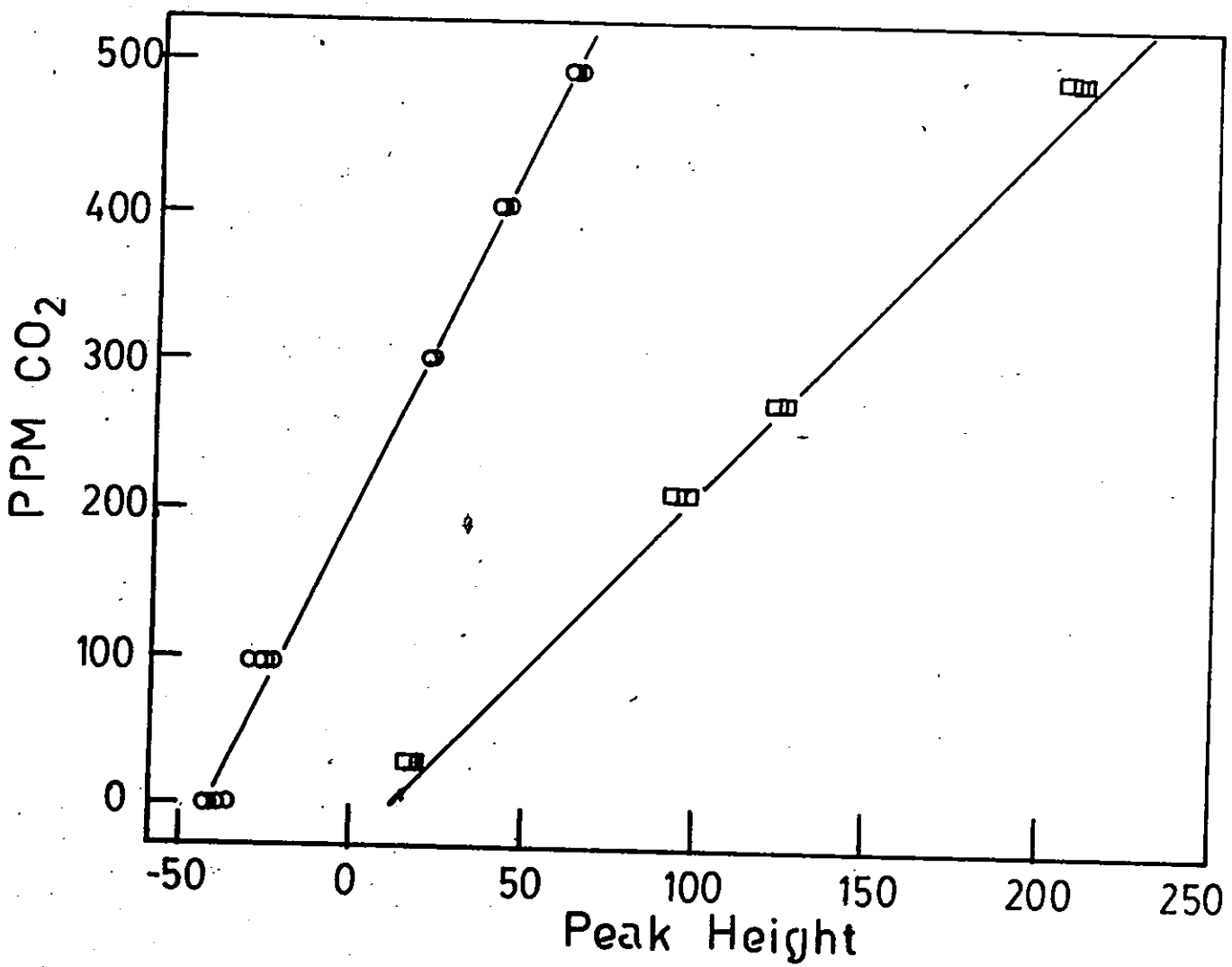
2. Introduction

2.1 Infra-red gas analysis theory

The most common method of measuring net photosynthesis in terrestrial plants involves monitoring the change in concentration of CO_2 in the air surrounding the plant with an infra-red gas analyzer (IRGA). The advantage of this technique is the ability of the IRGA to continually measure the CO_2 concentrations. Such common atmospheric gases as O_2 and N_2 do not absorb in the infra-red spectrum, while CO_2 is one of the most intensely absorbing gases (Jarvis et al. 1971). This absorbance is directly proportional to the concentration of CO_2 between 0 and 500 ppm (Figure 1). and the system is sensitive to the slight changes in CO_2 produced during photosynthesis.

This study used a Beckman 865 IRGA run in the differential mode. This instrument has two chambers. one containing the sample gas and the other containing a reference gas of known CO_2 concentration (Figure 2). An infra-red beam is passed through each of these chambers and is then measured by a detector. The output is the difference between the constant radiant energy transmitted through the reference cell and the continuously changing transmittance of

FIGURE 1: The relationship between IRGA output (peak height) and the CO_2 concentration of the standard gases used to calibrate the IRGA. A carrier gas of either 200 ppm(O) or 0 ppm (□) was used.


500
400
300
200
100
0
PPM CO_2
-50
0
50
100
150
200
250
Peak Height

the gases in the sample cell. This difference is amplified by the IRGA and is recorded as a millivolt output by a PDP-11 computer and a Rikadenki chart recorder.

Water vapour also strongly absorbs infra-red radiation and its absorption band overlaps considerably with that of CO_2 (Jarvis et al. 1971). Thus a difference in humidity between the sample and reference cells will result in a substantial error in the estimated CO_2 concentration (Bunce and Ward 1985). The Beckman 865 IRGA is sensitive to humidity differences, and this is best corrected by the removal of all moisture from the air before it is analyzed (Bunce and Ward 1985). Larson and Kershaw (1975a) modified the IRGA system such that the airstreams passed through a drierite column prior to its entrance to the reference or sample chambers of the IRGA. This technique was also used in this study to clear the air of H_2O vapour.

The design of the assimilation chamber may represent the "weakest link" in the IRGA system. The cuvette of Larson and Kershaw (1975a) was used to contain the thallus for the gas exchange measurements (Figure 2). This consisted of a clear glass cell with a detachable ground glass bottom. A thin layer of vacuum grease was placed between the cell and its ground glass bottom, thus providing an air tight seal which prevented any CO_2 exchange between the interior of the cuvette and the atmosphere (Larson and Kershaw 1975a) but allowed the experimenter to rapidly break the seal to remove

FIGURE 2: A schematic diagram of the IRGA system.

Ref : carrier gas supply

rc : reference cell

sc : sample cell

Sy : 5 ml syringe

St : serum stopper

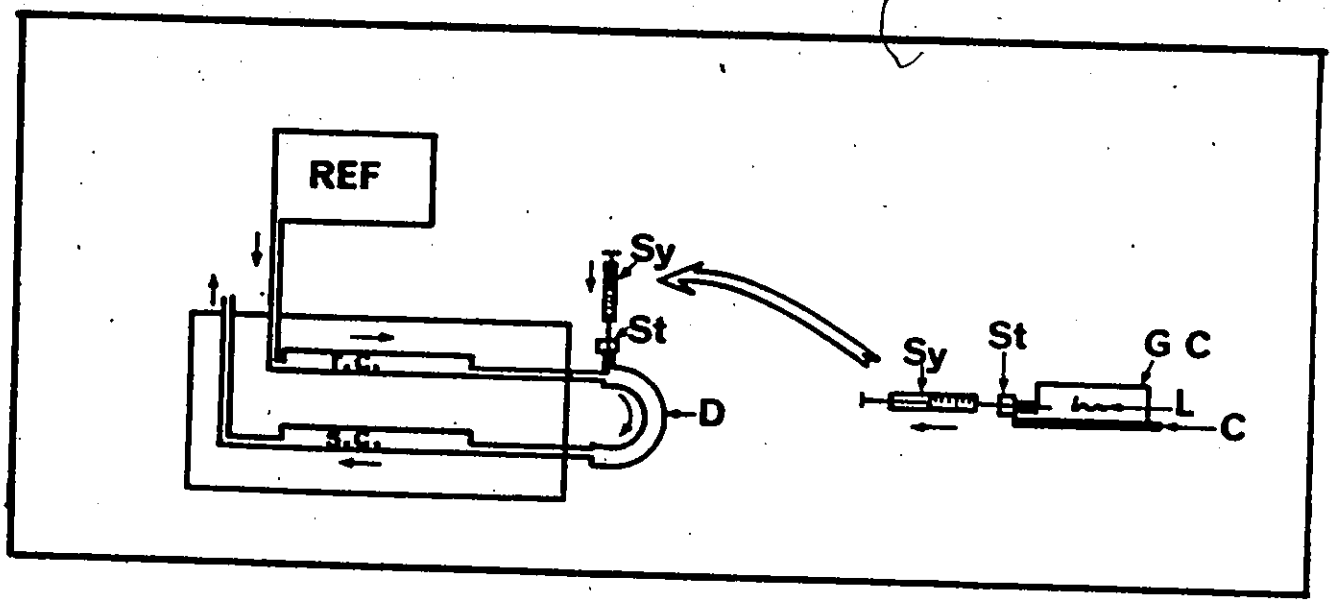
D : Dryrite column

GC : clear glass cuvette top

C : ground glass cuvette base

L : lichen thallus

(from Larson and Kershaw 1975a)


REF
Sy
St
D
Sy
St
G C
L
C

and replace the lichen samples (see Section 3.2.2). A stoppered injection port was attached to the side of the cell to allow the removal of air samples without opening the cuvette

Jarvis et al. (1971) have identified two major problems with the design of the assimilation chamber: ventilation and the greenhouse effect. The greenhouse effect is a direct result of the transmission properties of the material used for the cuvette. Glass is a poor conductor of heat (long wave radiation), but a good conductor of photosynthetically active radiation (PAR). This PAR causes a warming of the thallus in the cuvette. The thallus emitts long wave radiation, but this is reflected by the walls of the cuvette and results in further heating of the thallus. This short circuit continues until an equilibrium is established between the rate of radiant heating and the convective cooling through the cuvette wall (Jarvis et al. 1971). An increase in the rate of convective cooling. with the use of fans and placement of the cuvette in a growth chamber at a much lower ambient temperature, can partially compensate for the greenhouse effect. Thallus temperature was monitored continually during the gas exchange measurements (Section 3.2.4) and the growth chamber temperature was adjusted to maintain the thallus temperature within 1°C of the desired experimental temperature.

The air movement within the cuvette can also cause

serious problems with the estimation of net photosynthesis, as a still air boundary layer around the thallus will have a high resistance to CO_2 transport. Under natural conditions, the highly turbulent air flow around the thallus can maintain this boundary layer resistance at a low level. Jarvis et al. (1971) recommend that the air within the cuvette be mixed to limit the boundary layer resistance to 0.1 $s \cdot cm^{-1}$ for higher plants. Ventilation does not pose a problem for lichen research, although the boundary layer resistance within a cuvette under still air may be considerably higher than recommended above. Collins and Farrar (1978) estimated a boundary layer resistance of 0.6 $s \cdot cm^{-1}$ in Xanthoria, while Green and Snelgar (1981) report a very high resistance of 7.0 $s \cdot cm^{-1}$ for large thalli of Sticta and Pseudocyphellaria. Similar boundary layer estimates for P. rufescens are in the 0.4 to 0.6 $s \cdot cm^{-1}$ range. However, these resistances are insignificant when compared to a total thallus CO_2 diffusion resistance of 100 to 400 $s \cdot cm^{-1}$ (Collins and Farrar 1978; Green and Snelgar 1981; Snelgar et al. 1981). Larson and Kershaw (1975a) and Larson (1979) have shown experimentally that ventilation does not affect the rate of CO_2 fixation in Peltigera polydactyla and Umbilicaria, confirming that a high boundary layer resistance is not a problem with the lichen IRGA system.

Lichens differ from higher plants in two other aspects which complicate the measurement of net

photosynthesis: their poikilohydric nature and their low gas exchange rates. Three different IRGA systems have been developed to measure net photosynthesis and alleviate these problems to different extents.

The traditional open flow system has been used extensively in early work (Harris 1971; Kershaw 1972) and is still a favourite in some laboratories (Lange 1980; Lange and Matthes 1981). In this method, the gas exchange cuvette is connected directly to the sample cell of the IRGA. A continuous supply of air, containing a known and constant concentration of CO_2, is pumped through the cuvette to the IRGA and is then vented into the room. The flow rate is adjusted such that only a small change in CO_2 concentration occurs across the cuvette (approximately 20 ppm (Jarvis et al. 1971)) and is thus directly proportional to the net photosynthetic rate.

This technique is useful in that it provides an estimate of the net photosynthetic rate at a defined CO_2 concentration. However, it does have three severe disadvantages when used with lichens. The gas exchange rate of a lichen is relatively low, therefore to produce a measurable change in CO_2 concentration, either low flow rates must be used, or a large amount of material must be placed inside the cuvette. These flow rates are difficult to measure and maintain (Jarvis et al. 1971), and thus the estimated rate is inaccurate. A large sample size will lead

to the production of severe microclimate gradients within the cuvette. Thus the experiment cannot be run under well defined, repeatable conditions (Larson and Kershaw 1975a). In addition, the gas exchange rate in a lichen is extremely dependant on the size of the thallus used (Larson 1984b) and the larger sample sizes required for an open flow system make it difficult to control for this variation.

Gas exchange in lichens is also highly dependant on the moisture content of the thallus. The loss of moisture due to evaporation in the constantly flowing air stream prevents the measurement of gas exchange at one defined moisture content. This is complicated by the fact that an open flow system makes it nearly impossible to measure the water content of the thallus during the course of the experiment (Lange and Matthes 1981). In addition, the large amount of material required interacts with the windspeed, causing an uneven drying throughout the thallus. Thus the results of such a drying curve are difficult to interpret and relate to field situations (Harris 1976).

The third difficulty with an open flow system is that the number of samples or replicates that can be accommodated is severely restricted. Usually only one cuvette can be used for each IRGA and this imposes a severe constraint in the experimental design. However an elaborate switching device developed by Lange (1980) allows the simultaneous monitoring of up to four samples.

Larson and Kershaw (1975a) developed a gas exchange system specifically designed to alleviate the inherent problems of the open flow system. Their discrete sampling method, which is described in detail below (Section 3.2.2), completely separates the cuvette from the IRGA. Gas samples were taken from the cuvette at the beginning and end of an incubation and injected into the IRGA. Net photosynthesis was calculated from the difference in the CO_2 concentrations and the incubation time. This technique has been used extensively in lichen research (Snelgar et al. 1980; Link and Nash 1984a; Kershaw 1985), and has been adapted for mosses (Kershaw and Webber 1986 ; Busby and Whitfield 1978) and higher plants (Ehleringer and Cook 1980; Peterson and Zelitch 1982, 1985).

The major advantage of the discrete sampling method is that up to 20 cuvettes can be monitored in any one experiment. This allows for a much more complex experimental design, where secondary and tertiary interactions between temperature, light and moisture can be studied with a minimal time commitment (Larson and Kershaw 1975a). Additionally, the lichen thallus can be removed from the cuvette between incubations for weighing, allowing the moisture content of the thallus to be estimated for each gas exchange measurement.

Green and Snelgar (1981) and Lange (1980) have, however, criticized this method. They suggest that it

underestimates the net photosynthetic rate, due to a CO_2 limitation which occurs in the closed cuvette during the incubation period. This can cause a problem with some lichens, but due to their low rates of net photosynthesis, it will not seriously affect the results provided the incubation period is kept short (Larson and Kershaw 1975a; Nash et al. 1983; Link et al. 1984). It is important that the response of net photosynthesis to changing concentrations of CO_2 is determined for each new experimental species.

A third analysis method, using a closed loop gas exchange system, has recently been adapted for use in lichens (Green et al. 1981). In this method, the gas is continuously circulated between the cuvette and the IRGA and the IRGA generates a continuous trace of CO_2 exchange with time. The discrete sampling method of Larson and Kershaw (1975a) is actually a derivative of this technique, where the continuous trace is replace by a measure of the beginning and ending concentration of CO_2. The closed loop system has been used quite successfully in experiments designed to study the relationship between net photosynthesis and CO_2 concentration, and the role photorespiration may play in lichen metabolism (Snelgar and Green 1981; Snelgar et al. 1981; Coxson et al. 1982; Green and Snelgar 1982). However, large scale use of this system will be quite limited, as, with the open flow system, it is restricted to one cuvette per IRGA.

2.2 Photosynthetic-illumination curve theory

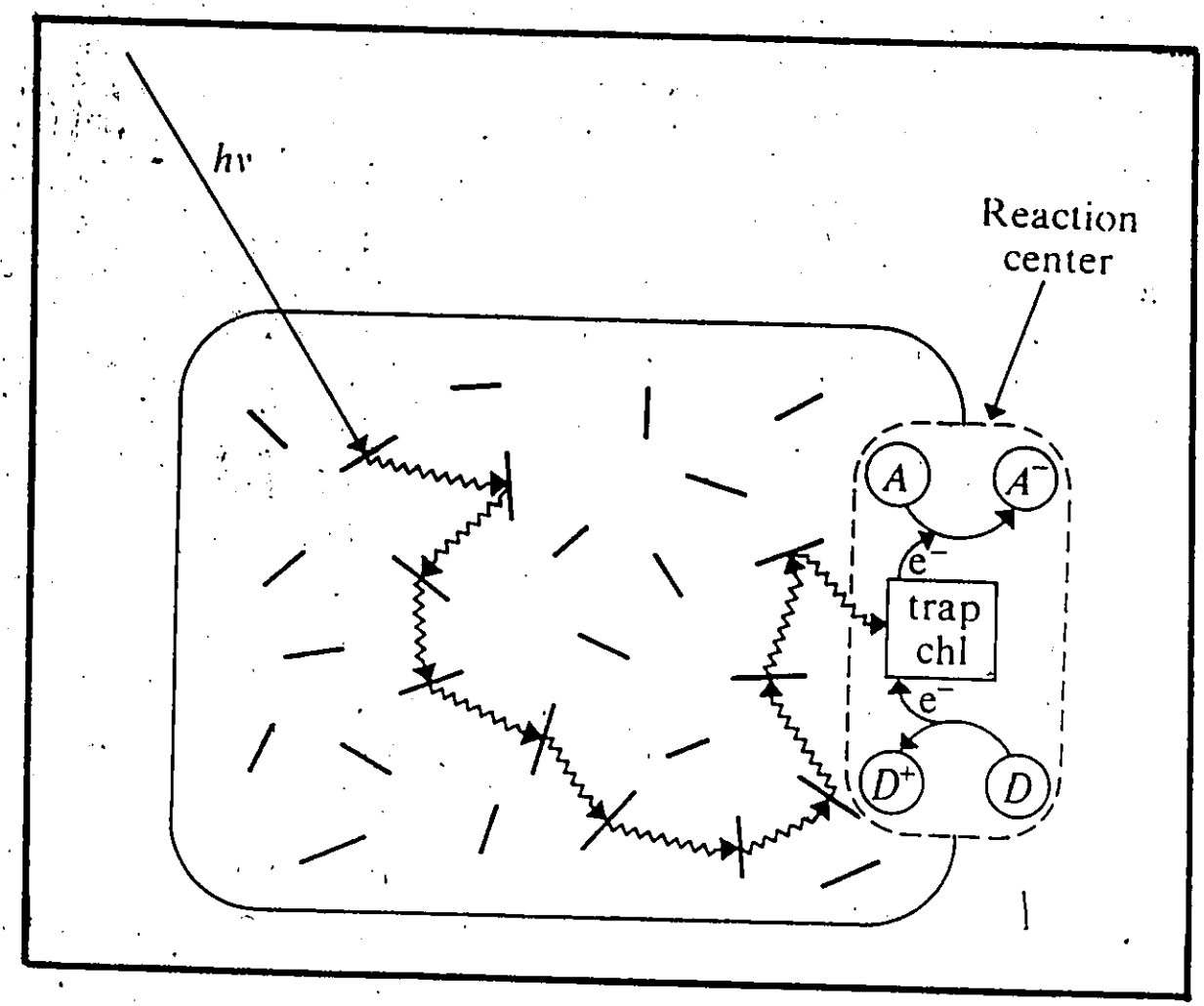
The photosynthetic units (PSU) in a blue-green cyanobacterium consists of the paired reaction centers (P_{680}, P_{700} and their associated proteins), a core of approximate 140 to 180 chlorophyll molecules and the light harvesting antenna pigments (Vierling and Alberte 1980). The light harvesting pigments consist of additional membrane-bound chlorophyll molecules, and the water soluble biliproteins which funnel energy primarily into the PS II reaction centers (Nobel 1983).

Any one of these pigments are capable of absorbing a photon and once absorbed, this energy is transferred from pigment to pigment through the system to a specializing chlorophyll dimer which forms the P_{680} or P_{700} reaction center (Figure 3). At the reaction center, this energy is used to remove an electron from the chlorophyll trap to an acceptor molecule on the photosynthetic electron transport chain (Nobel 1983). The flow of electrons through this chain produces the ATP and NADPH required for the Calvin cycle fixation of CO_2, while water is hydrolized to ultimately replace the electron lost from the chlorophyll.

The largest quantity of pigment molecules makes up the antenna system (Vierling and Alberte 1980) and this is responsible for most of the light energy absorbed. Thus a change in the size of this light harvesting antenna will

FIGURE 3: A schematic diagram indicating the transfer of light energy in the pigment system. A light quantum ($h\nu$) can be absorbed by any pigment, and this excitation is passed on to a special trap chlorophyll. In the reaction center, an electron (e-) is transferred from the trap chl to an acceptor molecule (A- in the reduced form). This electron is then replaced by another, coming from a suitable donor (D- in the oxidized form).

(from Nobel 1983)


hv
Reaction center
A
A⁻
e⁻
trap chl
e⁻
D⁺
D

affect the rate of net photosynthesis at subsaturating light intensities (Herron and Mauzerall 1972). The maximum, light-saturated rate of net photosynthesis is affected by the total number of reaction centers (Herron and Mauzerall 1972) and the turnover rate for these centers. The turnover rate is determined by the the capacity of the electron transport chain and Calvin cycle to utilize ATP and NADPH. This last process is a temperature dependant enzymatic reaction. Thus the light saturated rate is temperature dependant, while the light-limited photoprocesses are not (Prézelin 1981).

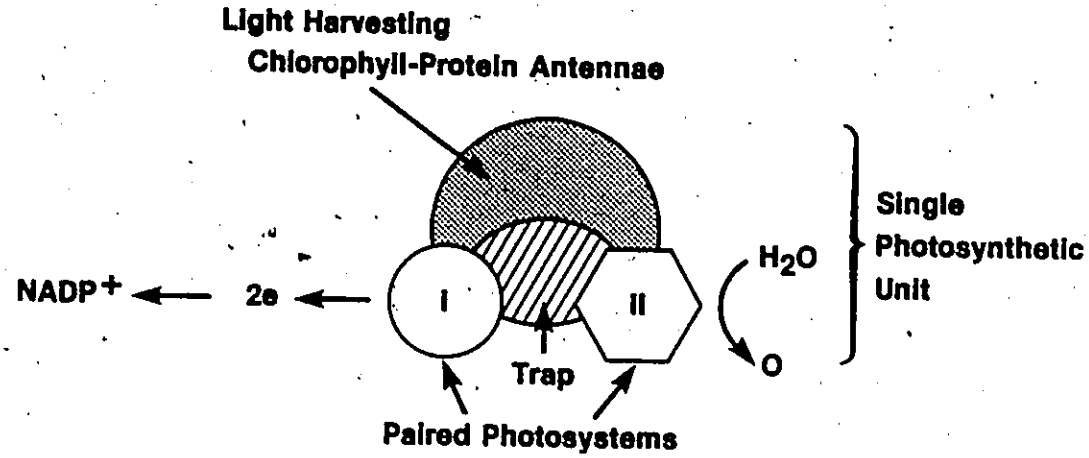
Four mechanisms were proposed by Prézelin (1981) to describe the changes in net photosynthetic capacity. These models are based on the pioneering work of Herron and Mauzerall (1972) on greening in Chlorella cells and concerns the dynamic changes in the PSU.

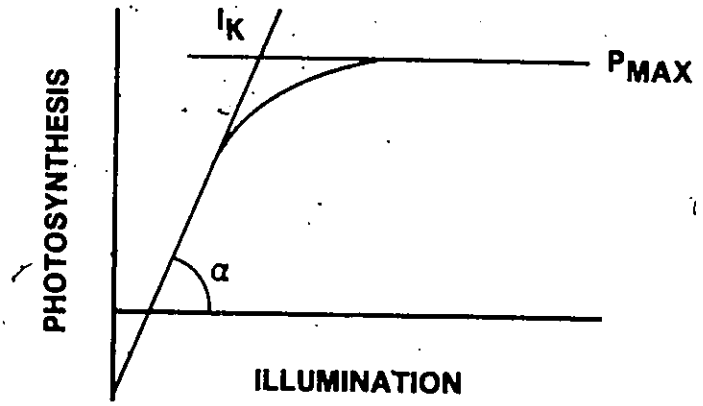
The models involve a simplification of the P-I curve into four components (Figure 4). The two major components are Pmax, the maximum light-saturated rate of photosynthesis and α, the slope of the linear light-limited portion of the P-I curve. α is equivalent to the quantum efficiency of photosynthesis and will be termed AQE (apparent quantum efficiency) in this study. Two additional parameters are derived from Pmax and AQE. I_k is the theoretical light intensity which saturates photosynthesis while $I_{k/2}$ is the light intensity which half saturates this process. Although AQE and Pmax are considered to be the best indicators of

FIGURE 4: The upper figure is a diagrammatic interpretation of a photosynthetic unit.

The lower figure represents a simplified photosynthetic-illumination curve, indicating the apparent quantum efficiency (α, calculated as the initial slope), the light saturation point (I_k) and the maximum rate of net photosynthesis (P_{max}).

(from Kershaw 1985)


Light Harvesting
Chlorophyll-Protein Antennae
NADP+
2e
I
II
Trap
Paired Photosystems
H2O
O
Single
Photosynthetic
Unit


IK
PMAX
PHOTOSYNTHESIS
α
ILLUMINATION

photosynthetic performance in algae (Coté and Platt 1984), I_k and $I_{k/2}$ are useful in determining the mechanism behind some of the more complicated changes in photosynthesis (Prézelin and Matlick 1980)

The most frequent change observed in algae involves an increase in the size of the PSU antenna system which occurs during an adaptation to low light intensity (Figure 5). When the gas exchange rate is calculated on a biomass basis, this increase in pigments result in a higher AQE. Similarly, $I_{k/2}$ will change due to the increased efficiency, but no change in Pmax is observed as the number of reaction centers have not changed (Prézelin 1981). However, when gas exchange is calculated on a pigment basis, a decrease in Pmax is expected. As it is unlikely that a 1:1 relationship exists between the quanta absorbed and the increased in PSU size, a slight decrease in AQE also occurs (Prézelin and Sweeney 1978; Ramus 1981; Richardson et al. 1983). A change in PSU size can also be identified by a disproportionate alteration in the pigment ratios (Herron and Mauzerall 1972; Vierling and Alberte 1980; Prézelin 1981). Such changes have been documented in several species of algae, mosses and vascular plants (Prézelin 1981; Kershaw and Webber 1986), but have not yet been observed in lichens (Kershaw 1985).

A second adaptation to low light involves an increase in the number of photosystems per cell (Figure 6). The increase in the number of reaction centers results in an

FIGURE 5: The theoretical changes which occurs in the P-I curve due to an increase in photosynthetic unit size, expressed either on a cellular basis (upper) or a chl basis (lower).

(from Kershaw 1985)

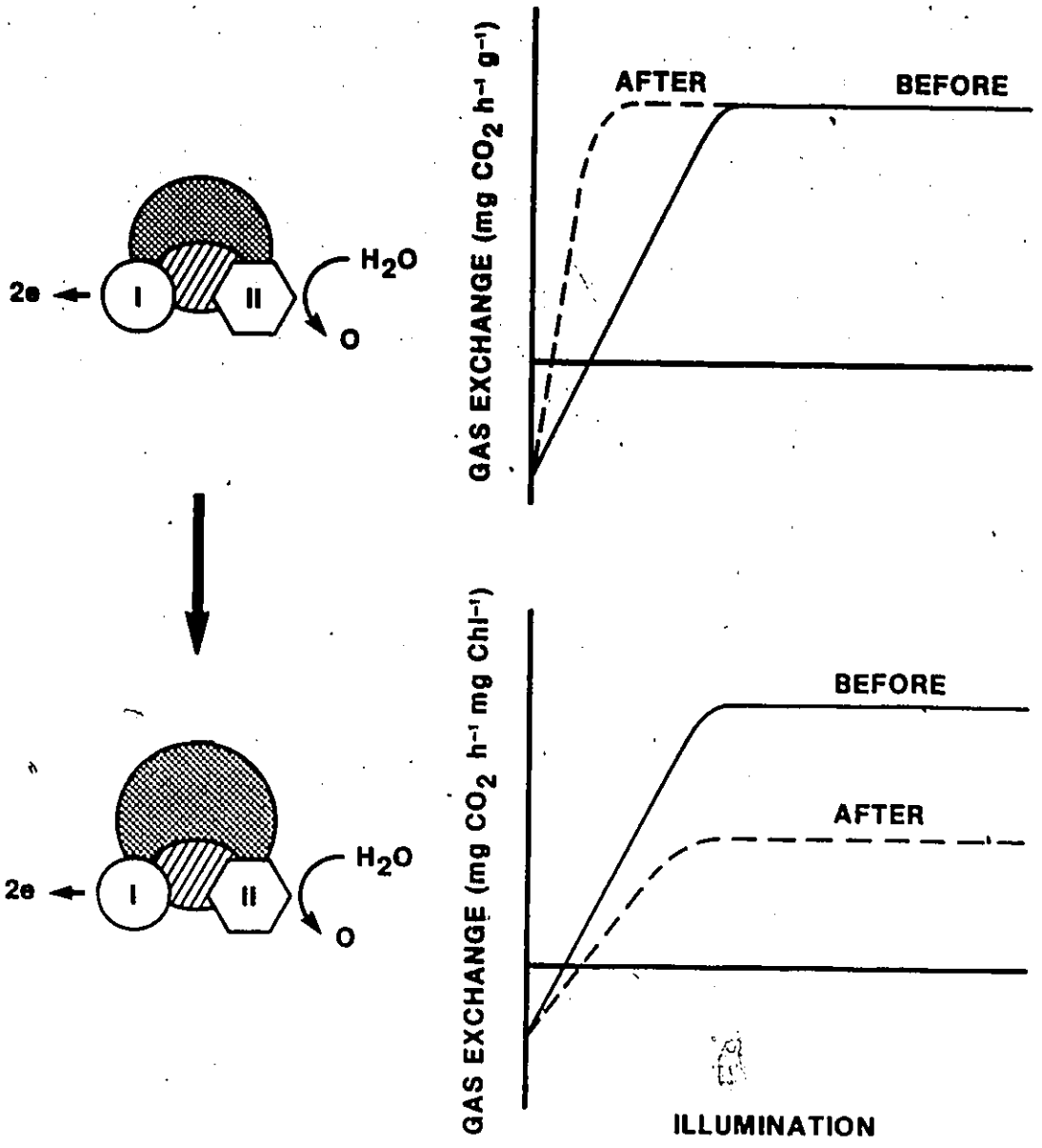
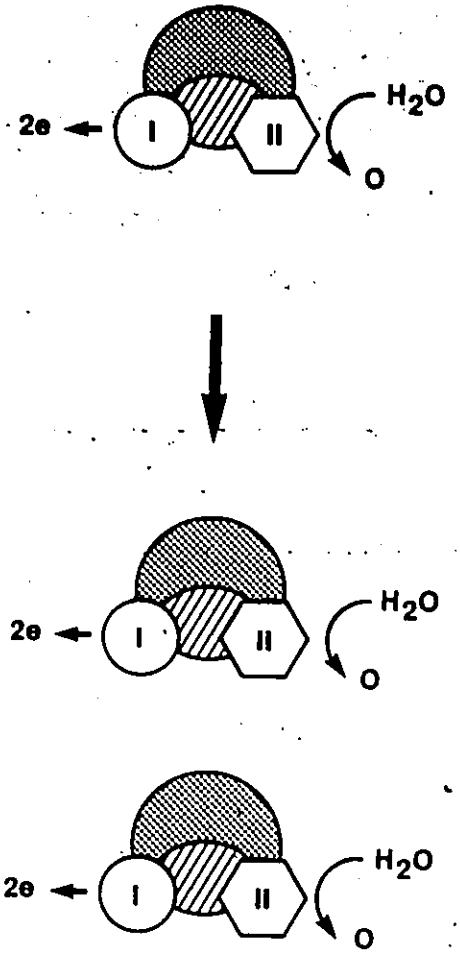
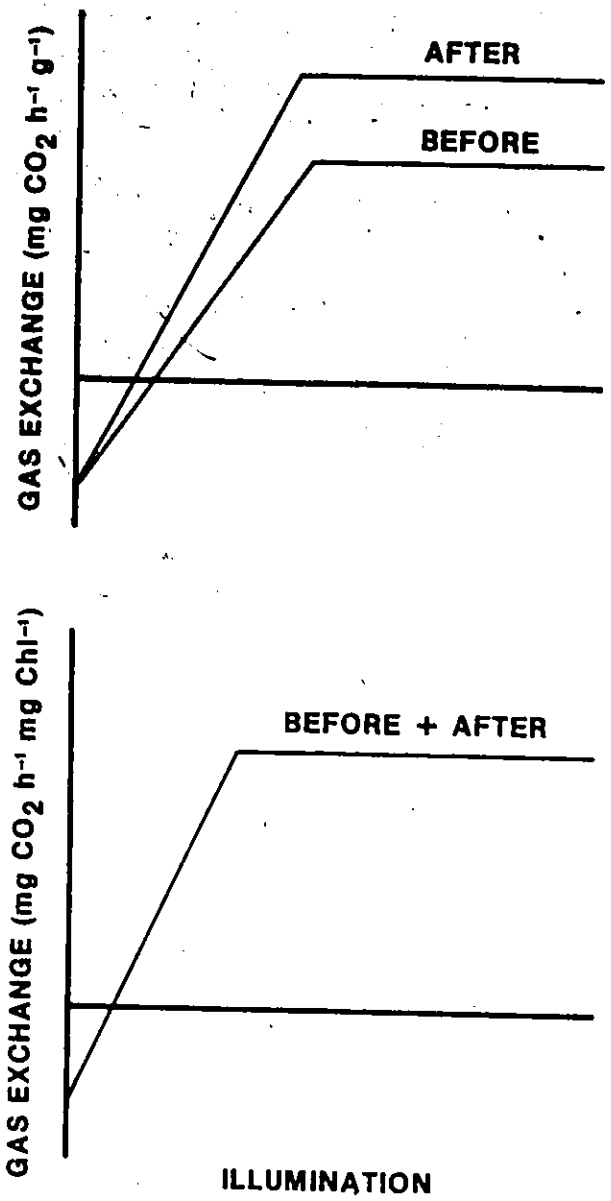

2e
I
II
H_2O
O
2e
I
II
H_2O
O
AFTER
BEFORE
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
BEFORE
AFTER
GAS EXCHANGE (mg CO_2 h^{-1} mg Chl^{-1})
ILLUMINATION

FIGURE 6: The theoretical changes which occur in the P-I curve due to an increase in photosynthetic unit density, expressed either on a cellular basis (upper) or a chl basis (lower).

(from Kershaw 1985)


2e
I
II
H_2O
O
2e
I
II
H_2O
O
2e
I
II
H_2O
O

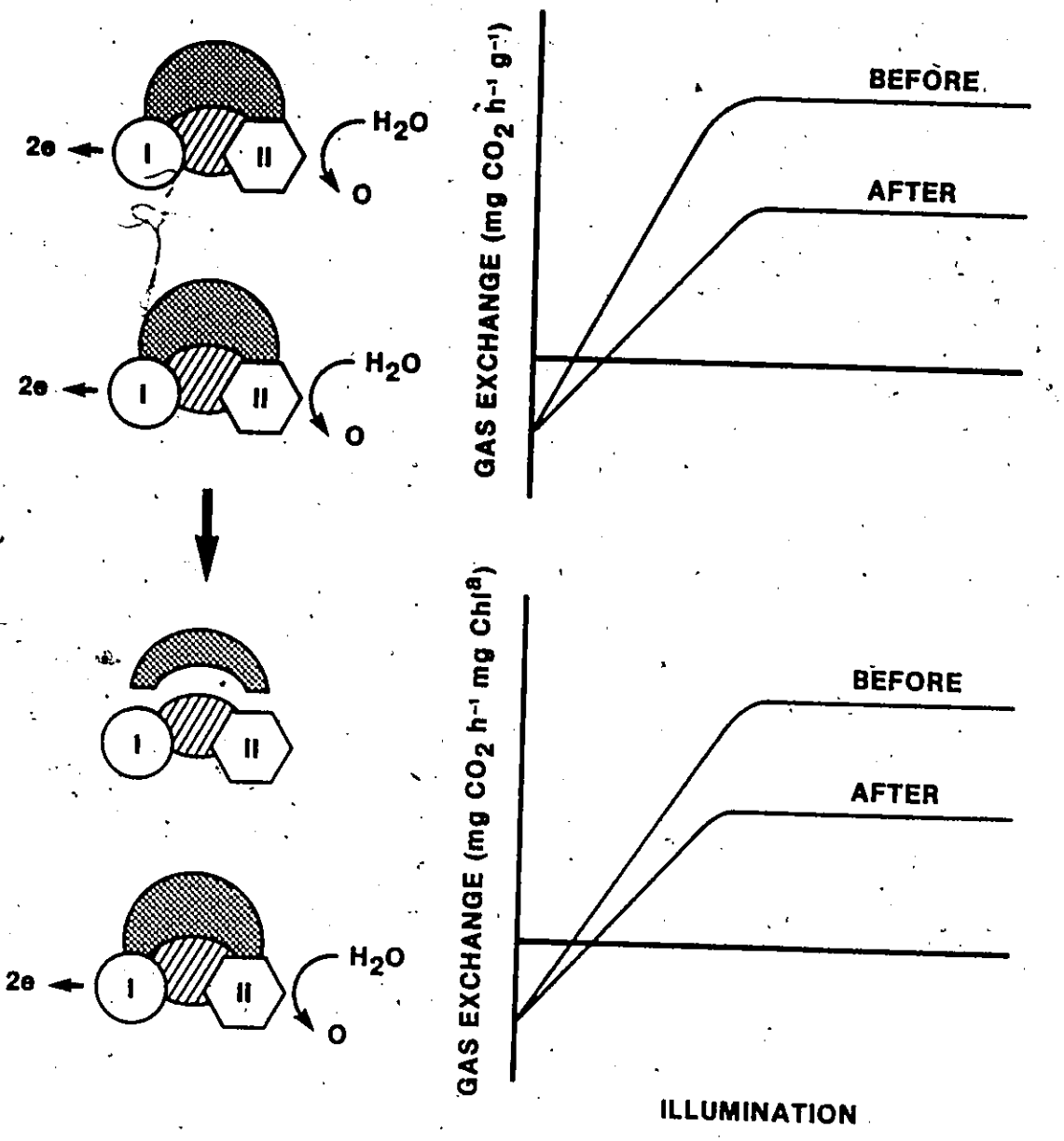

AFTER
BEFORE
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
BEFORE + AFTER
GAS EXCHANGE (mg CO_2 h^{-1} mg Chl^{-1})
ILLUMINATION

increase in Pmax, while the larger pigment area will increase the AQE when the gas exchange rate is referenced to a biomass basis. No changes are observed if the gas exchange rate is calculated on a pigment basis, and no change in the pigment ratios are expected (Prézelin 1981: Ramus 1981). Although changes in PSU density are infrequent in algae, they have been observed in several lichen species (Prézelin 1981; Kershaw et al 1983: MacFarlane et al. 1983: Kershaw and Webber 1984) and most likely reflect changes in algal density in the lichens.

A third change, which involves a circadian rythmn of photosynthesis, was observed in several species of dinoflagellates (Prézelin et al. 1977: Prézelin and Sweeney 1977). A decrease in AQE and Pmax were observed on both a biomass and pigment basis, but no change in I_k or $I_{k/2}$ occurred (Figure 7)(Prézelin and Matlick 1980). The changes observed on a biomass basis are identical to the pattern for the PSU density change, therefore Prézelin (1981) suggests that this is a change in the functional PSU density, more commonly called uncoupling. At any given time, energy transfer in a certain fraction of the PSU is disconnected from the photosynthetic electron transport chain and the light energy trapped by these systems is lost by fluorescence (Prézelin 1981). The site of this uncoupling is near the PS II reaction center (Samuelsson et al. 1983). Although the ecological significance of this uncoupling is unknown for the

FIGURE 7: The theoretical changes which occur in the P-I curve due to decrease in photosynthetic energy transduction (uncoupling), expressed either on a cellular basis (upper) or a chl basis (lower).

(from Kershaw 1985)

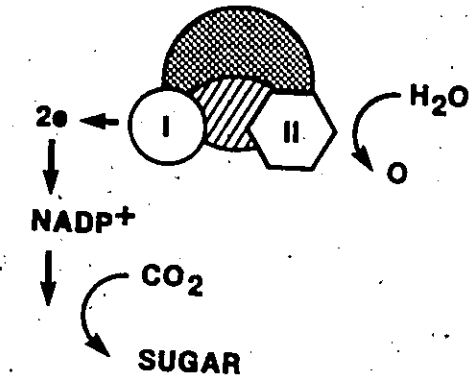

2e
I
II
H_2O
O
2e
I
II
H_2O
O
I
II
2e
I
II
H_2O
O
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
BEFORE
AFTER
GAS EXCHANGE (mg CO_2 h^{-1} mg Chl^a)
BEFORE
AFTER
ILLUMINATION

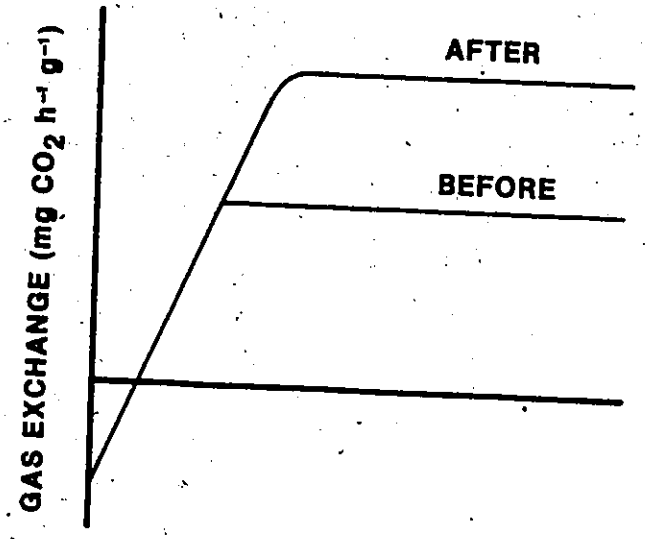
algae, it may be involved in the high temperature inhibition of photosynthesis in higher plants (Stidham et al. 1982; Downtown et al. 1984). The decrease in photosynthetic capacity observed during the winter in the lichens Peltigera praetextata, Cladonia rangiferina and C. stellaris may also be a result of this uncoupling (Kershaw et al. 1983; MacFarlane et al. 1983; Kershaw and Webber 1984).

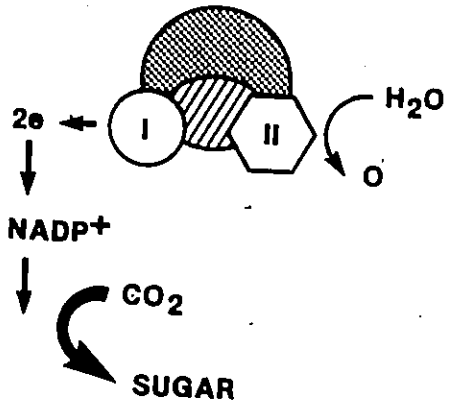
The last model proposed by Prézelin (1981) involves changes in the dark reaction of photosynthesis, presumably due to an alteration in the electron transport chain, Calvin cycle or photorespiration (Figure 8). There are no changes in cellular pigments, therefore the AQE is not altered. Due to the change in the dark reaction, Pmax is affected and therefore I_k and $I_{k/2}$ are also affected. A similar change is observed when the rates are calculated on a biomass or pigment basis. This pattern has been observed in the photoadaptation in Zea mays, Amaranthus palmeri and the symbiotic dinoflagellate Symbiodinium microadariaticum, and has been correlated to changes in the photosynthetic enzymes (Hatch et al. 1969; Chang et al. 1983). A decline in leaf nitrogen caused by drought stress has also produced this pattern (Ehleringer 1983). In addition, changes in Pmax with no concurrent changes in AQE or cellular pigment have been observed during the temperature acclimation in Larrea divaricata, Nérium oleander and wheat (Sawada and Mivachi 1974; Mooney et al. 1978; Badger et al. 1982).

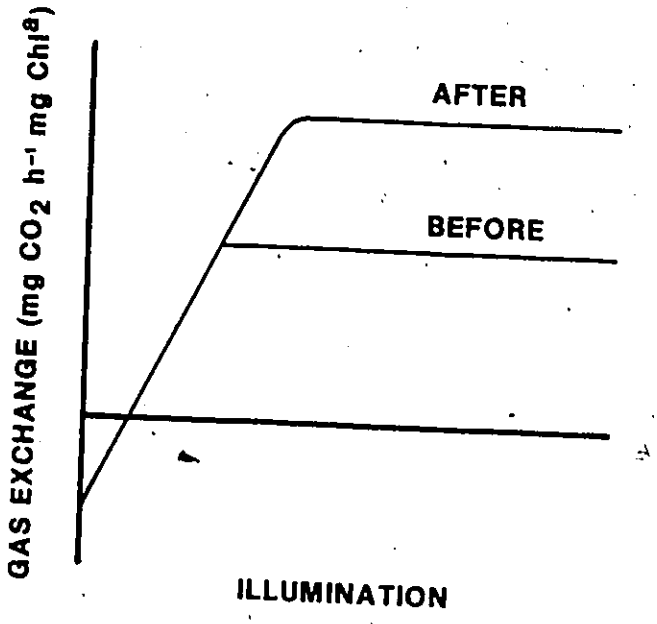
FIGURE 8: The theoretical changes which occur in the P-I due to an increase in the capacity of the photosynthetic enzyme reactions, expressed either on a cellular basis (upper) or a chl basis (lower).

(from Kershaw 1985)


2e
I
II
H_2O
O
NADP+
CO_2
SUGAR


AFTER
BEFORE
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})


2e
I
II
H_2O
O
NADP+
CO_2
SUGAR


AFTER
BEFORE
GAS EXCHANGE (mg CO_2 h^{-1} mg Chl^a)
ILLUMINATION

The response of plants to changes in the environment are very complicated. The four models proposed by Prézelin (1981) represent an oversimplification of the situation (Richardson et al. 1983). however, they do provide a provisional indication of the biochemical changes behind the observed physiological differences. Thus the models are helpful in indicating the direction which future research should follow.

In the following study, the seasonal photosynthetic patterns were initially characterized using a response matrix approach (a factorial combination of 5 light and 5 temperatures). The observed capacity change was then analysed via Prézelin's (1981) P-I curves. This suggested that the capacity change was caused by an alteration in the dark reactions (model 4, Figure 8).

3. THE GAS EXCHANGE METHODS

3.1 Material and site description

Peltigera rufescens (Weis) Mudd. is a foliose lichen which grows in a rosette form closely adhering to its soil or moss substrate. The lobes are small (1 cm), with upturned margins. The upper surface is white pruinose, while the whitish lower surface is veined, and has conspicuous tufts of rhizines (Hale 1969; Jahns 1983). Hale (1969) describes this species as the sun form of P. canina, thus it is alternately described as P. canina var rufescens. Dr. I. Brodo of the National Museum of Canada has confirmed the taxonomy of the populations used in this study, and voucher specimens are on deposit at the museum.

The morphology of this species is quite variable, ranging from small, highly infolded lobes at the most exposed sites, to a much broader and flatter form in the shadier areas. This study used thalli that were intermediate in this gradient, found growing amongst the grasses at the collection sites. This represented the most numerous fraction of the forms present, and was easier to work with than the more exposed ecotypes.

The Muskoka population was collected along a 40 km stretch of Highway 69 between Port Severn, Ontario and Muskoka county road 38. Extensive patches of this population are

found frequently on the granite outcrops along the roadside (Figure 9). These patches vary in size from individual rosettes of 15 cm in diameter to clumps up to 1.5 m.

The Churchill population was collected from the coastal raised beach system near the National Research Council rocket launch center at Churchill, Manitoba. The lichen was found growing alongside P. apthosa as small (10 cm) individual rosettes amongst the grasses on the young beaches (Figure 10).

Figure 11 shows the monthly mean climatic summaries from the weather stations nearest each of the respective collection sites (Environment Canada 1984 a,b). The Muskoka site is in a temperate region, with monthly maximum temperature above 20°C for four summer months and a record high temperature of 35°C. The monthly minimums are above freezing from May to October. Churchill is in a much cooler, subarctic climate. The mean monthly maximums never exceeded 20°C, although a record high of 34°C has been observed. Night temperatures are above freezing for only four months, and snow can be expected in any month except July and August. Precipitation is distributed fairly evenly throughout the year in both locations. The Muskoka region is considerably wetter, with an annual accumulation of 1000 mm as compared to 400 mm in Churchill.

FIGURE 9: (upper) A photograph of a Muskoka collection site.

(lower) A photograph of P. rufescens from the Muskoka collection site.

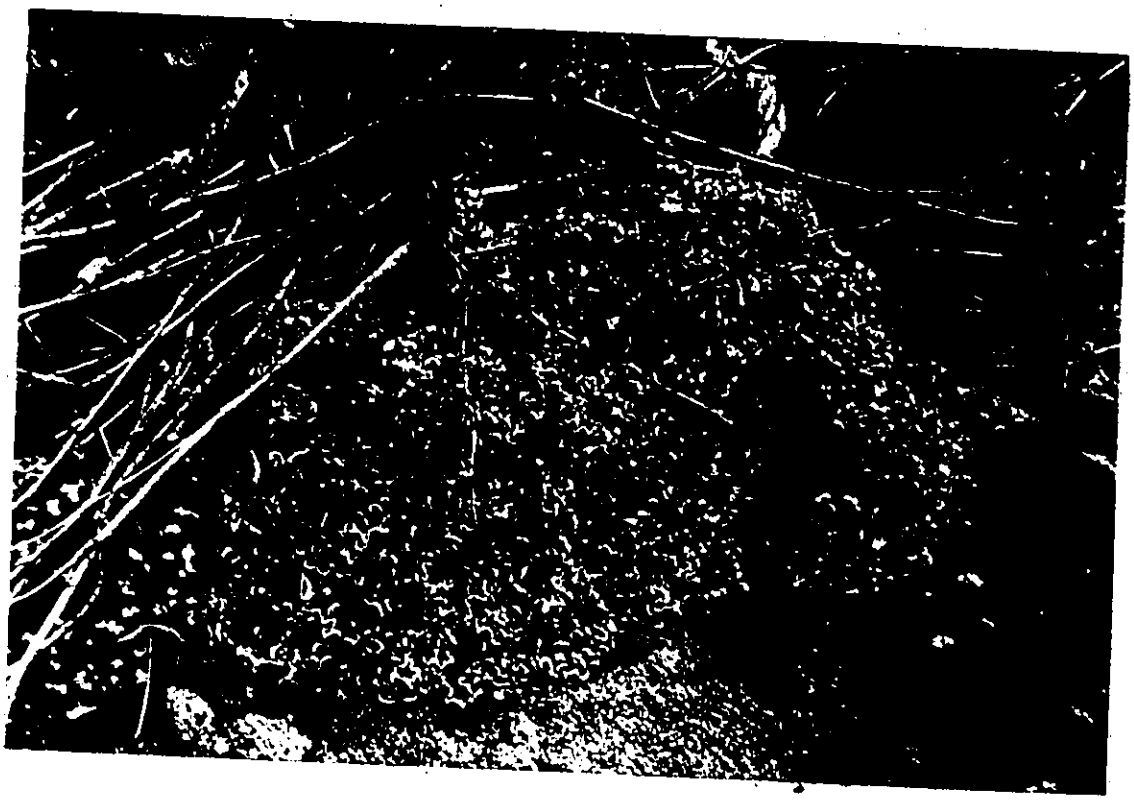
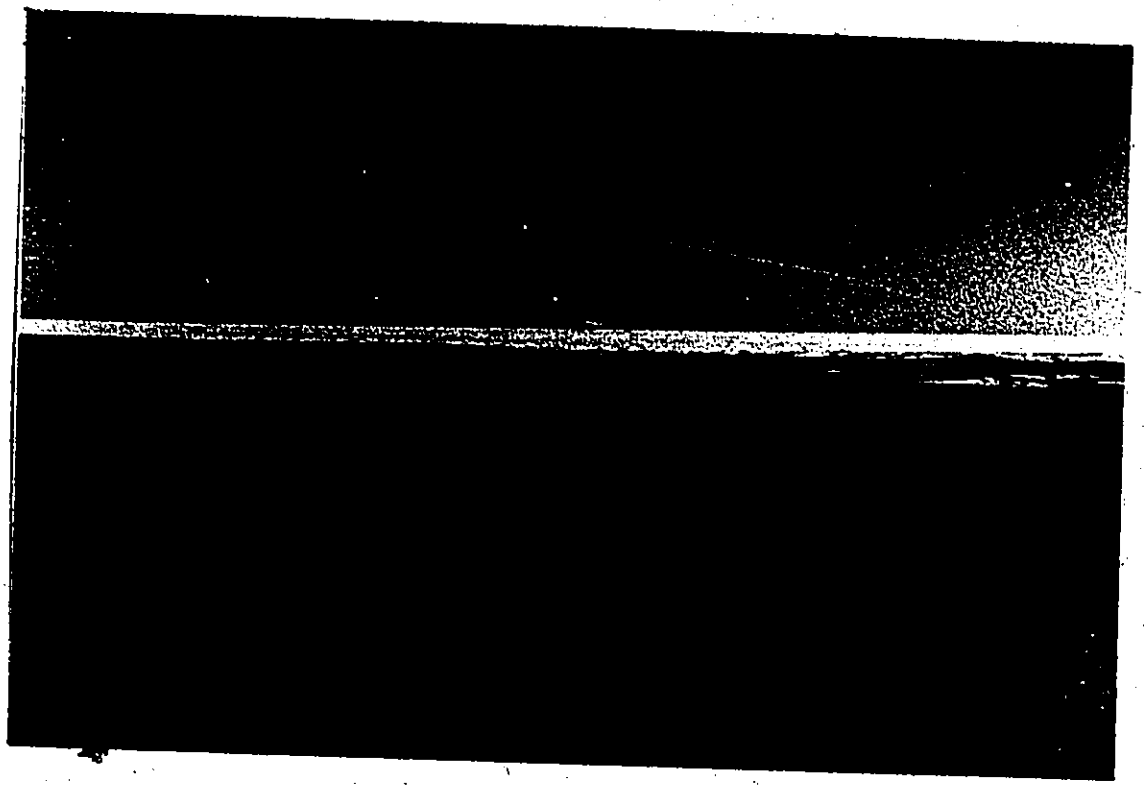


FIGURE 10: (upper) A photograph of a raised beach used for the Churchill collection site.

(lower) A photograph of P. rufescens from the Churchill raised beach system.



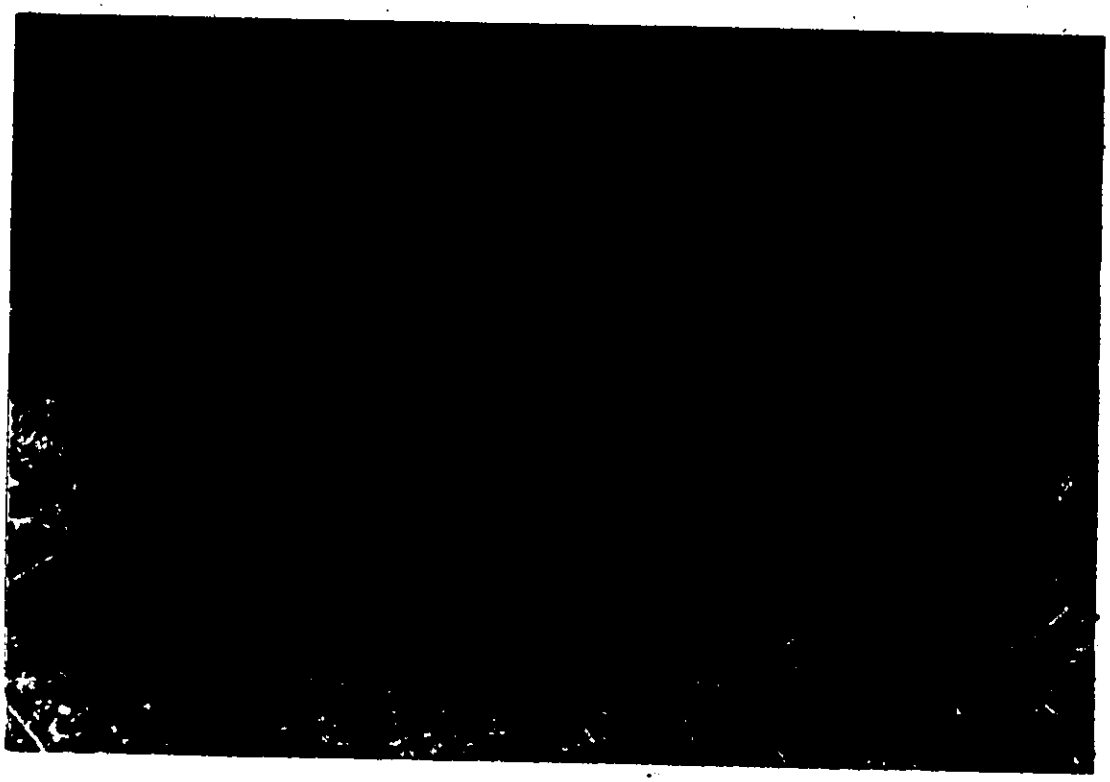
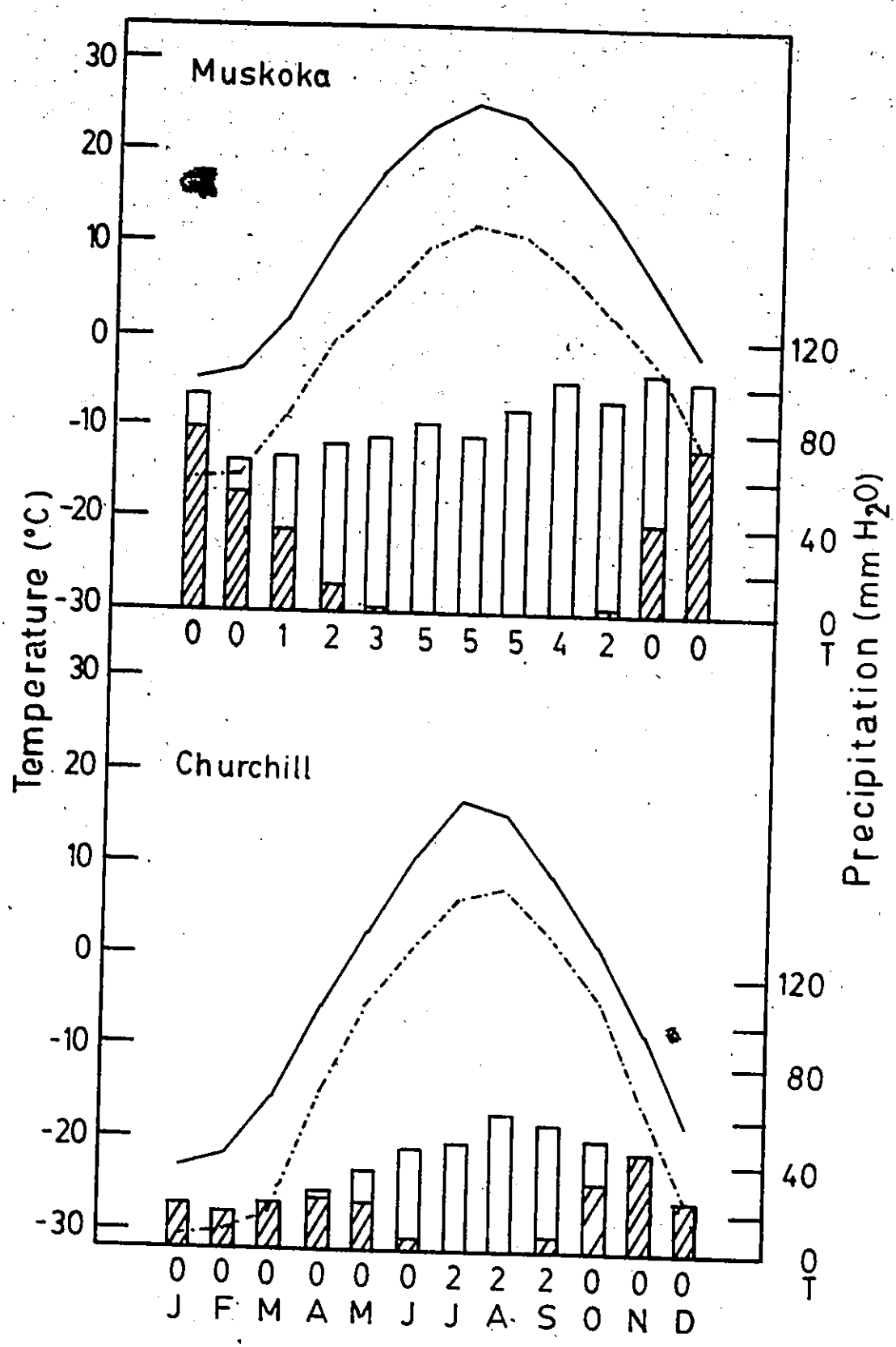


FIGURE 11: The 30 year monthly climatic summaries from the Muskoka (upper) and Churchill (lower) airports. The curves represent monthly minimum (—·—·—) and maximum (———) temperatures; the histograms represent the monthly precipitation (in mm H_2O equivalent) as snowfall (hatched) and rainfall (unhatched). The numbers on the line labelled T indicate the number of days that thunderstorms were recorded for each month.

(from Environment Canada 1984a,b)


Muskoka
Churchill
Temperature (°C)
Precipitation (mm H_2O)
30
20
10
0
-10
-20
-30
120
80
40
0
0 0 1 2 3 5 5 5 4 2 0 0
J F M A M J J A S O N D

3.2 Infra-red gas analysis

3.2.1 The measurement of net photosynthesis

A saturated lichen sample which has undergone the prescribed pretreatment conditions (Section 3.4) is trimmed to an appropriate size. Excess surface water is removed by gently blotting the thallus on a paper towel, and the lichen is placed on the ground glass bottom of the cuvette. This is placed in a conditioned air stream and the top of the cuvette is placed over the thallus and firmly pressed down on the base, forming an air tight seal. The airstream, from the departmental compressed air line, is conditioned by passing it through a water filled radiator connected to a Lauda circulating water bath. The temperature of the bath is adjusted such that the air temperature is within 1-2°C of the desired experimental temperature. Special care is taken to ensure that the cuvette has been sufficiently ventilated in this air stream, as this is used to control the initial CO_2 within the 200 ml cuvette at a level of 340 to 360 ppm.

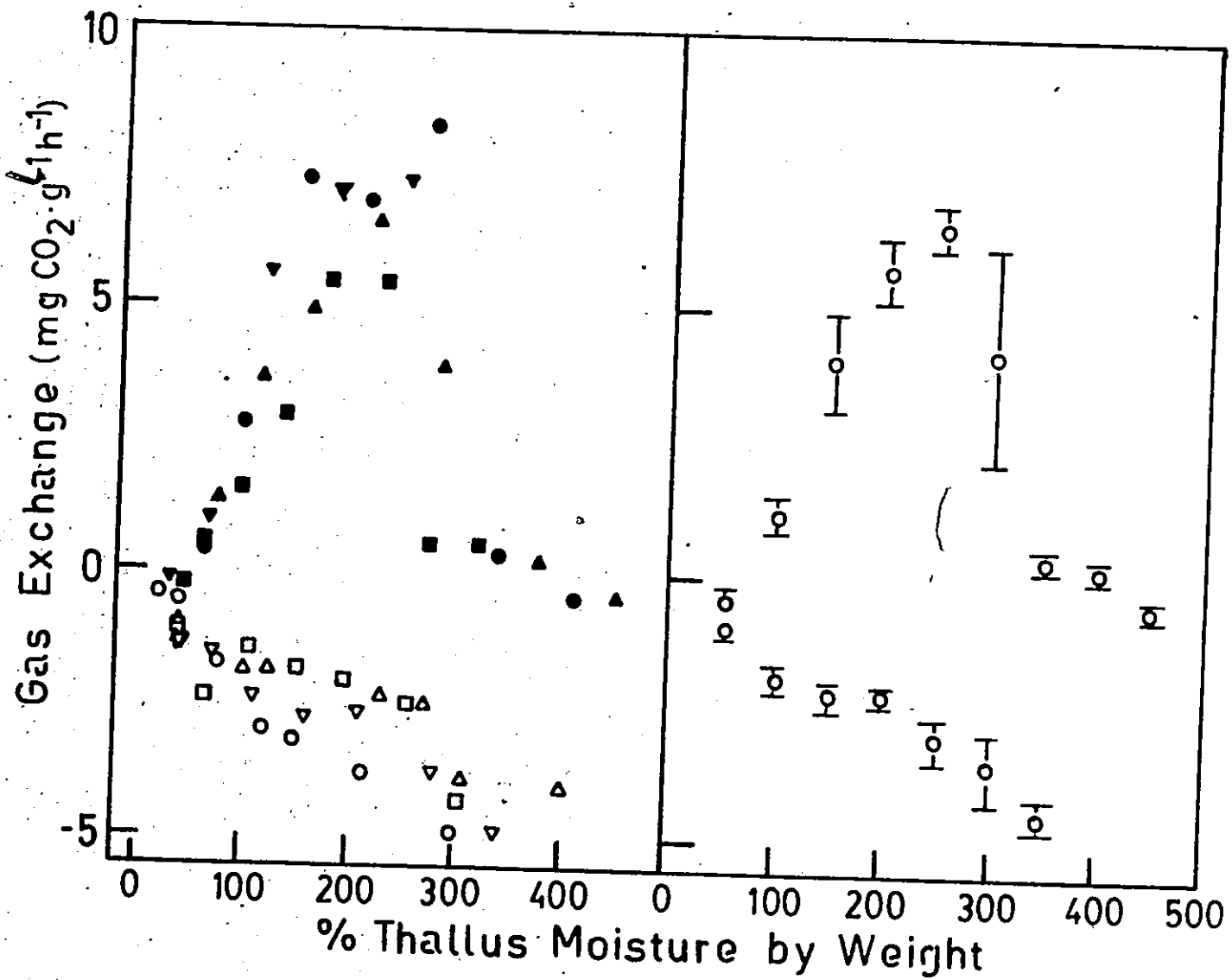
A 3 ml air sample is injected into the cuvette. The syringe is rapidly pumped 4 to 5 times to mix the air and a 3 ml sample is then removed for analysis. This air sample is injected into the carrier gas which flows through the sample cell of the IRGA (Figure 2). The IRGA sees this sample as a rapid increase in absorbance which peaks and then declines to the baseline absorbance of the carrier gas as the sample gas passes through the sample cell of the IRGA. The IRGA is

interfaced with a PDP-11 computer which determines the height of this peak and the time at which it occurs. The cuvette is then placed under the appropriate experimental conditions. Following an 8 to 10 minute incubation period, the air within the cuvette in again mixed by pumping the syringe and a second 3 ml air sample is removed and analysed. The seal in the cuvette is then broken, the lichen is removed and weighed, and the process is repeated until the lichen is dry. Normally 10 to 15 incubations are required for a complete drying curve. The gas exchange rate for any incubation is calculated from Eqn. 1.

$$mgCO_2 \cdot gm^{-1}h^{-1} = \frac{dCO_2 \times \text{Calib Coef} \times \text{cuvette volume} \times 1.09\times10^{-4}}{\text{incubation time} \times \text{dry weight}} \quad (1)$$

where dCO_2 is the difference in the IRGA output between beginning and end of the incubation and Calib Coef is the calibration coefficient (Section 3.2.2). To simplify the presentation of the data, a histogram is constructed by averaging the net photosynthetic rates over 50% moisture classes and plotting this against the upper limit of the moisture class (Figure 12). The thallus moisture content is expressed as the % weight of water in the thallus (Eqn. 2). The dry weight is measured following an overnight treatment at 80°C.

FIGURE 12: The relationship between net photosynthesis and thallus moisture content. The left figure is the raw data from 4 replicate thalli assayed at 35°C, 0 (open) and 800 $\mu E \cdot m^{-2}s^{-1}$ (closed). This raw data is then divided into 50% moisture classes and plotted as gas exchange (mean ± SE) against the upper moisture limit for each class, as shown in the right figure.


10
5
0
-5
Gas Exchange (mg $CO_2 \cdot g^{-1} h^{-1}$)
0
100
200
300
400
0
100
200
300
400
500
% Thallus Moisture by Weight

$$\% H_2O = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}} \times 100 \qquad (2)$$

3.2.2 Calibration of the IRGA

The IRGA was calibrated at the beginning of each day by injecting 3 ml sample of a prepared CO_2 balance N_2 mixture into the carrier gas. At the start of the study, a 200 ppm CO_2 balance N_2 mixture was used for the carrier, and the calibration gases contained 0, 100, 300, 400, and 500 ppm CO_2. The IRGA was set on range 3 for maximum sensitivity and the gain was adjusted such that the 500 ppm gas produced a full page deflection on a chart recorder set on the 100 millivolt range. The 0 and 100 ppm gases produced a negative peak, and the zero adjust on the recorder was changed to read this peak. Five injections per gas was used to construct the calibration curve. This curve is linear in the 0 to 500 ppm range (Figure 1) and repeated injections were accurate to within 5 ppm.

During the final two year of the study, the carrier gas was changed from 200 ppm to 0 ppm CO_2 balance N_2. Gases of 30, 100, 200, 300 and 500 ppm were then used to calibrate the IRGA. The response is still linear for this range (Figure 1), but the sensitivity was reduced.

3.2.3 The effects of CO_2 limitation on net photosynthesis

The effects of both above and below ambient concentrations of CO_2 were tested for several thallus moisture

contents and temperatures.

The lichen was dried to the desired moisture content, and then placed on a plastic weighing dish in the cuvette. Moist filter paper was placed on the bottom of the cuvette and the walls were sprayed with water. This produced a high humidity environment which buffered the lichen from drying during the experiment.

The cuvette was then flushed with either 0, 500 or 1750 ppm CO_2 balance air mixtures and the net photosynthetic rate was measured. The length of the incubation period was adjusted such that the change in CO_2 concentration during the incubation was less than 50 ppm. Unlike the normal assay procedure described above (Section 3.2.1), the lichen was not removed from the cuvette at the end of the incubation for the wet weight measure. Instead, the seal was not broken and a small volume of high CO_2 air was injected into the cuvette and was rapidly mixed. This raised the CO_2 concentration in the cuvette by 10 to 15 ppm.

A second incubation was then started, and this process was repeated. Usually 15 to 20 incubations could be obtained before the lichen dried appreciably. Thus a series of net photosynthetic measurements were obtained for overlapping CO_2 concentrations, covering the range of 0 to 500 and 500 to 1750 ppm CO_2. For the 500 to 1750 ppm range, the carrier gas in the IRGA was changed to 500 ppm CO_2, and the IRGA was calibrated by injections of 1, 2, and 3 ml samples of the 1750

ppm CO_2 balance air mixture.

The CO_2 limitation curves were carried out for thalli at moisture contents between 150 and 250% and over 350% water at a thallus temperature of 25 and 35°C and a light intensity of 800 µE. The Muskoka population of P. rufescens collected in June of 1982 was used for these experiments. In addition to the limitation curves, a complete drying curve run at a CO_2 concentration of 1750 ppm was obtained at 25 and 35°C at a light intensity of 800 $\mu E \cdot m^{-2} s^{-1}$.

3.2.4 Measurement of light and temperature

A metal halide light source was used in both the experimental and storage chambers. This light was sufficient to produce a light intensity of 1800 µE. A reostat dimmer was used for a coarse adjustment of light intensity, while the fine adjustments were made by altering the location of the cuvette within the growth chamber. By blocking part of the light source with grids or tinfoil, a continuous gradient of light between 20 and 1800 $\mu E \cdot m^{-2} s^{-1}$ could be created within the experimental growth chamber.

This light source was subject to rapid oscillations in intensity, which made it impossible to precisely describe the light conditions. These fluctuations range from ± 50 $\mu E \cdot m^{-2} s^{-1}$ at an average intensity of 1000 µE to ± 2 $\mu E \cdot m^{-2} s^{-1}$ at an intensity of 20 $\mu E \cdot m^{-2} s^{-1}$. The light was filtered through a 10 cm water bath to absorb the infra-red radiation. The light

intensity in the 400-700 nm wavelengths was measured with a Lamda quantum sensor placed inside a cuvette and recorded by a Licor LI-185a photometer.

Thallus temperature was measured using a small YSI series 500 thermistor pressed against the lower surface of a dry reference thallus. As the experimental thallus has to be removed from the cuvette frequently to obtain weight measurements, it was impractical to attach the temperature probe to the experimental thallus. An additional reference cuvette was set up under identical light conditions as the experimental cuvettes and the temperature was continuously monitored in this cuvette. The air temperature of the growth chamber was adjusted such that the temperature of this reference thallus was within 1°C of the desired temperature.

Placement of this thallus temperature probe at various locations with the growth chamber indicated that the temperature variation from location to location would be less than 1°C provided the light intensity of the positions did not differ by more than 200 μE.

There is a systematic error involved with the temperature measurement made in this manner. The reference thallus was dry, but the experimental thallus was at various water contents as mentioned above. Thus there would be a slight temperature difference between the reference and experimental thalli due to the effects of evaporative cooling. It was thought that a better experimental control, both within

and between runs, could be obtained if the temperature was referenced against a constantly dry thallus instead of a thallus continuously changing in moisture content.

3.3 Choices of experimental material

The measured net photosynthetic rate is very dependent on the choice of experimental material, being affected by the ratio of fungal to algal biomass and the physiological state of the lichen. Kärenlampi (1970) demonstrated that the distribution of chlorophyll (and hence algae) is highly stratified in the fruticose lichen Cladonia stellaris. Gas exchange in the thallus is highly correlated to this distribution. Net photosynthesis is highest near the young tips while the gas exchange in the basal portion of the podetia is dominated by fungal respiration (Nash et al. 1980; Lechowicz 1983).

This variation is also present in foliose lichens. Nash et al. (1980) found a seven-fold variation in the $^{14}CO_2$ fixation rate within the thallus of Parmelia separata. This lichen undergoes a radial growth pattern and the maximum fixation rates were observed near the thallus margin. Larson (1983) has also observed regions of high and low net photosynthetic capacity in thalli of Umbilicaria (which he suggests indicates the existence of several discrete populations of algae within the thallus). This variation in net photosynthesis did not follow any specific distributional

pattern within the thallus, but between- thallus variation was high and related to the size of the thallus (Larson 1986). Kershaw (1977a) has shown a similar phenomena in Peltigera polydactyla, where the larger thalli had lower rates of net photosynthesis and respiration. Thus the outcome of an experiment can be affected by the size of thallus used.

Link and Nash (1984a) used a prescreening method to reduce this individual variability. They measured the gas exchange rates of 20 replicates of Parmelia praesignis and then used the five thalli closest to the sample mean for the experiments. This would ensure that the response pattern obtained is representative of the population sampled and reduce the between run variation. However the possibility that the replicates will undergo a physiological change during the one to two weeks required to construct a gas response matrix would introduce a larger uncertainty in the results than the variation caused by selecting different thalli for each measurement.

The choice of experimental material in this study was thus highly subjective. Non-fruiting lobes the size of a thumb nail were picked from the margin of the rosettes. This corresponded to a thallus dry weight of 0.05 to 0.15 gm. With practice, between replicate variation could be minimized such that the standard error within any moisture class was less that 10% of the mean, and good replication between experiments could be obtained.

3.4. Standard pretreatment conditions

The storage conditions reported in the literature vary considerably, ranging from a situation where the material was kept on the laboratory benchtop, to storing the lichen in a freezer (Larson 1979), or in continuous darkness (Snelgar et al. 1981). The objective of this study was to monitor the changes in photosynthesis occurring throughout the year, therefore it was decided to store the material in growth chambers under a temperature and photoperiod regime that was similar to the field conditions for the season of collection.

The thalli were stored in an air dry state, which represented a thallus moisture content of approximately 12% in the summer and 25% in the winter. This does not parallel a field situation, as P. rufescens seldom remains dry for extended periods of time. Altering wet/dry periods are required to maintain the symbiosis (Tysiaczny and Kershaw 1979), but it was decided to store the lichens dry to simplify the experiment. This lichen could be maintained in this state for several weeks before any decline in net photosynthesis was observed.

The moistening of the lichen thalli at the beginning of an experiment does produce some physiological problems. Several researchers report a transient burst of high respiration when the thallus is wetted. The extent of this resaturation respiration is ecotype specific, lasting less than an hour in xeric species such as Chondropsis, Umbilicaria

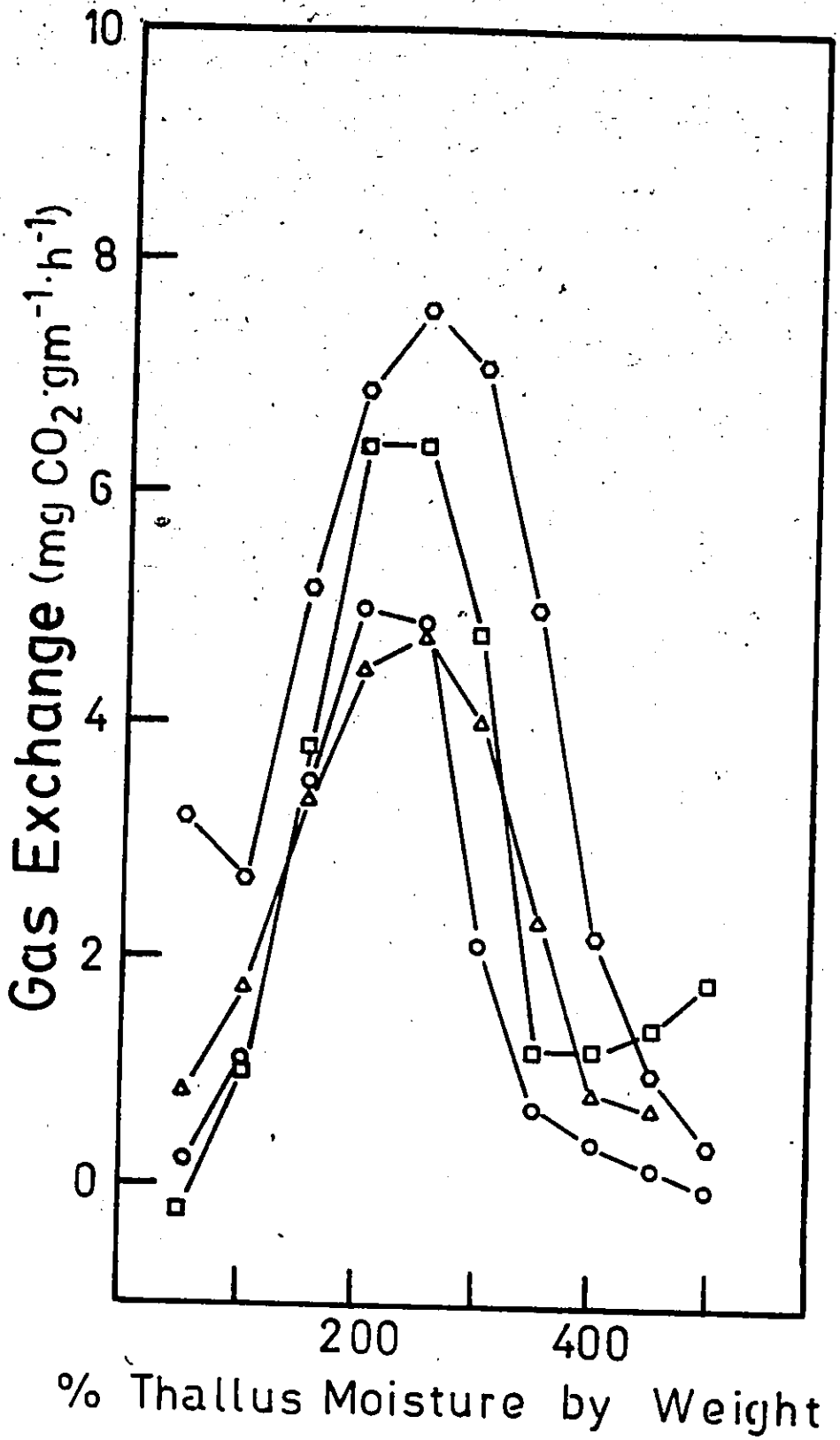
and Hypogymnia, up to 2 hours in Xanthoria and Parmelia, and 12 hours in a mesic population of Peltigera polydactyla (Rogers 1971; Smith and Molesworth 1973; Farrar and Smith 1976; Larson 1979; Link and Nash 1984b). The magnitude of this burst is strongly dependent on the previous history of the lichen (Brown et al. 1983; Link and Nash 1984).

In P. rufescens, net photosynthesis increased continuously during a 24h period following rewetting (Figure 13). Although a 24 hour presoak produced a higher activity, it was decided that an overnight presoak of 10 to 16 hours was adequate to allow the lichen to recover from the resaturation effects, in addition to being a convenient time interval experimentally. This presoak was done under temperature and photoperiods identical to the storage regime of the lichen collection, with one exception. When the night storage temperature was below freezing, the lichen was soaked at 2°C to prevent freezing.

3.5 The seasonal gas exchange matrix

A response matrix was constructed to describe the pattern of net photosynthesis in P. rufescens. This matrix was designed to show all possible interactions between thallus temperature, light intensity, thallus moisture content and season. A complete drying curve was run at all combinations of temperature (5, 15, 25, 35 and 45°C) and light (0, 300, 500, 800 and 1000 $\mu E \cdot m^{-2} s^{-1}$) for each seasonal collection.

FIGURE 13: The effects of resaturation on gas exchange. Thalli which had been stored air dry were rehydrated 2(Δ), 4(O), 12(□) or 24(O) hours prior to the measurement of net photosynthesis.


10
8
6
4
2
0
Gas Exchange (mg $CO_2 \cdot gm^{-1} \cdot h^{-1}$)
200
400
% Thallus Moisture by Weight

Five collections of the Muskoka population and three collections of the Churchill population were included (Table I,II) in the matrix.

Four replicate thalli were run at each light and temperature combination. To maintain experimental control of thallus temperature, the 0, 300 and 500 $\mu E \cdot m^{-2}s^{-1}$ light intensities were run simultaneously in one experiment, while the 800 and 1000 $\mu E \cdot m^{-2}s^{-1}$ intensities were run in a second experiment, usually on the same day. Thus the entire matrix could be completed in five days. The 15, 25 and 35°C assays were run in a walk-in growth chamber, while the 5 and 45°C assays were done in a reach-in chamber.

3.6 Photosynthetic-illumination curves

3.6.1 P-I Gas exchange method

The gas exchange matrix is useful in showing the interactions between several environmental factors, but it is not adequate to describe the detailed response of a single parameter. P-I curves have been used frequently to describe the photosynthetic response of algae, and a detailed study of these curves can be used to indicate the physiological mechanism behind any observed changes in net photosynthesis.

The P-I curves were constructed using thalli at optimal water contents according to the procedure described by Kershaw and Webber (1984). The light intensity within the experimental growth chamber was adjusted by the arrangement of

TABLE I: The seasonal storage conditions for Muskoka population of *P. rufescens*.

Collection date	Thallus temperature night	day	photoperiod hours per day
April 7, 1981	-5	2	12
June 25, 1981	21	33	16
September 21, 1981	8	15	12
January 6, 1982	-2	1	9
November 30,1982	-3	2	8

All collections were stored at a light intensity of 800 to 1000 $\mu E \cdot m^{-2} s^{-1}$.

TABLE II: The seasonal storage conditions for Churchill population of *P. rufescens*.

Collection date	Thallus temperature		photoperiod
	night	day	hours per day
July 16, 1982	15	20	15
September 9, 1982	3	10	12
October 27, 1982	-5	3	8

All collections were stored at a light intensity of 800 to 1000 $\mu E \cdot m^{-2} s^{-1}$.

shelf heights, grids and tin foil such that a range of intensities between 20 and 1800 $\mu E \cdot m^{-2} s^{-1}$ could be obtained. The walls and top of the cuvettes were sprayed with water, and a moist filter paper was placed on the bottom of the cuvette to buffer the rate of evaporation from the thalli. The lichens were allowed to dry until their moisture content was at near optimum levels. The P. rufescens thallus undergoes a colour change as it dries (from dark green to grey pruniose), and the optimum moisture content corresponds to the point when the light grey speckles start to appear. This colour change, along with periodic checks of the net photosynthetic rate at 800 μE made as the lichen dried out, were used to judge the time at which optimal net photosynthetic rates could be obtained. At this time the lichen placed in the humidified cuvette and net photosynthesis was measured as the cuvette was brought through a sequential increase in light intensity of 0, 25, 50, 75, 100, 150, 200, 250, 300, 400, 500, 800, 1000, 1400 and 1600 $\mu E \cdot m^{-2} s^{-1}$. Two measurements of net photosynthesis were made at each light level. The growth chamber temperature was constantly adjusted during the experiment to ensure that thallus temperature did not vary by more than 1.5°C from the experimental temperature.

Net photosynthesis was measured in a similar manner as the CO_2 limitation curves (Section 3.2.3). The cuvette was not opened between most incubations, and a small volume of CO_2 enriched air was injected into the cuvette at the beginning of

each incubation to bring the concentration to near ambient levels. Two or three thalli were placed inside each cuvette. This allowed the incubation times to be shortened to 3 minutes, thus the experimental time was kept short and the thalli did not dry out substantially during the measurements.

AQE was calculated from a linear regression between 0 and 200 μE, while light saturated rates are reported as the average of the measurements recorded at 800, 1000, 1400 and 1600 μE

3.6.2 Extraction of the photosynthetic pigments

The water soluble biliproteins and the acetone soluble chlorophyll was extracted from the same thallus following a procedure modified from Hampton (1973). Following the completion of the P-I curves, the lichen samples were allowed to dry overnight in a desiccator. The weights were recorded and the thalli were frozen in liquid nitrogen and ground with a mortar and pestle. To this was added 30 ml of a 0.01 M sodium phosphate buffer, pH 6.5. This was homogenized in a glass piston type tissue homogenizer, sonicated for 20 minutes and placed on an aliquot mixer in a cold room. The following morning this mixture was centrifuged for 20 minutes at 20,000 xg. The absorbance of the supernatant was measured at 562, 615, and 650 nm, and corrected for background absorbance measured at 750 nm. The phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC) contents ($mg \cdot gm^{-1}$) were calculated

using equations 3, 4, and 5 from Bennett and Bogorad (1973).

$$PC = \frac{(A_{618} - 0.74 \times A_{592}) \times \text{extract volume}}{5.34 \times \text{dry weight}} \quad (3)$$

$$APC = \frac{(A_{592} - 0.208 \times A_{618}) \times \text{extract volume}}{5.09 \times \text{dry weight}} \quad (4)$$

$$PE = \frac{A_{592} - 2.41 \times PC - 0.849 \times APC \times \text{extract volume}}{9.62 \times \text{dry weight}} \quad (5)$$

The pellet was resuspended in 30 ml of 80% acetone, sonicated for 20 minutes and placed on the mixer in the cold room. The following morning, this mixture was centrifuged for 20 minutes at 20,000xg. The chlorophyll content ($\mu g \cdot g^{-1}$) was calculated from the absorbance at 663 nm (corrected for background absorbance at 750 nm) using the formula of Lorenzen (1967) (Eqn 6).

$$Chl = \frac{26.73 - A_{663} \times \text{extract volume}}{\text{dry weight}} \quad (6)$$

3.7 Laboratory induced acclimation experiments.

The seasonal net photosynthetic response matrices for the Muskoka population indicates that this plant may undergo large seasonal changes in its photosynthetic capacity (Figure

19). A series of experiments were undertaken in controlled laboratory conditions to determine the plasticity of this response, the potential rate at which this change may occur and the environmental triggers for this change. Temperature and photoperiod were the only environmental parameters considered. The acclimation experiments were performed on lichen thalli collected from Muskoka in April, June, and July of 1981, January, August, October and November of 1982, and July and September of 1985.

The experimental protocol was similar in all studies, although the actual storage conditions varied. The lichen material was initially stored in the air dry state for one week under the temperature and photoperiod conditions similar to that observed in the field at the time of collection. This is referred to as the control, or day 0, conditions. The lichens were then placed under the experimental conditions at 9 AM. They were kept in an air dry state throughout the experiment, except for the overnight presoak required for the measurements of net photosynthesis . Thus a gas exchange value reported for day 1 of an experiment represents a lichen thallus maintained under the experimental conditions for 12 hours in an air dry state, and 12 hours in a saturated state, while day 18 represents material stored for 17.5 days dry and one half day saturated. The reversibility of the experimentally induced responses were tested at the end of each series by returning the experimental material to control

conditions and then measuring the net photosynthetic rate. Table III contains the details of the control and experimental storage conditions for all the acclimation experiments.

TABLE III: Storage conditions used to study the laboratory induced acclimation in the Muskoka population of *P. rufescens*

Collection date	treatment	time (days)	Storage (°C)	(h)	assay temperature
April 7,81	Control	9	-5/2	12	5,15,25,35
	to warm	7	15/25	12	5.15.25,35
June 25,81	Control	8	21/33	15	35.45
	to short	4	21/33	9	35.45
	to cold	4	0/8	15	35.45
	to winter	5	0/8	9	35.45
	to control	5	21/33	15	35.45
July 21,81	Control	7	17/35	15	35
	to short	var	15/30	9	35
	to cold	var	-1/5	15	35
	to winter	var	-1/5	9	35
	to long	var	-1/5	15	35
	to warm	var	15/30	9	35
	to control	var	17/35	15	35

Continued ...

Jan. 6,82	Control	6	-2/1	9	35
	to long	var	-2/0	15	35
	to warm	var	15/19	9	35
	to summer	var	15/19	15	35
August 1,82	Control	7	25/35	16	25,35
	to winter	10	2/5	8	25,35
Oct. 5,82	Control	7	25/35	16	25,35
	to winter	15	-2/8	8	25,35
	to control	5	25/35	16	35
Nov. 30,82	Control	13	-3/2	8	25,35
	to summer	9	25/35	16	25,35
July 29,98	Control	7	35/32	15	35
	to winter	var	-2/2	9	35
Sept. 20,85	Control	10	25/30	15	35
	to winter	var	-2/1	8	35
	to control	3	25/35	15	35

1 time under the described conditions prior to the measurement of net photosynthesis and/or shift to next experimental treatment. Var indicates that the experiment was a time series.

2 night/day thallus temperature in °C and the storage photoperiod in hours of light per day.

4 RESULTS

4.1 Interaction of thallus moisture and CO_2 concentration with gas exchange

Moisture relationships play a crucial role in the metabolic processes of poikilohydric plants. Although lichens do not have structures such as cuticle or stomata, they do have several morphological modifications which affect water uptake, storage and loss (Kershaw 1985). These modifications are not of direct consideration in this study, but the interaction between moisture, CO_2 diffusion and the net photosynthetic rate do deserve some comment.

The changes in the rate of net photosynthesis which occur during drying are complex (Figure 12). At extremely high moisture contents, an efflux of CO_2 is observed. This efflux declines as the thallus dries, and a positive carbon uptake is achieved at water content of 400%. Net photosynthesis increases to a maximum at moisture contents near 200%, then rapidly drops as the thallus dries out further until it again becomes negative below 50% moisture contents. This gas exchange pattern, common to several Peltigera species (Kershaw 1977a; Kershaw and MacFarlane 1980; Lange and Matthes 1981), as well as Sticta latifrons, Usnea sp., Umbilicaria deusta, Cladonia stellaris, and C. rangiferina (Larson 1979; Snelgar et al. 1980; MacFarlane et

al. 1983; Kershaw et al. 1983), is the result of a complex interplay between respiration, photosynthesis and CO_2 diffusion resistance. Temperature and light intensity does not affect the moisture response of net photosynthesis significantly (Figure 16, 19).

Respiration has a lower moisture threshold than photosynthesis (Figure 12), which accounts for the CO_2 efflux at low moisture contents (Lange 1980). Respiration then increases with thallus moisture. This increase is linear at temperatures of 35 and 45°C, but shows a saturation response at 5, 15 and 25°C (Figure 16, 19). This difference may represent a low temperature inhibition of the biochemical processes involved.

It was originally thought that the depressed rate of net photosynthesis at thallus saturation was a result of the high respiration rates at these moisture contents (Kershaw 1977a). However a more recent study using $^{14}CO_2$ techniques has shown that this is not the case (Tysiaczny and Kershaw 1979), and the depressed rate of net photosynthesis is now best explained as a CO_2 limitation phenomena. Snelgar et al. (1981) have demonstrated that the thallus resistance to CO_2 diffusion is greatly increased by high moisture contents, due to the flooding of internal air passages and the formation of a film of water on the thallus surface (Green and Snelgar 1982; Coxson et al. 1983).

The degree of CO_2 limitation caused by this increased

thallus resistance is easily seen in Figure 14. Net photosynthesis at optimum levels of thallus hydration gradually decreases as the experimental CO_2 concentration declines from 1750 to 250 ppm. After this point, net photosynthesis becomes increasingly limited by the CO_2 concentrations, with gas exchange becoming negative below 70 ppm. A considerably different pattern is observed in a fully hydrated thallus. Net photosynthesis responds linearly to CO_2 concentrations at 25°C, and rates comparable to optimum values were not obtained until the cuvette CO_2 concentration exceeded 1500 ppm. Gas exchange is even further depressed at thallus saturation when the temperature is increased to 35°C (Figure 14), where the CO_2 compensation point is not reached until 750 ppm.

Green and Snelgar (1982) have previously demonstrated this phenomena, and Lange and Tenhunen (1981) suggest that a high concentration of CO_2 could be used to eliminate this depression. This would greatly simplify some experimental procedures (such as a P-I curve) by eliminating the moisture dependence of net photosynthesis. A complete drying curve for P. rufescens, determined under an atmosphere containing 1750 ppm CO_2 is presented in Figure 15. At 25°C. this concentration of CO_2 is sufficient to increase the net photosynthetic rate at thallus saturation to near optimum levels, but it cannot overcome the combined effects of high respiration and high CO_2 diffusion resistance at 35°C.

FIGURE 14: The relationship between net photosynthesis and CO_2 concentration in P. rufescens assayed at 35°C (left), and 25°C (right) and at optimal thallus hydration (upper) and in a saturated thallus (lower).

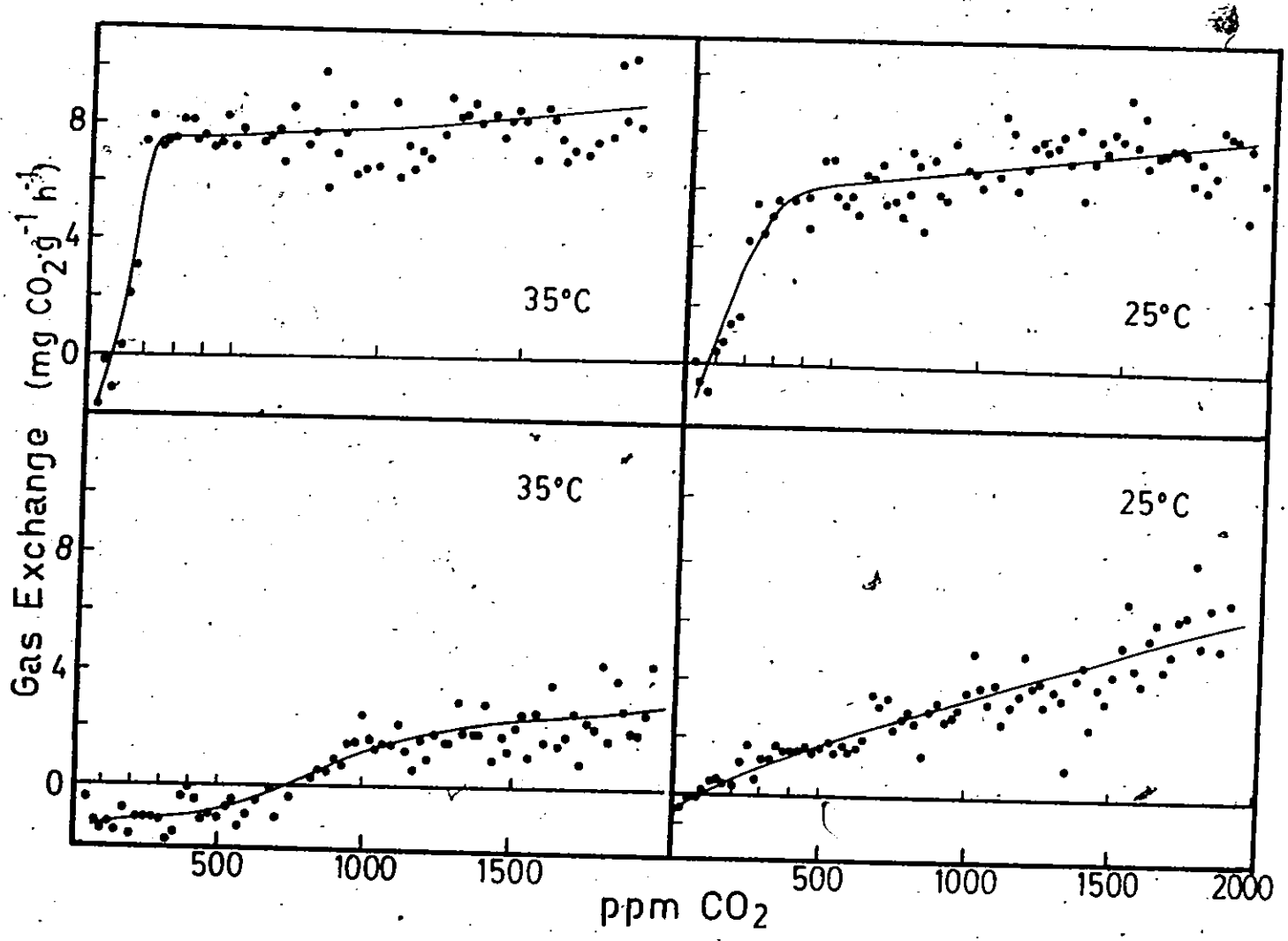
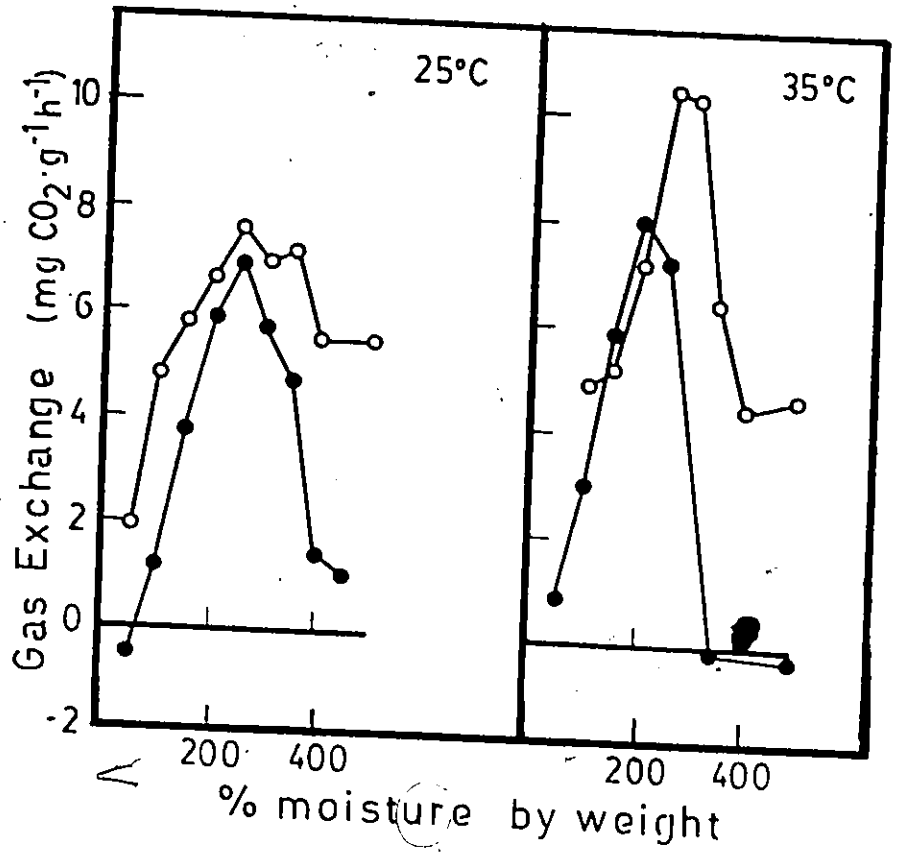

Gas Exchange (mg $CO_2 \cdot g^{-1} h^{-1}$)
8
4
0
35°C
25°C
35°C
25°C
500
1000
1500
500
1000
1500
2000
ppm CO_2

FIGURE 15: The relationship between the degree of thallus hydration and mean rates of gas exchange in *P. rufescens*, at 25°C, 800 $\mu E \cdot m^{-2}s^{-1}$ (left); 35°C, 800 $\mu E \cdot m^{-2}s^{-1}$ (right); and under either normal ambient CO_2 concentrations (●), or 1750 ppm CO_2 (O). Values are for 50% class intervals. SE less than 0.8 mg $CO_2 \cdot h^{-1}g^{-1}$.


25°C
35°C
Gas Exchange (mg $CO_2 \cdot g^{-1} h^{-1}$)
10
8
6
4
2
0
-2
200
400
200
400
% moisture by weight

4.2 The seasonal net photosynthesis responses

The combined effects that thallus moisture, temperature, light and season have on the gas exchange in the Churchill population is shown in Figure 16. In July, net photosynthesis (measured at 1000 μE) was little different at temperatures of 25, 35 and 45°C, rates at optimum moisture contents being 3.9, 4.4 and 3.2 mg $CO_2 \cdot gm^{-1}h^{-1}$ respectively. Gas exchange was slightly reduced at 15°C (2.4 mg $CO_2 \cdot gm^{-1}h^{-1}$), and dropped to one third of the 35°C rates at 5°C. The highest rate of net photosynthesis was observed in the September collection at 35°C.

The temperature effects decrease as light intensity was lowered, with very similar rates recorded at 300 $\mu E \cdot m^{-2}s^{-1}$ for temperatures between 5 and 35°C for all collections. However, at 45°C the 300 and 500 $\mu E \cdot m^{-2}s^{-1}$ interaction show depressed rates relative to the other cells in the matrix. This may indicate a possible stress response at this temperature.

A different presentation of the Churchill data is shown as a 3 dimensional plot in Figure 17 for the July and November collections. Here the temperature optimum is clearly shown to be 35°C in both collections, and a slight temperature effect is observed for the lower light intensities. The light saturation points appears to shift from 300 $\mu E \cdot m^{-2}s^{-1}$ at 5°C to 800 $\mu E \cdot m^{-2}s^{-1}$ at the higher temperatures. Temperature effects are not significant below

FIGURE 16: The seasonal gas exchange response matrix for the Churchill population of *P. rufescens* at 0, 300, 500, 800 and 1000 $\mu E \cdot m^{-2} s^{-1}$ and 5, 15, 25, 35 and 45°C, in July (▲), September (O) and November (●). SE less than 0.8 mg $CO_2 \cdot h^{-1} g^{-1}$.

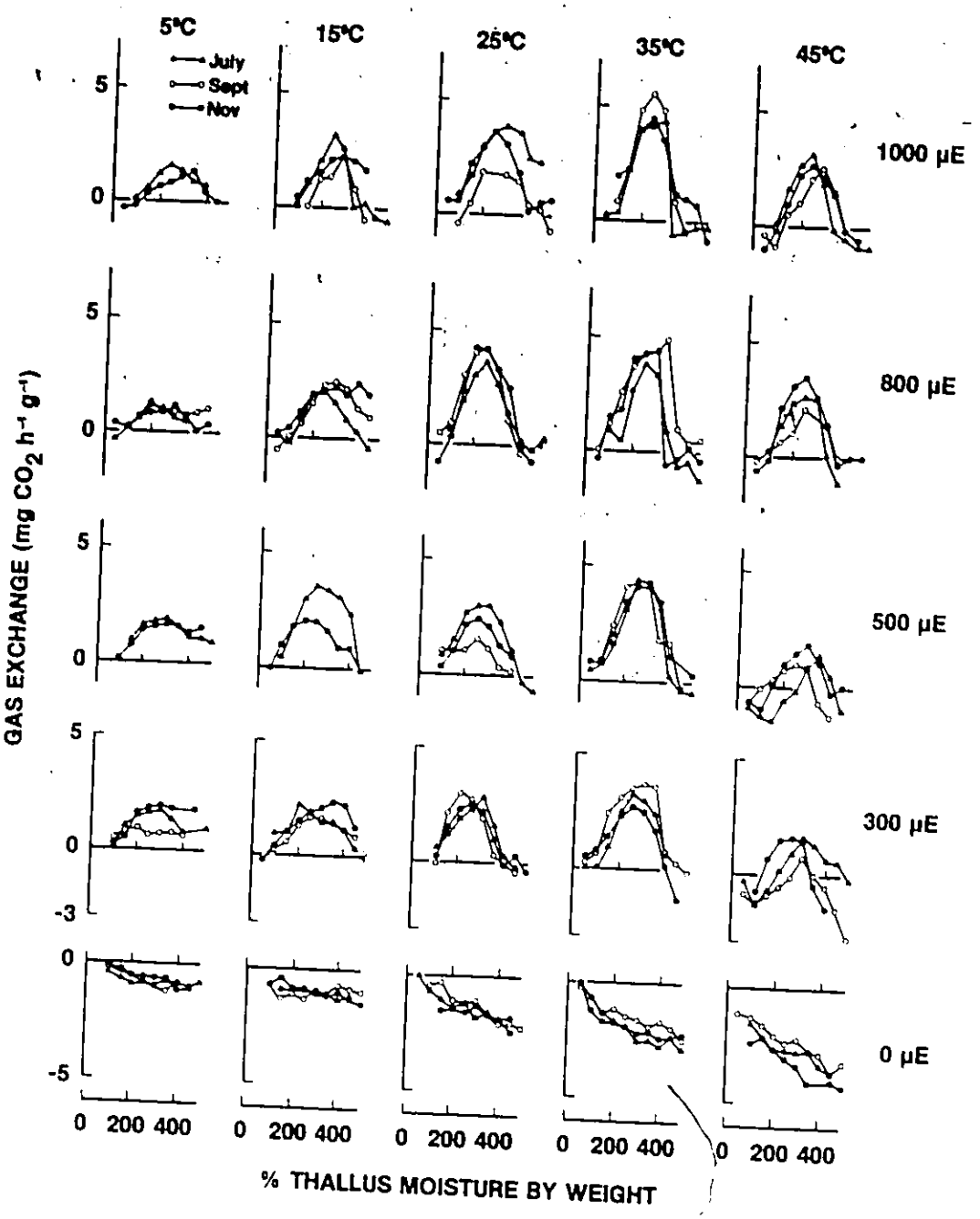
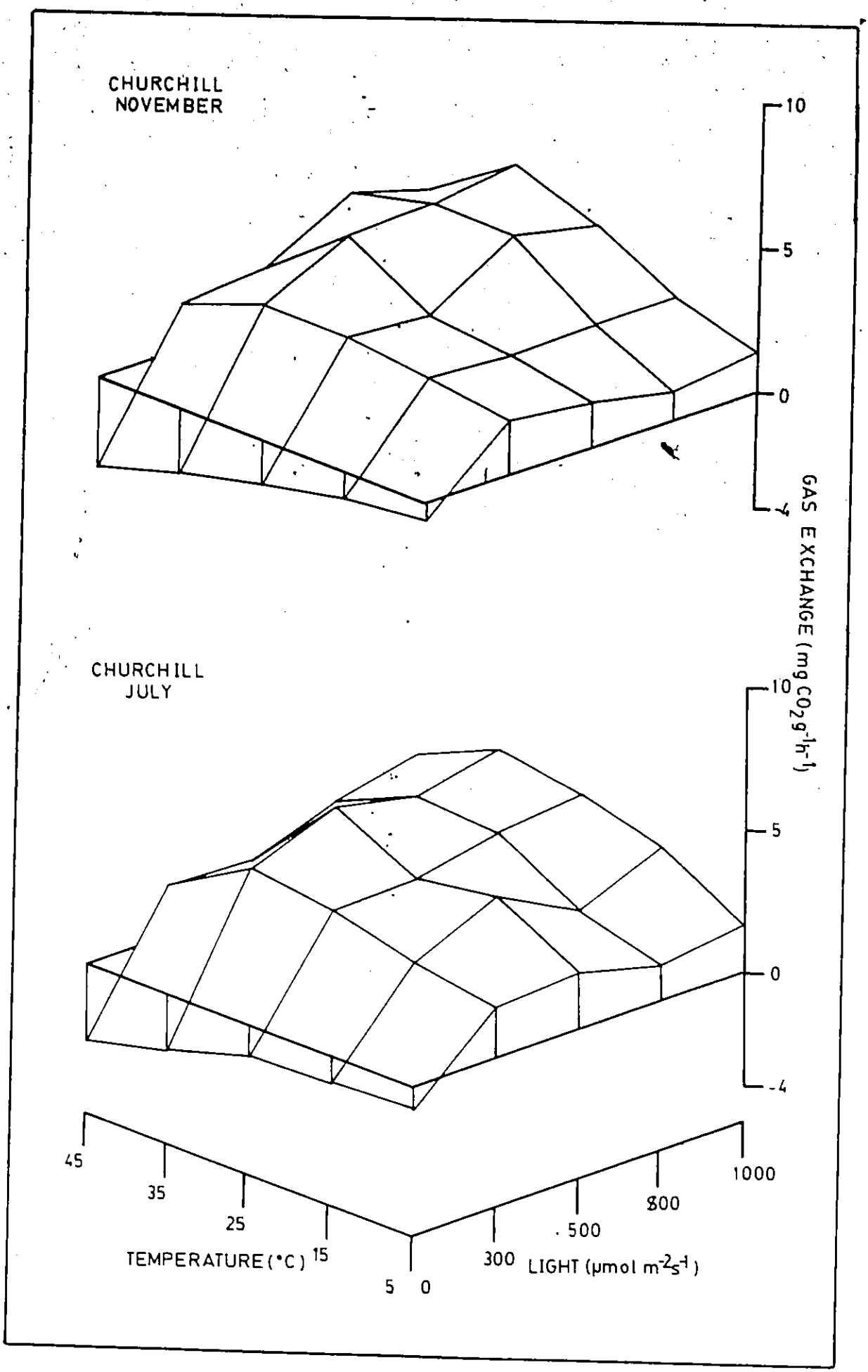

5°C
15°C
25°C
35°C
45°C
July
Sept
Nov
1000 µE
800 µE
500 µE
300 µE
0 µE
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
% THALLUS MOISTURE BY WEIGHT

FIGURE 17: Three dimensional plot of the relationship between light, temperature and net photosynthesis in the July and November collections of the Churchill population of P. rufescens. The net photosynthetic capacities given for the 200-250% thallus moisture class.


CHURCHILL
NOVEMBER
10
5
0
-4
CHURCHILL
JULY
10
5
0
-4
GAS EXCHANGE (mg CO_2 $g^{-1}h^{-1}$)
45
35
25
15
5
0
TEMPERATURE (°C)
300
500
800
1000
LIGHT (μmol $m^{-2}s^{-1}$)

a light intensity of 300 $\mu E \cdot m^{-2}s^{-1}$, as the AQE calculated from the P-I curves do not vary significantly for the temperatures between 15 and 35°C (Figure 18, Table IV). The lower AQE observed for the 15°C curve disappeared when the net photosynthetic rate was corrected for the chlorophyll content of the thallus, possible indicating that the differences were due to slight between replicate variation in algal content.

Respiration increases with the assay temperature, from a low of approximately -1 mg $CO_2 \cdot gm^{-1}h^{-1}$ at 5°C to between -3 and -4 mg $CO_2 \cdot gm^{-1}h^{-1}$ at 45°C.

There were no seasonal effects observed in the Churchill population (Figure 16). The net photosynthetic response to thallus moisture, temperature and light intensity were very similar in the July, September and November collections, and no significant difference in the magnitude of the rates were observed.

The response of the Muskoka population, however, did contain a pronounced seasonal capacity change (Figure 19). In December, net photosynthetic rates at optimum thallus water content reached 7.7 mg $CO_2 \cdot gm^{-1}h^{-1}$ at 25°C and under 1000 $\mu E \cdot m^{-2}s^{-1}$ illumination. Rates declined slightly at the higher temperatures (5.8 mg at 35°C and 4.8 mg $CO_2 \cdot gm^{-1}h^{-1}$ at 45°C), and were severely depressed at 15 and 5°C (3.3 mg and 2.1 mg $CO_2 \cdot gm^{-1}h^{-1}$). The temperature response for the January collection was similar, with maximum rates of 2.2.

FIGURE 18: The P-I curve for the September collection of the Churchill population of P. rufescens, assayed at 15(Δ), 25(□) and 35°C(○) and optimum thallus moisture content. Gas exchange rates (±SE) are expressed on a dry weight (upper) and Chl (lower) basis.

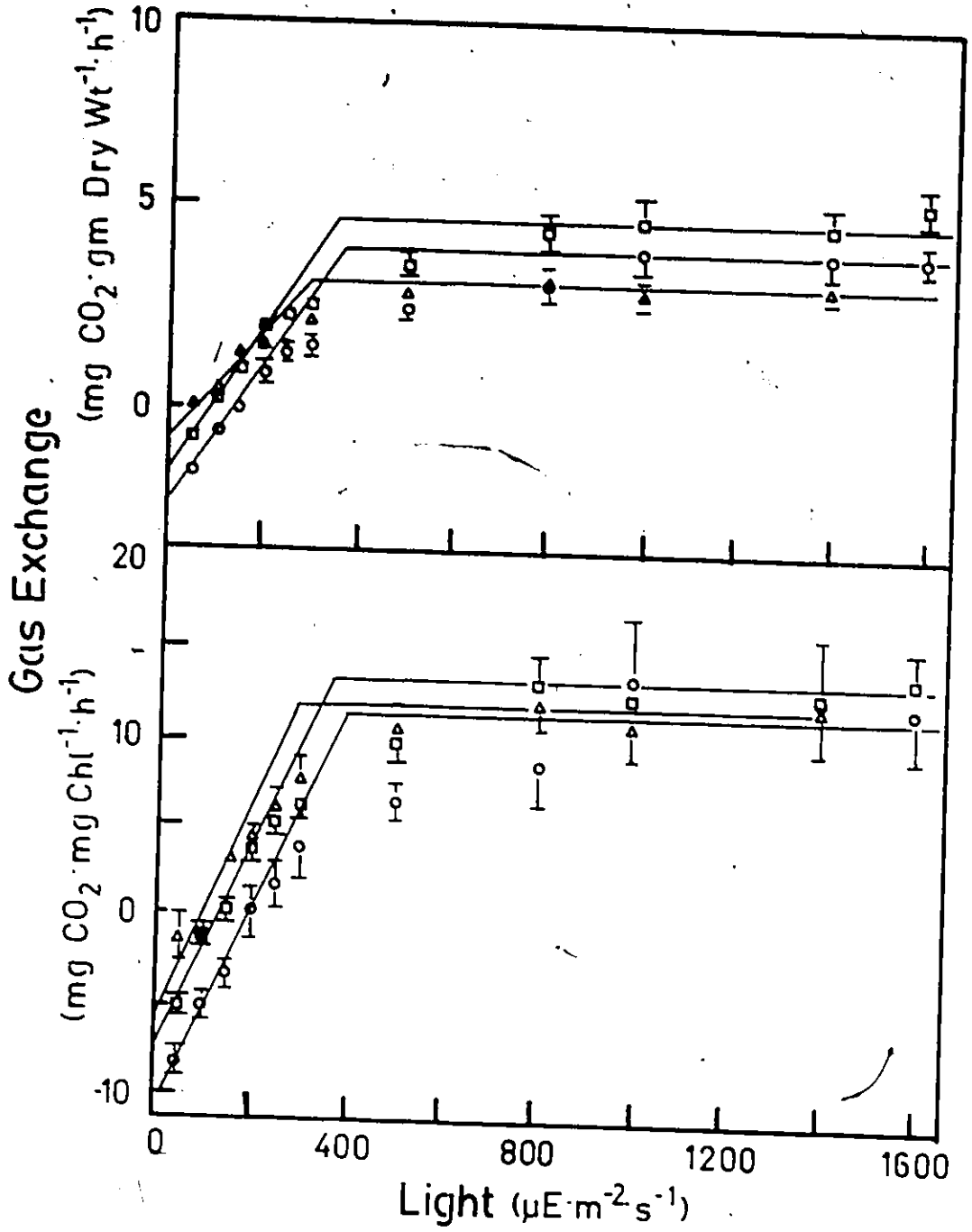
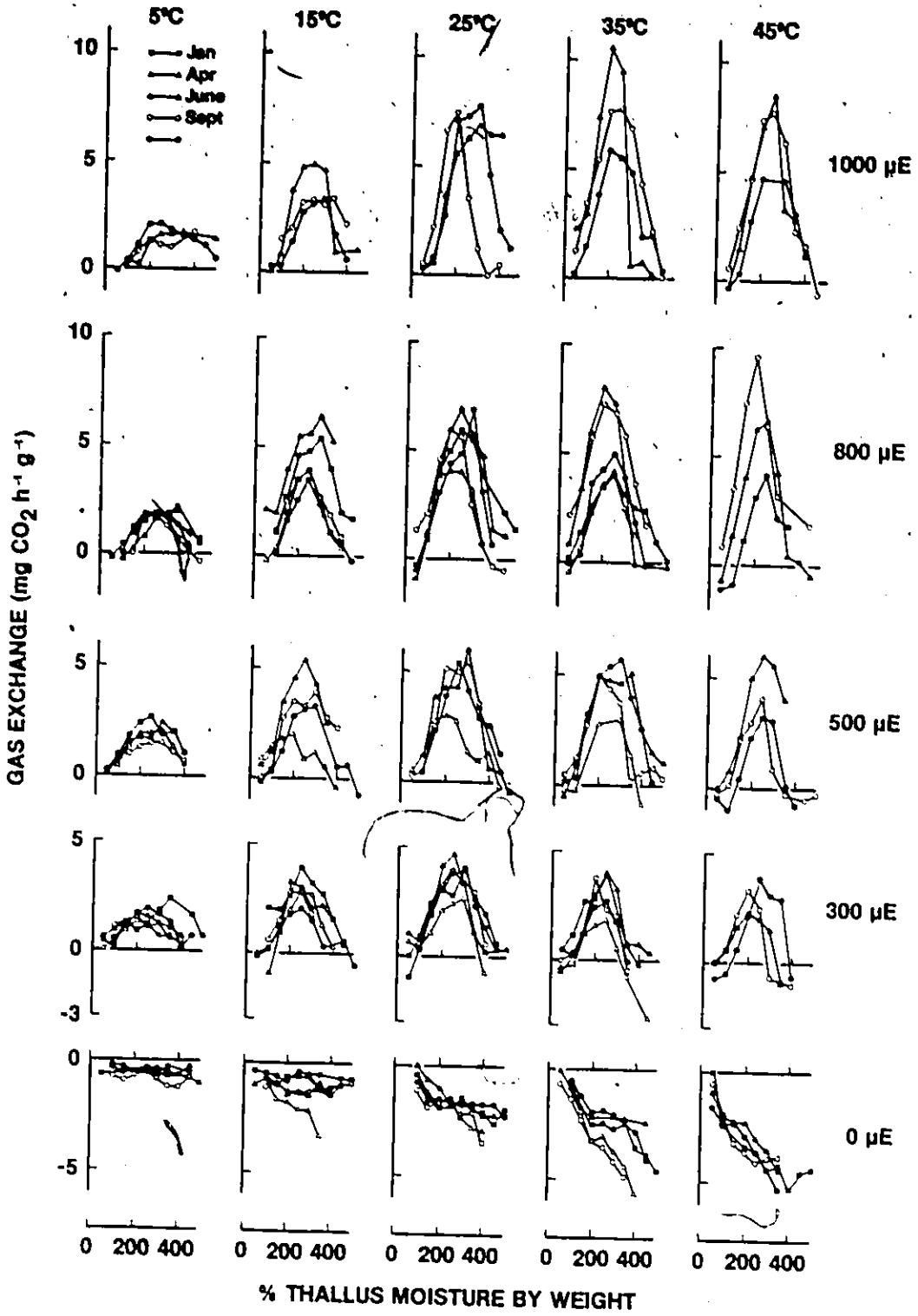

Gas Exchange
(mg CO_2·gm Dry Wt^{-1}·h^{-1})
10
5
0
20
(mg CO_2·mg Chl^{-1}·h^{-1})
10
0
-10
0
400
800
1200
1600
Light (μE·m^{-2}·s^{-1})

TABLE IV :The summaries of the seasonal P-I curves for *P. rufescens*

storage	assay temperature		A AQE	A Pmax	B AQE	B Pmax	n
Muskoka							
Aug. 1982	summer	35	0.031±.002	8.40±.2	0.109±.007a	29.6±1.	8
		25	0.027±.002	6.15±.2	0.095±.007ab	21.6±.8	5
	winter	35	0.033±.002	5.25±.2	0.126±.008a	20.0±.6	5
		25	0.023±.002	5.30±.3	0.088±.011ab	20.6±.2	4
Oct. 1982	summer	35	0.032±.001	8.07±.3	0.073±.007b	18.2±.6	8
		25	0.019±.003	5.85±.2	0.035±.006c	12.0±.8	4
	winter	35	0.028±.002	4.80±.2	0.053±.005cd	7.0±.2	7
		25	0.023±.002	5.78±.2	0.033±.003c	8.4±.4	4
Dec.	winter	35	0.029±.001	4.18±.2	0.081±.007b	13.0±.9	4
		25	0.023±.001	4.90±.3	0.061±.003d	12.9±.9	4
Churchill							
Sept. 1982		15	0.014±.002	2.79±.1	0.061±.007cd	11.7±.9	4
		25	0.020±.003	4.34±.3	0.057±.006cd	13.1±.8	5
		35	0.019±.002	3.46±.2	0.057±.013cd	11.6±1	4

AQE and Pmax (mean±SE) are expressed on a (A) dry weight ($mgCO_2 \cdot gm^{-1}h^{-1}$) or on a (B) chlorophyll ($mgCO_2 \cdot mg\ Chl^{-1}h^{-1}$) basis. The letters indicate which AQE are statistically different at the P=0.05 level (t-test).

FIGURE 19: The seasonal gas exchange response matrix for the Muskoka population of P. rufescens at 0, 300, 500, 800 and 1000 $\mu E \cdot m^{-2} s^{-1}$ and 5, 15, 25, 35 and 45°C, in January (■), April (Δ), June (▲), September (O) and November (●). SE less than 0.8 mg $CO_2 \cdot h^{-1} g^{-1}$.


5°C
15°C
25°C
35°C
45°C
Jan
Apr
June
Sept
1000 µE
800 µE
500 µE
300 µE
0 µE
GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
% THALLUS MOISTURE BY WEIGHT

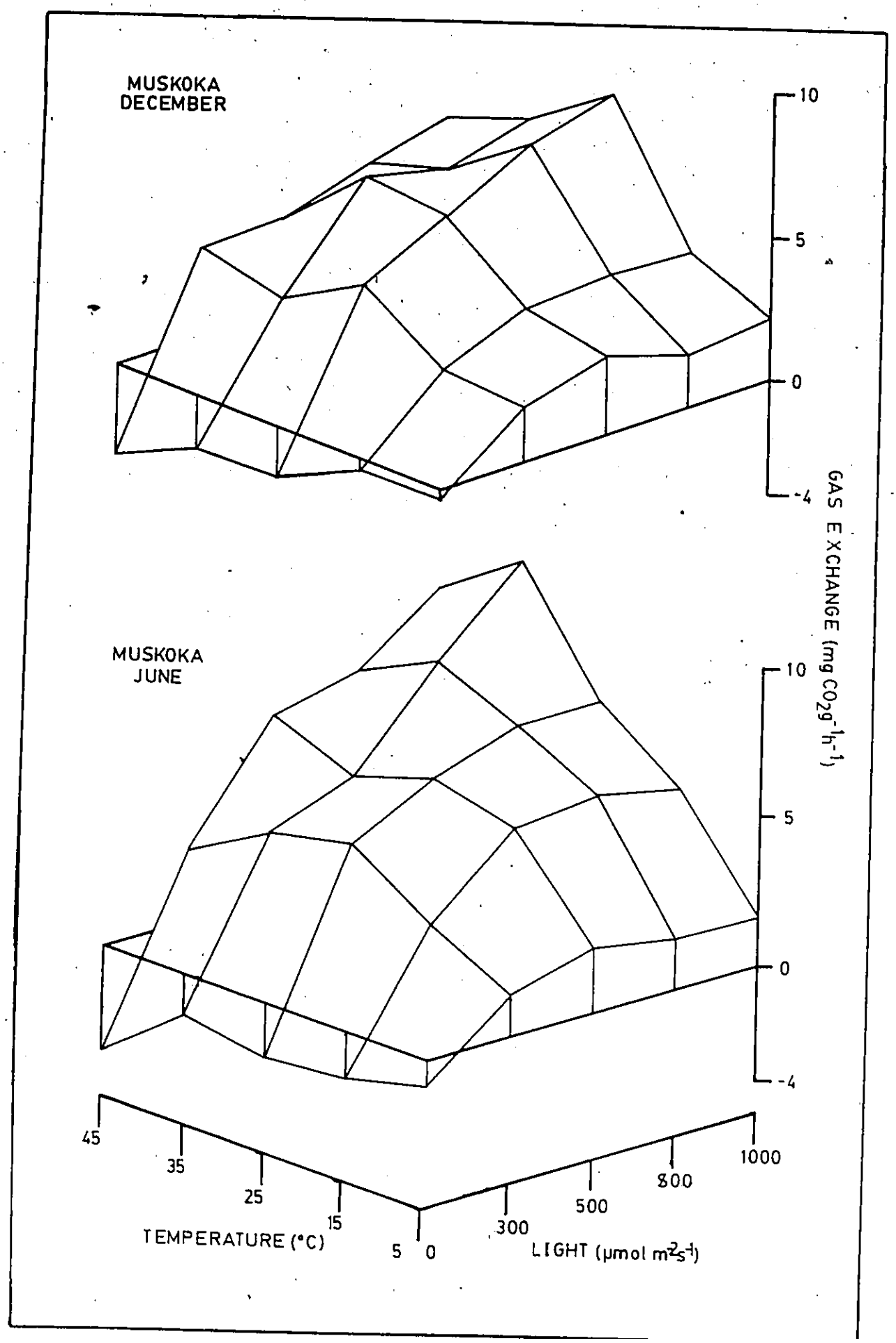
5.4, 5.9 and 3.9 mg $CO_2 \cdot gm^{-1}h^{-1}$ at 5, 15, 25 and 35°C and with 800 $\mu E \cdot m^{-2}s^{-1}$ illumination. However, by June the temperature optimum was now evident at 35°C, due to an increase in the photosynthetic capacity at both 35 and 45°C to approximately 10.5 and 7.5 mg $CO_2 \cdot gm^{-1}h^{-1}$, respectively, under the highest levels of illumination used. The pattern of gas exchange observed in September was similar to the June response, while the April collection followed the December and January pattern.

A three dimensional diagram of the June and December collections of the Muskoka population is presented in Figure 20. The seasonal shift in the temperature optimum from 25°C in the winter to 35°C in the summer is very evident in this figure. A somewhat higher rate of net photosynthesis was also apparent at 15°C for the June collection, but this increase did not appear consistently throughout the study (Figure 19) and it is thought that this was not a component of the seasonal change.

Respiration was not affected by the change in seasons (Figure 19). Rates increased steadily from values near -1 mg at 5°C to -4 to -5 mg $CO_2 \cdot gm^{-1}h^{-1}$ at 45°C.

Although there appears to be no seasonal changes in the gas exchange rates in the Muskoka population at light intensities below 500 $\mu E \cdot m^{-2}s^{-1}$, the light intervals used to construct the seasonal matrix were not adequate to cover photon flux densities below 300 $\mu E \cdot m^{-2} \cdot s^{-1}$. Thus a more

FIGURE 20: Three dimensional plot of the relationship between light, temperature and net photosynthesis in the June and November collections of the Muskoka population of P. rufescens. The net photosynthesis capacities given for the 200-250% thallus moisture class.


MUSKOKA
DECEMBER
10
5
0
-4
MUSKOKA
JUNE
10
5
0
-4
GAS EXCHANGE (mg $CO_2 g^{-1} h^{-1}$)
45
35
25
15
5
0
TEMPERATURE (°C)
300
500
800
1000
LIGHT (μmol $m^{-2} s^{-1}$)

detailed examination of the light effects on net photosynthesis are necessary to determine if changes in the rate at subsaturating light are involved in the seasonal response. To determine this, a P-I curve was determined from material collected in August and November of 1982. This was assayed at a temperature of 25°C, where no seasonal changes should be observed, and 35°C, where the seasonal capacity change was observed in the seasonal matrices (Figure 19).

From this P-I curve (Figure 21), it appears that AQE does not change seasonally. A value of 0.031 ± 0.002 was observed in August at 35°C, while in November a slope of $0.029 \pm .001$ was recorded (Table IV). Gas exchange at saturating light intensities did follow the seasonal pattern, with a 50% reduction in Pmax observed between August ($8.40 \pm .2$ $mgCO_2$) and November ($4.18 \pm .2$ $mgCO_2$) (Figure 21, Table IV). Pmax and AQE did not change at 25°C between summer and winter. The slope of the P-I curve at this temperature was $0.023 \pm .002$ in August, while an identical value of $0.023 \pm .001$ was calculated for the November collection. The respective Pmax values of $6.15 \pm .2$ and $4.90 \pm .3$ $mgCO_2$ at 25°C were also within the range predicted from the seasonal matrices.

There was, however, a difference in the pigment content between these collections (Table V,VI), with the August collection having a lower chlorophyll content (284 ± 11 $\mu g \cdot gm^{-1}$ compared to 380 ± 19 $\mu g \cdot gm^{-1}$ in November). When the gas exchange rates were recalculated, correcting for these

FIGURE 21: The P-I curve for the August (□) and November (O), collections of the Muskoka population of P. rufescens. Gas exchange rate (±SE) at 35 (upper) and 25°C (lower) are expressed on a dry weight (left) and Chl (right) basis.

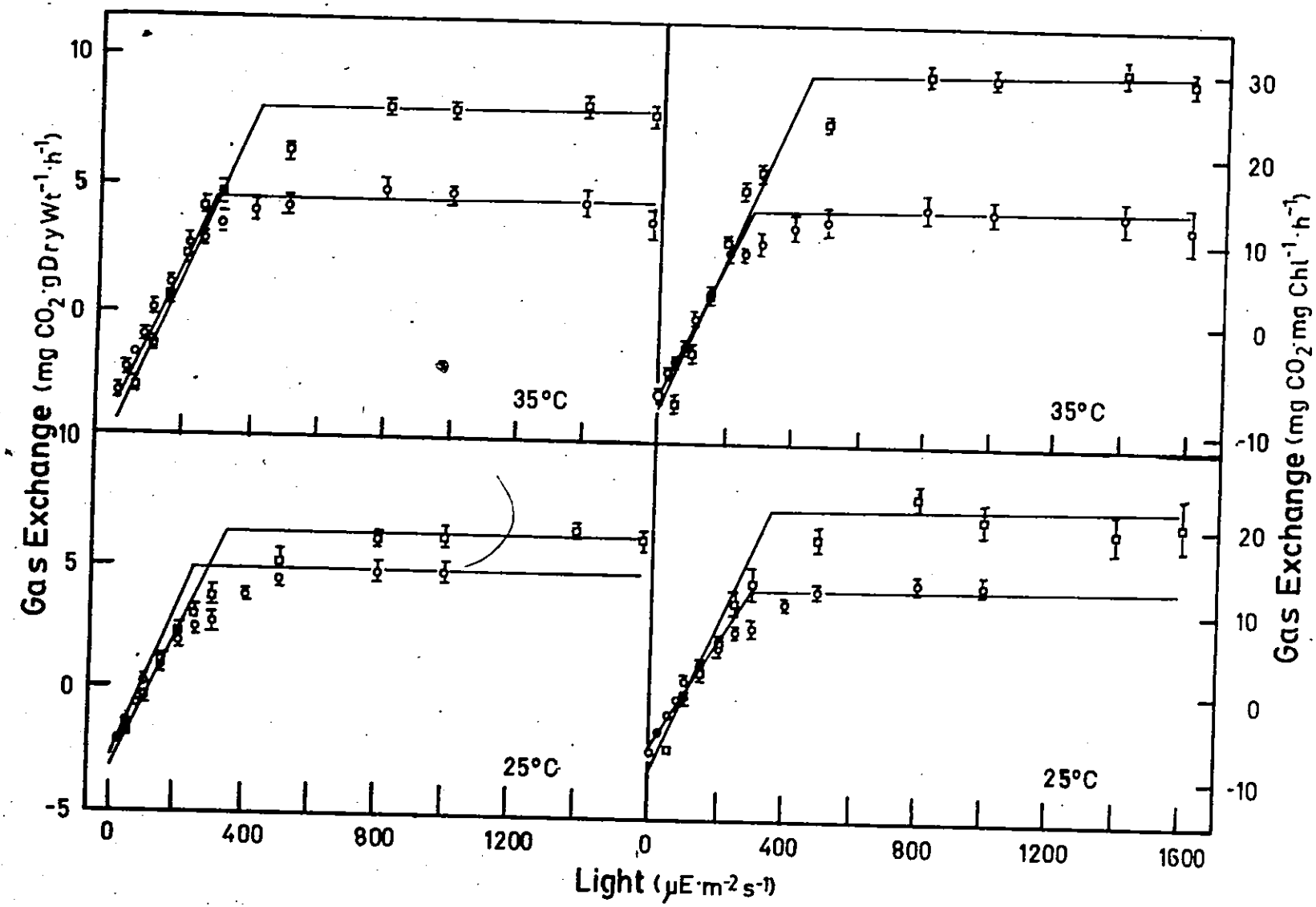

Gas Exchange (mg CO_2·gDryWt^{-1}·h^{-1})
Gas Exchange (mg CO_2·mg Chl^{-1}·h^{-1})
Light (μE·m^{-2} s^{-1})
35°C
35°C
25°C
25°C
10
5
0
-5
30
20
10
0
-10
400
800
1200
1600

TABLE V: The chlorophyll contents of the Muskoka and Churchill populations of *P. rufescens* in 1982.

Population	Collection	Storage	Chlorophyll	n
			$\mu g \cdot gm^{-1}$	
Muskoka	August	summer	284±11a	9
		winter	262±11a	6
Muskoka	October	summer	488±23b	8
		winter	686±51c	7
Muskoka	November	winter	380±19d	8
Churchill	September		243±24a	15

Comparisons (t-test) differing significantly at the P=0.05 level are indicated by different letters.

Table VI: The biliprotein contents of the Muskoka and Churchill populations of *P. rufescens* in 1982.

Population	collection	storage	PE	PC	APC	n
Muskoka	August	summer	1.50±.04a	1.95±.11a	1.16±.03a	10
		winter	1.85±.09b	2.36±.11b	1.48±.06b	8
Muskoka	November	winter	1.64±.16b	3.34±.28c	1.52±.20ab	8
Churchill	September		1.93±.2b	1.62±.22a	0.98±.07a	8

phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC) concentrations are in $mg \cdot gm^{-1}$.

Comparisons (t-test) differing significantly at the P=0.05 level are indicated by different letters.

pigment differences, a decrease in AQE was observed in the November collection (Figure 21).

Seasonal changes in pigments are not uncommon in lichens. Harris (1971) observed changes in the algal density in Parmelia caperata and P. sulcata. while Orus and Estevez (1984) and Kershaw and Webber (1984) observed changes in pigment contents which also most likely represent change in algal density. Variations in pigments between sun and shade ecotypes are also common (Rundel 1972; Hampton 1973; Kappen 1983; Kershaw et al. 1983). The chlorophyll content in P. rufescens (Table V) is lower that the 700 $\mu g \cdot gm^{-1}$ reported for P. canina (Hampton 1973; Bergman and Hällbom 1982), but similar to that reported by Kershaw and Webber (1984) in P. praetextata. P. rufescens does have a higher biliprotein content that other lichenized Nostoc. especially the phycocyanin and allophycocyanin pigments (Table VI; Bergman and Hällbom 1982; Czeczuga 1982). Few differences in accessory pigments were observed between the Churchill and Muskoka population (Table VI), but the Churchill population did have a lower chlorophyll content (Table V).

4.3 Laboratory induced changes in the Muskoka population.

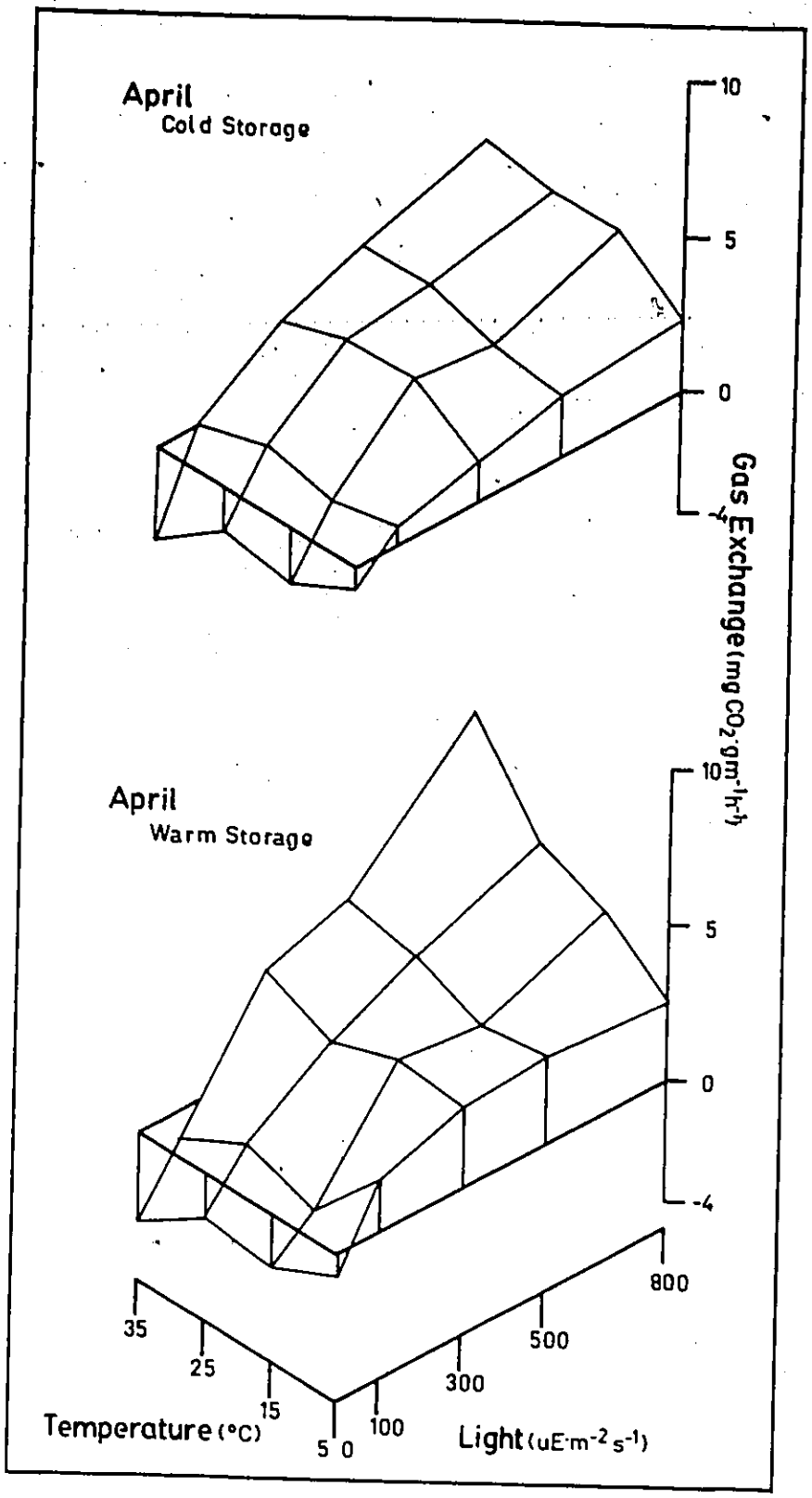
From the response matrix (Figure 19) the similarities between the June and September collections, and the January, April and December collections make it apparent that that the Muskoka population undergoes a seasonal

change which can best be represented as a summer-winter adaptation, without the intermediate spring or fall patterns observed by Kershaw (1977a) in P. praetextata and P. polydactyla. Kershaw (1977b) and Kershaw and MacFarlane (1980) have indicated that gas exchange rates could be manipulated in the laboratory by altering the temperature and photoperiod under which the thalli were stored. A detailed study of the Muskoka population was undertaken to determine if the observed seasonal changes could be induced, and which environmental parameters act as keys for the change.

P. rufescens, collected from Muskoka for the April 1981 matrix, was stored under a cold temperature regime of -5/2°C night/day with a 12 hour photoperiod. After nine days in this regime, some of the thalli were placed under a warm storage of 15/25°C with the same 12 hour photoperiod and seven days later the gas exchange matrix was repeated. Figure 22 compares the gas exchange patterns under these two storage conditions. The changes in storage temperature induced a response similar to the seasonal change in net photosynthesis described above (Figure 19,20). Gas exchange rates at 35°C, 800 $\mu E \cdot m^{-2} s^{-1}$ went from 4.2±.25 $mgCO_2$ under the cold temperature to 8.2±.5 under the warm temperature regime. As with the observed seasonal pattern, no differences in the gas exchange rates occurred at the lower light levels or temperatures.

Unlike the April results, a change in storage

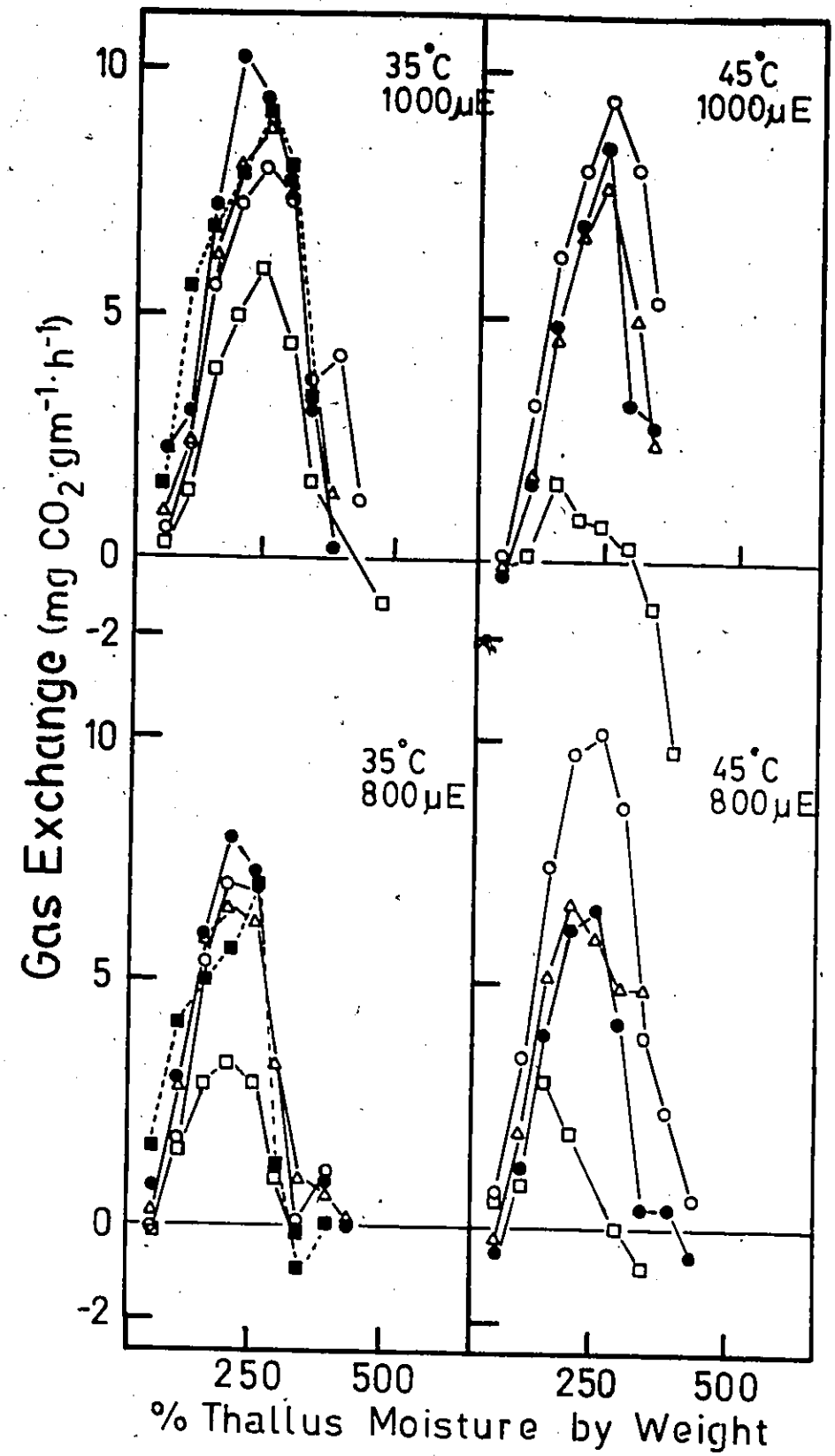
FIGURE 22: A three dimensional diagram showing the relationship between net photosynthesis, light and temperature measured at optimal thallus moisture content in the April 1981 collection of the Muskoka population of P. rufescens. The material was stored for 9 days at a night/day temperature of -5/2°C (upper) and then transferred to a temperature of 5/25°C (lower) for 7 days prior to the gas exchange measurements. A 12 hour photoperiod was used for both storage regimes.


April
Cold Storage
10
5
0
-4
Gas Exchange (mg CO2 gm-1 h-1)
April
Warm Storage
10
5
0
-4
35
25
15
5
0
100
300
500
800
Temperature (°C)
Light (uE m-2 s-1)

temperature could not induce a winter response in the June 1981 Muskoka collection. In this experiment, material stored under summer conditions of 21/33°C night/day with a 15 hour photoperiod, were placed under a cold regime of 0/8°C with the same 15 hour photoperiod. The gas exchange rates under this cold regime remained similar to the control values (Figure 23). Similarly, a decrease in the photoperiod from 15 to 9 hours had no effect on the measured rates after 4 days. A change in both the temperature and daylength could, however, induce a winter response (Figure 23). Four days in a winter regime of 0/8°C, and a 9h photoperiod was sufficient to lower the rates at 35 and 45°C, and 1000 $\mu E \cdot m^{-2}s^{-1}$ from the summer value of 7.46±.8 and 6.60±.4 mg CO_2 to values of 5.97±1.1 and 1.90±.9 mgCO_2, characteristic of the winter response. A similar decline in net photosynthesis was observed at these assay temperatures under an illumination of 800 $\mu E \cdot m^{-2}s^{-1}$ (Figure 23). Thus it is apparent that it requires both a change in temperature and daylength to induce a winter response in P. rufescens collected during the summer months. This induced change was readily reversible. When the material was returned from the winter to the summer storage regime, gas exchange values at 35°C, 800 and 1000 $\mu E \cdot m^{-2}s^{-1}$ increased to 7.13±1.1 and 8.92±.7 mgCO_2 respectively.

The rate of this experimentally induced change was tested in July, 1981 and again in September of 1985. Similar

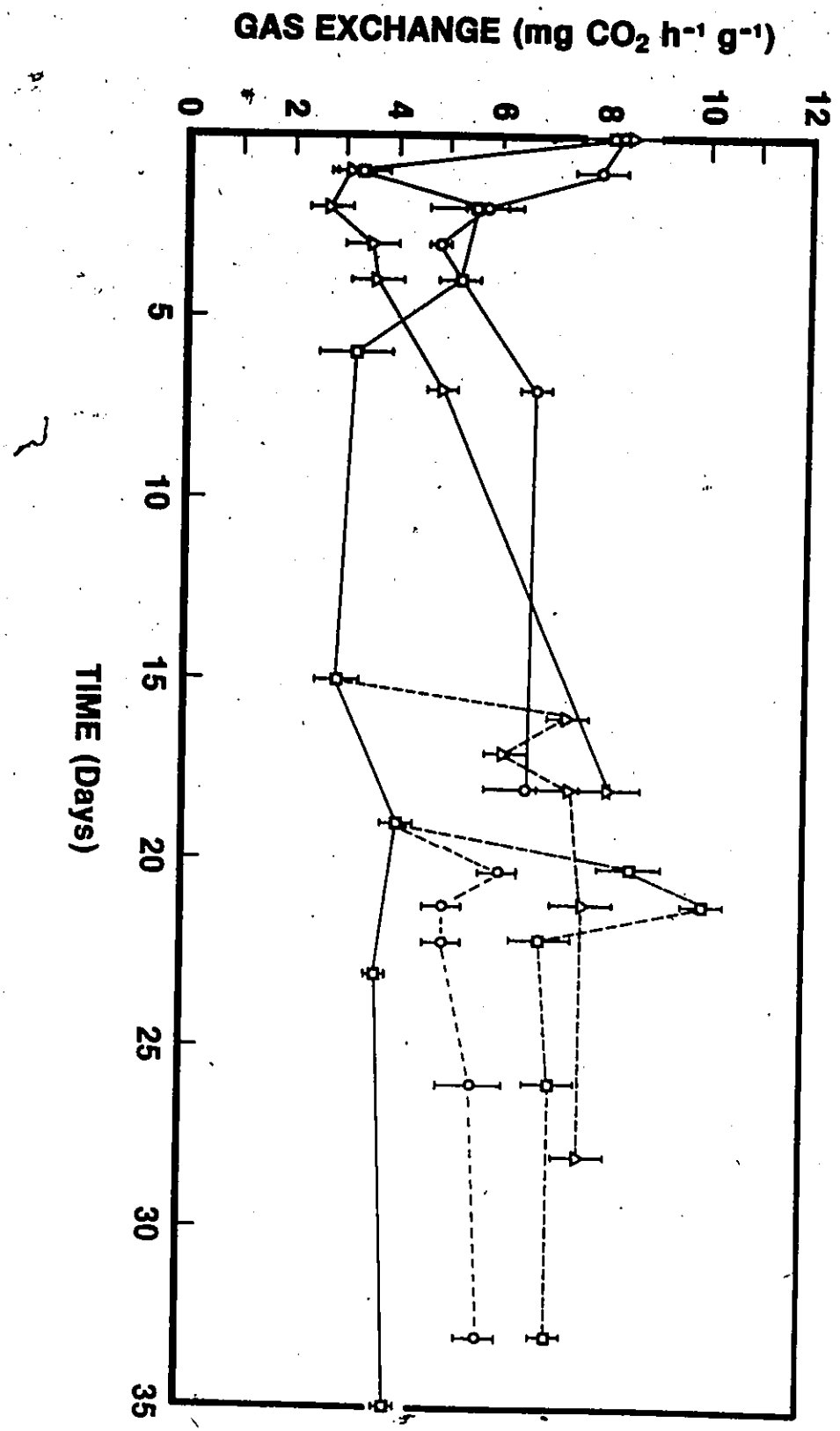
FIGURE 23: The effect of storage conditions on the gas exchange rate in a June 1981 collection of the Muskoka population of P. rufescens. Thalli which had been stored for 8 days under summer conditions (21/33°C N/D; 15 h photoperiod) (●—●) were transferred to different storage regimes. Gas exchange was measured after 4 days storage at low temperature with a long photoperiod (0/8°C. 15 h) (Δ): and warm temperature with a short photoperiod (21/33°C, 9 h) (○): and 5 days storage under winter conditions (0/8°C. 9 h) (□). The winter stored material was then transferred back to a summer regime and net photosynthesis was measured after 5 days (■ – –■). Gas exchange rates were assayed at 35 (left) and 45°C (right) and 800 (lower) and 1000 (upper) $\mu E \cdot m^{-2} s^{-1}$.


35°C
1000µE
45°C
1000µE
35°C
800µE
45°C
800µE
10
5
0
-2
10
5
0
-2
250
500
250
500
Gas Exchange (mg $CO_2 \cdot gm^{-1} \cdot h^{-1}$)
% Thallus Moisture by Weight

to the results obtained in June, a change to low temperature or short daylength alone could not induce a stable winter response (Figure 24). Gas exchange was, however, affected by an alteration in either of these storage conditions. For example, when the lichen was shifted from a summer storage regime of 17/35°C, 15h photoperiod to a cold temperature of -1/5°C with the same 15h photoperiod, gas exchange rates at 35°C and 800 $\mu E \cdot m^{-2} s^{-1}$ rapidly declined from the control value of 8.3±.7 $mgCO_2$ to 3.1±.4 mg CO_2 on the first day in the new temperature regime. Gas exchange rates remained at these levels until day 7, and then gradually returned to a value of 8.2±.6 mg CO_2 by day 18 (Figure 24), which is similar to the control rate of 6.8±.5 recorded for day 19. A change in the photoperiod alone did not have as drastic an effect on the rates, but the pattern was similar: rates declined to 4.8±.2 mg CO_2 by day 4, and then returned to near control values of 6.7±.3 $mgCO_2$ on day 7.

An alteration in both the temperature and photoperiod to a winter regime of -1/5°C, 9h photoperiod produced a complicated pattern in the Muskoka population. The gas exchange rates during the first four days in winter storage showed a pattern similar to the single shift experiments. Rates declined from a value of 8.3±.7 $mgCO_2$ on day 0 to 3.3±.5 mg CO_2 on day 1, and then recovered to a value of 5.5±.4 $mgCO_2$ by day 4 (Figure 24). Gas exchange then declined and a stable winter rate of 3 to 4 $mgCO_2$ was

FIGURE 24: The time course of low temperature acclimation in the Muskoka population of P. rufescens. Material collected in July 1981 and initially stored under a summer regime of 17°C night/35°C day with a 15 h photoperiod were transferred to one of three storage regimes: low temperatures with a long photoperiod (-1/5°C, 15 h) (Δ——Δ), warm temperatures with a short photoperiod (15/30°C, 9 h) (O——O), and winter (-1/5°C, 9 h) (□—□). Material maintained under winter conditions for 15 or 19 days were then returned to the summer condition (□- -□), or the two intermediate regimes (warm temperatures with short photoperiod (O- -O), cold temperature with long photoperiod (Δ- - -Δ)), or retained under full winter conditions (□——□). Gas exchange rates at 35°C, 800 $\mu E \cdot m^{-2} s^{-1}$ (M±SE) are given for the 200-250% thallus moisture class.


GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
0
2
4
6
8
10
12
TIME (Days)
5
10
15
20
25
30
35

obtained from days 6 to 41. When this experiment was repeated in September of 1985 (Figure 50), a similar initial crash of the gas exchange rates, followed by a recovery to control levels, and then a decline and final stabilized at winter rates of gas exchange, was observed.

This stable winter rate, inducible only by a change in both temperature and daylength, is quite reversible. The gas exchange rate, when material which had been kept under winter conditions for 19 days and then returned to the summer storage regime of warm temperature (17/35°C) and long photoperiods (15 h), increased from 4.1±.3 to 8.6±.6 mgCO_2 on the first day and remained at this level for the duration of the experiment (Figure 24). Unlike the induced winter process, which could not be achieved by a single alteration in photoperiod or temperature, a stable net photosynthetic rate similar to the summer controls could be obtained when the winter-stored material was shifted to a regime of either warm temperature, short photoperiod or cold temperature, long photoperiod regime (Figure 24).

In contrast to the ability to acclimate the Muskoka population of P. rufescens in the spring, summer and fall, experiments in the winter could not induce a summer response. In January of 1982, shifting the material from winter (-2/1°C, 9h photoperiod), to a summer storage condition of 15/19°C night/day and a 15 hour photoperiod caused a rapid decline in net photosynthesis to a respiration dominated

value of $-1.17\pm.1$ $mgCO_2$ at 35°C, 800 $\mu E \cdot m^{-2}s^{-1}$ and optimum moisture contents (Figure 25). Gas exchange reached the compensation point by day 15, and recovered to the control winter values by day 23. At no time during the 28 day experiment did material placed under the summer storage regime achieve net photosynthetic rates comparable to the summer values. A single change in daylength or storage temperature produced a similar pattern (Figure 25). When this experiment was repeated in late November of 1982, a similar depressed rate of gas exchange was observed in the lichens kept in summer storage conditions for 9 days (Figure 26).

The previous acclimation experiments have, for the most part, involved inducible changes in the gas exchange rates at high levels of illumination only. The seasonal capacity change observed in the Muskoka population (Figure 19.20) did not indicate that alterations in the gas exchange below a light intensity of 800 $\mu E \cdot m^{-2}s^{-1}$ would occur. However, the possibility that the laboratory induced acclimation might include changes at the lower light intensities could not be discounted.

The P-I curves for a laboratory induced acclimation in August of 1982 are shown in Figure 27. Light saturated rates at 35°C followed the previously described pattern, declining from $8.40\pm.2$ in the summer to $5.25\pm.2$ $mgCO_2$ under the winter storage regime. AQE was not affected by the

FIGURE 25: The time course of high temperature acclimation in the January 1982 collection of the Muskoka population of *P. rufescens*. Replicates initially stored under winter (-2° night/1°C day, 9 h photoperiod) conditions were transferred to storage regimes of warm temperature with short photoperiod (15/19°C N/D, 9 h) (O), low temperatures with long photoperiod (-2/0°C, 15 h) (□), and summer (15/19°C, 15 h) (Δ). Gas exchange values (±SE) are given for the 200-250% thallus moisture class, and assayed at 35°C, 0 and 800 $\mu E \cdot m^{-2} s^{-1}$.

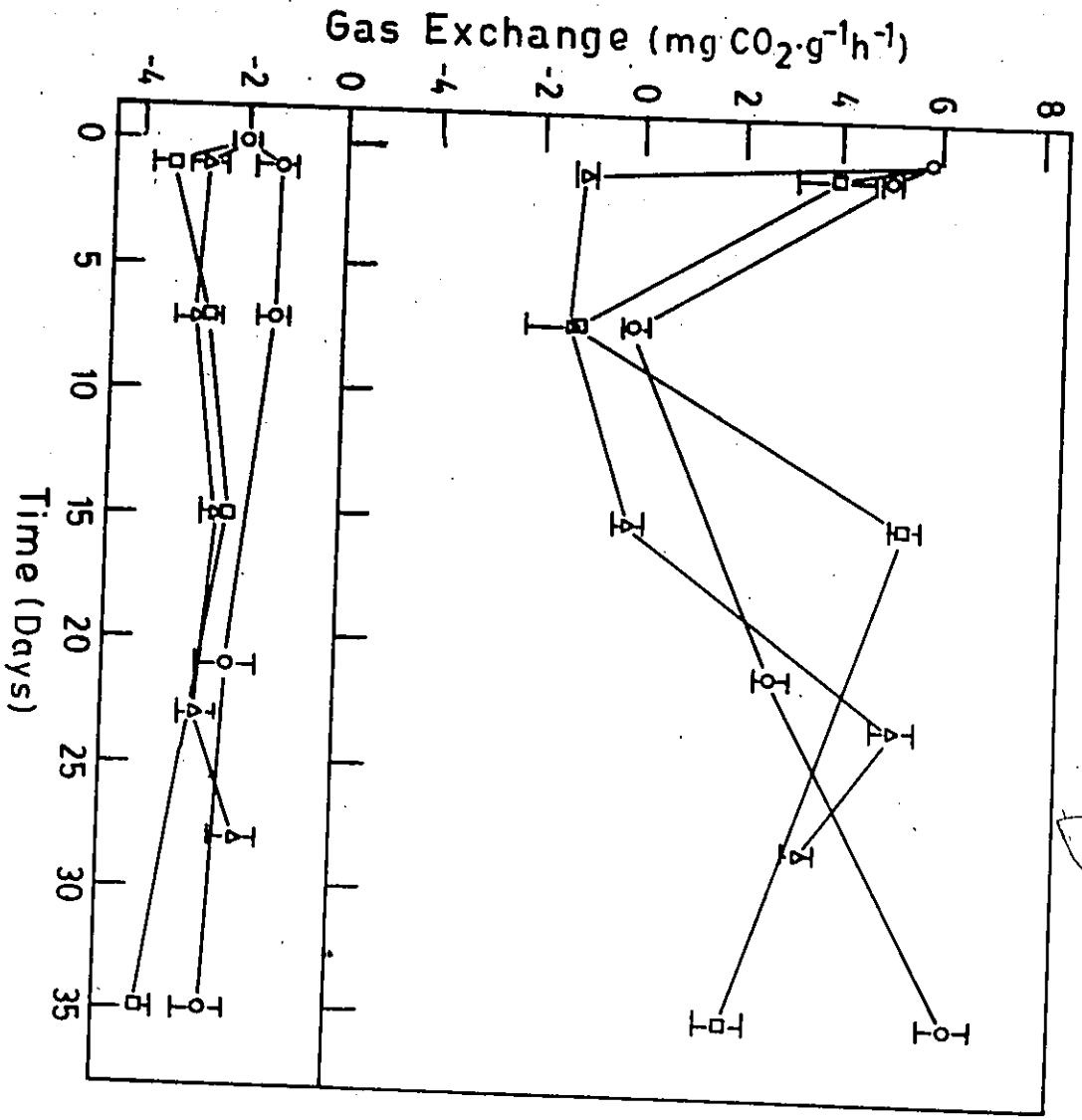

Gas Exchange (mg $CO_2 \cdot g^{-1} h^{-1}$)
-4
-2
0
-4
-2
0
2
4
6
8
Time (Days)
0
5
10
15
20
25
30
35

FIGURE 26: A high temperature acclimation in the November 1982 collection of the Muskoka population of P. rufescens. Material was stored in a winter regime of -3° night/2°C day with a 9 hour photoperiod (O) was transferred to a summer regime of 25/35°C N/D with a 16 hour photoperiod (□). Gas exchange rates were assayed at 35°C, 800 $\mu E \cdot m^{-2} s^{-1}$ following 13 days storage in the winter regime and 9 days storage in the summer regime.

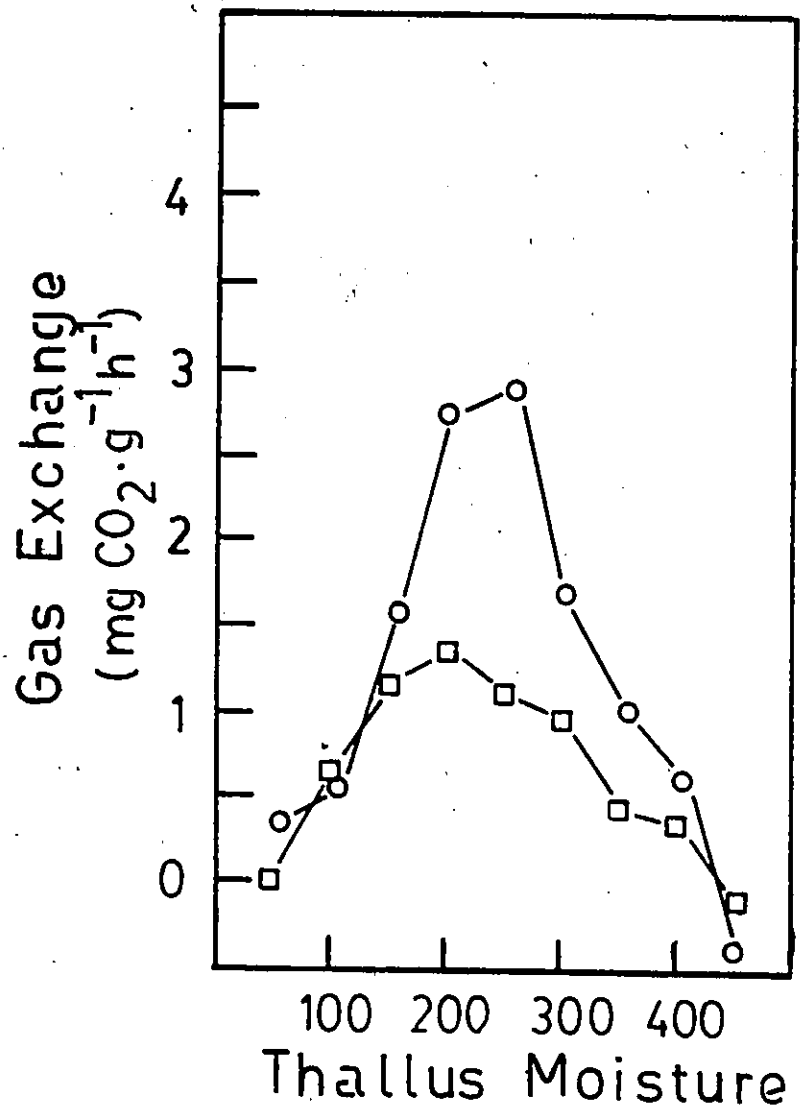
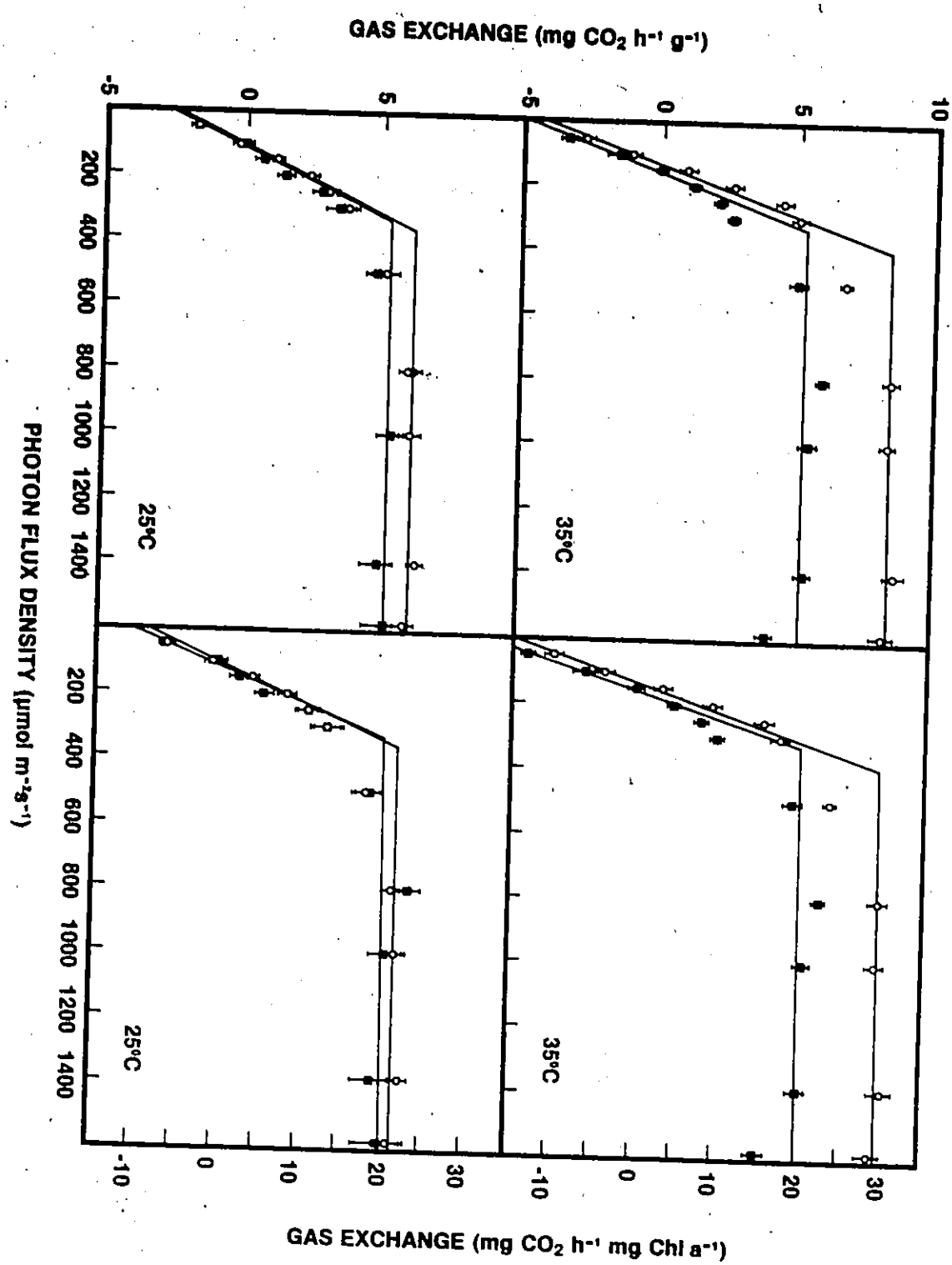

Gas Exchange
(mg $CO_2 \cdot g^{-1} h^{-1}$)
0
1
2
3
4
100
200
300
400
Thallus Moisture

FIGURE 27: P-I curve for an August 1982 collection of the Muskoka population of P. rufescens stored under summer (25°C night/35°C day, 16 h photoperiod) (O) and winter (-1/5°C, 8 h) (■) conditions for 7 and 10 days respectively. Gas exchange rate (±SE) at 35 (upper) and 25°C (lower) are expressed on a dry weight (left) and Chl (right) basis.

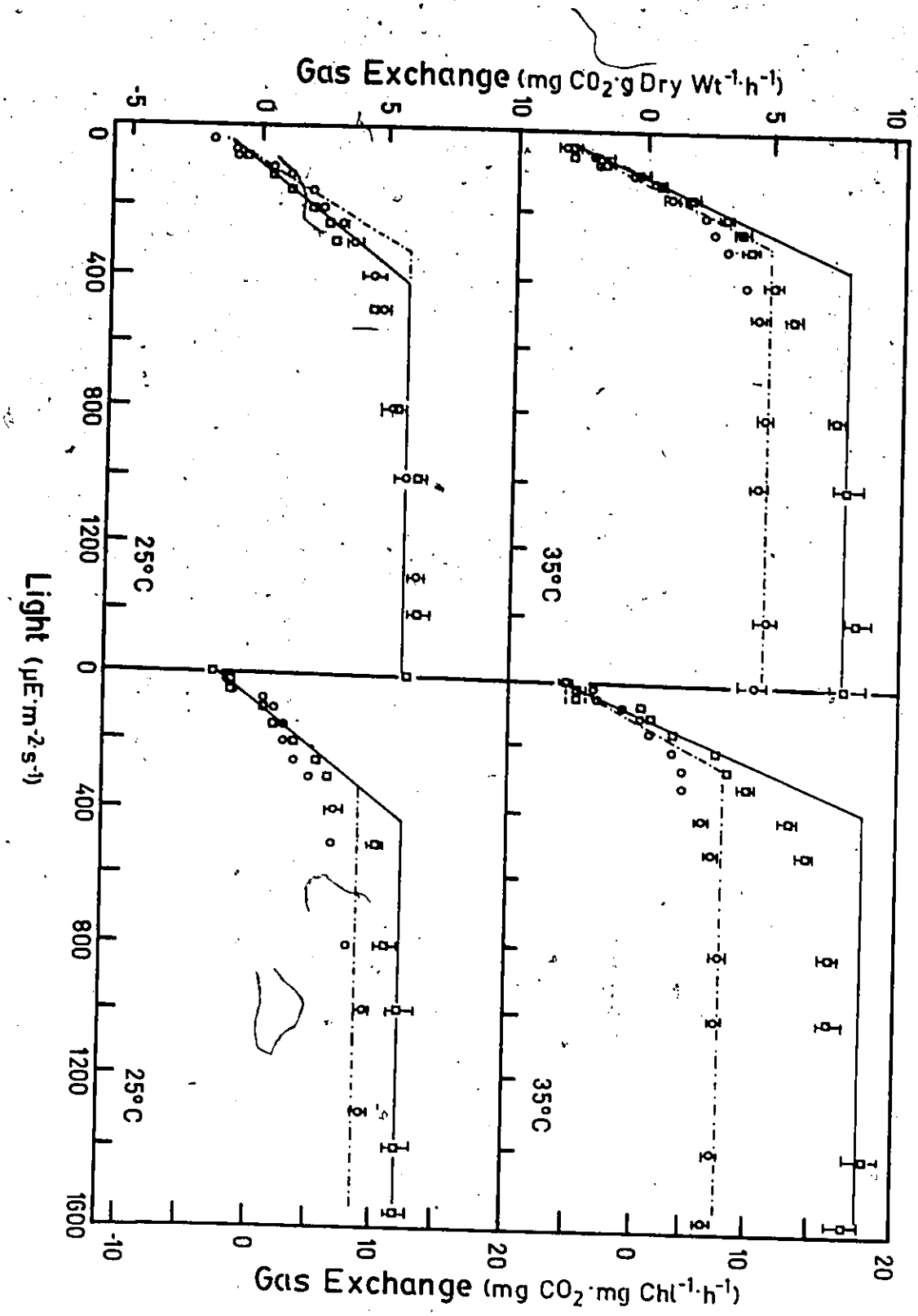

GAS EXCHANGE (mg CO_2 h^{-1} g^{-1})
PHOTON FLUX DENSITY (µmol $m^{-2}s^{-1}$)
GAS EXCHANGE (mg CO_2 h^{-1} mg Chl a^{-1})
25°C
35°C
25°C
35°C

storage conditions, with the summer slope of 0.031±.002 being very similar to the induced winter value of 0.033±.003 (Table IV). Neither of these slopes differed significantly to the AQE of the December 1982 collection of 0.029±.001. No inducible changes in either Pmax or AQE were observed at an assay temperature of 25°C.

A similar pattern was observed when the P-I curves were repeated in October, 1982 (Figure 28, Table IV). The light saturated rates of net photosynthesis measured at 35°C declined from 8.07±.3 to 4.80±.2 mgCO_2 when the material was acclimated to winter storage conditions for 15 days. No concurrent changes in AQE (0.032±.001 and 0.028±.002 for the summer and winter stored material respectively) were observed, and, as in August, no changes in either parameter were observed when the P-I curves were assayed at 25°C (Figure 28, Table IV).

A similar pattern was obtained when the parameters of the P-I curve were calculated on a chlorophyll basis (Table IV). In August, net photosynthesis at 35°C decreased from 29.6±1.0 mgCO_2·mg Chl^{-1} to 20.0±.6 mg CO_2·mg Chl^{-1} when the thalli were placed under a winter storage regime. AQE, when calculated on a chlorophyll basis, was not affected by the storage conditions, with a slope of 0.109±.007 in the summer and 0.126±.008 for the winter. This slightly higher, although not statistically different, slope may reflect the larger biliprotein content of the winter stored material

FIGURE 28: P-I curve for an October 1982 collection of the Muskoka population of P. rufescens stored under summer (25°C night 35°C day . 16 h photoperiod) (□) and winter (-2/8°C N/D. 8 h) (○) conditions for 7 and 15 days respectively. Gas exchange rate (±SE) at 35 (upper) and 25°C (lower) are expressed on a dry weight (left) and Chl (right) basis.


Gas Exchange (mg CO2·g Dry Wt-1·h-1)
-5
0
5
10
0
5
10
0
400
800
1200
25°C
35°C
Light (μE·m-2·s-1)
0
400
800
1200
1600
25°C
35°C
-10
0
10
20
0
10
20
Gas Exchange (mg CO2·mg Chl-1·h-1)

(Table VI). No differences in net photosynthesis or AQE were observed at 25°C.

In October, the slope of the P-I curve at 35°C was affected by the storage regime. The thalli stored under winter conditions had a significantly higher chlorophyll content than those under the summer regime (Table V). No changes in AQE was observed at 35°C when gas exchange was calculated on a dry weight basis. However, when expressed on a chlorophyll basis, the higher pigment content of the winter stored material resulted in this material having a significantly lower AQE (0.053±.005) than the summer stored material (0.073±.007). Results at 25°C paralleled the dry weight pattern, with no inducible changes in AQE or Pmax observed on a chlorophyll basis.

5. Discussion of the gas exchange studies

The temperature response of net photosynthesis in the Churchill population of P. rufescens was totally unexpected (Figure 16). Plants growing in arctic or alpine environments commonly have low temperature optima for net photosynthesis, with an upper temperature compensation point below 30°C. Temperature optima of 14°C has been reported for subarctic populations of Parmelia disjuncta and Alectoria ochroleuca (Larson and Kershaw 1975c; Kershaw and Watson 1983) while an alpine population of Rhizocarpon superficiale had maximal rates of net photosynthesis between 1 and 14°C (Coxson and Kershaw 1983). Similarly, low temperature optima have been reported for several arctic mosses and the sedge Carex aquatilis (Oechel 1976; Chapin and Oechel 1983). Antarctic lichens tend to have temperature optima near 0°C (Kappen 1983; Kappen and Friedmann 1983; Ino 1985).

On this basis, the Churchill population, with a temperature optimum of 35°C and an upper temperature compensation point in excess of 45°C (Figure 16), seems to be rather unsuited for a subarctic environment. However, at 5°C and under a high photon flux density, gas exchange rates of 1.3 to 1.6 $mgCO_2 \cdot gm^{-1}h^{-1}$ could be expected (Figure 16). This rate is comparable to, or much higher than, the net photosynthetic capacity of the species of Cladonia, Cetraria,

and Alectoria that will eventually replace P. rufescens in the raised beach succession (Kershaw 1975; Kershaw et al. 1983; Adams 1971a,b; Tegler and Kershaw 1980).

The environmental conditions of these raised beaches are quite harsh. The relatively low soil moisture combines with a high radiant energy input to produce an excessively hot microclimate in the boundary layer. Thallus temperatures are therefore expected to be much higher than the corresponding air temperature would suggest. Kershaw and Watson (1983) have measured the thallus temperatures of a Churchill population of Parmelia disjuncta. They observed a thallus temperature of 15°C above the ambient air temperature in this black crustaceous lichen which grows on rock outcrops near the raised beach system used in this study. Air temperatures during the summer months at Churchill are in the low teens (Figure 11), thus even a moderate boundary layer warming of 10°C would result in near maximal rates of net photosynthesis throughout a large part of the growing season for P. rufescens.

The literature reports on a general trend that species growing in a cooler environment have a lower temperature optimums. It is apparent that the Churchill population of P. rufescens does not fit into this generalization. Kershaw (1983) has suggested that adaptations to microclimatic conditions should also be taken into consideration. The high temperature optimum and upper

compensation limit of the Churchill population could represent an adaptation to life in the severe microclimate associated with the young raised beaches where it occurs, and not the general response expected in the Churchill area. Similarly the high (25°C) temperature optimum observed for grasses from arctic islands (Addison and Bliss 1984) and the low optimum (20°C) for several hot desert lichens (Lange 1969; Nash et al. 1982) represent species which are adapted to specific microclimatic niches. Scholander et al. (1952) has also observed that the respiration rates in several lichen species did not correspond with the general latitudinal trends. It may be that the stress tolerant strategy (Grime 1979) of the lichen restricts it to habitats where the microclimate contrasts with the macroclimate, thus obscuring any general trend.

The lack of any seasonal change in the rate of net photosynthesis in this population was also quite surprising. Although several of the earlier studies which indicated a high acclimation potential in arctic lichens (Kershaw 1975; Larson and Kershaw 1975b,c) are now questionable (Brown and Kershaw 1984; Kershaw 1984), other studies have shown that seasonal capacity changes are possible in subarctic populations (Kershaw and Smith 1978; Tegler and Kershaw 1980; Kershaw and Watson 1983). In addition, the seasonal changes in net photosynthesis and nitrogenase activity observed in temperate populations of P. praetextata and P. polydactyla

(Kershaw 1977a; MacFarlane and Kershaw 1977; Kershaw and Webber 1984) and the changes observed in the Muskoka population of P. rufescens (Figure 19) suggest that the ability to seasonally adjust net photosynthetic capacity may be widespread in Peltigera. The data from Churchill indicated that this was not necessarily the case (Figure 16), and now it is important to reemphasize the findings of Harris (1971), Rundel (1972), Kershaw (1975), Larson and Kershaw (1975c) and MacFarlane et al. (1983) that large ecotypic variation may exist within the populations that form a species, which makes the extrapolation from one population to another rather tenuous.

The net photosynthetic capacity in the winter collections of Muskoka and Churchill were similar at 800 $\mu E \cdot m^{-2} s^{-1}$ (Figure 16,19). However, when the photon flux density was increased to 1000 $\mu E \cdot m^{-2} s^{-1}$, the Muskoka population had a consistently higher rate at all temperatures above 5°C. This larger capacity may have resulted from the higher chlorophyll content in the winter Muskoka population (Table V), as the photosynthetic capacities between the populations are similar when referenced to a chlorophyll basis (Table IV).

The similarities between the Churchill response matrix and that of the winter Muskoka population are paralleled in higher plants. Pearcy (1977) has shown that the winter response pattern of a Death Valley population of

Atriplex lentiformis is similar to the pattern exhibited by a permanently cool adapted coastal population of this species. Berry and Björkman(1980) suggested that the adaptational potential to a cool environment is often limited, as the increase in photosynthetic machinery required to overcome the low temperatures would incur too much of an energy cost. Additionally, arctic and alpine environments are often nutrient poor, thus there may be insufficient nitrogen available for the required structures (Chapin and Oechel 1983). However, seasonal acclimation cannot be excluded in the Churchill lichens, as a large increase in net photosynthetic capacity has been observed in Parmelia disjuncta. (Kershaw and Watson 1983).

Recent studies (Kershaw and Webber 1984; Brown and Kershaw 1985; Scott and Larson 1985) have indicated that large year to year variation can be observed in the seasonal patterns of net photosynthesis exhibited by a single population of lichen. The Churchill matrix (Figure 16) consisted of three collections covering a five month period in 1982 (Table II). This sampling is lacking a mid-winter collection and is thus inadequate to fully describe the changes which occurred in 1982, let alone determine if the response observed in that year was typical of other years. Much more work is needed, involving collections between January and May and several spot checks of the matrix for an additional one or two years, before it can be conclusively

stated that the Churchill population does not exhibit any seasonal changes in photosynthesis or respiration.

Reciprocal transplant studies between the Muskoka and Churchill sites would be beneficial in determining if the Churchill population has the potential to acclimate in the Muskoka environment. Similarly, if the Muskoka population, moved to the Churchill beach system, did not show its characteristic summer-winter capacity changes, it may indicate that the Churchill environment is lacking some of the correct environmental signals which initiate and maintain the summer acclimated stated.

The seasonal change observed in the Muskoka population was consistently observed in 1981, 1982, 1984 and 1985 (Figures 19, 24, 50, Table IV). However, in 1983, the net photosynthetic rate remained in the winter pattern during the summer months (Brown and Kershaw 1985). The winter of 1982/83 was atypical of the Muskoka area, being much warmer, with considerably less snowfall (Scott and Larson 1985). This may have altered some of the environmental signals which induce the summer capacity change, or the lower snowcover could have induced a cold stress in the lichen which interfered with the capacity change.

During the summer, there was a dramatic increase in the net photosynthetic capacity in the Muskoka population on a dry weight basis. This change was observed only in the 35 and 45°C cells of the matrix (Figure 19) and did not involve

changes in AQE or pigments (Tables IV, V, VI). Prézelin (1981) has suggested that the mechanism for this type of change involves an alteration in the dark reactions of photosynthesis (Figure 8). As no changes in the light limited rates of net photosynthesis could be induced experimentally, or observed seasonally (Figures 21, 27, 28, Table V), no changes in the light reaction were involved in this acclimation process. There were changes in the thallus pigment contents (Tables V, VI), which resulted in a decrease in AQE from August to November when this value was corrected for the chlorophyll content in the thallus (Figure 22). It was surprising that this change in pigment did not result in an alteration in the P-I curve when expressed in a dry weight basis. This suggested that the additional pigments in the October and November collections were uncoupled from the electron transport chain (Figure 7, Prézelin 1981).

Uncoupling of the photosynthetic units is thought to be responsible for the lower net photosynthetic capacity observed in winter collections of P. praetextata, Cladonia stellaris and C. rangiferina (MacFarlane et al. 1983: Kershaw et al. 1983: Kershaw and Webber 1984). A 50% reduction in the rate of net photosynthesis at all experimental light and temperature combinations was observed in these three species during the winter months. Although the pattern of this uncoupling (change in AQE and Pmax on both a dry weight and pigment basis) was identical to that observed in several

dinoflagellates (Prézelin et al. 1977), the time scale of these events is considerably different. The reversible uncoupling in the dinoflagellates follows a circadian rythmn (Prézelin and Sweeney 1977; Prézelin and Matlick 1980) while the event in the lichens occur seasonally. If this uncoupling is identical to that observed in the algae, an increase in fluorescence would be expected (Prézelin and Sweeney 1977). Fluorescence techniques have just recently been adapted for use in lichen research (Jensen and Feige 1986) and it will be useful to define the changes seen in Peltigera and Cladonia with this technique.

The seasonal changes observed in the Muskoka population have not been previously documented in a lichen, and are not typical of higher plants either. This pattern involved a doubling of the photosynthetic capacity, but only at high light and temperature levels (Figure 19). This resulted in a concurrent shift in the temperature optimum from 25°C to 35°C.

The cold temperature adaptations in Caloplaca trachyphylla resulted in an increased capacity at 7°C. with no changes in AQE (Coxson and Kershaw 1984) and probably represented the cold adaptation equivalent to the warm adaptation seen here. A similar phenomena may occur in Umbilicaria deusta, although it is difficult to determine this from the sparse data matrix presented in Larson (1980). The seasonal response observed in U. mammulata (Larson 1980)

and Stereocaulon paschale (Kershaw and Smith 1978) involves an increase in capacity at 28 and 35°C respectively, but this is concurrent with an increase in AQE and is thus not comparable to the Muskoka population. A decreased respiration rate at 28°C in the summer was largely responsible for the changes observed in U. mammulata (Larson 1980), while the pattern seen in S. paschale may represent a stress response to the 35°C assay in the winter. Several researchers (Sheridan and Ulik 1976; Pearcy et al. 1977; Armond et al. 1978; Mooney et al. 1978; Stidham et al. 1982; Downtown et al. 1984) indicate that a high temperature stress results in a decreased AQE. This explanation is plausible, as seasonal change in the resistance to temperature stresses in Cladonia rangiferina has been previously documented (Tegler and Kershaw 1981), but more experimentation is required to verify it.

In P. disjuncta, a seasonal change in Pmax which does not involve alterations in AQE or respiration has been documented (Kershaw and Watson 1983). However, this differs from the Muskoka population in that the capacity change is observed at all the experimental temperatures. This most likely reflects a quantitative increase in concentrations of the enzymes which limit the dark reaction of photosynthesis while the P. rufescens pattern can be best described as a qualitative change in the temperature response of the rate limiting steps.

The acclimation process in higher plants tend to involve a simultaneous alteration in net photosynthesis capacity at both high and low temperatures (Berry and Björkman 1980). An increase in the photosynthetic capacity at high temperatures has, as its cost, a decrease in capacity at low temperatures and vice versa. High temperature adaptation seems to involve an increase in the thermal stability of the electron transport system (Pearcy et al. 1977; Armond et al. 1978; Badger et al. 1982; Downtown et al. 1984) while a cold temperature adaptation is due to an increase in enzyme concentration (Chabot et al. 1972; Pearcy 1977; Badger et al. 1982). A pattern similar to the Muskoka population has been observed in the C4 graminiod Buchole dactyloides (Monson et al. 1983), the perennial herb Polygonum bistortoides (Mooney and Shropshire 1967), and the winter annuals Erigeron canadensis and Lactuca scariola (Regehr and Bazzaz 1976). while a cold adaptation similar to Caloplaca trachyphylla has been reported for Bouteloua gracilis (Monson et al. 1983).

Recently there has been a considerable debate in the literature as to what seasonal changes actually constitute an acclimation. Larson (1980) used Prosser's definition of acclimation as "an adaptation resulting in the constancy or homeostatic control of some vital process". This definition suggests that, for a complete seasonal acclimation, the amount of CO_2 fixed at low temperatures during the winter is equivalent to the amount fixed at the high temperatures of

the summer months. This has been interpreted, in the sense of a gas exchange matrix, to require a shift in the temperature optimum which either paralleled the seasonal change in thallus temperature (Larson and Kershaw 1975 b,c; Kershaw 1975, 1977a), or resulted in similar CO_2 fixation rates at the respective seasonal growth temperatures (Smith and Hadley 1974; Mooney 1980). In this sense, the changes in capacity observed in the Muskoka population of P. rufescens cannot be considered acclimation. There is no increase in photosynthetic capacity in the January, April and December material which would result in a CO_2 fixation rate at 5°C similar to the 8 to 10 $mgCO_2 \cdot gm^{-1}h^{-1}$ fixed at 25 to 45°C in the summer (Figure 19). For this strict definition, acclimation to seasonal changes in temperature has only been observed in Caloplaca trachyphylla (Coxson and Kershaw 1984), while acclimation to variations in light intensity occurred in P. praetextata, Cladonia stellaris and C. rangiferina (Kershaw et al. 1983; MacFarlane et al. 1983; Kershaw and Webber 1984).

To describe changes such as that observed in the Muskoka population of P. rufescens. terms such as restricted. incomplete or partial acclimation have been used (Larson 1980; Kershaw and MacFarlane 1980, 1982). Larson (1980) is correct in stating that these terms are misleading, as they suggest that the changes observed represent an incomplete adaptation to the environment, while they may actually

represent an incomplete understanding of the plant's ecology by the experimenter. In the Muskoka region of Canada, summer thunderstorms are a common feature and following these conditions P. rufescens will be frequently exposed to quite high levels of illumination, under ambient temperatures near 25°C (Figure 11). At the soil surface these ambient temperature and radiation conditions may result in thallus temperatures in excess of 30°C, despite the concurrent evaporative cooling (MacFarlane and Kershaw 1980; Kershaw 1983). With the summer increase in net photosynthesis at high temperatures, a considerable level of carbon gain can be expected to occur following these thunderstorms.

The decrease in the photosynthetic capacity at these temperatures during the winter most likely represents a energy-saving strategy. The thallus is unlikely to experience temperatures in excess of 20°C during the late fall and early spring (Figure 11), therefore the photosynthetic machinery required for these high rates are redundant. Raven (1984) and Kershaw (1984) have discussed acclimation in the context of a cost/benefit approach. Richardson et al. (1983) have identified two costs in acclimation; a capital cost (the energy required to construct the additional pigments, enzymes and structural supports necessary for the acclimation) and a maintenance cost (the energy required to maintain these structures in a functional state). For acclimation to be a viable strategy, the energy

gains must be higher than the costs involved. The Muskoka population seems to reduce this maintenance cost in the winter by reducing the redundant photosynthetic capacity at high temperatures. The thallus remains active throughout the winter, while the light intensities under a snowpack are insufficient to allow for a net carbon gain (Crittenden and Kershaw 1979; Scott and Larson 1985). Therefore, although temperatures are at near freezing levels, a continual respiratory drain on the carbon reserves will occur. Any adaptation which would reduce this maintenance respiration, but still allow maximal CO_2 fixation rates during snowmelt, should be considered an effective acclimation strategy.

In this regard, a better working definition of acclimation is that proposed by Mooney et al. 1978.

> "The ability of a given genotype to change its photosynthetic capacity characteristics in an adaptive manner, in response to changes in environmental conditions such as light, temperature or water regime".

Within this framework, acclimation has been demonstrated for the species mentioned above, and additionally in Stereocaulon paschale, Parmelia disjuncta, the Muskoka population of P. rufescens and possibly Evernia prunastri and Umbilicaria deusta (Kershaw and Smith 1978; Larson 1980; Kershaw and Watson 1983; Orus and Estevez 1984), and numerous species of higher plants (Berry and Björkman 1980).

It is uncertain if the decrease in net photosynthetic capacity observed in winter collections of P. polydactyla, P. praetextata, Cladonia stellaris and C. rangiferina (Kershaw 1977a; Kershaw et al. 1983; MacFarlane et al. 1983) should be considered as acclimation or not. The uncoupling of the photosynthetic units could be considered beneficial if it decreased the probability of photoinhibition of the apparatus during the winter, or was necessary to maintain a metabolic balance between the fixation and utilization rates of carbon (Sveinbjörnsson and Oechel 1983). In contrast, the decrease in net photosynthetic capacity may be the byproduct of a winter hardening process, and thus the rate change would not be an adaptation, although the hardening process would be. Similarly, the seasonal changes in respiration rates observed in several lichens (Lechowicz and Adams 1974; Kershaw and MacFarlane 1980; Larson 1980; Tegler and Kershaw 1980) are also difficult to interpret as beneficial, neutral or harmful. Until the mechanism behind the change and the results of the change are known on a physiological and ecological basis, it will be impossible to decide if such changes are adaptive.

Raven (1984) has constructed an energy budget for the adaptation of phytoplankton to light. He compared the increase in ATP and NADPH produced due to the increase in quantum efficiency to the ATP and NADH required to construct the additional chlorophyll, carotenoids, bilipigments,

membranes and proteins necessary for the adaptations. In all cases but one, the energy gain was shown to outweigh the capital costs involved (Raven 1984). The one exception was the classes of algae which use biliproteins as their antenna pigments. For this case, Raven (1984) suggested that the energy cost of the additional phycobilisomes could not be compensated for by the increase in net photosynthetic capacity at low light intensities. The advantage of the phycobilisome system is in a reduction in the maintenance cost. The thylakoid membranes required to support the structure is not as leaky as the membrane systems in other algae, therefore less energy is required to maintain the ionic balance across this membrane (Raven 1984).

Such an energy budget is required to determine the significance of temperature adaptation in lichens and other plants. Carbon gain can be easily estimated using $^{14}CO_2$ or IRGA techniques, but the capital and maintenance costs associated with respiration are difficult to estimate. Very little is understood about the physiology and ecology behind respiration in lichens (Kershaw 1985) and until this gap in our knowledge is filled, realistic estimates for maintenance costs will be impossible.

The modeling of net photosynthesis has been one technique used to estimate the importance of acclimation. Larson (1980) has suggested that modeling is beneficial in showing how even a slight difference in the net

photosynthetic response, when compounded over a season, can result in a large difference in carbon accumulation. This approach has been used infrequently in lichen studies (Lange et al. 1977; Paterson et al 1983; Link and Nash 1984a,b), while only Harris (1971) has attempted to estimate the benefit of adaptation to the growth and distribution of a lichen population.

In higher plant studies, this technique has often shown that seasonal acclimation to temperature does not result in an increase carbon gain, and in this respect it would be best if the plant remained cool adapted through the entire growing season (Lange et al 1978; Forseth and Ehleringer 1982). In these studies, the temperature optimum for net photosynthesis was often higher than the corresponding leaf temperature (Lange et al. 1975; Berry and Bjorkman 1980). For example, the temperature optimum of the C_4 succulent Hammada scoparia shifted from 28°C in March, to 41°C in August and back to 28°C in September (Lange et al. 1974). The mean daily leaf temperature ranged from 20°C in March to 30°C in August (Lange et al. 1978). If the net photosynthetic pattern had not acclimated, but remained at its spring and fall optimum of 28°C throughout the season, the mean leaf temperature would be similar to the net photosynthetic optimum in August, instead of being 10 degrees lower, resulting in a higher carbon gain through the season.

The importance of this acclimation was not to

increase carbon gain, but decrease the likelihood of high temperature damage to the photosystems (Forseth and Ehleringer 1982). It is generally accepted that the primary acclimation to high temperature in higher plants is an increase in the thermal stability of the photosystems, with the change in net photosynthesis as a side effect of this process (Pearcy et al. 1977; Mooney et al 1978; Armond et al. 1978; Badger et al. 1982; Downtown et al 1984). It is recognized in the lichen literature that the distribution of a population is governed by its ability to withstand extremes in environmental conditions (Kershaw and MacFarlane 1980; MacFarlane and Kershaw 1980; Scott and Larson 1985, 1986). Although the photosynthetic acclimation potential of the population plays a minor role in determining plant distribution (Kershaw and Smith 1978), the possible carbon gain cannot be overlooked in some situations (Kershaw and Watson 1983; Coxson and Kershaw 1984).

The Muskoka population of P. rufescens has an extremely high tolerance to thermal stress during the summer (MacFarlane and Kershaw 1980), but not many studies on the seasonal changes in this important property have been undertaken. Although it cannot be ruled out, it is unlikely that the observed change in photosynthetic capacity may be due to an increase in the stress resistance. Such changes in higher plants have involved alterations in AQE (Pearcy et al. 1977; Armond et al 1978; Badger et al. 1982) and no such

change was observed during either the seasonal or experimentally induced acclimation in P. rufescens (Figure 21, 27, 28, 50, Table IV). Therefore the observed change is different from the stress adaptation in higher plants and may be a specific alteration in net photosynthetic capacity to allow a higher CO_2 gain in the hot summer months.

The ability to experimentally induce the acclimation response provided an opportunity to study this process in much greater detail than field observations alone would allow. As indicated previously (Section 3.3) the measured gas exchange rate is highly dependant upon the choice of material used in the experiment. Thus it is important to distinguish between variations due to choice of material within an experiment with that between experiments, between collections and between seasons. The repeated observations of the summer/winter pattern over several years in the Muskoka population is a strong indication that the observed changes are indeed a seasonal phenomena and not an artifact of the large between-collection variation. Additional confirmation of this pattern was provided by the ability to induce the acclimation response within one collection of the lichen material.

The laboratory induced response is similar to the naturally occurring seasonal change in the following two aspects. First, the induced response involved changes at 35 and 45°C only. Absolutely no inducible changes in the gas

exchange rates were observed at 5, 15, and 25°C (Figures 22, 26, 27). Secondly, the induced response did not involve a change in AQE (Figure 27, 28, 50). These similarities suggest that a comparable mechanism is responsible for both the laboratory induced and the naturally occurring seasonal change.

There is considerable variation in the gas exchange rates during the initial four days of the acclimation process. When the lichen is placed into a different storage regime, an immediate decrease in net photosynthetic capacity occurs, followed by either a complete or partial recovery to the original rate (Figure 24, 50). This crash in the rates of net photosynthesis was observed in four separate experiments, indicating it may be a real phenomenon. A change in temperature had a larger effect than photoperiod, but an alteration in either parameter produced this response (Figure 24). Kershaw (1977b) observed a similar pattern in P. polydactyla when he attempted to winter adapt a July collection. It was originally thought that this crash resulted from an uncoupling of the photosynthetic units (Prézelin 1981). However, later experiments indicated that this was not correct, as no change in AQE on either a chlorophyll or dry weight basis was observed (Figure 50). This indicates that the crash is produced by an alteration in the dark reactions (Figure 8), but data will be presented in the following section which indicates that the change

occurring has a different cause from the process occurring later in the acclimation sequence.

This initial crash is best described as a short term stress response caused by a rapid change in environmental conditions. The physiological or ecological consequences of this phenomenon are uncertain, but it does present some important experimental implications. The normal collection and transportation of the lichens for the experiment will represent a sudden change in environmental conditions and cause a similar stress response. It is therefore important to allow time for a collection to stabilize in the laboratory storage conditions prior to the commencement of any data collection or manipulation.

Berry and Björkman (1980) suggest that an acclimating plant will continuously track the environmental change. A complete temperature acclimation can occur in a period of one to two weeks in Nerium oleander and Ledum groenlandicum (Smith and Hadley 1974; Badger et al. 1982), while capacity changes in wheat can be completed within one day (Sawada and Miyachi 1974). Similar rapid (1 day) changes have been observed in Peltigera and Cladonia (Kershaw 1977b; MacFarlane et al. 1983; Kershaw et al. 1983) while the low temperature adaptation in Caloplaca trachyphylla is completed within seven days (Coxson and Kershaw 1984)

It is difficult to determine the actual time course that the acclimation process in the Muskoka population of P.

rufescens followed, as it is complicated by the initial crash. For the July 1981 study (Figure 24), it appears that the change occurred gradually during the first five days after the lichen is placed in a cold temperature, short photoperiod storage regime. In September of 1985 (Figure 50) the change occurred between days 4 and 10. This rate of change is similar to that observed in Caloplaca and Nerium (Badger et al 1982; Coxson and Kershaw 1984) and suggests that a similar process is occurring in these species.

The conditions required to induce a stable response also changed during the season. In April (Figure 22) the collection was stored under a 12 hour photoperiod and a change in storage temperature was sufficient to induce the acclimation response. However, in June or July (Figure 23,24), with the material stored under a summer photoperiod of 15 hours, a change in temperature alone could not induce the winter net photosynthetic rates at 35 and 45°C. Similarly, a decrease in the photoperiod to 9 hours while maintaining the storage temperature at the summer level could not induce the winter response. During the summer, a stable winter net photosynthetic capacity could only be generated by a simultaneous change in temperature and photoperiod (Figures 23,24).

Kershaw (1977b) was the first to indicate that the ability to induce an acclimation response had a significant seasonal component. In P. polydactyla and P. praetextata.

the shift from high summer photosynthetic capacity to a low winter rate could only be induced in the spring or fall. At this time, photoperiod effects were not considered important in the acclimation process and therefore temperature was the only variable considered. The results for P. rufescens parallel Kershaw's (1977b). An alteration in the photoperiod did result in a transient crash in net photosynthesis similar to that observed in Figure 24 (Kershaw and MacFarlane 1980). They interpreted this as a stress response.

The seasonal changes observed in Nerium oleander and Larrea divaricata (Mooney et al 1978; Badger et al. 1982) could be induced in the laboratory by growing the plants at different temperatures only. Similarly, the induced acclimation seen in Atriplex lentiformis, Ledum groenlandicum, Heliotropium curassavicum and Carex aquatilis (Smith and Hadley 1974; Pearcy 1977; Mooney 1980; Chapin and Oechel 1983) did not involve changes in photoperiod. The study of Lange et al. (1975) was the only one found which suggested that photoperiod may be involved in the control of the acclimation response in higher plants.

Lichens appear to be different in this respect. Photoperiod is definitely one of the environmental parameters which controls the acclimation process in P. rufescens (Figure 23,24), P. praetextata, Cladonia stellaris, C. rangiferina and Caloplaca trachyphylla (Kershaw et al. 1983; MacFarlane et al. 1983; Coxson and Kershaw 1984; Kershaw and

Webber 1984). In all of these studies, a stable acclimation could not be induced during the summer or winter without the appropriate changes in the photoperiod.

These studies have another important common finding: an alteration in photoperiod and temperature could not increase the net photosynthetic capacity in the naturally acclimated lichen. Thus in the Peltigera's and Cladonia's, laboratory induced changes can only go from the high summer capacity to the low winter capacity. An increase in photosynthetic capacity could not be induced by storing winter collections under the appropriate summer regime (MacFarlane et al. 1983; Kershaw et al. 1983; Kershaw and Webber 1984). In the Muskoka population of P. rufescens, a shift to the summer storage conditions of a December collection caused an immediate decline in CO_2 fixation in one day (Figure 25). A change in photoperiod or temperature caused a similar decline within one week (Figure 25), and it required 15 to 20 days for the material to recover from this shock.

In Caloplaca, the seasonal pattern is an increase in net photosynthetic capacity at low temperatures during the winter . A similar pattern to that observed in P. rufescens is seen, where the low summer capacity can be induced in the winter, but the high winter rate could not be induced in the summer (Coxson and Kershaw 1984).

The significance of this inability to experimentally

increase the net photosynthetic capacity during the winter (or summer in Caloplaca) is uncertain. The induced winter acclimation in P. rufescens is readily reversible (Figure 24, 50), suggesting that this experimental acclimation is not totally comparable to the naturally occurring process. It is possible that the photosynthetic machinery required for this high photosynthetic capacity remained intact, but inoperable, during the induced acclimation and was thus present to restore activity in the lichen when it was returned to the summer regime. This machinery could have been dismantled during the month prior to the January and November 1982 collections for which the summer acclimation was attempted. Either the resynthesis of this material may not have been possible in the air dry thallus, or additional environmental parameters (besides temperature and light) are required to induce the summer response in a winter collection.

Although the biochemical mechanism behind the seasonal acclimation cannot be determined strictly from the gas exchange work, several researchers have presented models where the net photosynthetic changes can be predicted from the biochemical alterations (Prézelin 1981; Ramus 1981; Richardson et al. 1983). Thus a change in photosynthetic unit density, size or function, or an alteration in the dark reaction each result in a different but predictable change in the photosynthetic-illumination curves (see Section 2.2).

The P-I curves from the seasonal and laboratory

induced acclimation all show the same pattern. The light saturated net photosynthetic rate at 35°C declines when the lichen is winter adapted, but there is no change in AQE (Figures 27, 28). An identical pattern is shown when the results of the induced acclimation experiments are expressed on either a chlorophyll basis or a dry weight basis, while there is a slight change in AQE observed on a chlorophyll basis during the seasonal acclimation (Table IV). Prézelin (1981) has discussed this pattern, and suggests that it is produced by a change in the dark reactions of photosynthesis.

Such changes have been observed infrequently in algal systems, but are more common in higher plants. The adaptation to change in light intensity in the dinoflagellate symbiont of the coral Montipora verrucosa and the free living dinoflagellate Gonyaulax polyedra have shown this pattern (Prézelin and Sweeney 1978; Chang et al. 1983). Mooney and Shropshire (1967) were the first to suggest that temperature acclimation may involve enzymatic changes. The changes in the light saturated rate of net photosynthesis occurring during temperature acclimation have been correlated to the RuBP carboxylase concentration in Atriplex lentiformis, Oxyria digyna, Amaranthus palmeri, and several C4 species (Hatch et al. 1969; Chabot et al. 1972; Pearcy 1977; Usuda et al. 1984), while the activity of FBPase is related to the acclimation process in Nerium oleander (Badger et al. 1982)

Part II: Enzyme Studies

6. INTRODUCTION

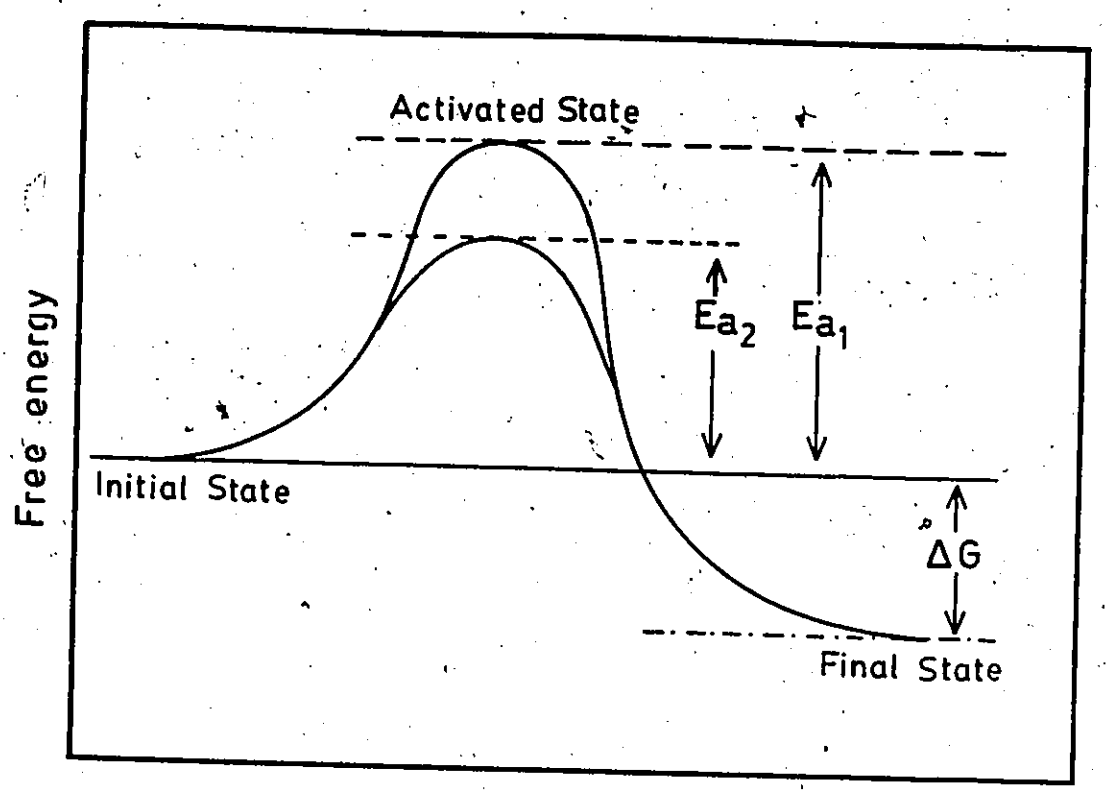
The analysis of the seasonal gas exchange pattern in the Muskoka population of P. rufescens (Figure 19)indicates that the biochemical basis of the observed seasonal change is most likely enzymatic. Seasonal changes in enzymes are quite common, being reported in several species of plants and poikilothermic animals. Such changes could involve quantitative adjustments in the amount of enzyme present Küppers and Weidner 1980; Badger et al. 1982). or qualitative changes affecting the enzyme's thermal stability. activation energy (Ea) or substrate affinity (Somero and Hochachka 1971).

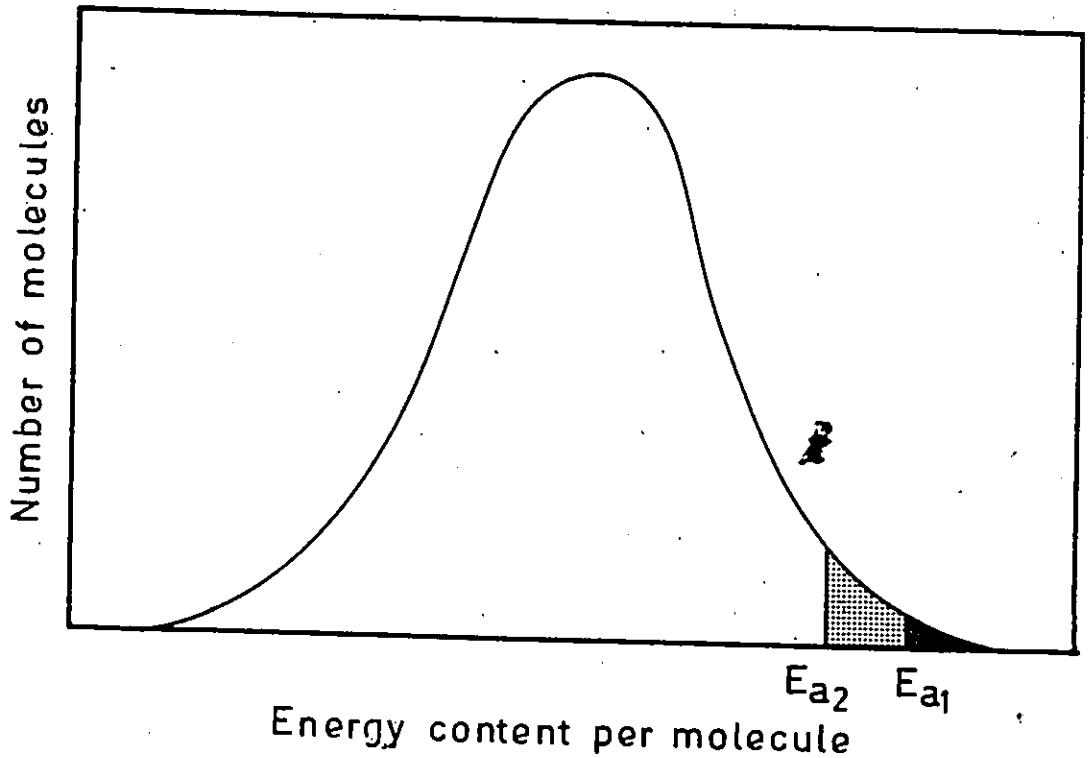
It is intuitively easy to understand how an increase in the thermal stability of an enzyme can be adaptive. For any give temperature (or time) the amount of enzyme present depends on its relative rate of synthesis and degradation. Any qualitative change in the enzyme which increases its stability (decreases the rate of degradation) at higher temperatures will result in a net increase in the concentration of that enzyme, producing a higher catalytic capacity at that temperature.

The activation energy can be considered as the amount of energy required to start the reaction (Figure 29). During

FIGURE 29: The upper figure illustrates the change in energy as molecules undergo a chemical reaction. The E_a can be considered as the energy required to raise the system from the initial (substrate) energy level to the transition state level. The lower diagram gives the normal energy distribution of the substrate molecules, with the shaded areas indicating which molecules have sufficient energy to undergo the reaction. A decrease in the E_a of the reaction increases the percentage of molecules that have sufficient energy to undergo the reaction.

(modified from Stryer 1981)


Activated State
Free energy
E_{a_2}
E_{a_1}
Initial State
ΔG
Final State
Progress of the reaction


Number of molecules
E_{a_2}
E_{a_1}
Energy content per molecule

the course of a reaction, the molecules involved pass through a transition state which has a higher energy content that either the substrate or product molecules (Stryer 1981). The energy difference between this transition state and the substrate acts as a barrier to the reaction. Only a very small fraction of the substrate molecules present have sufficient kinetic energy to overcome this barrier. A slight decrease in this E_a will result in a large increase in the number of molecules able to react (Figure 29), and thereby increase the flux through that enzyme.

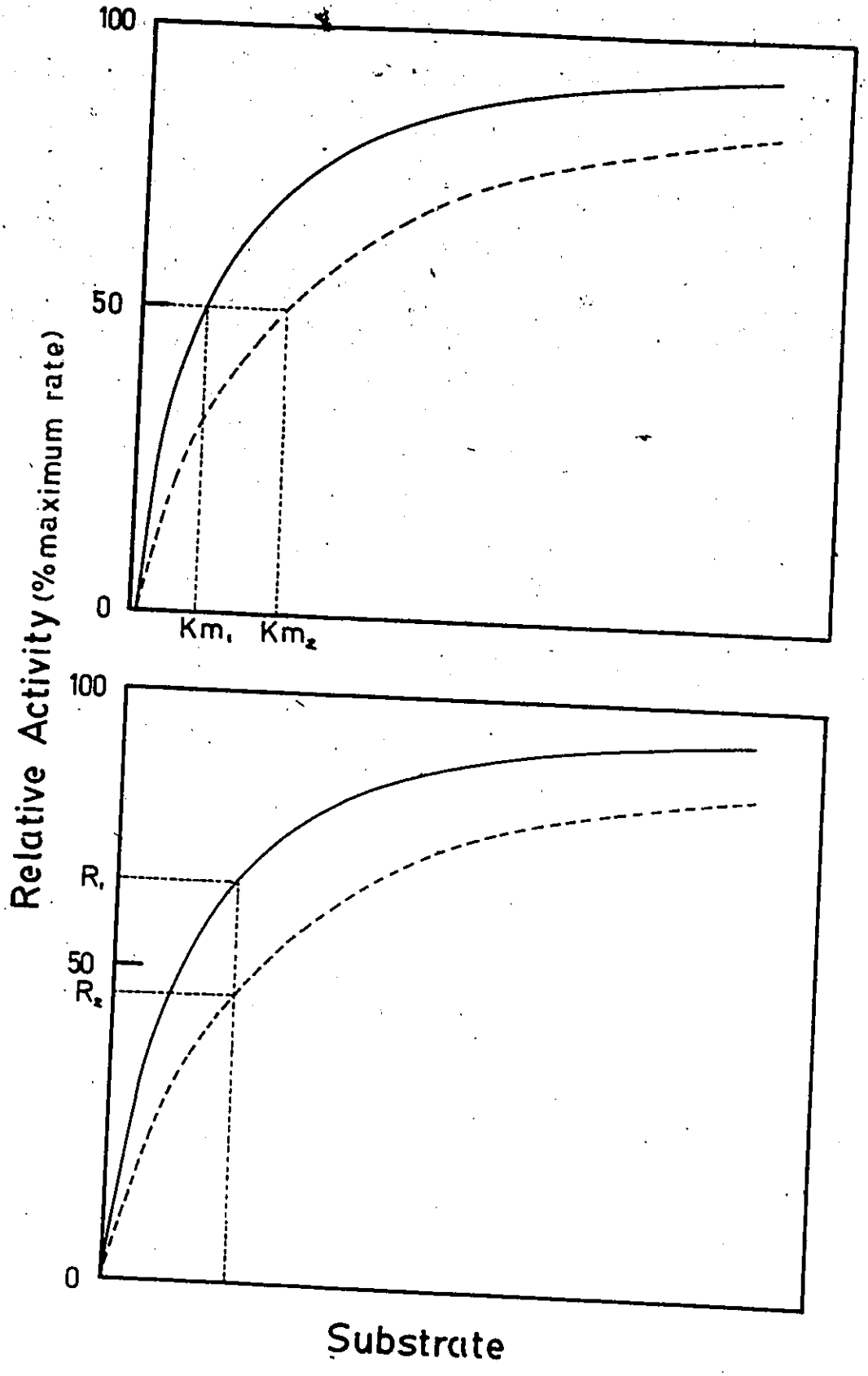
Another important parameter is the substrate affinity. This is often measured as the substrate concentration which half saturates the rate of the reaction (Figure 30). If the enzyme follows Michaelis-Menten kinetics (Eqn. 7, where v is the flux at substrate concentration s, and V is the maximum rate of that enzyme), the substrate affinity is negatively correlated to the Michaelis constant, K_m.

$$v=Vs/(K_m+s) \qquad (7)$$

The importance of K_m in the acclimation process is often overlooked (Graham and Patterson 1982). Most physiological substrate concentrations are lower than the K_m of the enzyme. Therefore a slight change in substrate affinity can result in a large change in velocity (Figure

FIGURE 30: A plot of the reaction rate as effected by substrate concentration for an enzyme which follows Michaelis-Menten kinetics. A decrease in substrate affinity (from K_{s1} to K_{s2}) will result in a decrease in enzyme activity (from R_1 to R_2).

(modified from Somero 1975)


100
50
0
Km1
Km2
Relative Activity (% maximum rate)
100
R1
50
R2
0
Substrate

30). The regulation of enzyme activity occurs via positive and negative effector molecules which operate through their effects on K_s, therefore it is important for the enzyme to maintain its K_s at a concentration where it can respond optimally to changes in substrate and effector concentration (Somero 1975). In cases when the K_s is too low relative to the concentration of the substrate, the fine control exhibited by effector molecules will not have a large effect on the reaction rate. As K_s is strongly affected by temperature, a plant experiencing large seasonal temperature fluctuations must be able to adjust its substrate affinity to maintain the proper levels of control over its metabolism (Somero and Hochachka 1971).

McNaughton (1972,1974) studied the relationship of K_s and E_s to temperature in several geographically diverse populations of Typha latifolia. He observed a trend in which these parameters tended to be insensitive to changes in temperature within the range found in their "natural" environment. Somero and Hochachka (1971), studying the response of K_s to temperature in several species of fish and invertebrates adapted to different thermal habitats have observed one consistent trend. For all the enzymes studied, the K_s was the lowest (ie. substrate affinity highest) at the lowest temperature which that organism was likely to experience in the field. Such trends indicate the importance of adaptive shifts in E_s and K_s over a wide

temperature range and therefore indicate the potential importance of these parameters in seasonal adaptation to temperature.

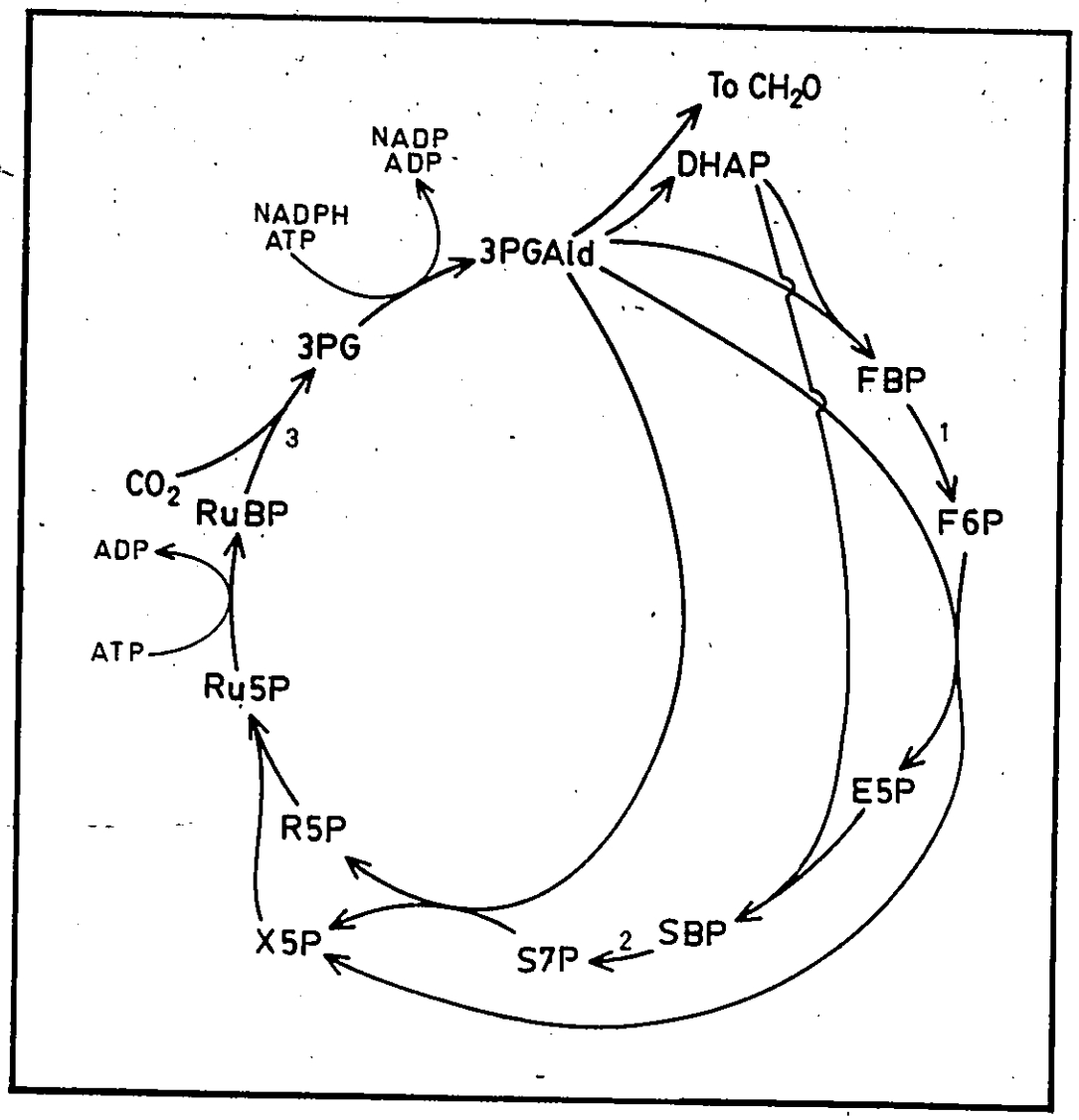
The organism, therefore, has several options available to modify its enzymatic rate, either through a simple change in the concentration of the enzyme, or a modification of the enzyme's structure which will affect its activation energy, substrate affinity or thermal stability. However, for a change to be beneficial to the organism, it must effect the overall flux through the metabolic cycles. This would only happen if the enzymatic change involved the rate-limiting step in that cycle.

The observed gas exchange patterns clearly indicate a change in the temperature response of the Calvin cycle. Based on studies of <u>in vitro</u> activity, three enzymes of this cycle were identified a possible rate limiting steps (Latzko and Kelly, 1979); ribulose-1,5-bisphosphate carboxylase (RuBP CO_2ase), which catalyzes the initial CO_2 fixation step, and the two phosphatases, sedoheptulose-1,7-bisphosphatase (SBPase), and fructose-1,6-bisphosphatase (FBPase), which are involved in the regeneration of RuBP (Figure 31). Improvements in the <u>in vitro</u> assay techniques have now shown sufficient activity for RuBP CO_2ase and FBPase extracts to account for the rate of CO_2 fixation, leaving SBPase as the only Calvin cycle enzyme where <u>in vitro</u> activity cannot match its <u>in vivo</u> activity. The studies of Bassham and Krause

FIGURE 31: The Calvin cycle. The reaction catalyzed by FBPase (1), SBPase (2) and RuBP CO_2ase (3) are possible rate-limiting steps in this cycle.

3PG	3-phosphoglyceric acid
3PGALD	3-phosphoglyceraldehyde
CH_2O	Carbohydrate
DHAP	Dihydroxyacetone phosphate
FBP	Fructose-1,6-phosphate
F6P	Fructose-6-phosphate
E5P	Erythrose-5-phosphate
SBP	Sedoheptulose-1,7-phosphate
S7P	Sedoheptulose-7-phosphate
R5P	Ribose-5-phosphate
X5P	Xyluose-5-phosphate
RuBP	Ribulose-1,5-phosphate

(modified from Lehninger (1982))


To CH_2O
NADP
ADP
DHAP
NADPH
ATP
3PGAld
3PG
FBP
1
3
CO_2
RuBP
F6P
ADP
ATP
Ru5P
E5P
R5P
X5P
S7P
2
SBP

(1969) on the metabolite levels during steady state photosynthesis in Chlorella also indicate that the steps catalyzed by these three enzymes were energetically unfavourable and most likely reflect bottlenecks in the cycle.

RuBP CO_2ase activity has been correlated to CO_2 fixation rates in several species (Hatch et al. 1969; Chabot et al. 1972; Sawada et al. 1974; Senger and Fleischhacker 1978; Perchorowicz et al. 1981; Makino et al. 1983; Tsel'nicher et al. 1983; Usuda et al. 1984). Researchers who suggest that it is the carboxylation reaction which controls the rate of net photosynthesis in C_3 plants base this on observations that under ambient CO_2 concentrations, carbon dioxide limits net photosynthesis (Kelley et al. 1976; Leegood 1985). However, under optimal moisture conditions in P. rufescens, net photosynthesis is not limited by CO_2 at ambient concentrations above 200 ppm (Figure 14). Therefore it is unlikely that the carboxylase limits the flux through the Calvin cycle in this lichen.

Other studies have indicated that the regeneration of RuBP limits CO_2 fixation in cyanobacteria (Pelroy et al. 1976; Karagouni and Slater 1979) and spinach (Stitt et al. 1980; Dietz and Heber 1984). It is most likely that this regeneration is limited by FBPase activity (Pelroy et al. 1976; Portis et al. 1977; Karagouni and Slater 1979; Dietz and Heber 1986). Badger et al. (1982) studied the changes in

activity of six photosynthetic enzymes during temperature adaptation in Nerium oleander and found that the FBPase was the only enzyme correlated to the change in CO_2 fixation rates. The following study was undertaken to determine if changes in the properties of the photosynthetic FBPase correlates with the observed seasonal and laboratory-induced changes in net photosynthesis in Peltigera rufescens.

Fructose-1,6-bisphosphatase (EC 3.1.3.11) is an enzyme that hydrolyzes the C1 phosphate ester of fructose-1,6-bisphosphate producing fructose-6-phosphate and Pi. The FBPase involved in photosynthetic carbon assimilation is found in the stromal fraction of the chloroplasts (Latzko et al. 1974). Another form of this enzyme, located in the cytosol is involved in gluconeogenesis (Zimmermann et al. 1978). In addition, a nonspecific acid phosphatase has also been characterized which demonstrates FBPase activity (Racker and Schroeder 1958). As this study involves a detailed analysis of the photosynthetic FBPase, the presence of multiple enzymes poses a problem which requires a separation technology that can specifically isolate this photosynthetic enzyme. Fortunately, in addition to the above mentioned compartmental differences, the three enzymes differ significantly in their response to pH, $MgCl_2$ concentration and inhibitors (Table VII). Therefore, once each form has been isolated, it can be easily identified.

The acid phosphatase is active over a pH range of 5.5

TABLE VII: A summary of the kinetic properties of the different FBPases reported in the literature.

Reference	pH range optimum	pH range limits	Half saturation $MgCl_2$ (mM)	Half saturation FBP (mM)	Inhibited by AMP	Inhibited by FR-2, 6-P	Inhibited by FBP	Activated by DTT
PHOTOSYNTHETIC (SPINACH)								
1	8.0	<7.5	-	-	-	-	NO	YES
2	-	-	0.7	0.085	-	-	-	YES
3	8.7	<8.2	3	1.1	-	NO	NO	-
4	-	-	2	0.1	-	-	NO	YES
5	-	-	1	-	NO	-	NO	YES
6	8.0	<7.4	-	0.2	NO	-	NO	-
7	-	-	2	0.033	NO	-	-	YES
8	-	-	1	3.0	-	-	NO	YES
9	-	-	5	0.08	YES	-	NO	YES
10	8.7	<7.5	3	0.085	NO	-	-	YES
11	8.5	<7.8	2	0.03	NO	-	NO	-
PHOTOSYNTHETIC (CYANOBACTERIAL)								
12	-	-	-	0.013	YES	-	NO	YES
13	8.0	-	12	1.7	-	-	NO	YES
14	8.0	<7.0	1	0.088	YES	-	NO	-
15	8.0	<7.0	5	0.06	YES	-	NO	YES

Continued on the next Page....

CYTOSOLIC								
16	-	-	1	0.003	YES	YES	YES	-
10	7.5	<6.6	1	0.025	YES	-	-	NO
17	7.5	<6.0	0.1	0.003	YES	-	YES	-
18	7.0	-	-	0.005	YES	YES	-	NO
19	7.5	-	0.15	0.06	NO	-	NO	-
20	7.5	<6.0	0.17	0.003	YES	YES	YES	-
21	-	-	-	0.005	YES	YES	YES	-
ACIDIC FBPase								
18	7.0	>8.0	0	ANY BP	YES	NO	-	NO
11	6.5	>8.0	0	SBP,F6P	-	-	-	-
19	7.0	>8.0	0	-	-	-	-	-
P. RUFESCENS								
FRACTION A	6.0	>7.5	0	0.23	YES	NO	NO	NO
FRACTION B	9.0	<7.5	2	0.039	YES	-	-	YES

SYMBOLS: < no activity below pH > no activity above pH

- not determined BP bisphosphate

REFERENCES: 1. Schürmann & Wolosiuk 1978; 2. Corley & Wolosiuk 1985; 3. Lazaro et al. 1975; 4. Pradel et al. 1981; 5. Buchanan et al. 1971; 6. Preiss et al. 1967; 7. Charles & Halliwell 1980; 8. Schürmann et al. 1976; 9. Zimmermann et al. 1976; 10. Latzko et al. 1974; 11. Racker & Schroeder 1958; 12. Gerbling et al. 1984,1986; 13. Schmidt 1981; 14. Bishop 1979; 15. Udvardy et al. 1982; 16. Herzog et al. 1984; 17. Zimmermann et al. 1978; 18. Pharr & Huber 1984; 19. Woodrow et al. 1982; 20. Stitt et al. 1985; 21. Cséke et al. 1982.

to 8.0, and this activity does not require $MgCl_2$ (Racker and Schroeder 1958; Woodrow et al. 1982; Pharr and Huber 1984). Both the cytosolic and photosynthetic FBPase are active at alkaline pH. The cytosolic enzyme has a pH optimum of 7.5 to 8.0 (Latzko et al. 1974; Zimmermann et al. 1978). The photosynthetic FBPase has a slightly higher pH optima but, more importantly, no activity is observed below pH 7.5 (Preiss et al. 1967; Lázaro et al. 1975; Zimmermann et al. 1976). In contrast to the acid phosphatase, the other enzymes have an absolute requirement for $MgCl_2$. The cytosolic enzyme is inhibited by AMP, fructose-2,6-phosphate (Fr-2,6-P) and high concentrations of FBP, while the photosynthetic enzyme is not (Latzko et al. 1974; Zimmermann et al. 1978; Udvardy et al. 1982; Stitt et al. 1985). In addition, the photosynthetic enzyme is one of the light activated enzymes in the Calvin cycle. Therefore, unlike the other FBPases, its activity is enhanced by dithiothreitol (DTT) and this activation involves a lag period of up to 20 minutes (Rosa and Whatley 1984). Based on these characteristics (Table VII) the three types of FBPases can be easily identified once they have been separated.

7. METHODS

7.1 FBPase assay

7.1.1 The phosphate assays

There are two different assays that can be used to measure FBPase activity. One assay detects the fructose-6-phosphate (F6P) produced, while the other assay involves a colourimetric determination for PO_4^{-2} released during the reaction.

The phosphate assay was a two step procedure. FBPase activity was allowed to proceed in a buffer consisting of:

3.0 mM fructose-1,6-bisphosphate

10.0 mM $MgCl_2$

20.0 mM Dithiothreitol

100 mM Tris-HCl, pH 8.5

in a final volume of 1 ml. The reaction was started with the addition of a volume of the crude extract from P. rufescens equivalent to 100 µg of protein. The reaction was allowed to proceed at 25°C for 20 minutes, and then stopped with the addition of 100 µl of a 12.5% (w/v) solution of tricarboxylic acid.

In the second step, the phosphate formed is reacted with ammonium molybdate to form a coloured solution which is measured spectrophotometrically. Three variations of this phosphate assay were tested; the methods of Buchanan 1975;

Chen et al. 1956; and Allen et al. 1974.

1. Buchanan 1975:

Reagents:

ammonium molybdate 6.6 gm in 100ml

H_2SO_4 9 N(1:2 dilution of Conc.acid)

ferrous sulfate 2 gm in 25 ml H_2O containing 0.5 ml of 0.9 N H_2SO_4

Assay:

0.5 ml ammonium molybdate solution

0.5 ml 9 N H_2SO_4

0.5 ml ferrous sulfate solution

1.0 ml sample and water

incubate at room temperature for 5 min.

measure the absorbance at 660nm.

2. Allen et al. 1974:

Reagents:

ammonium molybdate 1.25 gm in 10 ml H_2O add 14 ml H_2SO_4 and H_2O to a final volume of 50 ml.

stannous chloride 0.1 gm into 1 ml HCl and add H_2O to a final volume of 50 ml. Heat to dissolve, make fresh each day.

Assay:

0.5 ml ammonium molybdate solution

0.5 ml stannous chloride solution

1.0 ml sample

incubate 30 minutes at room temperature

measure the absorbance at 700nm

3. Chen et al. 1956:

Reagents:

6 N H_2SO_4	18 ml conc acid diluted to 108 ml
ammonium molybdate	2.5 gm in 100 ml water
ascorbic acid	10 gm in 100 ml water
reagent C	1:2:1:1 H_2SO_4:H_2O:molybdate: ascorbic acid, make fresh daily

Assay:

1 ml sample

1 ml reagent C

incubate 1.5-2.0 hours at 37°C

read absorbance at 820nm

An assay of the crude extract, determined from the method of Racker and Schroeder (1958) (Section 7.1.2) indicated that FBPase activity is approximately 10 pmol·μg^{-1} min^{-1}. A 20 minute assay using 100 µg of enzyme would

therefore produce 200 nmol of phosphate. The lower detection limits observed for the phosphate assays of Buchanan (1975) (250 nmol) and Allen et al. (1974) (50 nmol) (Table VIII) were thought too be to low to be useful. The third method of Chen et al. (1956) was the most sensitive, with a lower detection limit of 2.5 nmol. However, it reacted with an unidentified compound in the crude extract producing a precipitate which interfered with the assay. Therefore, the phosphate assay method was rejected in favour of the continuous spectrophotometric method which measures F6P production.

7.1.2 The continuous assay

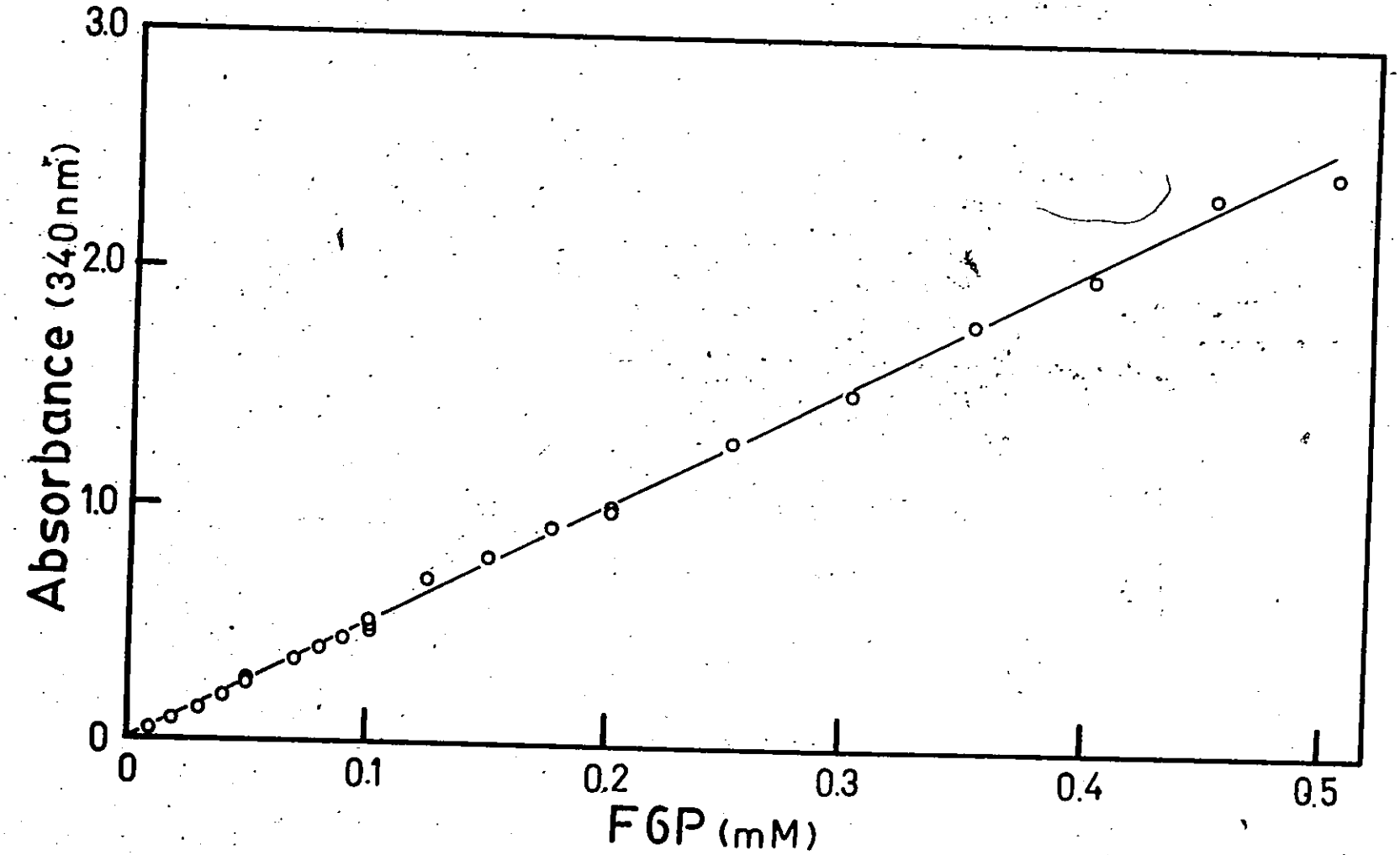
This assay, derived from Racker and Schroeder (1958) is a continuous assay for the F6P produced by the FBPase reaction. Phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G6PDH) are used to link the the FBPase reaction to the reduction of NADPH (Eqn. 8). The F6P produced is converted to Glucose-6-PO_4 by PGI. This is then dehydrogenated by G6PDH and the NADPH generated is measured at 340nm. The temperature regulated Varian DMS-100 spectrophotometer used, measures changes in optical density accurately to 3 decimal places, resulting in an assay sensitive to µM changes in F6P concentration (Figure 32). The concentration of F6P produced is calculated from Eqn. 8.

Table VIII: Test of the phosphate assays

	Phosphate assay tested		
	Buchanan	Allen	Chen
Observed detection limits			
low	250 nmol	50 nmol	2.5 nmol
high	>1000	>1000	100
Regression equation			
slope	8869	8509	77.65
intercept	307.9 nmol	-17.8 nmol	-3.62 nmol
R2	0.87	0.99	0.99

phosphate range tested: 2.5 to 1000 nmol in 1 ml

FIGURE 32: The calibration curve for the continuous FBPase assay.


3.0
2.0
1.0
0
Absorbance (340nm)
0
0.1
0.2
0.3
0.4
0.5
F6P (mM)

$$F6P(mM) = 0.198 * OD_{340nm} - 0.00086 \quad (8)$$

The standard assay used for the photosynthetic FBPase consisted of:

3.0 mM fructose-1,6-bisphosphate

1.0 mM NADP

10.0 mM $MgCl_2 \cdot 7H_2O$

20.0 mM Dithiothreitol

4 units phosphoglucose isomerase$\cdot ml^{-1}$

4 units glucose-6-phosphate dehydrogenase$\cdot ml^{-1}$

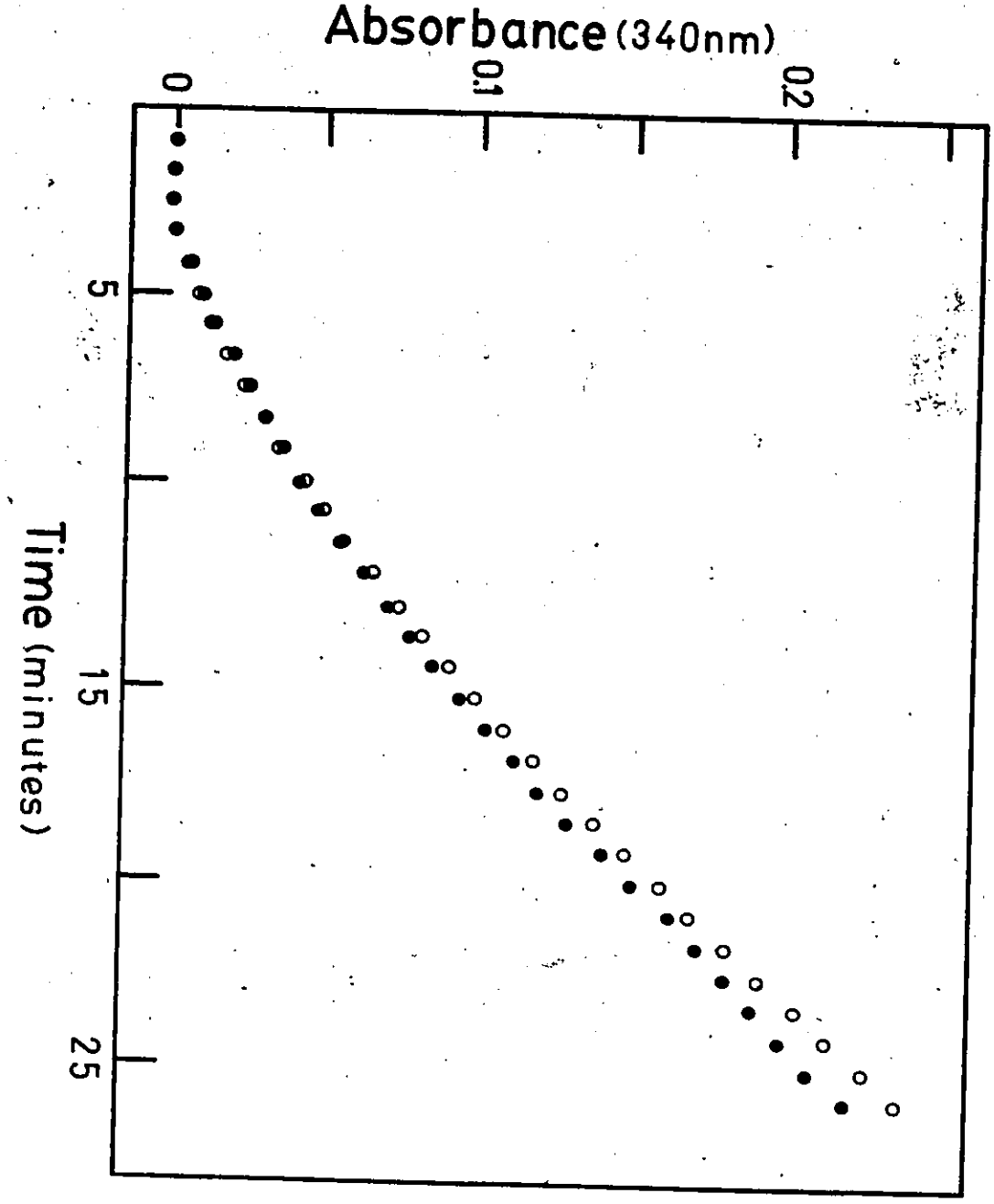
100 mM Tris-HCl, pH 8.5

in a final volume of 1 or 3 ml. The reaction was started with the addition of a sufficient amount of FBPase to produce a change in absorbance of 0.001 to 0.005 units$\cdot min^{-1}$.

This assay has the major advantage in that the FBPase activity is continuously monitored. This is important for the photosynthetic enzyme, where its light activation properties could result in substantial lag periods before maximum activity was observed (Figure 33). A continuously monitored assay provides the experimenter with adequate data to determine when the lag period has ended and therefore which points along the progress curve should be used in calculating the maximum rate activity.

However, a problem can occur if the activity of the coupling enzymes (PGI and G6PDH) are insufficient to maintain

FIGURE 33: The effects of a DTT pretreatment on the activity and lag period of the Fraction B enzyme. The change in absorbance with time of the enzyme pretreated with 20 mM DTT for either 24h (●) or 0h (O) was assayed in the presence of 20 mM DTT at pH 8.5.


Absorbance (340nm)
0
0.1
0.2
Time (minutes)
5
15
25

an equilibrium with the FBPase reaction. This is most likely to occur when the FBPase activity is very low or very high. To overcome this problem, a high concentration (4 units·ml^{-1}) of each coupling enzyme was used (the quantities normally used for this assay are 1 to 2 units·ml^{-1} (Latzko et al. 1974; Charles and Halliwell 1980; Pharr and Huber 1984)). As an additional check, assays were run with varying concentrations of the coupling enzymes near the limits of the assay conditions likely to be used(5, 25 and 40°C and FBP concentrations of 0.02 and 3.0 mM). The results (Table IX) show that the rate of the reaction is independent of the concentration of coupling enzymes between 1 and 8 units·ml^{-1}, indicating a concentration of 4 units·ml^{-1} is more than adequate under these conditions.

Two additional problems with this assay were observed during the course of this study. Three of the lichen extracts contained a compound which reacted with the DTT in the assay causing a change in absorbance at 340 nm. This required the inclusion of a reaction blank containing all the assay elements except FBP in the reference cell of the double beam spectrophotometer to correct for this continual change in absorbance. This corrected out the side reaction with DTT, but produced a noisier baseline for the FBPase reaction. The second problem concerned the water-soluble accessory photosynthetic pigments in the cyanobiont. These biliproteins absorb at 340 nm. This produced a high

Table IX: The effects of varying the amount of PGI and G6PDH in the continuous assay of FBPase.

Temperate (°C)	PGI+G6PDH Units ml^{-1}	FBPase Activity $\mu mol\ F6P \cdot mg^{-1} min^{-1}$	
		0.02 mM FBP	3.0 mM FBP
5	1	0.036	0.33
	2	0.033	0.31
	4	0.033	0.28
	6	0.054	0.28
	8	0.029	0.32
25	2	--	6.1
	4	--	6.3
	8	--	6.6
40	1	5.47	20.25
	2	5.49	21.69
	4	5.85	22.38
	6	6.78	17.62
	8	7.38	21.73

background absorbance for some of the assays which decreased the sensitivity of the spectrophotometer. Therefore the 5°C assay temperatures, which, due to low enzyme activity required a higher concentration of extract, tended to show more variation.

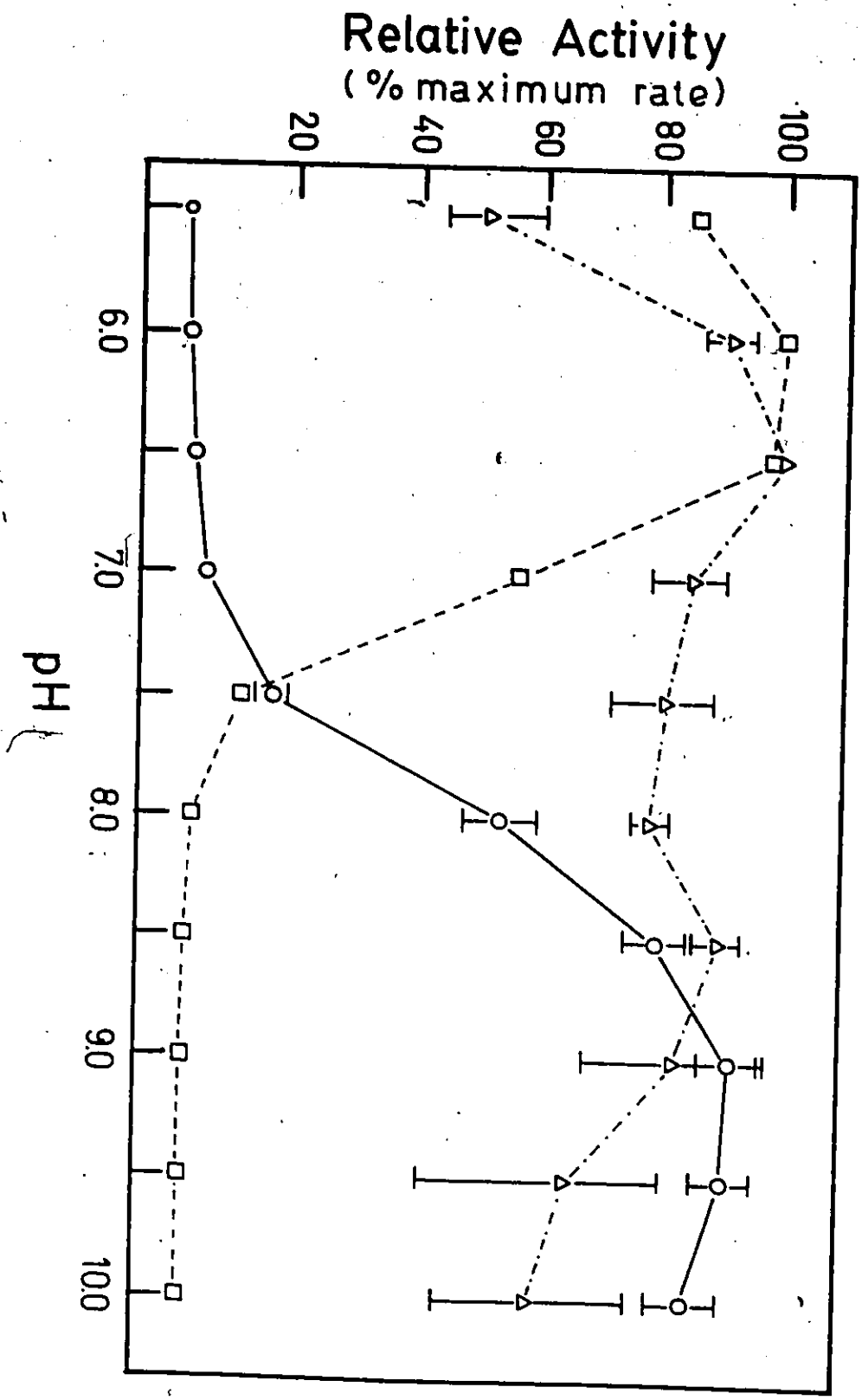
7.1.3 Variations of the standard assay

The detailed characterization of the FBPases from P. rufescens required some alterations to the standard assay conditions. In such cases, the assays are identical to the one above (Section 7.1.2) with the exception of the specific changes listed in the respective figure and table legends.

The relationship between enzyme activity and $MgCl_2$ was tested by varying the $MgCl_2$ concentration from 0.0 to 10.0 mM. The pH curves were assayed using 100 mM PIPES buffer from pH 5.5 to 7.0 and 100 mM Tris-HCl from pH 7.5 to pH 10.0. The preliminary analysis of the pH curves of the FBPase activity in the crude extract (Figure 34) indicated a bimodal response to pH, with local optima at pH 6.5 and 8.5. This was tentatively thought to indicate the presence of two FBPases in the extract. Therefore assays were routinely done at pH 6.5 and pH 8.5 during the course of the purification process to determine the fate of these enzymes.

The effect of AMP on the activity of the purified FBPases was studied using two concentrations of this

FIGURE 34: The relationship between pH and FBPase activity of the crude extract (Δ), Fraction A (□) and Fraction B (O) enzymes from the Muskoka population of P. rufescens.


Relative Activity
(% maximum rate)
20
40
60
80
100
pH
6.0
7.0
8.0
9.0
10.0

inhibitor (0.20 and 2.0 mM AMP). In addition, the inhibitory effects of Fr-2,6-P were also tested. This compound acts as a competitive inhibitor of the cytosolic FBPase (Cséke et al. 1982; Herzog et al. 1984; Pharr and Huber 1984; Stitt et al. 1985). The extracts were tested for inhibition using an Fr-2,6-P concentration of 2 µM at 3 different substrate concentrations (0.03, 0.30, and 3.0 mM FBP). In addition, the combined inhibitory effects of 2 µM Fr-2,6-P and 0.2 mM AMP was tested.

The activation of the photosynthetic FBPase by 20 mM DTT was studied for three extractions of this enzyme. The $S_{0.5}$, V and activation time in the standard assay (containing 20 mM DTT) were compared to assays which did not contain any DTT.

The $S_{0.5}$ for FBP was calculated using a 50% dilution series of substrate concentrations from 0.313 to 0.010 mM FBP. Reactions were normally carried out in a total volume of 1.0 ml with a sufficient dilution of the purified FBPase to produce an adequate change in absorbance. Unfortunately, some assays (the lower FBP concentrations at the higher assay temperature) became substrate-limited too quickly to obtain a good estimate of the velocity. The assay volume was therefore increased to 3.0 ml in the 35, 40 and 45°C assays to overcome this problem.

The assays for substrate affinity and the Arrhenius plots were performed over a temperature range of 5 to 45°C in

5° intervals. All assays for the acclimation time series (Section 7.7.1) were at 35°C. All other assays were at a temperature of 25°C.

7.2 Enzyme extraction

7.2.1 The extraction buffer

Previous attempts at the isolation of enzymes from lichen tissue have been hindered due to severe technical problems associated with this plant (MacFarlane et al. 1983). Lichens contain high concentrations of secondary compounds, most of which are phenolic derivatives. These can complex with proteins, denaturing them (Kelley and Adams 1977). In addition, the low protein content in lichens makes it difficult to obtain sufficient quantities of purified enzyme for detailed kinetic studies (Vicente and Requeva 1984). This is compounded by their robust cell walls, which resist most types of mechanical disruption (Fahselt 1980) and may require harsh chemical digestion which also denatures proteins (Fahselt and Jancey 1977). Each of these problems must be dealt with before a successful isolation is achieved.

Phenolic compounds interfere with enzyme activity in several ways. Strong hydrogen bonds can form between the hydroxyl groups of the phenol and the oxygen atoms in the protein (Loomis 1974). The carbon rings of the phenol can also interfere with the hydrophobic interactions of the enzyme. Thus the enzyme's tertiary structure, which is

dependent on these interactions, is disrupted and the catalytic efficiency is decreased. Phenols can also be oxidized by cellular phenolases to produce highly reactive quinones which may undergo covalent bonding with the thiol and amino residues of a protein (Loomis 1974). Normally these compounds are sequestered (extracellularly on the lichen cell walls and intracellularly in vacuoles) and do not cause problems until the cell is disrupted during the extraction procedure.

The problems related to phenolic derivatives can be minimized chemically by the addition of protective compounds in the extraction buffer, or by removing these compounds prior to the extraction of the protein. The latter method has been used in several lichen studies (Fahselt and Jancey 1977; Cifuentes et al. 1981; Fahselt and Hageman 1983; Hageman and Fahselt 1984), and involves soaking the thalli in several changes of acetone for 3 hours. This method was rejected due to the time commitment and the uncertainty of the physiological effects that a 3 hour acetone treatment would have on the light activation process of the FBPase.

Chemical protection of the protein during extraction has been successfully used in other lichen studies (MacFarlane et al. 1983; Kershaw et al. 1983; Legaz and Vicente 1983; Brown and Kershaw 1985) and with other plants that contain high phenol levels (Kelley and Adams 1977; Loomis 1974).

The extraction buffer used in this project is a variation of the buffer developed by MacFarlane et al. (1983), consisting of:

20 mM 2-mercaptoethanol	1.39 $ml \cdot l^{-1}$
20 mM sodium metabisulfite	3.95 $gm \cdot l^{-1}$
30 mM PIPES buffer, pH 6.6	9.07 $gm \cdot l^{-1}$

Insoluble polyvinylpolypyrrolidone (PVPP) (0.5 $gm \cdot gm$ dry thallus $wt.^{-1}$) was mixed with the lichen prior to the addition of the extraction buffer. The PVPP removes phenolic compounds by absorption (Loomis 1974). The metabisulfite complexes with the phenols (Kelley and Adams 1977) and also inhibits the quinone-producing phenolase reaction (Loomis 1974). Thiol compounds such as mercaptoethanol and DTT have been shown to protect several enzymes from lichen acids (Cifuentes et al. 1981; Legaz and Vicente 1983). In addition, the reducing abilities of the mercaptoethanol is sufficient to maintain the photosynthetic FBPase at its current state of activation without causing any additional activation (Schmidt 1981; Leegood and Walker 1982).

7.2.2 Isolation of *Nostoc* from *P. rufescens*

Plants contain several enzymes capable of hydrolizing FBP (Latzko et al. 1974; Pharr and Huber 1984). The preliminary study of the crude extract from *P. rufescens* indicated the presence of at least two distinct FBPases (a Mg^{2+} dependent alkaline phosphatase and a Mg^{2+} independent

acid phosphatase)(Figure 35). As this project was specifically concerned with the photosynthetic enzyme, an initial step involving the separation of the algae from the mycobiont was attempted as this would be the best method for isolating this photosynthetic enzyme.

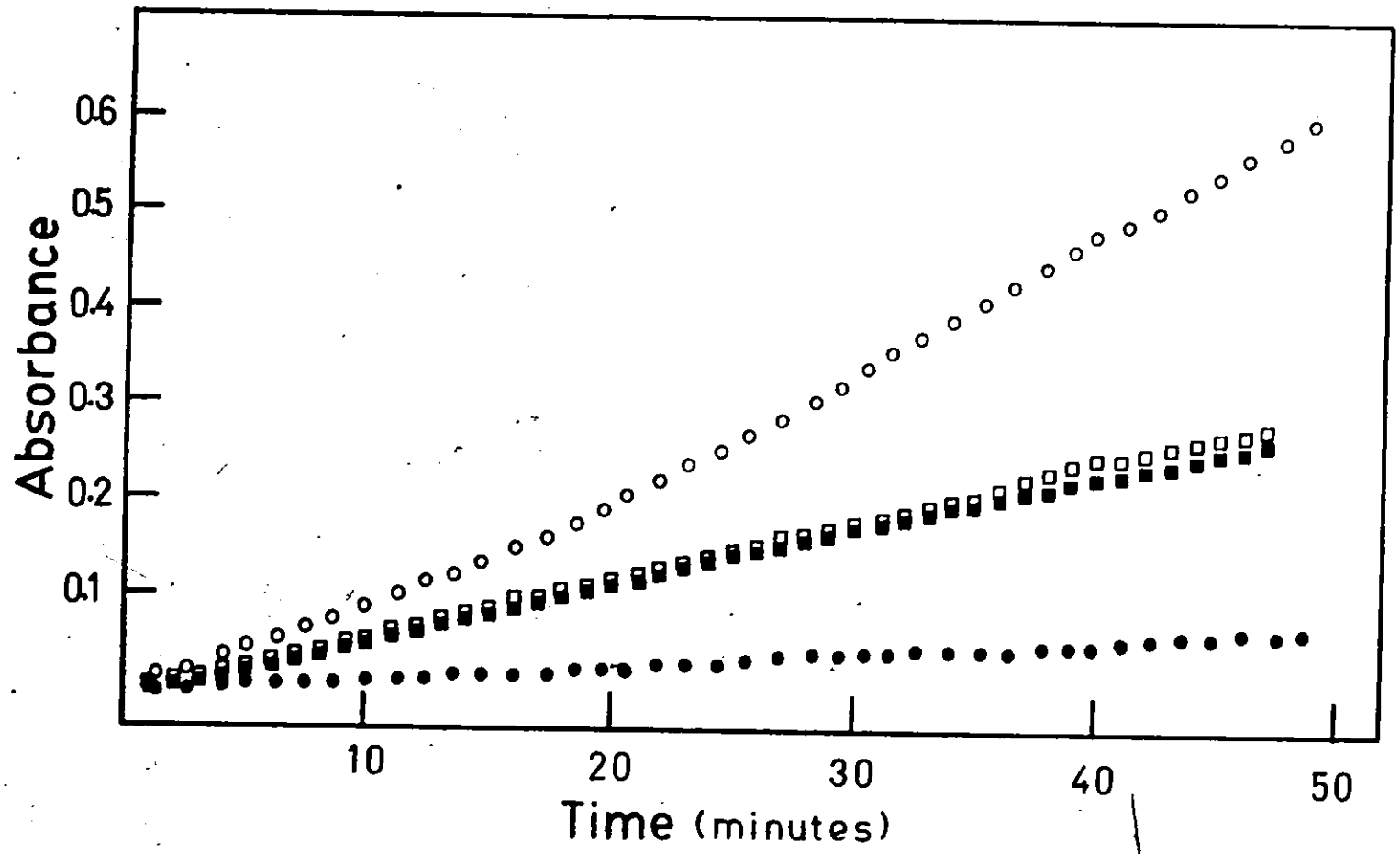
7.2.2.1 The sucrose method

Kershaw (Kershaw and Millbank 1970; Kershaw et al. 1979) isolated Coccomyxa and Trebouxia from P. aphthosa and Parmelia caperata using a stepwise density centrifugation method. The lichen was gently shredded with a blender in a 10% sucrose solution. This was further processed in a glass piston homogenizer, layered on an 85% sucrose solution and centrifuged for 30 min at 2000xg (Kershaw and Millbank 1970). The algae collected at the 10/85% sucrose interface, while the fungal component migrated into the 80% sucrose. This method was attempted with P. rufescens but was unable to separate the algae from smaller fungal fragments. Similar results were reported by Kershaw et al.(1979) upon attempts to obtain a pure isolation of Nostoc from Peltigera praetextata.

7.2.2.2 The differential centrifugation method

Smith's laboratory developed a differential centrifugation method which was successful in isolating Nostoc from P. polydactyla (Drew and Smith 1967; Green and Smith 1974). This method is based on the principle that the

FIGURE 35: The change in absorbance with time in the crude extract from the Muskoka population of P. rufescens assayed at pH 6.5 (□, ■) and 8.5 (○, ●) in the presence (open) and absence (closed) of 10.0 mM $MgCl_2$.


Absorbance
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
Time (minutes)

more dense fungal fragments will pellet faster than the algal cells when under a low centrifugal force. The thalli were gently ground in a mortar and pestle with 10 $ml \cdot gm^{-1}$ of the extraction buffer and then homogenized an additional 5 minutes in a motorized tissue homogenizer. This suspension was loaded into a 250 ml beaker and spun in a refrigerated Beckman TJ 6 centrifuge for 2 minutes at 50xg, and then an additional 5 minutes at 2000xg. The algae formed a thin green layer on the top of the pellet. This layer was carefully removed and resuspended in a small volume of extraction buffer. The remainder of the pellet was also resuspended and the above centrifugation procedure was repeated until no further algae could be removed.

The combined algal fractions retained a large amount of fungal contaminants. Repeated purification with the above centrifugation method did not result in a pure algal fraction, as large fungal fragments were still present after 5 cycles of centrifugation. (This was based on a subjective observation using a light microscope). This is in contrast to the result of Green and Smith (1974), who report that this process could produce a Nostoc isolation with only a few small fungal fragments.

7.2.2.3 The Percoll method

Price et al. (1978) purified the cyanobacterium Oscillatoria using isopycnic density centrifugation on

Percoll gradients. In this procedure, cellular fragments centrifuged at low speeds will migrate though a preformed density gradient until they reach a solution density equal to their own. This method was used to further purify the algal extract obtained from the first differential centrifugation step described in section 7.2.2.2.

A nonlinear Percoll gradient was formed by centrifuging a solution of:

2.5 M sucrose	10 ml
Percoll	68 ml
H_2O	12 ml

at 30,000xg for 1 hour in a refrigerated Sorval RC-2B centrifuge. The algal extract was gently lavered on top of this and then centrifuged at (200xg) for 20 minutes in the Beckman TJ-6 centrifuge. The algae banded at a density of 1.06-1.08 $gm \cdot ml^{-1}$. (Density was measured using Pharmacia Density Marker Beads.) The algae were removed from the Percoll using a pipette and washed several times in the extraction buffer. Very little fungal contaminants were present in the purified fraction.

The isolated Nostoc was primarily single cells, with a few 2 cell chains. Green and Smith (1974) reported similar results from their isolation procedure. Nostoc usually exists as a long chain of cells, therefore the absence of chains in the purified extraction indicated the possibility of severe cell breakage during the process. The 45 gm of

lichen used in this isolation procedure produced approximately 1 ml of purified algae. This yield is very poor and unsatisfactory, considering other studies on the cyanobacterial FBPase used 50 to 250 gm fresh weight of algal cells (Schmidt 1981; Yee et al. 1981).

The pH curve of the FBPase extracted from the algae isolated above showed a biphasic response to pH, with a relatively high activity at pH 7.0. The photosynthetic FBPase is inactive at this pH (Zimmermann et al. 1976), therefore it appears that this algal separation did not produce a pure photosynthetic FBPase. As the yield in the algal purification was poor, and did not result in a contaminant free photosynthetic FBPase, this method was abandoned in favour of the chemical and chromatographic separation method described below.

7.2.3 The protein extraction method

Fourty to sixty gm(air dry weight) of non-fruiting lobes were carefully dissected from the lichen mat and soaked overnight (16 h) in distilled water. 10 $ml \cdot gm^{-1}$ of extraction buffer (Section 7.2.1) and PVPP (0.5 $gm \cdot gm^{-1}$) were added to the drained thalli and this was ground with a mortar and pestle. The slurry was further processed for 20 minutes with a motorized tissue homogenizer (Janke and Kunkel Ultra-Turrax). The resulting suspension was sonicated for a total of 20 minutes, and then centrifuged in a swinging

bucket rotor on a Beckman TJ 6 refrigerated centrifuge for 20 minutes at 1500xg. The supernatant was reserved and the pellet was resuspended in approximately 300 ml of the extraction buffer, sonicated an additional 20 minutes and centrifuged again. The supernatants were combined and the final pellet was discarded.

The initial pretreatment conditions (overnight soak under the lichens current storage condition) was maintained in the experimental protocol to ensure that the enzyme results are comparable to the previous net photosynthetic studies in most aspects (Section 3.4). Due to the large quantity of material required for the enzyme extraction compared to that required for the gas exchange studies, the experimenter was forced to use material from the older parts of the rosette which would not have been included in the gas exchange studies. Therefore the lichen material used in the enzyme work was more variable and may not be totally physiologically comparable with the gas exchange studies, but this could not be avoided (Section 3.3).

The protein extraction was started at approximately 9AM. Thus the lichen material had at least one full hour of exposure to light prior to the extraction. The photosynthetic FBPase is a light activated enzyme (Buchanan 1980) and the kinetic parameters of the activated enzyme are very different from the inactive form (Schurmann and Wolosiuk 1978; Charles and Halliwell 1980). The light pretreatment

ensures that the extracted FBPase is in a state of activation similar to its normal daytime activity. The presence of 2-mercaptoethanol in the extraction buffer and DTT in the assay should prevent the enzyme from being oxidized into its inactive state while the removal of the enzyme's activator, thioredoxin, in the first step of the purification procedure (see Section 7.2.4) will prevent any further activation of the enzyme (Schürmann et al. 1976). Thus the photosynthetic FBPase in vitro form should be comparable to its in vivo state.

7.2.4 FBPase purification

The photosynthetic FBPase was purified in a five step procedure modified from Buchanan et al. (1971). All stages in the separation were done at 5°C.

1. The crude extract was acidified to pH 4.5 by the addition of 1.0 M acetic acid. This formed a green precipitate, which was collected by centrifugation (1500xg for 30 minutes) and resuspended in 150 ml of the extraction buffer.

2. Ammonium sulfate (243 $gm \cdot l^{-1}$) was added to bring the solution to 40% saturation. The solution was allowed to sit for 30 minutes and then it was centrifuged in a Sorval RC 2B centrifuge at 20,000xg for 15 minutes. The greenish pellet was discarded and the pink supernatant was brought up to 80% saturation with additional ammonium sulfate (285 $gm \cdot l^{-1}$).

After 30 minutes, this was centrifuged as above, the yellow supernatant was discarded and the pink pellet was resuspended in a minimal volume (15 ml) of extraction buffer.

3. This was chromatographed overnight on a 75x2.5 cm column of Sephadex G-100 (previously equilibrated with the same extraction buffer) at a flow rate of 18 $ml \cdot h^{-1}$. Fractions of 3 ml were collected and assayed for FBPase activity. Fractions high in activity chromatographed behind the void volume and prior to the pink phycoerythrin pigments.

4. These fractions were combined and loaded onto a 10x1.6 cm column of DEAE Sephacel equilibrated with the extraction buffer. A 0 to 0.3 M linear NaCl gradient was produced in 300 ml of extraction buffer and used to elute the protein from the column at a flow rate of 18 $ml \cdot h^{-1}$.

5. Fractions were assayed and those containing FBPase activity were combined and concentrated by ultrafiltration through an Amicon YM 30 membrane.

As mentioned above, preliminary studies on the FBPase activity in crude extracts from P. rufescens indicate the likelihood of several FBPases (Figure 34, 35). Each step in the isolation procedure was assayed at a pH near the optimum for the photosynthetic FBPase (pH 8.5), and at pH 6.5, where the photosynthetic enzyme is inactive (Zimmermann et al 1976; Schürmann and Wolosiuk 1978; Table VII). The purification procedure was developed to maximize the activity at pH 8.5 relative to that at pH 6.5 (the pH 8.5:pH 6.5 activity

ratio). Summary tables for each extraction are found in appendix A. Table X contains a relative summary of the extraction procedure and purification progress.

The acid precipitation (step 1) was used by Buchanan et al. (1971) and Schmidt (1981) to separate the photosynthetic FBPase from its activator, thioredoxin. Approximately 60% of the total protein came out of solution in this step (Table X). This protein contained half of the FBPase activity assayed at pH 8.5 and one quarter of the activity assayed at pH 6.5. In spite of the large loss of enzyme activity at pH 8.5, this step was included as it eliminates a large percentage of the nonphotosynthetic (pH 6.5) FBPase activity and as a volume reduction technique.

The ammonium sulfate fractionation (step 2) served two purposes. The 40% ammonium sulfate treatment removed a greenish compound (possibly a lipid) that bound tightly to Sephadex and thus interfered with the subsequent chromatography steps. This step also purified the enzyme by removing 80% of the remaining protein (Table X). FBPase activity in this lichen is inhibited by low concentrations of ammonium sulfate, making it difficult to measure the increase in specific activity for this step. Overnight dialysis of 1 ml of this fraction against 2 l of extraction buffer did not remove sufficient ammonium sulfate to reduce the inhibition. During one extraction (15) a small volume of the 40-80%

TABLE X. Summary of the Fraction B FBPase Purifications from the Muskoka population of *Peltigera rufescens*.

Step	Volume (ml)	Relative Total Protein	Relative FBPase Activity pH 8.5		Relative FBPase Activity pH 6.5		Relative Ratio of Specific Activity
			Total	Specific	Total	Specific	
CE	900	100	100	100	100	100	100
1	190	59±5	52±10	102±18	25±4	49±9	279±60
2	13	8±1	53	683	15	186	362
3	55	3±.5	19±3	754±166	3±1	106±24	1229±600
4	15	1±1	6±1	3963±886	1±.4	235±92	2877±960

Relative summary of extractions #11 to 20.

Ratio is $\frac{\text{total activity assayed at pH 8.5}}{\text{total activity assayed at pH 6.5}}$

See Appendix for individual summarizes.

STEPS: CE Crude extract

1 Acid precipitate

2 40-80% ammonium sulfate fraction

3 Sephadex G-100

4 DEAE Sephacel

ammonium sulfate fraction was desalted on a Sephadex G-25 column and the results indicate that no FBPase activity measured at pH 8.5 was lost in this step (Table X).

The Sephadex G-100 chromatography (Step 3) separated the remaining FBPase activity into two fractions (Figure 36). A large proportion of the total protein eluded in the void volume, while the majority of the activity assayed at pH 8.5 eluted behind the void volume. Fractions high in this activity were combined and further purified by ion exchange chromatography (step 4). The second FBPase peak was enriched in activity at pH 6.5. These fractions were usually discarded. For two extractions (20,21) these fractions where combined, designated as the Fraction A enzyme and characterized without any further purification.

The ion exchange chromatography (Step 4) of the fractions high in ph 8.5 activity also produced two peaks(Figure 37). The leading peak had a very high specific activity when assayed at pH 8.5, and no FBPase activity when assayed at pH 6.5. These fractions were pooled, concentrated and analysed as the Fraction B enzyme. The trailing peak, containing some activity at pH 6.5 was discarded.

The activity of the purified FBPase was not stable when stored at 5°C. The addition of bovine serum albumin to a concentration of 5% and freezing the enzyme in small aliquots at -15°C maintained high activity for several months.

FIGURE 36: The elution pattern of FBPase activity by a Sephadex G-100 column. Enzyme activity was assayed at pH 6.5 (□---□) and pH 8.5 (○-·-·-○). Protein concentrations (Δ——Δ) were calculated from the absorbance at 280 and 260nm using the equation:

$$\mu g \cdot ml^{-1} protein = 1.55A_{280nm} - 0.76A_{260nm}$$

(from Cornish-Bowden 1979)

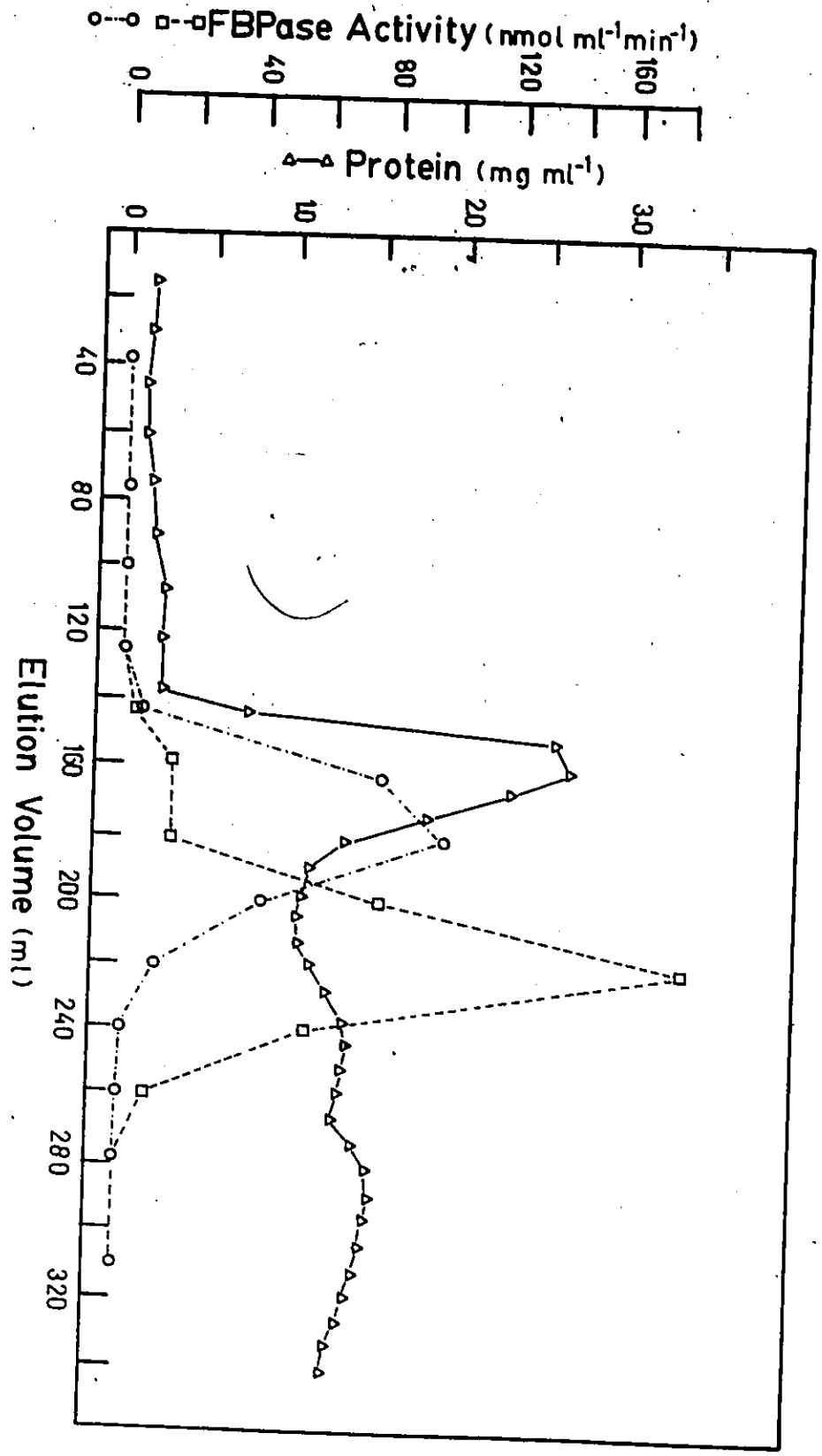
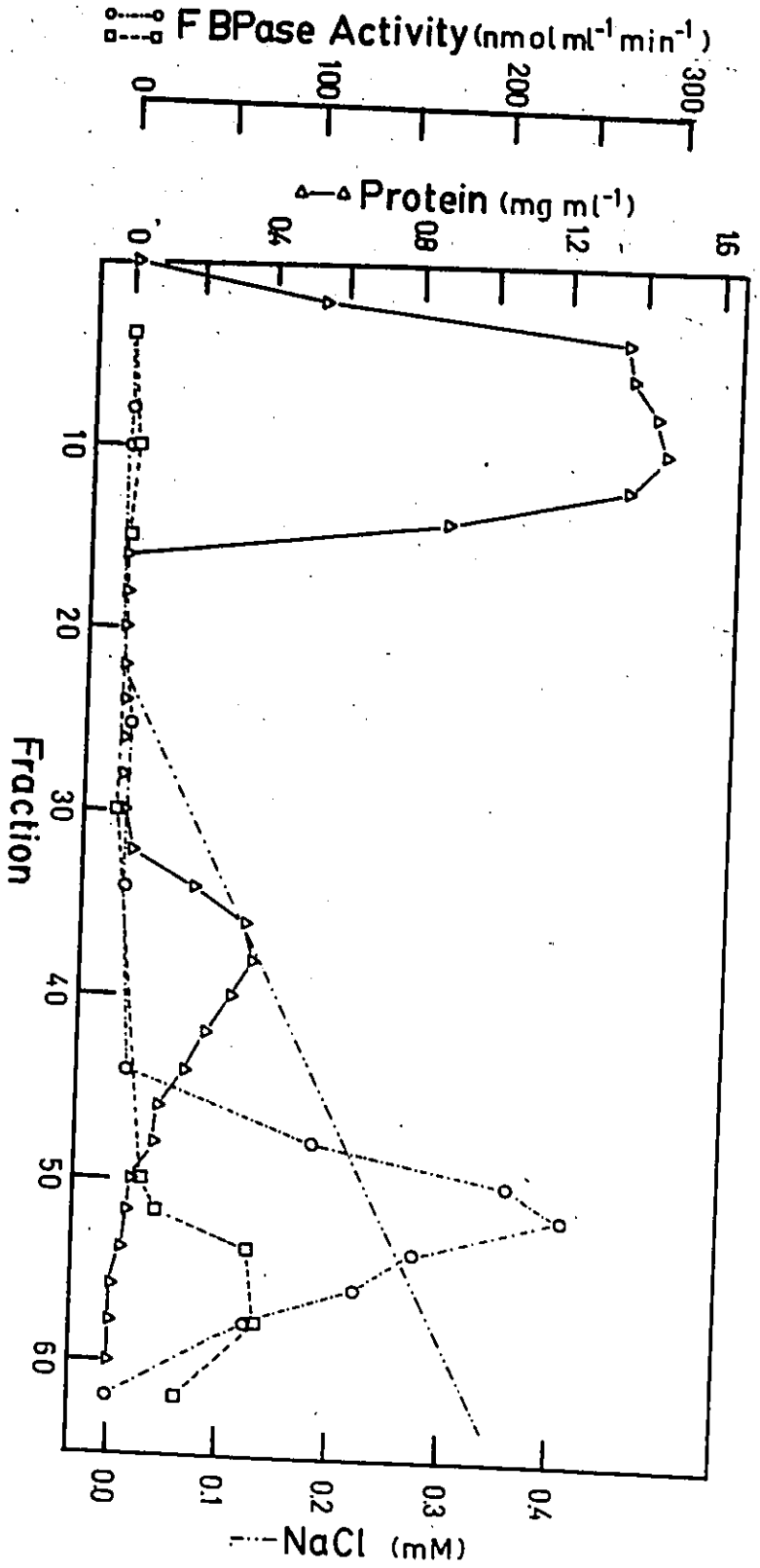

FBPase Activity (nmol ml⁻¹min⁻¹)
0
40
80
120
160
Protein (mg ml⁻¹)
0
10
20
30
Elution Volume (ml)
40
80
120
160
200
240
280
320

FIGURE 37: The pattern of FBPase activity eluted from a DEAE Sephacel column by a NaCl gradient (—·—·—·—). Enzyme activity was assayed at pH 6.5 (□ - - □) and pH 8.5 (○-·-·-○). Protein concentrations (Δ——Δ) were calculated as in Figure 36.


FBPase Activity (nmol ml⁻¹ min⁻¹)
0
100
200
300
Protein (mg ml⁻¹)
0
0.4
0.8
1.2
1.6
Fraction
10
20
30
40
50
60
0.0
0.1
0.2
0.3
0.4
NaCl (mM)

7.2.5 The protein assay

The protein content of each solution was determined using the Coomassie Blue binding assay from Biorad laboratories, which is a modification of the assay of Bradford (1976). For this assay, 3.5 ml of a 20% (v/v) solution of the Biorad dye concentrate was mixed with 100 µl of an appropriate dilution of the protein sample and the colour development was observed at 595 nm. This assay is linear for protein concentrations between 0.2 and 1.2 $mg \cdot ml^{-1}$. A standard curve using 5 dilutions of BSA was included with each assay, and the protein content reported is the mean of 3 to 6 assays for each sample.

7.3 Estimation of FBPase substrate affinity.

7.3.1 Estimation of the initial rates.

Calculation of the parameters of the Michaelis-Menten equation requires an accurate estimate of the enzyme velocity (v) at several known substrate concentrations (s). Three methods are primarily used to determine these initial rates: drawing a tangent to the progress curve at time 0, or calculating v from either a polynomial regression or an integrated form of the Michaelis-Menten equation which has been fitted to the progress curve. The tangent method is the most frequently used (Cornish-Bowden 1975), but is the least accurate, especially when the progress curve departs from linearity rather quickly (Nimmo and Atkins 1978).

The treatment of the progress curve as an integrated Michaelis-Menten equation provides the best estimate for the initial velocity (Cornish-Bowden 1975; Nimmo and Atkins 1978). However, neither of these methods could be used because they assume that the progress curve achieves maximal velocity at the beginning of the reaction. This was not the case in this study. A considerable lag period existed before maximal velocity was reached (Figure 33, 35), due to the slow activation of the photosynthetic FBPase.

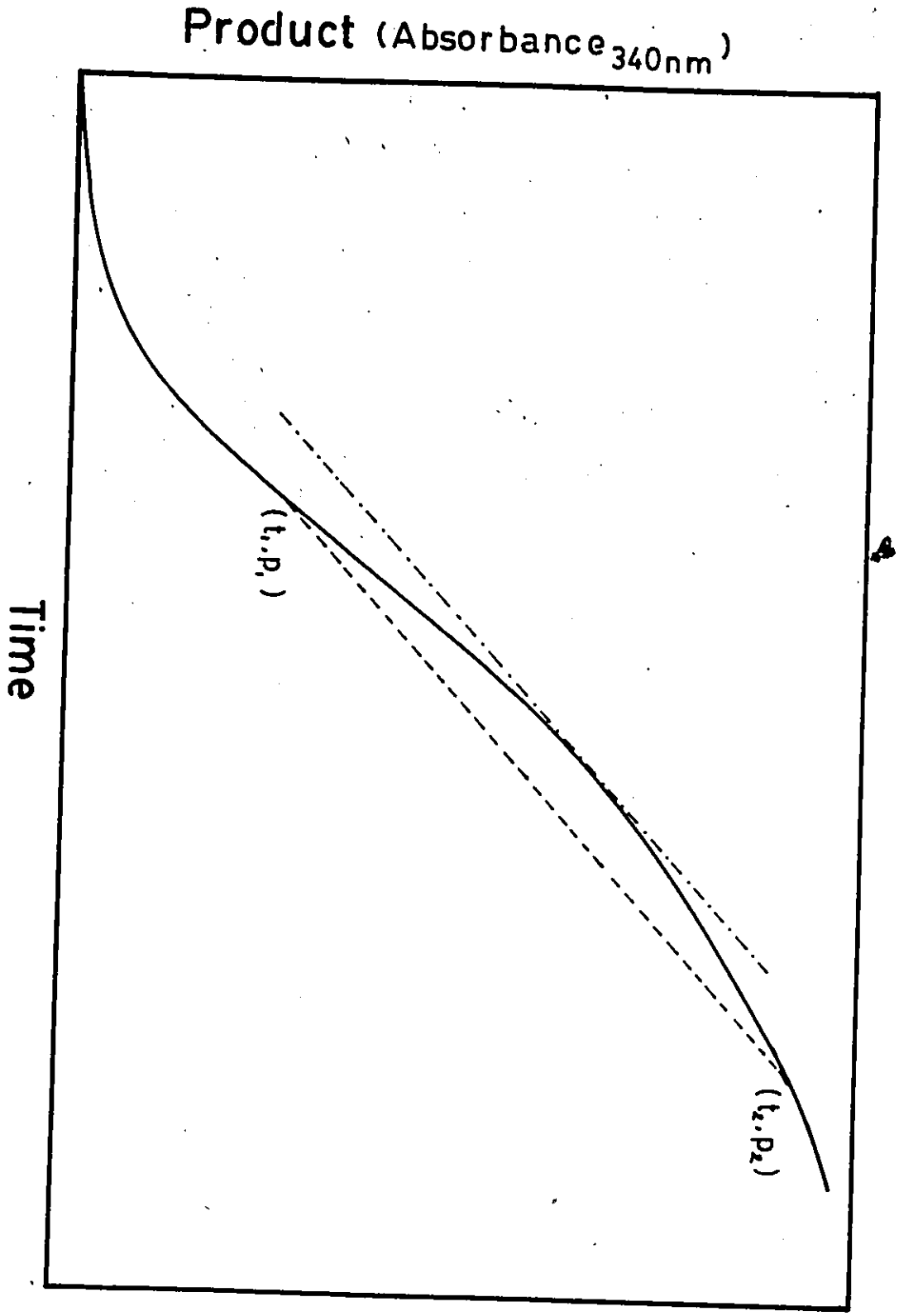
The chord method of Waley (1981) was the only technique found that could provide an estimate of both the enzyme velocity and substrate concentration in the middle of a progress curve. In this method, a line (chord) is drawn between two points in the progress curve (Figure 38). It is assumed that the reaction follows Michaelis-Menten kinetics between these points and an integral form of the Michaelis curve is used to estimate the substrate concentration corresponding to the velocity measured by the chord. For two data points (s_1, t_1) (s_2, t_2) the chord represents a velocity of:

$$(s_1 - s_2) / (t_1 - t_2) \qquad (9)$$

and this velocity corresponds to the tangent to the progress curve at a substrate concentration of:

$$(s_1 - s_2) / \ln(s_1 / s_2) \qquad (10)$$

FIGURE 38: The estimation of initial rates by the chord method of Waley (1981). The rate of reaction is calculated as the slope between two points along the progress curve (- - - -). A tangent is drawn parallel to this chord (-·-·-·-), and the substrate concentration which corresponds to this instantaneous rate is calculated from Eqn. (11). See appendix 2 (page 275) for examples of the application of this method to some representative raw data sets.


Product (Absorbance$_{340nm}$)
Time
(t_1, p_1)
(t_2, p_2)

calculated from the integrated Michaelis-Menten equation Waley 1981). The FBPase assay used measures the formation of the product (F6P), therefore the substrate concentration is estimated from equations (11);

$$s = (P_2-P_1)/\ln[(s_0-P_1)/(s_0-P_2)] \qquad (11)$$

where the product concentrations (P_1,P_2) are calculated from the absorbance at 340 nm using Eqn. (8) from section 7.1.2. This method provided an estimate for the velocity of the reaction and the concentration of substrate at which this velocity occurs. This estimate represents one (s,v) point for the calculation of the Michaelis parameters from the direct linear plot described in section 3.2.3.

The chord method also allows the experimenter to pick a section of the assay's progress curve where the enzyme is fully activated and thus its rate is maximal. This, however, does introduce a subjective input by the experimenter, but it cannot be avoided. An additional problem is that both the rate and substrate concentration are estimated values, and thus the errors involved in these estimates may result in larger errors in the subsequent calculations of K_m and V. The relative errors in the estimation of s can be reduced by drawing the chord as close to the beginning of the reaction as possible, decreasing the systematic errors that affect the calculation.

7.3.2 Estimation of the Michaelis parameters.

7.3.2.1 The linear transformations

The most recognized method for the estimation of K_m and V involves one of three linear transformations of the Michaelis-Menten equation: the double reciprocal plot (Lineweaver-Burk) (Eqn. 12), the Hanes plot (Eqn. 13), or the Hofstee plot (Eqn. 14), of which the Lineweaver-Burk plot is the most frequently used

$$1/v = 1/V + K_m/Vs \qquad (12)$$

$$s/v = K_m/V + s/V \qquad (13)$$

$$v = V - K_m v/s \qquad (14)$$

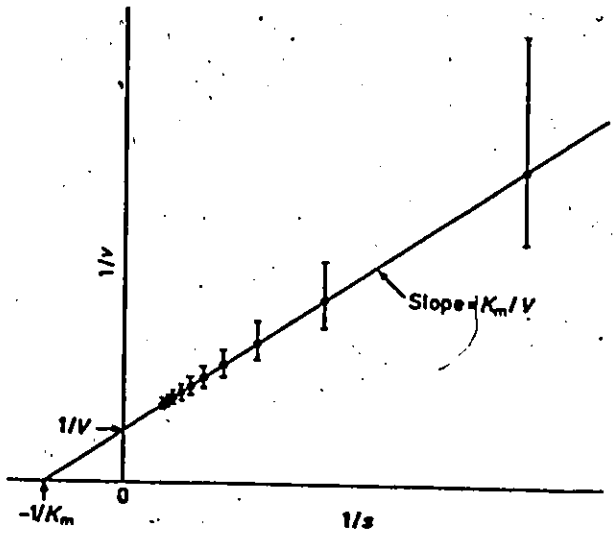
(Dowd and Riggs, 1965). More recently, the use of least square iteration estimates (Wilkinson 1961) and the direct linear plot (Eisenthal and Cornish-Bowden 1974) have increased in popularity. The estimates from each of these methods can vary considerably and are highly dependent on the type of errors in the data.

The linear transformations have several statistical problems associated with them. Estimates using either a least square regression or visual best line of fit depend on uniform variance in the Y-axis. This is not true for any of the transformations (Figure 39) (Cornish-Bowden 1979). The reciprocal 1/v used in the Lineweaver-Burk and Hanes plots places a large emphasis on the small velocity measurements,

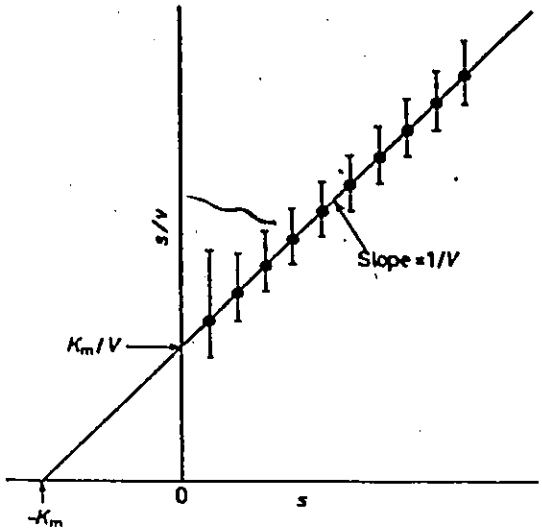
FIGURE 39: The (a) Lineweaver-Burk; (b) Hanes and (c) Hofstee transformations of the Michaelis-Menten equation. The error bars represent a uniform error in the velocity of ±0.05V.

(from Cornish-Bowden 1979)

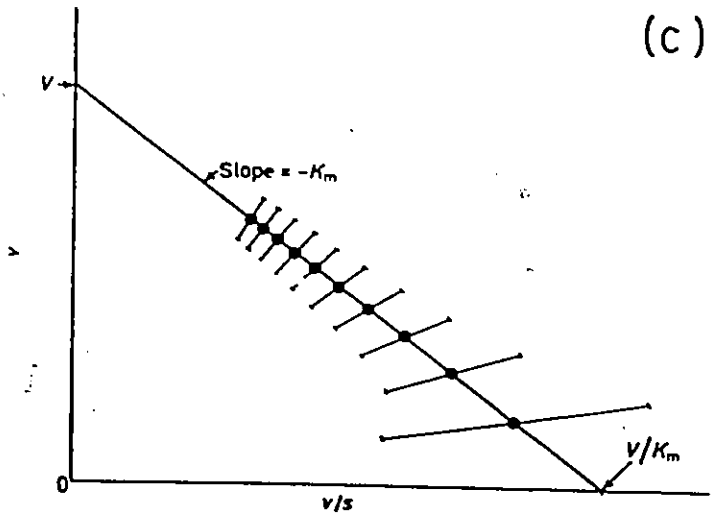
(a)



(b)



(c)



ones which are the most likely to experience a high % error (Dowd and Riggs 1965), while the appearance of an estimated parameter (v) on both sides of the Hofstee equation results in a built-in positive correlation between the axes, making a linear regression statistically invalid (Dowd and Riggs 1965). Tests of the three methods using computer simulated data have indicated how poorly the Lineweaver-Burk plot estimates the K_m, even when errors in the velocity measurements are very small (Dowd and Riggs, 1965; Atkins and Nimmo 1975). The Hanes and Hofstee plots seem to give comparable results but neither provide as good estimates of the Michaelis constants as the techniques of Wilkinson (1961) or Eisenthal and Cornish-Bowden (1974) when the data set contains outlier points (Dowd and Riggs 1965; Atkins and Nimmo 1975). (An outlier point is defined as a data point with a variance that is much larger than expected.)

7.3.2.2 The iterative procedure

The technique of Wilkinson (1961) uses an iterative procedure to calculate values for K_m and V that minimizes the sum of squares error between the observed data and predicted results. Provisional estimates for the Michaelis constants are calculated from a least squares regression of a properly weighted linear transformation. These provisional estimates are then refined by fitting the values to a bilinear regression of the Michaelis equation and its first derivative

(Wilkinson 1961). This method gives a better estimate for K_m and V_{max} than any of the above mentioned techniques when the variation in the velocity measurements are small (Cornish-Bowden and Eisenthal 1974; Atkins and Nimmo 1975; Currie 1982). However, when the variance tends to be larger, the direct linear plot is by far the superior method (Cornish-Bowden and Eisenthal 1974; Atkins and Nimmo 1975; Currie 1982).

7.3.2.3 The direct linear plot

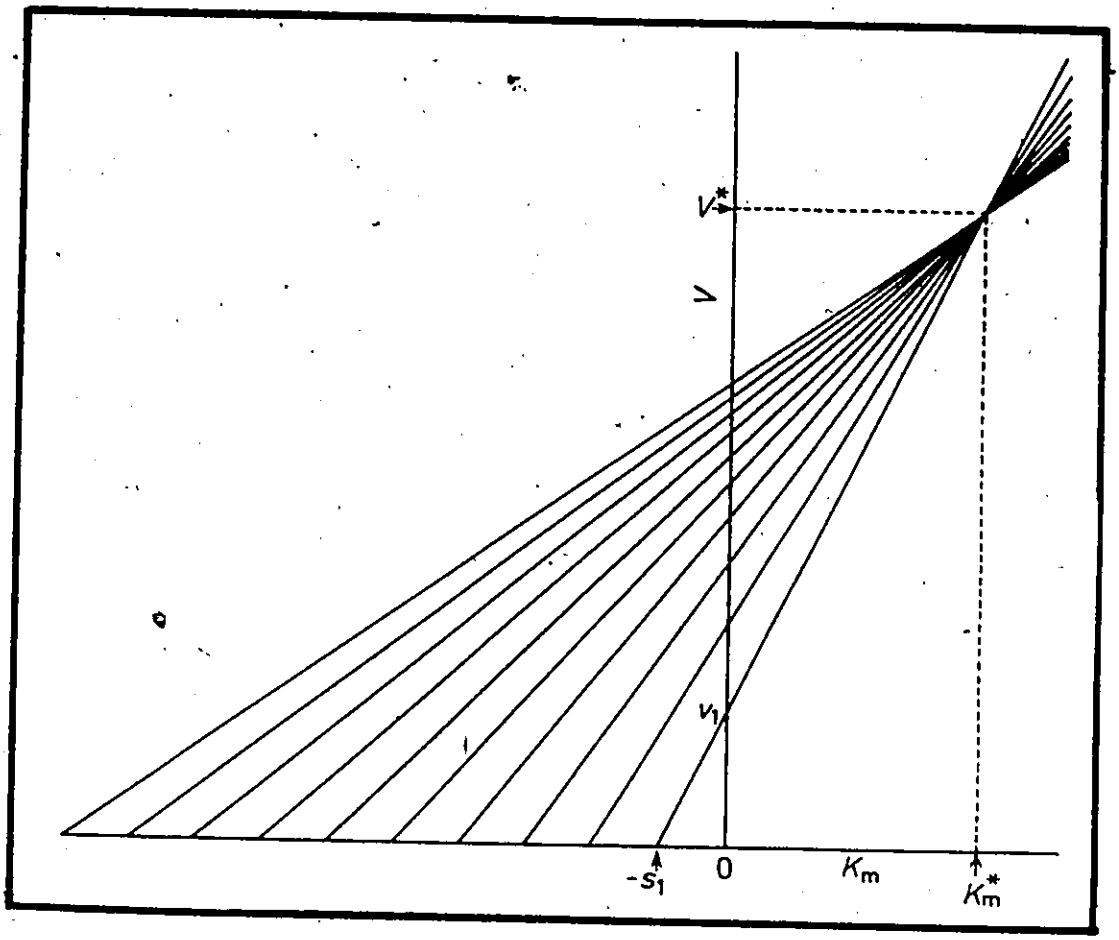
The direct liner plot is based on another transformation of the Michaelis-Menten curve (Eqn 15)(Eisenthal and Cornish-Bowden 1974). Each data point (s,v)

$$V = s + vK_m/s \qquad (15)$$

is used to construct a straight line on an X-Y axis of K_m and V with intercepts (-s,0) and (0,v)(Figure 40). This line represents all values of V and K_m which satisfy the Michaelis equation for the observed point. A second line is drawn from another data point and the intersection of these two lines represent the unique value of K_m and V which satisfy both points. The intercepts can be calculated directly from the data using equations 16 and 17 (Cornish-Bowden and Eisenthal 1974). For n observations, 1/2n(n-1) provisional estimates

FIGURE 40: Direct linear plot of V against K_m. Each line represents one observation (s,v), and is drawn with intercepts -s on the abscissa and v on the ordinate. The point of intersection gives the co-ordinates of the values of K_m and V (K_m and V on the diagram) that best represents the data.

(from Cornish-Bowden 1979)


V*
V
v1
-s1
0
Km
Km*

are then obtained for K_m and V. The median of these values is the best estimate of K_m and V for the data set (Cornish-

$$V = (s_1 - s_2) / (s_1/v_1 - s_2/v_2) \quad (16)$$

$$K_m = (v_2 - v_1) / (v_1/s_1 - v_2/s_2) \quad (17)$$

Bowden and Eisenthal 1974).

The advantage of this technique over any other is that, since it uses the median intercept for the estimates of V and K_m, it is relatively insensitive to outlier points. (A few bad intercepts will not affect the median value by much.) The linear transformations produce estimates that are extremely sensitive to outlier points at low substrate concentrations (Dowd and Riggs 1965) and this results in biased estimates for K_m and V. The iterative procedure is considerably less sensitive to outliers (Atkins and Nimmo 1975) and performs as well as the direct linear plot when the outliers are in the middle concentration ranges. However, when the errors are at the extremes of concentration, (where they are more likely to occur), the direct linear plot provides the best estimates of the Michaelis constants (Cornish-Bowden and Eisenthal 1974; Atkins and Nimmo 1975). Therefore this method was chosen for this study.

A later refinement to the direct linear plot was included to minimize the effects of errors in the estimates of substrate concentration and to reduce the problem of

replicate observations (Cornish-Bowden and Eisenthal 1978). Replicate observations at the same substrate concentration produce an intercept on the K_m (X) axis, thus providing an incorrect estimate of K_m=s and V=0. In this case, parameters predicted from replicate observations at the same substrate concentration were eliminated from the data set before the median value was determined. This problem did not arise frequently in this study, as the calculation of s from the individual progress curves using the chord method (Eqn. 10, Section 7.3.1) is unlikely to result in identical values, even from assays with the same initial substrate concentration. However, a more serious problem is introduced, as the data will include several points with nearly similar substrate concentrations. It is possible that the errors involved in estimating velocity and concentration are larger than the actual difference between them. This may result in the incorrect ranking of the data points, producing a negative estimate for both K_m and V (Figure 41). Too many of these points would result in a median that underestimates the Michaelis parameters. This can be corrected by treating these incorrect estimates as infinitely large instead of negative values (Cornish-Bowden and Eisenthal 1978). This "fudging" of the median value initially seems inappropriate, but makes sense once the basis behind the type of error that leads to an intercept in the third quadrant of the plot is understood (Figure 41). These errors can occur when the

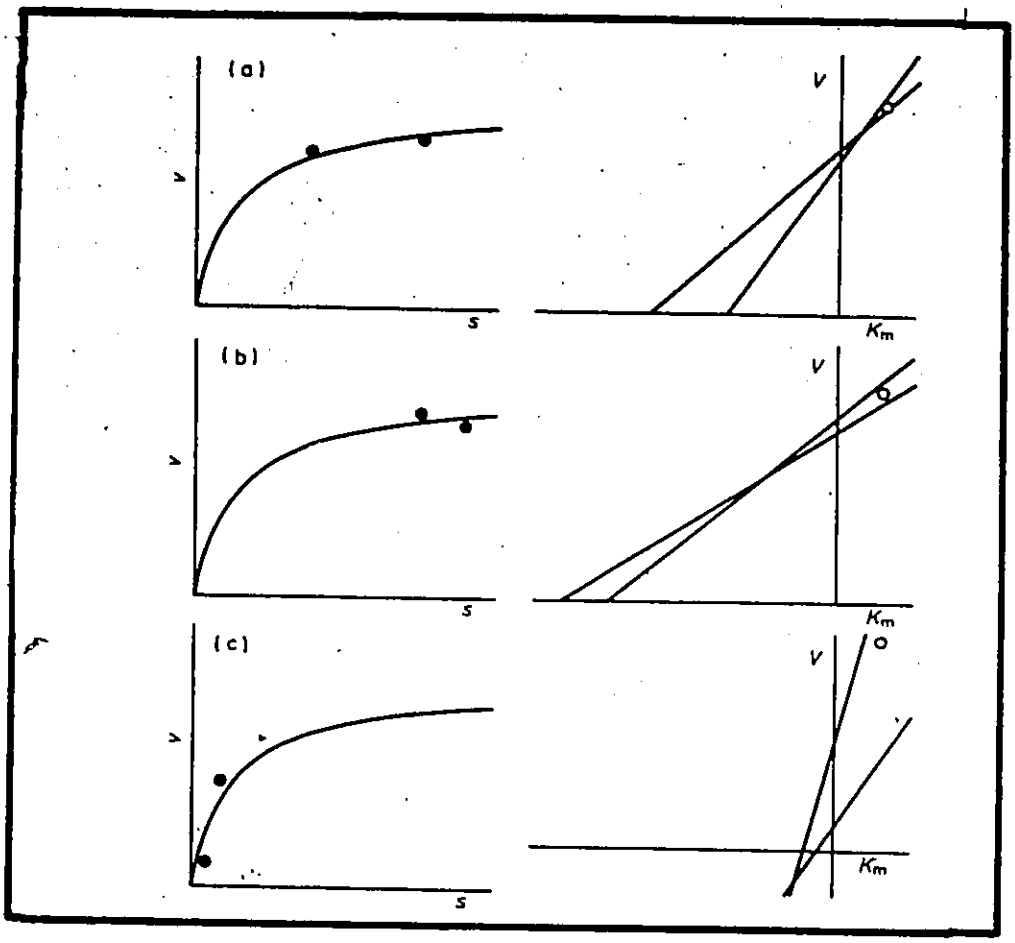
FIGURE 41: An explanation of why intersections may occur in the second or third quadrants of the direct linear plot. The diagrams on the left shows two experimental points on a Michaelis plot of v against s. The diagrams on the right present the the corresponding direct linear plot, with an open circle (o) to indicate the values of K_m and V used in calculating the curves shown on the left.

(a) The 'normal' case, with an intersection in the first quadrant.

(b) Intersections in the second quadrant occur when both s values are large compared with K_m, and both v values are similar in magnitude to V, but are incorrectly ranked.

(c) Intersections in the third quadrant occur when one (and often both) of the s values is small compared with K_m, and at least one of the v values is small compared to V.

(from Cornish-Bowden 1979)


(a)
v
s
V
Km
(b)
v
s
V
Km
(c)
v
s
V
Km

substrate concentration tested is much smaller than the enzyme's true K_m, and thus the velocity is much smaller than V. Incorrect ranking of these points due to experimental inaccuracies at measuring v and s could cause an intercept predicting negative values of K_m and V, but the actual values of K_m and V are extremely large compared to the observed s and v, and should be treated accordingly (Cornish-Bowden 1979).

7.3.3 Experimental design for estimating the Michaelis constants

The direct linear plot is most effective when the substrate concentrations follow a geometric sequence between 1/3 K_m and 2 K_m (Currie 1982). The literature reports a K_m of 0.03 to 0.20 mM FBP for the photosynthetic enzyme (Table VII). Therefore six substrate concentrations, in a 50% dilution series from 0.313 to 0.010 mM FBP, were used (0.313, 0.157, 0.0778, 0.039, 0.020, 0.010 mM). The Varian DMS-100 spectrophotometer is equipped with a cell changer that will contain 5 cuvettes. Five simultaneous assays were run covering either the 0.010 to 0.157 mM FBP or the 0.020 to 0.313 mM FBP ranges. Three to five sets of assays (covering both ranges) were run at each temperature for each extraction. The chord method (Eqn. 9, 10) was used to calculate the v and s points for each of the assays (15 to 25 points) and estimates for $S_{o.5}$ and V were calculated from these using the modified direct linear plot (Eqn. 16, 17).

The degree of activation of the FBPase could be dependant upon temperature and FBP concentration (Corley and Wolosiuk 1985). As this may affect the measured activity, the Michaelis constant (K_m) cannot be calculated in theory. Therefore $S_{0.5}$ is used to describe the half-saturation concentration.

7.4 Estimation of E_a.

Activation energy was calculated from the Arrhenius plot of log v against 1/T (in degrees kelvin). The slope of this line, calculated from a least square linear regression, is $-E_a/2.303*R$, where R is the gas constant (8.83 $J \cdot mol^{-1}K^{-1}$) (Cornish-Bowden 1979). The E_a was calculated over the temperature intervals from 5 to 45°C using the data collected for the substrate affinity curves. Two activation energies were calculated; one for the V values predicted from the direct linear plot, representing the E_a of the substrate saturated enzyme; and one from the observed velocities at 0.020 mM FBP, representing a more realistic *in vivo* substrate concentration.

7.5 *In vitro* thermal stress

The stability of the Fraction B FBPase to *in vitro* extremes in temperature was tested in the purified extracts. Aliquots of the FBPase were subjected to several freeze-thaw cycles (0,1,2,3,4,5,6,10 and 12), and then the remaining

activity was assayed at 15 and 35°C. Other samples were heated for varying lengths of time at 50°C, and then the activity was assayed at 15 and 35°C.

7.6 Plant material and storage conditions

The Muskoka population of Peltigera rufescens was collected from rock outcrops in the Port Severn area of Ontario during the winter and summer of 1985, transported back to McMaster University and stored in an air dry state in controlled environmental growth chambers. The collection dates and gas exchange rates measured at 35°C and 800 $\mu E \cdot m^{-1} s^{-1}$ are reported in Table XI. Material collected from December to April was stored under winter conditions of -2/5°C night/day with a nine hour photoperiod, while those collected from May to August were stored under summer conditions of 25/32°C with a daylength of 15 hours (Brown and Kershaw 1984). The lichen was allowed to equilibrate to these storage conditions for one week prior to any experimentation (Section 3.4). Extractions 11 to 16 were from the winter collections of P. rufescens, while extractions 17 to 20 were from material collected in the summer.

7.7 Laboratory induced acclimation

The time course of temperature acclimation was followed in a collection of P. rufescens made during September, 1985. Lichen thalli stored air dry under summer

Table XI: The collection dates and CO_2 exchange rates for the Muskoka population used in the seasonal studies of the Fraction B FBPase

Extraction	Date of Collection	Net Photosynthetic rate*
11	November 7, 1984	ND
12	November 7, 1984	3.6±0.58
13	January 15, 1985	ND
14	February 26, 1985	1.9±
15	March 21, 1985	3.7±.3
16	March 21. 1985	3.7±.29
17	April 17, 1985	9.2±.4
18	May 14, 1985	6.4±.3
19	May 14, 1985	ND
20	July 18, 1985	9.9±.7

* The net photosynthetic rate (mean ±SE in $mg \cdot CO_2 gm^{-1} h^{-1}$) for the optimum moisture class measured at a light intensity of 800 $\mu E \cdot m^{-2} s^{-1}$ and a temperature of 35°C. ND indicates that no measurement was taken for that extraction. Extractions 11-16 were classified as winter and 17-20 were classified as summer.

conditions of 25/32°C night/day with a 15 hour photoperiod were moved to winter conditions of -2/1°C and a 9 hour photoperiod. This treatment had previously been shown to induce a winter pattern of photosynthetic response (Section 4.3). Following an overnight soak in distilled water under the same storage conditions, the enzymes were extracted by grinding 1 gm of lichen in 15 ml of extraction buffer and 0.7 gm of PVPP according to the method in section 7.2.3. The substrate affinity of FBPase was measured at 35°C in the crude extracts at a pH of 8.5.

The changes in the P-I curves during the acclimation process were measured with a Beckman 865 infra-red gas analyzer at 35°C using the discrete sampling method of Larson and Kershaw (1975a), as described in Section 3.6. Apparent quantum efficiency was calculated from a least square linear regression of the gas exchange values from 0 to 100 $\mu E \cdot m^{-2} s^{-1}$. Light saturated rates of net photosynthesis were assayed at 800 $\mu E \cdot m^{-2} s^{-1}$.

8. FBPase RESULTS

8.1 Characterization of the Fraction A FBPase

The Fraction A enzyme, isolated from extraction #20, had a specific activity of 0.43 $\mu mol \cdot mg^{-1} min^{-1}$ (Table A10). This represented a 5-fold purification over the activity in the crude extract and was obtained with a yield of only 3%.

This enzyme had a pH optimum at pH 6.0. Its catalytic activity declined rapidly with increasing alkalinity. Very little activity (<0.04 $\mu mol \cdot mg^{-1} min^{-1}$) observed above a pH of 7.5 (Figure 34).

The activity of this enzyme was independent of $MgCl_2$ concentration in the assay between 0.0 and 10.0 mM (Figure 42), and had a relatively low substrate affinity, with a K_m of 0.228±0.028 mM FBP (Figure 43). 2.0 mM AMP caused a 60% reduction in the catalytic activity of the enzyme, while a AMP concentration of 0.2 mM caused a slight inhibition of 20% (Table XII).

Figure 44 is a progress curve of enzyme reaction over the initial 35 minutes of an assay. The activity of this enzyme is constant with time and there are no lag effects, a characteristic of enzyme's that can be activated by DTT.

The activity of the Fraction A enzyme had a biphasic response to temperature. The Arrhenius plots of the log of enzyme activity against the inverse of temperature had a

FIGURE 42: The effect of $MgCl_2$ concentration on the activity of the purified Fraction A (□, assayed at pH 6.5) and Fraction B (○, assayed at pH 8.5) enzymes isolated from the Muskoka population of *P. rufescens*.

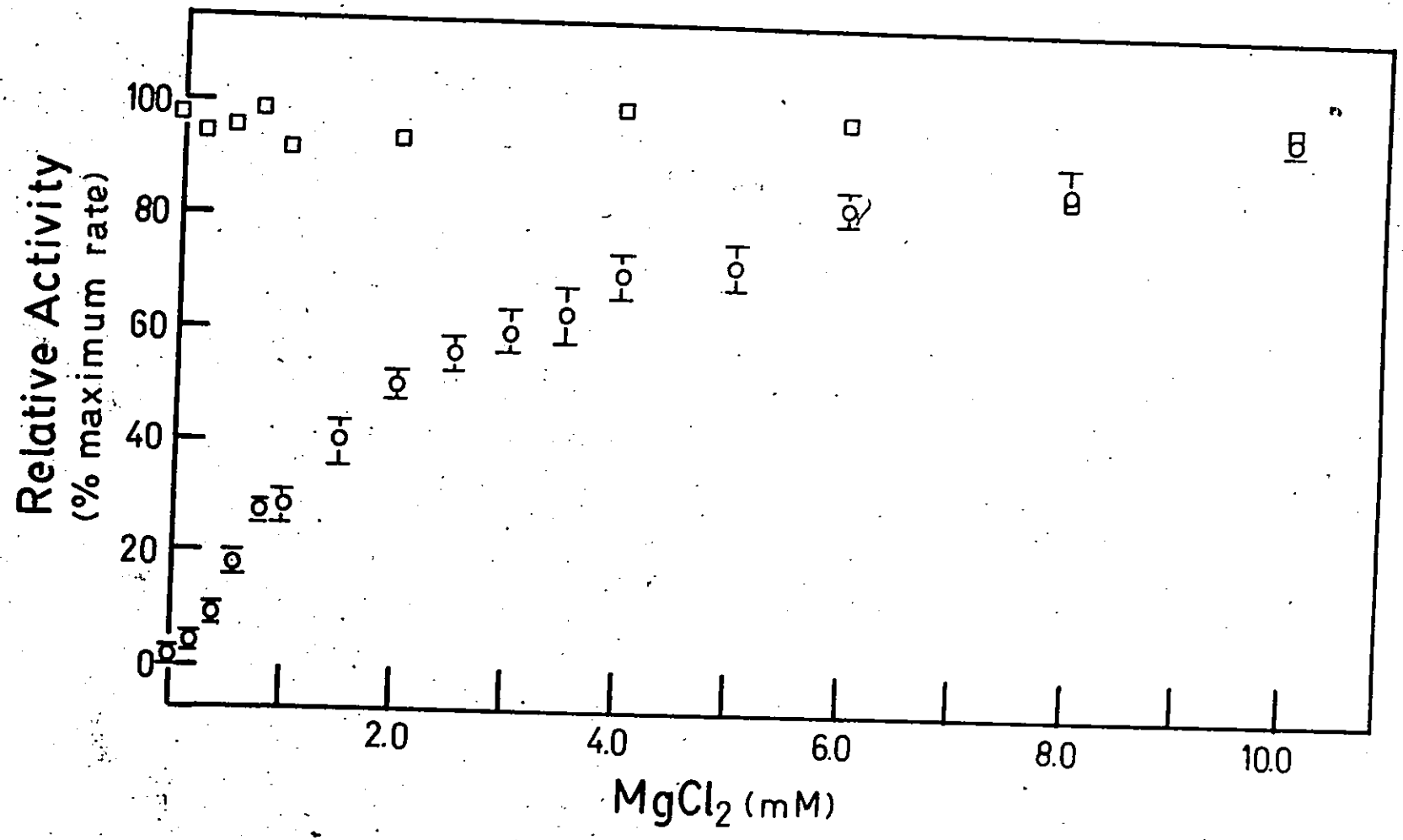

Relative Activity
(% maximum rate)
100
80
60
40
20
0
2.0
4.0
6.0
8.0
10.0
MgCl2 (mM)

FIGURE 43: The effect of FBP concentration on the activity of the purified Fraction A (□ - - □, assayed at pH 6.5) and Fraction B (○ - - ○, assayed at pH 8.5) enzymes isolated from the Muskoka population of P. rufescens.

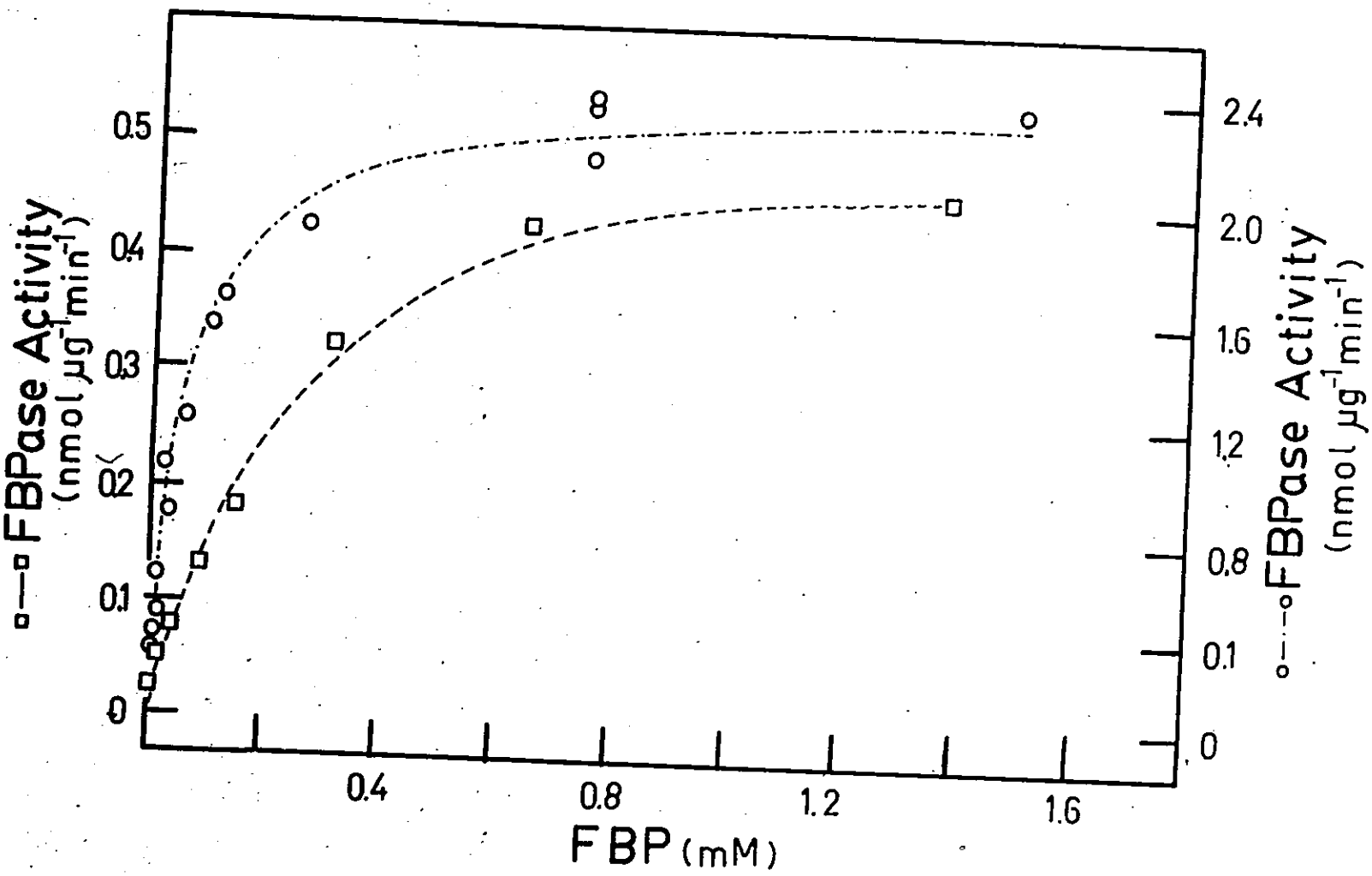
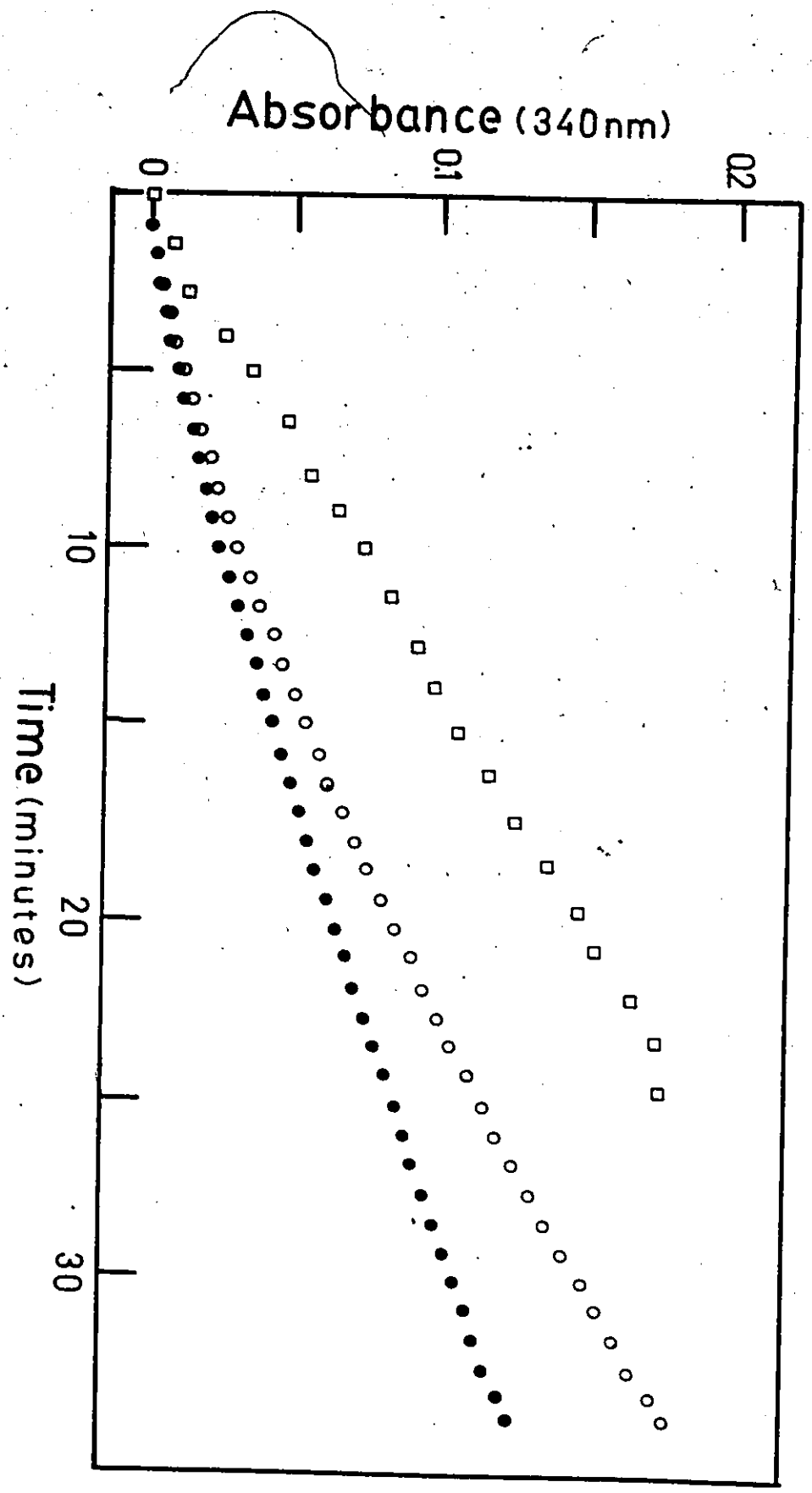

8
0.5
0.4
0.3
0.2
0.1
0
2.4
2.0
1.6
1.2
0.8
0.1
0
0.4
0.8
1.2
1.6
FBP (mM)
FBPase Activity (nmol μg⁻¹min⁻¹)
FBPase Activity (nmol μg⁻¹min⁻¹)

TABLE XII: The effect of AMP on the activity of the FBPases purified from the Muskoka population of P. rufescens.

Fraction	Concentration of AMP (mM)	% inhibition mean±SE
A	0.2	20
	2.0	61±1
B	0.2	56±7
	2.0	71±2.6

FIGURE 44: The effect of DTT concentration on the FBPase activity. The change in absorbance with time was monitored at a FBP concentration of 0.313 mM in an assay containing either 0.0 mM DTT (closed) or 20 mM DTT (open) at pH 6.5 (□ ,Fraction A) or pH 8.5 (○ . ● ,Fraction B).


Absorbance (340nm)
0
0.1
0.2
Time (minutes)
10
20
30

discontinuity at 25°C (Figure 45). The E_a below this temperature was 122 $kJ \cdot mol^{-1}$, while catalysis at the higher temperatures is more energetically efficient, with an E_a of 72.3 $kJ \cdot mol^{-1}$.

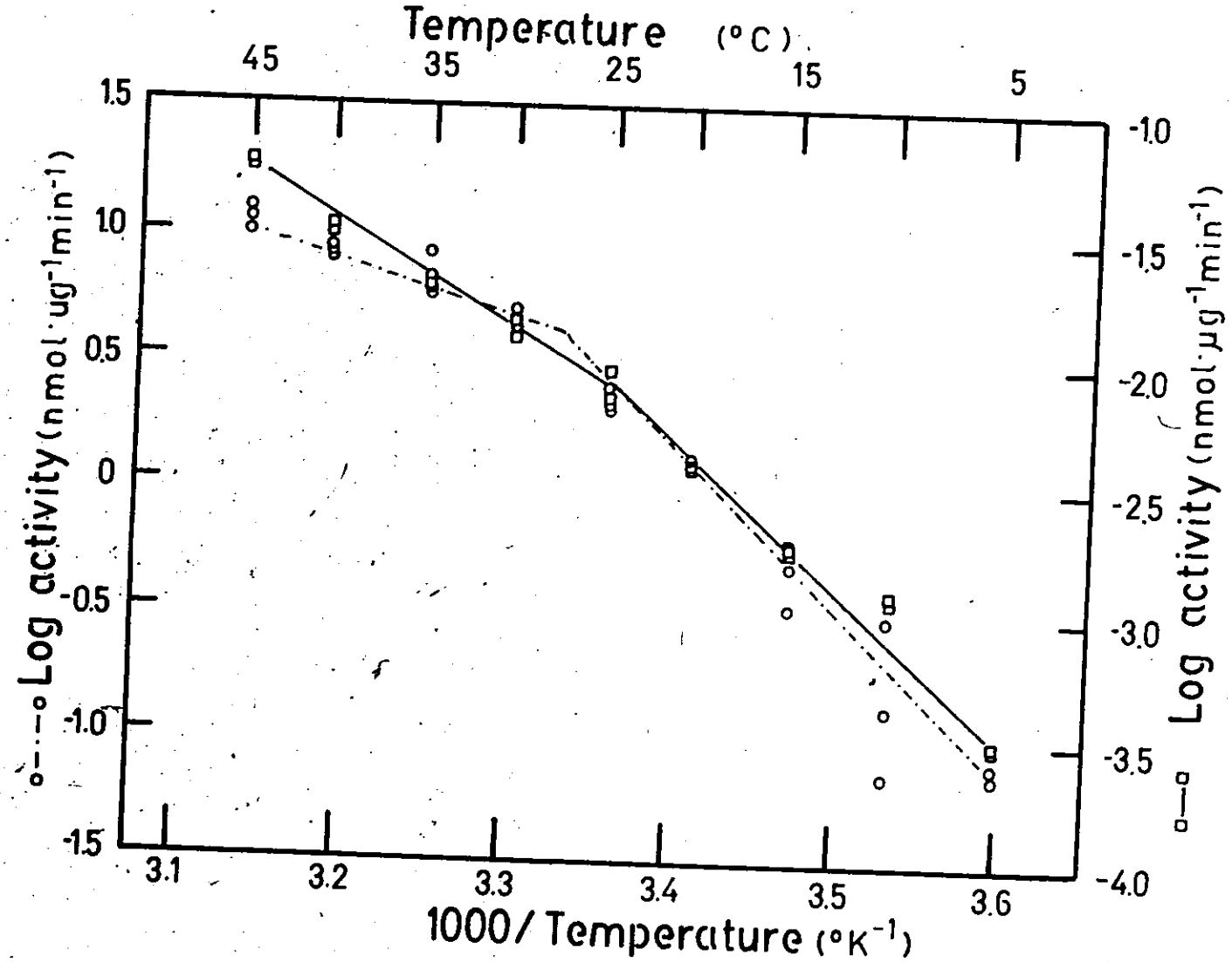
8.2 Characterization of the Fraction B FBPase

The Fraction B enzyme was obtained from extracts 12 through 20 at a much higher purification than the Fraction A enzyme. Specific activity for the purified enzyme ranged from a low of 0.441 $\mu mpl \cdot mg^{-1} min^{-1}$ in extract # 13 to a high of 9.5 $\mu mol \cdot mg^{-1} min^{-1}$ in extract 17 (Table A1-A10). This was similar to the specific activities obtained in studies of cyanobacteria by Schmidt (1981, 0.88 $\mu mol \cdot mg^{-1} min^{-1}$), Bishop (1979, 2.81 $\mu mol \cdot mg^{-1} min^{-1}$) and Udvardy et al. (1982, 8.1 $\mu mol \cdot mg^{-1} min^{-1}$), but lower than the 37.4 $\mu mol \cdot mg^{-1} min^{-1}$ obtained by Gerbling et al. (1985). The average purification of the Fraction B enzyme was 40-fold, with a recovery of 6% of the activity in the crude extract assayed at pH 8.5 (Table X).

The Fraction B enzyme differed significantly from the Fraction A FBPase in all of the above mentioned characteristics except the activation energy. It was active only at alkaline pH, with no activity observed below pH 7.5. The optimum pH for activity is 9.0 (Figure 34).

This enzyme was totally dependent on Mg^{2+} for activity (Figure 42), and exhibited a sigmoidal response to

FIGURE 45: The Arrhenius plots for the Fraction A (□——□) and Fraction B (○-·-○) FBPases isolated from the Muskoka population of P. rufescens.


Temperature (°C)
45
35
25
15
5
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
Log activity (nmol·ug⁻¹min⁻¹)
Log activity (nmol·µg⁻¹min⁻¹)
3.1
3.2
3.3
3.4
3.5
3.6
1000/ Temperature (°K⁻¹)

this cofactor, with a half-saturated activity ($S_{0.5}$) occurring at 2.0±0.15 mM $MgCl_2$, and near maximal activity at 10 mM. The enzyme activity responds hyperbolically to changes in substrate concentration, with a $S_{0.5}$ of 0.039±0.009 mM FBP at 25°C (Figure 43).

The Fraction B enzyme is more sensitive to AMP inhibition than Fraction A (Table XII). An AMP concentration of 0.2 mM resulted in a 56% inhibition of enzyme activity. Fr-2,6-P has been recently shown to act as a competitive inhibitor of the cytosolic FBPase (Cséke et al. 1982; Herzog et al. 1984; Pharr and Huber 1984). Table XIII shows the effect of 2 µM Fr-2,6-P on the activity of the Fraction B enzyme. There is no significant inhibition of enzyme activity at any of three FBP concentrations tested (0.03, 0.3, 3.0 mM). However, there was a slight synergistic effect between Fr-2,6-P and AMP for two of the four extracts tested (Table XIII).

The activity of the Fraction B FBPase is also affected by DTT. Enzyme assays show a considerable lag period, and maximal rates are not observed until 15 to 20 minutes into the assay (Figure 33, 44). This lag could not be eliminated by a 24 hour preincubation with 20 mM DTT (Figure 33)(Zimmermann et al. 1976). However, the omission of DTT from the assay reduces the maximum velocity of the enzyme by 3-fold (Table XIV, Figure 44) but had no effect on the $S_{0.5}$ for FBP or the length of the lag period.

TABLE XIII: The inhibitory effects of 2 μM Fr-2,6-P and 0.2 mM AMP on the activity of the Fraction B FBPase.

Extraction	Inhibitor	FBP concentration		
		3.0 mM	0.3 mM	0.03mM
16	Control	2.98±0.21	4.21±0.11	2.18±0.03
	AMP	2.86±0.21		
	F-2,6-P	2.94±0.19	3.93±0.19	2.02±0.03 *
	F-2,6-P+AMP	2.26±0.01		
17	Control	8.30±0.36	8.57±0.12	5.68±0.22
	AMP	2.86±0.27		
	F-2,6-P	7.43±0.33	7.76±0.19	6.32±0.21
	F-2,6-P+AMP	1.75±0.14 **		
18	Control	3.62±0.09		
	AMP	3.35±0.18		
	F-2,6-P	3.22±0.21		
	F-2,6-P+AMP	3.20±0.07		
20	Control	2.80±0.05		
	AMP	1.04±0.04		
	F-2,6-P	2.63±0.04 *		
	F-2,6-P+AMP	0.73±0.02 **		

* significantly less than control. P>0.05 (t-test)

** Significantly less than inhibition by AMP alone. P>0.05

Table XIV: The effects of DTT on the Michaelis-Menten parameters of the Fraction B FBPase from three purifications.

Extraction	Parameter	Assay condition		t-test
		20mM DTT	no DTT	
		mean±SE	mean±SE	
17	$S_{0.5}$(mM)	0.043±0.005	0.045±0.004	0.299
	Vmax	10.28±0.82	2.78±0.26	8.68 ***
	n	5	5	
18	$S_{0.5}$	0.058±0.006	0.045±0.009	1.6
	Vmax	5.21±0.49	2.29±0.17	5.67 ***
	n	4	4	
20	$S_{0.5}$	0.033±0.005	0.049±0.003	2.54 *
	Vmax	2.39±0.13	0.93±0.14	7.79 ***
	n	5	4	

* P>0.05

*** P>0.01

As mentioned above, there was no difference between the Arrhenius plots of the Fraction A and B enzyme. The E_a measured between temperatures of 5 to 25°C for the Fraction B enzyme was 103.9 $kJ \cdot mol^{-1}$, while the E_a for the higher temperature interval is 77.4 $kJ \cdot mol^{-1}$.

The nonlinearity of the Arrhenius plot (Figure 45) is contradictory to the theories on the temperature dependence of enzymes, as this plot should be linear throughout the entire temperature range (Cornish-Bowden 1979). A temperature dependence of the lag period could affect the shape of the Arrhenius plot. Corley and Wolosiuk (1985) has observed such a temperature dependence on the activation of the photosynthetic FBPase. However, it is unlikely that the nonlinearity observed in this study is the result a of a temperature effect on the activation of the Fraction B enzyme, as the same pattern was observed for the Fraction A enzyme, which demonstrated no lag or activation effects. (Figure 33, 45). Nonlinear Arrhenius plots have also been reported for the non-photosynthetic FBPase, malate dehydrogenase, glycolate oxidase, glutamate oxaloacetate transaminase and PEP carboxylase (Behrisch 1969; McNaughton 1972; Simon 1979c; Davidson and Simon 1981, 1983; Simon et al. 1984a,b). The study of Corley and Wolosiuk (1985) showed a linear Arrhenius plot for the photosynthetic FBPase between 0 and 30°C, with an E_a of 60 $kJ \cdot mol^{-1}$. The plots for the Fraction A and B FBPase could also be considered linear

within this temperature range (Figure 45), however the E_a is considerably higher.

8.3 Seasonal changes in the Fraction B FBPase

The Fraction B enzyme appeared similar, in many respects, to the photosynthetic FBPase isolated from other cyanobacteria and spinach (Table VII). Therefore, a detailed study of the difference between this enzyme isolated from summer (extractions 17 to 20) and winter (extractions 11-16) collections of P. rufescens was undertaken to determine if changes in this enzyme paralleled the observed seasonal change in net photosynthesis.

8.3.1 Seasonal effects on total FBPase activity

There was no seasonal difference in the total amount of protein that could be extracted (Table XV). In addition, the total FBPase activity assayed in the crude extract at pH 8.5 or pH 6.5 did not change on a seasonal basis (Table XV).

8.3.2 The seasonal $S_{0.5}$-temperature relationship

The substrate affinity of the Fraction B FBPase showed a strong interaction with temperature and season (Figure 46). The enzyme extracted from the winter collections had a minimum $S_{0.5}$ of 0.028 mM FBP at 5°C. Substrate affinity then decreased as the temperature increased, reaching a maximum $S_{0.5}$

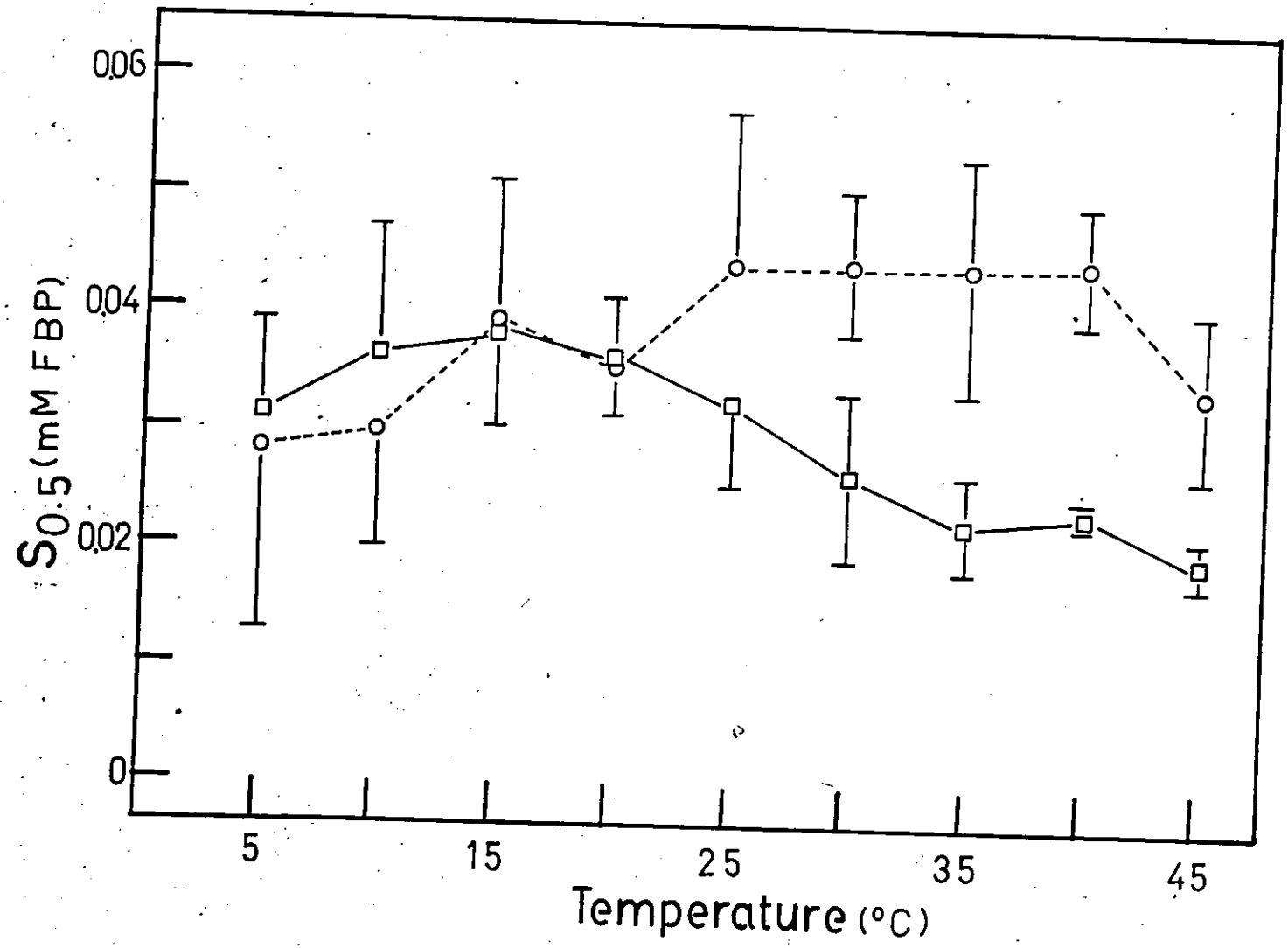
Table XV: Seasonal effects on the the amount of extractable protein and total FBPase activity from the Muskoka population of *P. rufescens*.

	Summer			Winter			
	mean	SE	n	mean	SE	n	t-test
Protein content[1]	17.62	1.9	4	15.97	1.25	4	0.73
FBPase activity[2]							
pH 8.5	1.42	0.43	3	1.44	0.33	4	0.003
pH 6.5	1.04	0.12	3	1.18	0.19	5	0.54

1 Total protein extracted, mg protein·gm dry wt of thalli^{-1}

2 Total enzyme activity, µmol F6P·gm dry wt of thalli^{-1}·min^{-1} at 25°C.

FIGURE 46: The relationship between substrate affinity (mean±SE) and temperature for FBPase isolated from summer (□——□, n=4) and winter (O - - -O, n=6) collections of the Muskoka population of *P. rufescens*.


0,06
0,04
0,02
0
$S_{0.5}$ (mM FBP)
5
15
25
35
45
Temperature (°C)

of 0.044 mM at 40°C. The summer material had similar affinities for FBP at assay temperatures less than 25°C. However, at temperatures above this, the $S_{0.5}$ decreased, reaching a minimum $S_{0.5}$ of 0.019 mM at 45°C. This results in a 2-fold difference in substrate affinity between the summer and winter forms of this enzyme at temperatures of 30°C and above. Figure 47 shows the plot of enzyme activity against substrate concentration for a $S_{0.5}$ of 0.020 and 0.045 mM, FBP, values characteristic of the summer and winter enzyme at the higher assay temperatures. It is apparent that this difference in affinity can result in a substantially higher rate of reaction in the summer form of this enzyme.

8.3.3 The seasonal changes in E_a

There was also a change in the activation energy of the Fraction B FBPase on a seasonal basis. The relationship between the activity of the enzyme and temperature is complex. The Arrhenius plots are nonlinear, with a distinct break occurring near 25°C (Figure 45). When calculated from Vmax (representing substrate-saturated rates), the E_a in the temperature range of 5 to 20°C was significantly lower in the summer collection than the winter collection (93.3 compared with 130 $kJ \cdot mol^{-1}$) (Table XVI). No significant differences were observed between the summer and winter for the E_a in the 30 to 45°C range. However, when the E_a was calculated from the activity measured at 0.020 mM FBP the pattern was

FIGURE 47: A comparison of the Michaelis-Menten substrate saturation curve for an enzyme with $S_{0.5}$=0.020 (——) and $S_{0.5}$= 0.045 (- - - -), which are comparable to the summer and winter forms of the Muskoka Fraction B FBPase at 35°C.

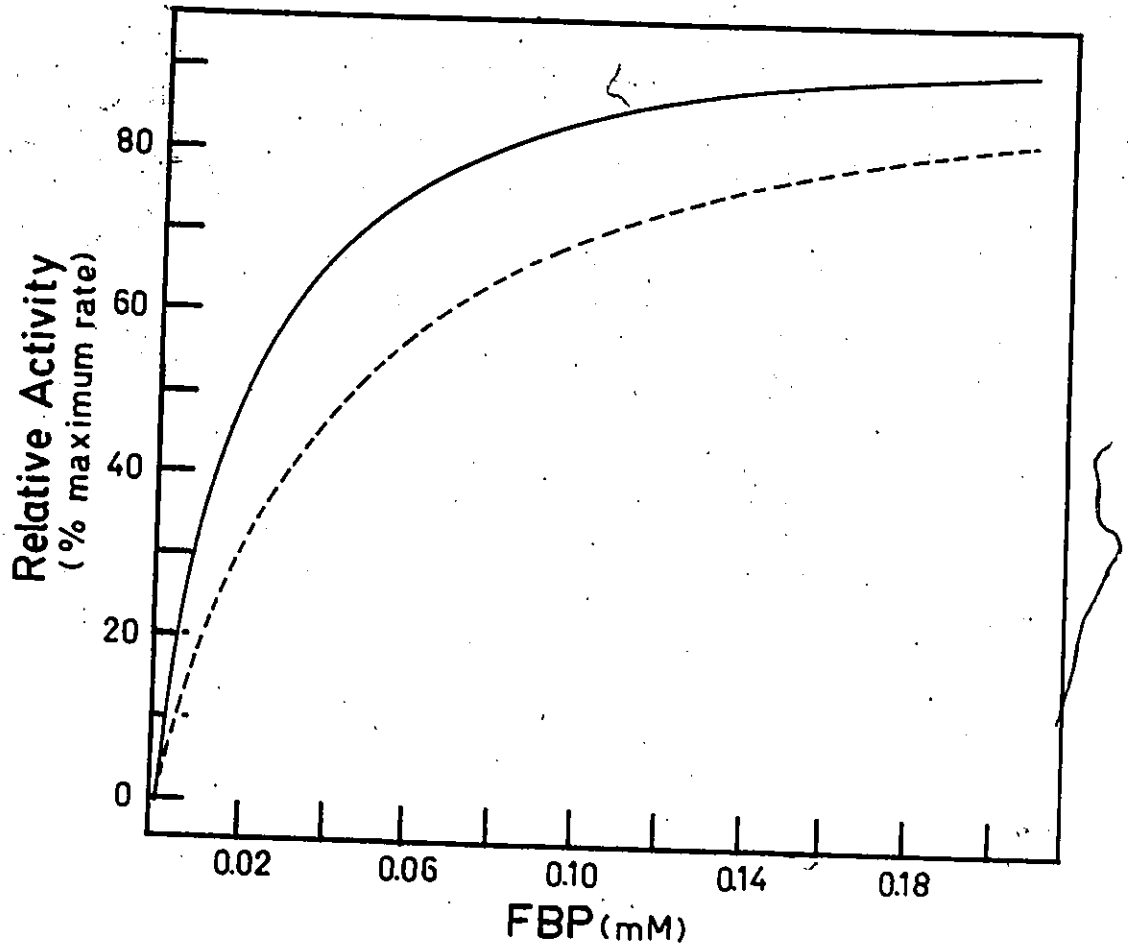

Relative Activity
(% maximum rate)
80
60
40
20
0
0.02
0.06
0.10
0.14
0.18
FBP (mM)

Table XVI: Seasonal changes in activation energy of the Fraction B FBPase isolated from the Muskoka population of *P. rufescens*.

E_a Calculated from Vmax

Season	Temperature	E_a	t-test
Summer	5-20°C	93.3±15	
Winter		130.0±7.8	2.353 *
Summer	30-45°C	58.9±11	
Winter		82.8±12	2.306

E_a Calculated at 0.020mM FBP

Season	Temperature	E_a	t-test
Summer	5-25°C	90.4±16	
Winter		110.0±5.6	1.31
Summer	30-45°C	62.3±4.3	
Winter		91.8±9.4	2.41 *

* P>0.05, df=8, t-test

E_a ($kJ \cdot mol^{-1}$), mean±SE

reversed. No seasonal changes were observed between 5° and 25° (E_a of 90.4±16 and 110±5.6 $kJ \cdot mol^{-1}$ for the summer and winter) and the E_a decreased significantly from 91.8±9.4 in the winter to 62.3±4.3 $kJ \cdot mol^{-1}$ in the summer at temperatures above 25°C (Table XVI).

8.3.4 Seasonal effects on *in vitro* thermal stability

8.3.4.1 High temperature denaturization

The rate of *in vitro* inactivation at high temperatures was studied in three winter (#13, 15 and 16) and three summer (#17, 18 and 19) extractions of the Fraction B enzyme. Table XVII reports the time of heating required to reduce the enzyme activity by 50%. Enzyme activity was assayed at 15 and 35°C following the heat stress. The Fraction B enzyme was found to be very sensitive to *in vitro* high temperatures stress, requiring only 3 minutes at 50°C to reduce its catalytic activity by 50%. There were no differences in the heat stability of the summer and winter extractions or between the enzymes assayed at 15 and 35°C.

8.3.4.2 Low temperature denaturization

The response of the Fraction B enzyme to repeated freeze-thaw cycles was very interesting, but unfortunately this was studied in only 4 of the extractions and the results are inconclusive. The winter extractions (13 and 15) showed similar denaturation patterns at the 15 and 35°C assay

TABLE XVII: The effects of high temperature (50°C) denaturation on the activity of the Fraction B FBPase isolated from the Muskoka population of *P. rufescens*.

Assay Temperature °C	Minutes required for 50% loss in enzyme activity						t-test
	Winter			Summer			
	mean	SE	n	mean	SE	n	
35	3.16	0.88	3	3.25	0.5	3	0.008
15	5.83	3.08	3	2.67	0.2	3	1.02

temperatures, with a 50% loss in enzyme activity occurring at or before the fourth freeze-thaw cycle (Figure 48). Extracts 16 and 17 behaved quite differently. FBPase activity measured at 35°C was unaltered by 10 freeze-thaw cycles. When assayed at 15°C, enzyme activity increased 3 to 5 fold during the first 3 cycles, and then remained high for the duration of the experiment (Figure 48). This may represent a seasonal change. Extraction 16, from a March 21st collection of P. rufescens, was stored and classified as a winter sample due to its low rate of net photosynthesis at 35°C, 800$\mu E \cdot m^{-2}s^{-1}$ (Table XI). It is possible that this material had started to summer acclimate at the time of collection, and the increase in stability of extract 16 and 17 may represent an adaptation to summer conditions, but more experimentation will have to be done before any conclusive statements can be made.

8.4 Laboratory induced changes in the Fraction B FBPase

The seasonal change in substrate affinity observed in the Fraction B FBPase could be mimicked in the laboratory by altering the storage conditions of the lichen (Figure 49a). Material collected in September of 1985 showed a $S_{0.5}$ (0.026 mM FBP) characteristic of the summer population at 35°C. This material was then shifted to winter storage conditions. The substrate affinity of the crude extract assayed at pH 8.5 remained unchanged for four days, and then gradually

FIGURE 48: The effect of repeated freezing and thawing on the activity of the purified Fraction B enzyme from the Muskoka population of P. rufescens. For each freeze-thaw cycle, the purified extract was frozen at -15°C for 10-14 hours and then allowed to thaw at room temperature for 2 hours. FBPase activity was assayed at 15 and 35°C for extractions 13 (○), 15 (□), 16 (△) and 17 (⬡) and are plotted relative to the activity prior to the initial freezing (Cycle 0).

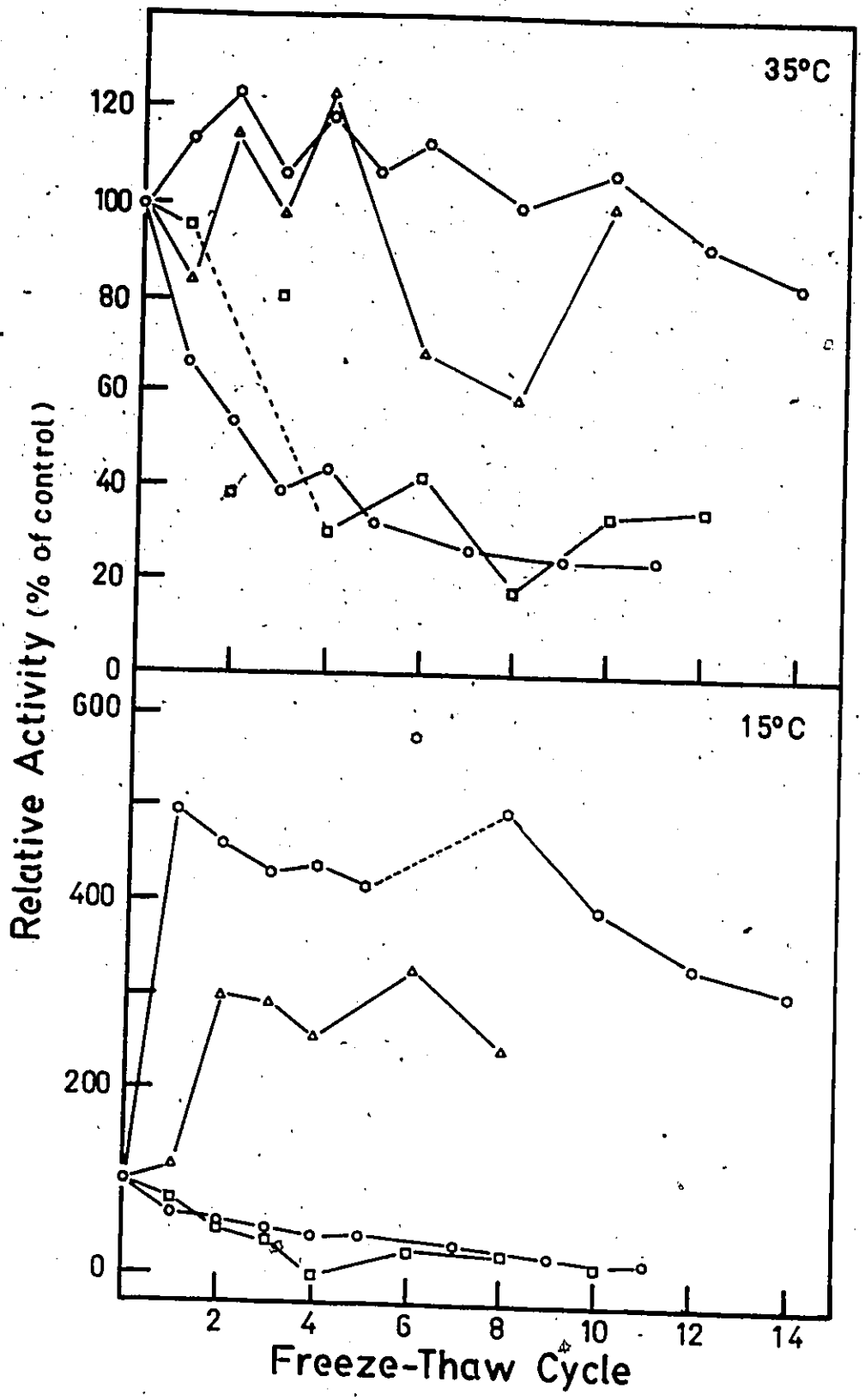
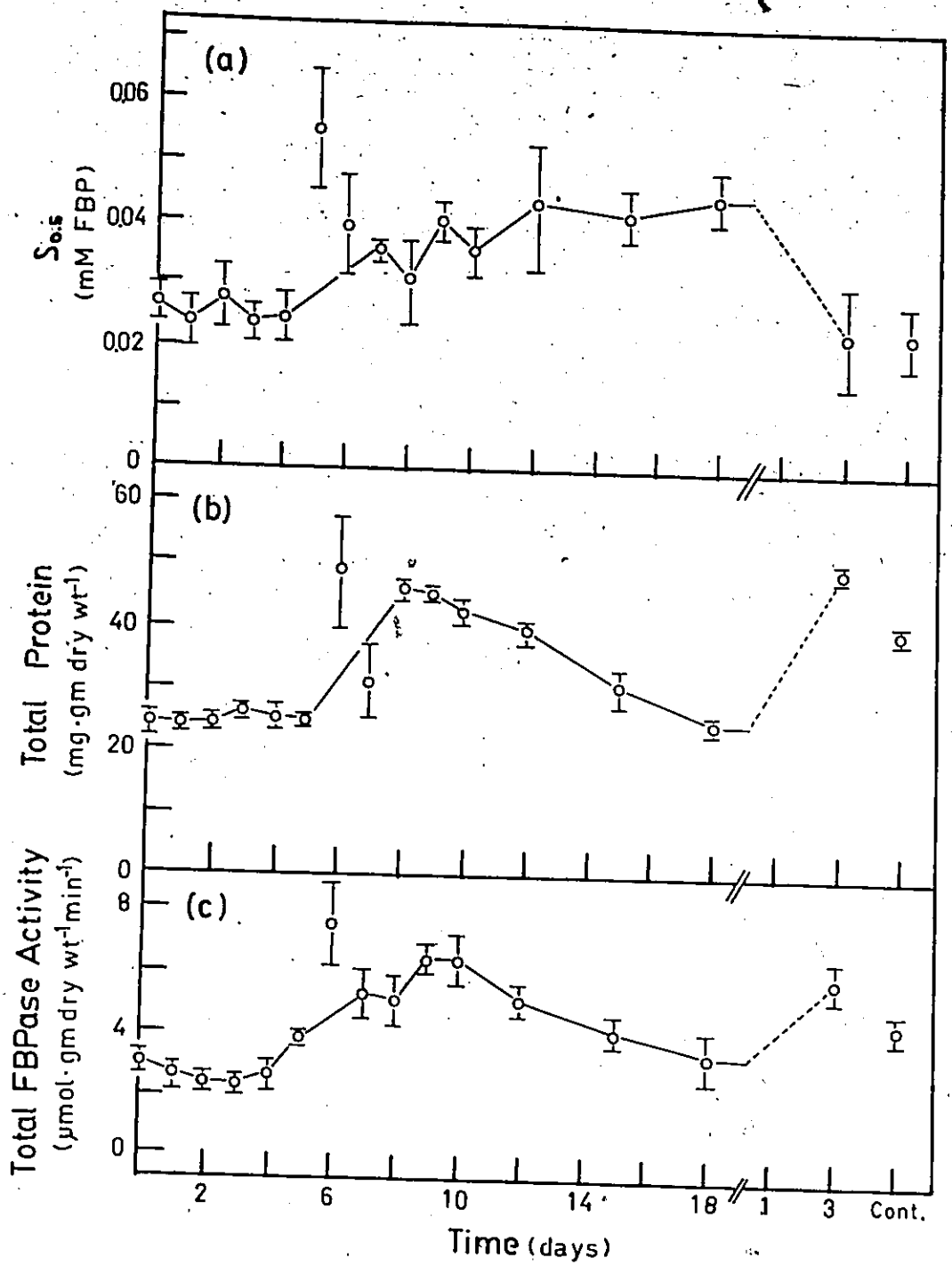

35°C
15°C
Relative Activity (% of control)
120
100
80
60
40
20
0
600
400
200
0
2
4
6
8
10
12
14
Freeze-Thaw Cycle

FIGURE 49: The change in substrate affinity (49a), total protein (49b) and total FBPase activity (49c) in crude extracts from the Muskoka population of P. rufescens undergoing a laboratory induced winter acclimation. Lichens collected in September 1985 and stored under summer conditions (25°C night/30°C, 15h photoperiod) were moved to winter conditions (-2/1°C N/D, 8h) on Day 0, and then returned to summer on Day 19. Each point represents the mean±SE of 3 replicate extractions measured at 35°C. Control material (Cont.)maintained under summer conditions was assayed on Day 24.


(a)
$S_{0.5}$ (mM FBP)
0.06
0.04
0.02
0
(b)
Total Protein (mg·gm dry wt^{-1})
60
40
20
0
(c)
Total FBPase Activity (μmol·gm dry wt^{-1}min^{-1})
8
4
0
2
6
10
14
18
1
3
Cont.
Time (days)

increased to a $S_{0.5}$ of 0.043±0.014 mM FBP on day 12, a value similar to the winter $S_{0.5}$ of 0.044 mM.

The specific activity of the FBPase assayed at pH 8.5 did not change in the crude extract during this induced acclimation, however there was an doubling in the total protein content between days 5 and 8 (from 24.39±0.97 to 46.7±1.5 mg·gm dry wt^{-1}) (Figure 49b). The protein content then gradually decreased, returning to its initial level by day 18. This resulted in a transient increase in total FBPase activity during the period when the substrate affinity was changing (Figure 49c).

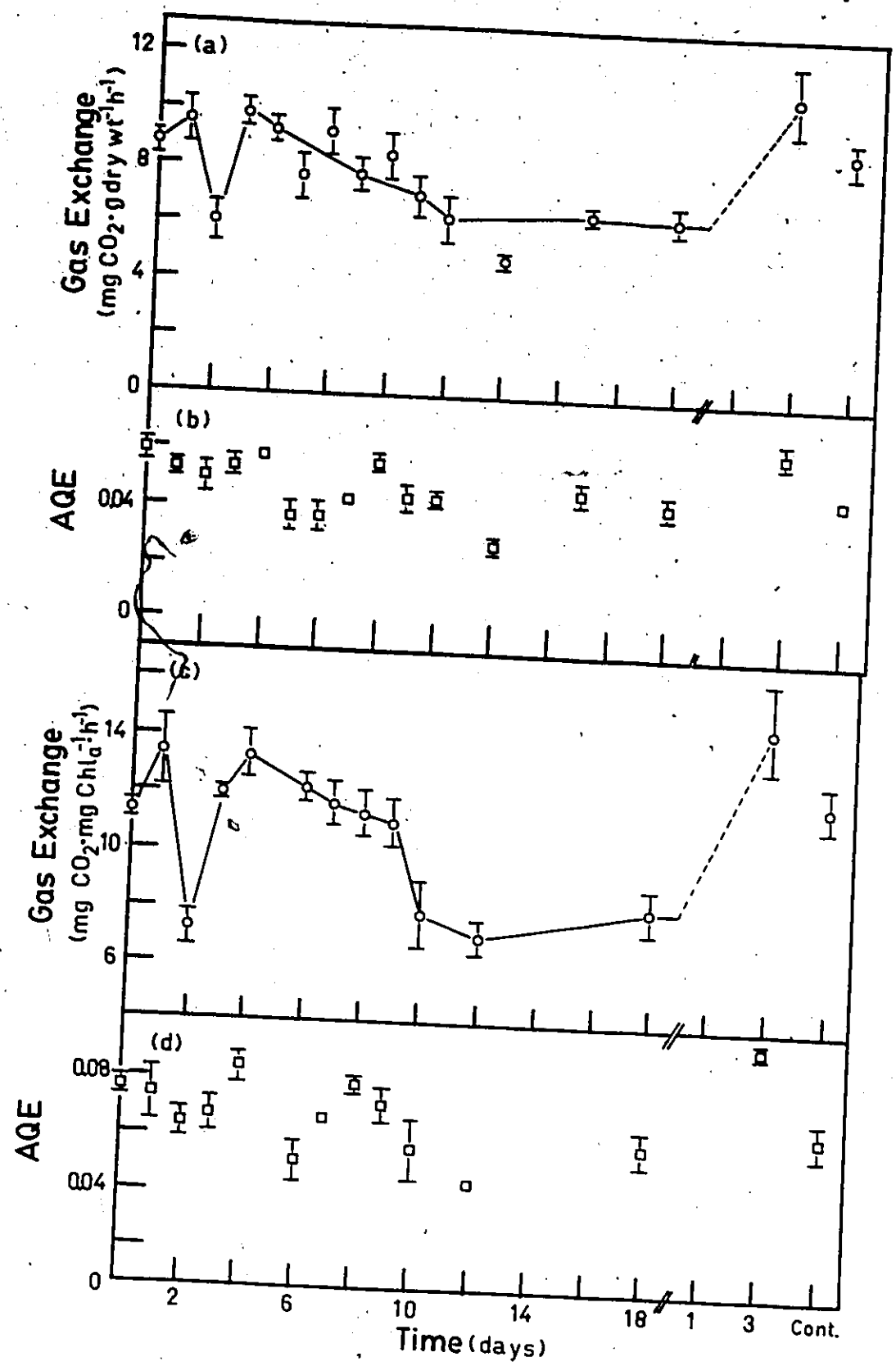
Although it required two weeks for a laboratory-induced acclimation to produce a stable change in $S_{0.5}$, the process can be quickly reversed (Figure 49a). Substrate affinity returned to summer levels within three days of transferring the lichen back into summer conditions. Total FBPase activity and total protein still remained higher than the characteristic summer level (Figure 49b,c) indicating that there may be some metabolic shifts occurring on day 3. It is apparent from this radical difference in the time course, and the previous inability to experimentally induce a summer acclimation in a winter population of *P. rufescens* (Figure 25, 26), that warm temperature acclimation is not a strict reversal of the cool acclimation process, and further studies are required to uncover the mechanisms involved.

The induced change in substrate affinity was

paralleled by a change in the light-saturated rate of photosynthesis. Gas exchange in the September collection was typical of the summer rates previously measured (Figure 21), with a rate of 8.7 mg $CO_2 \cdot gm^{-1}h^{-1}$ at 35°C, 800 $\mu E \cdot m^{-2}s^{-1}$ (Figure 50a). As indicated previously (Figure 24), net photosynthesis decreased on the 2nd day of winter storage, then rapidly returned to the summer level. This rate then gradually declined over a ten day period to a value of 6 mg $CO_2 \cdot gm^{-1}h^{-1}$, slightly higher than the December rate of 4.19 mg $CO_2 \cdot gm^{-1}h^{-1}$ measured in Figure 21. As in the case of FBPase affinity, this rate change was readily reversible, increasing to 10.8 $CO_2 gm^{-1}h^{-1}$ following 3 days of summer storage (Figure 50a). A similar pattern was observed when the gas exchange rate was based on a chlorophyll content (Figure 50c), indicating that the change in net photosynthesis cannot be the result of an environmentally-induced alteration in the algal density.

Apparent quantum efficiency, when measured on either a dry weight or chlorophyll basis, was variable, but does not show any definite trends during the experiment (Figure 50b,d). It is important to note that the 'crash' in net photosynthesis observed on day 2 was not correlated to a change in apparent quantum efficiency, chlorophyll content or FBPase substrate affinity, and thus results from a different process than the changes observed after day 3.

FIGURE 50: The change in light saturated (800 $\mu mol \cdot m^{-2} s^{-1}$) net photosynthesis (50a,c) and apparent quantum efficiency (AQE,50b,d), assayed at 35°C and calculated on a per thallus dry weight basis (50a,b) and on a per mg Chl basis (50c,d) during the acclimation experiment of Figure 49..


(a)
Gas Exchange
(mg CO_2 · g dry wt^{-1} h^{-1})
12
8
4
0
(b)
AQE
0.04
0
(c)
Gas Exchange
(mg CO_2 · mg Chl_a^{-1} h^{-1})
14
10
6
(d)
AQE
0.08
0.04
0
2
6
10
14
18
1
3
Cont.
Time (days)

9. DISCUSSION

9.1 The FBPases

The Muskoka population of Peltigera rufescens demonstrated a seasonal change in response of net photosynthesis to temperature (Figure 19). The gas exchange rate at 5, 15 and 25°C remained seasonally constant, while the rate measured at 35 and 45°C during the summer was approximately 2-fold higher than the rates obtained at these temperatures in the winter. A comparison of the photosynthetic-illumination curve of the summer- and winter-collected lichen provided a provisional interpretation of the mechanism behind this capacity change, namely an alteration of the flux through the Calvin cycle (Figure 21). This was presumably caused by a change in a rate-limiting step in this cycle, probably the reaction catalyzed by FBPase (Pelroy et al. 1976; Portis et al. 1977; Karagouni and Slater 1979; Badger et al. 1982).

Evidence obtained from the hydrolysis of FBP in crude extracts indicated the presence of several phosphatases in P. rufescens (Figure 34, 35). These have been purified into two components. Fraction A is a Mg^{2+}-independent acid phosphatase while the Fraction B enzyme is a Mg^{2+}-dependent alkaline phosphatase (Figures 34, 42). The literature reports that three different enzymes capable of hydrolizing

FBP are present in higher plants (a photosynthetic and a cytosolic FBPase and a nonspecific acid phosphatase) and their properties are summarized in Table VII.

9.1.1 The Fraction A FBPase

The Fraction A enzyme from P. rufescens was most similar to a nonspecific acid phosphatase isolated from the leaves of spinach and soybeans (Racker and Schroeder 1958; Pharr and Huber 1984), and from the roots of corn and pea (Woodrow et al. 1982). This enzyme had a pH optimum of 7. with no activity above pH 8. It did not require magnesium for its activity, and could dephosphorylate any sugar diphosphate. Similarly, the Fraction A enzyme from P. rufescens was active only below pH 7.5, with an optimum at pH 6.5 (Figure 34), and does not require $MgCl_2$ (Figure 42). Although the substrate specificity of the Fraction A enzyme could not be tested with the assay used, the high Km observed (0.23 mM FBP) suggested it may not be specifically involved in FBP metabolism.

9.1.2 The Fraction B FBPase

9.1.2.1 Comparison with the spinach enzymes

The photosynthetic FBPase has been shown to exist in two interconvertable forms. The enzyme undergoes a pH-dependent conversion between an inactive dimer and tetramer (Lazaro et al. 1975; Schürmann et al. 1976; Zimmermann et al.

1976). The *in vivo* activation of the tetrameric form is regulated by light via a ferredoxin-thioredoxin activation system (Schürmann *et al.* 1976; Pradel *et al.* 1981). Light energy generates a flux of electrons through the photosynthetic electron transport chain, causing the reduction of ferredoxin. This reducing power is enzymatically transferred, with the aid of a ferredoxin-thioredoxin reductase, to the protein thioredoxin, which then reduces two disulfide bridges in the FBPase, causing a conformational change and a large increase in enzyme activity. This process can be mimicked *in vitro* using DTT as the reductant (Zimmermann *et al.* 1976; Rosa and Whatley 1984). However, this is a slow process, with lag periods lasting up to 20 minutes. FBP and Mg^{2+} also act as activators, resulting in a very complex process with seven different kinetic states possible (Rosa and Whatley 1984). This light activation process is the reason that several researchers consider the FBPase to be an important regulatory and rate-limiting enzyme in the Calvin cycle (Pelroy *et al.* 1976; Stitt *et al.* 1980; Dietz and Heber 1984).

The activated photosynthetic enzyme isolated from spinach chloroplasts had a pH optimum above pH 8.0, with no activity observed below pH 7.5 (Table VII)(Latzko *et al.* 1974; Lazaro *et al.* 1975; Zimmermann *et al.* 1976). It responded sigmoidally to Mg^{2+} concentration, with a $S_{0.5}$ of 1.0 to 3.0 mM $MgCl_2$ (Buchanan *et al.* 1971; Charles and

Halliwell 1980; Pradel et al. 1981; Corley and Wolosiuk 1985). Literature reports on the response of this enzyme to substrate concentration have been controversial, with some researchers recording a sigmoidal response (Preiss et al. 1967; Zimmermann et al. 1976; Pradel et al. 1981; Corley and Wolosiuk 1985), while others found a hyperbolic response (Charles and Halliwell 1980). In either case, half maximal velocities were achieved at concentrations in the range of 0.03 to 0.08 mM FBP.

The third plant enzyme characterized, isolated from the leaves of spinach, was cytosolic FBPase, thought to be involved in glucose metabolism. This cytosolic FBPase had a much higher affinity for FBP, with a K_m an order of magnitude lower than either the photosynthetic FBPase (Lazaro et al. 1974; Zimmermann et al. 1978) or the Fraction B FBPase isolated from P. rufescens (Table VII). Activity of this enzyme required Mg^{2+}, with a $S_{0.5}$ of 0.1 to 1.0 mM (Zimmermann et al. 1978; Herzog et al. 1984) and was optimal around pH 7.5 to 8.0, exhibiting a rapid decline as pH dropped below 7.0 (Latzko et al. 1974; Zimmermann et al. 1978; Stitt et al. 1985).

The cytosolic enzyme differed considerably from the photosynthetic FBPase in its regulatory properties (Table VII). AMP acted as a noncompetitive inhibitor of enzyme activity (Stitt et al. 1985), causing a 20 to 40% inhibition at AMP concentrations in the 0.2 to 2.0 mM range (Latzko et

al. 1974; Cséke et al. 1982; Pharr and Huber 1984; Stitt et al. 1985). Fr-2,6-P was a competitive inhibitor, with a 2 μM concentration inhibiting 90% of the activity at a FBP concentration of $5K_m$, and a 50% inhibition at a FBP concentration of $40K_m$ (Cséke et al. 1982; Herzog et al. 1984; Pharr and Huber 1984; Stitt et al. 1985). The inhibition by Fr-2,6-P also acted synergistically with AMP. Concentrations of substrate in excess of 20 K_m (approximately 0.1 mM FBP) could also inhibit enzyme activity by up to 50% (Cséke et al. 1982; Herzog et al. 1984; Stitt et al. 1985). The cytosolic enzyme was not activated by DTT (Latzko et al. 1974).

The Fraction B enzyme isolated from P. rufescens was more similar to the spinach photosynthetic enzyme than to the cytosolic enzyme. It is active at alkaline pH (Figure 34), its affinity for FBP ($S_{0.5}$=0.039 mM) and $MgCl_2$ ($S_{0.5}$=2.0 mM) was within the range reported for the photosynthetic enzyme, and it was activated by DTT (Figure 44, Table XIV). However, contrary to what has been observed with the photosynthetic enzyme, pretreatment with DTT did not decrease the lag period, nor increase the enzyme's substrate affinity (Charles and Halliwell 1980; Rosa and Whatley 1984). In addition, the inhibition by AMP is not a characteristic of the photosynthetic FBPase (Buchanan et al. 1971; Udvardy et al. 1982). However, the lack of inhibition by Fr-2,6-P and high concentrations of FBP is evidence that the Fraction B enzyme is not cytosolic in origin or function (Table XIII).

9.1.2.2 Comparison with the cyanobacterial enzymes

The phycobiont of P. rufescens is a cyanobacterium (Nostoc). The photosynthetic FBPases isolated from other cyanobacteria (Anacystis and Synechococcus) appear to be quite similar to the Fraction B enzyme in many respects (Table VII)(Bishop 1979; Schmidt 1981; Udvardy et al. 1982). Activity was restricted to an alkaline pH, with an optimum at pH 8.0. Bishop (1979) reported hyperbolic kinetics for both FBP and Mg^{2+} with K_m of 0.088 and 0.95 mM respectively. Udvardy et al. (1982) reported a similar K_m of 0.06 mM for FBP, however they observed a sigmoidal curve with $MgCl_2$, with a $S_{0.5}$ of 5 mM. In contrast to the spinach photosynthetic enzyme, but closely corresponding with the Fraction B enzyme, FBPase activity in these cyanobacteria was sensitive to AMP concentrations in the 0.2 to 2.0 mM range (Table VII)(Bishop 1979; Udvardy et al. 1982).

Gerbling et al. (1984) reported that two FBPases, were present in Synechococcus leopoliensis (= Anacystis nidulans). They were both active at pH 8.0, but differed in AMP sensitivity and electrophoretic characteristics. Their Form B enzyme, which accounted for 95% of the total FBPase activity, was more similar to the P. rufescens Fraction B enzyme, showing an inhibition by 2.0 mM AMP. This enzyme undergoes a dimer-tetramer conversion, dependent upon DTT, FBP and Mg^{2+} in vitro (Gerbling et al. 1985), which is

similar to the spinach photosynthetic enzyme (Zimmermann et al. 1976). It was uncertain which form of the FBPase was involved in the Calvin cycle in Synechococcus, but Gerbling et al. (1985, 1986) suggested that it was Form B, due to its similarity to the spinach photosynthetic enzyme.

Thus the Fraction B enzyme in P. rufescens has many characteristics in common with the photosynthetic FBPases isolated from cyanobacteria and spinach, and is almost certainly the enzyme involved in the photosynthetic carbon metabolism in this lichen. However, the lack of a cytosolic FBPase is somewhat puzzling. It is possible that the Fraction B enzyme serves a dual role in the phycobiont, as suggested by Bishop (1979) for Anacystis, but this would not explain the apparent lack of a cytosolic fungal FBPase. The FBPases isolated from the yeasts Saccharomyces cerevisiae and Candida utilis (Funayama et al. 1979) appear to be similar to the cytosolic enzyme described above (Table VII) and thus different than either the Fraction A or B enzymes isolated in this study. However, it is most probable that this cytosolic enzyme was lost during the isolation process, since the crude extract had a high enzyme activity at pH 7.5 which was not observed in either of the purified enzymes (Figure 34), adding considerable support to this theory.

9.1.3 FBPase as a rate limiting step in the Calvin cycle

The total FBPase activity extracted from the thallus

was 1.4 μmol F6P·gm dry wt.$^{-1}$min^{-1} (measured at 25°C and pH 8.5)(Table XV). For every two CO_2 molecules fixed, one molecule of FBP must be metabolized to regenerate the RuBP (Lehninger 1982). Thus, there is sufficient FBPase activity in the crude extract to support a gross photosynthetic rate of 7.5 mg CO_2gm^{-1}h^{-1}. Net photosynthesis at 25°C, 800 and 1000 μE·m^{-2}s^{-1} ranges from 6 to 8 mg CO_2·gm^{-1}h^{-1} (Figure 19). When the respiratory load of 2 mg CO_2·gm^{-1}h^{-1} is taken into consideration, it is apparent that the maximum FBPase rate measured in vitro is not sufficient for the observed rate of gas exchange.

One possibility is that the level of FBPase in the cells is only just sufficient for the observed rate of photosynthesis, and that this enzyme may be limiting carbon fixation.

There is little information available on the in vivo concentration of metabolites for Nostoc. The assay conditions were designed to maximize in vitro activity, which should be sufficient to reproduce the in vivo rates, provided no inactivation or denaturization occurred during the extraction and storage of the enzyme. As mentioned above, the activation of the photosynthetic FBPase is a complicated process. The presence of mercaptoethanol in the extraction buffer and DTT in the assay buffer should prevent the inactivation of the enzyme (Gerbling et al. 1985) and, as the specific activity of the purified Fraction B enzyme was

similar to those reported elsewhere (Bishop 1979; Schmidt 1981; Udvardy et al. 1982), it is unlikely that the low activity in the crude extract is a result of an inappropriate assay method. The possibility that denaturization occurred during the protein extraction cannot be eliminated, although several preventative measures were undertaken (Section 7.2.1).

9.2 Temperature adaptations

Terrestrial plants, especially those growing in exposed habitats, must operate in highly variable temperature regimes, ranging from the rapid and unpredictable short term fluctuations due to sunfleck activity, to the more predictable diurnal and seasonal patterns. Some plants, notably the short lived annuals and ephemerals, and those with solar tracking abilities, can avoid some of these temperature fluctuations, but many plants are faced with the difficult task of maintaining an internal homeostasis while experiencing wide fluctuations in a major enzymatic rate determining factor, temperature.

McNaughton (1972,1974), while studying the temperature effects on the enzyme kinetic parameters of MDH isolated from several widely distributed populations of Typha latifolia, proposed that plants have evolved a thermal insensitivity to temperature regimes similar to their native environment. Rate effects caused by short term temperature

fluctuations are partially compensated for by a positive relationship between K_s and temperature (Somero and Hochachka 1971; Somero 1975). Thus, as temperature increases, substrate affinity decreases, lessening the temperature dependence of the reactions (Behrisch 1969).

Attempts to maintain homeostasis on a longer, seasonal basis can involve both quantitative and qualitative changes. Adaptation to low temperature can be obtained simply through a change in total enzyme content, as observed in Laminaria hyperborea (Küppers and Weidner 1980), Nerium oleander (Badger et al. 1982), Triticum aestivum (Sawada et al. 1974) and Arabidopsis thaliana (Simon et al. 1983). For a totally quantitative change in enzyme concentration, it is expected that its effect would be observed at all the temperatures studied. For P. rufescens, the change in CO_2 fixation is only observed at high temperatures (Figure 19). No seasonal changes in either the total protein or total FBPase content in the thallus were observed (Table XV). Thus the seasonal change must be of a qualitative nature. Such changes have been observed affecting the substrate affinity, activation energy and thermal stability of the enzyme.

9.2.1 Thermal stability

As changes in thermal stability are possible on a population basis, they may also be important for temperature acclimation on a seasonal basis. Increased thermal stability

has been correlated to high temperature adaptation in several species. McNaughton (1974) has found a strong positive correlation between the stability of malate dehydrogenase (MDH) and the mean temperature of the hottest month in several widely distributed populations of Typha latifolia. Liu et al. (1978) have also observed differences in the thermal stability for this enzyme in populations of this species. The association between habitat temperature and thermal stability of MDH has also been reported for Lathyrus japonicus (Simon 1979b) and Phaseolus sp. (Kinbacher 1970), as well as for phospho-enol pyruvate (PEP) carboxylase in populations of Echinochloa crusgalli (Simon et al. 1984a). In contrast, Terri and Peet (1978) and Davidson and Simon (1981) found no correlation between habitat and thermal stability of MDH in two populations of Potentilla glandulosa and eleven populations of Spirodela polyrhiza respectively. However, the populations in the latter study had been maintained in culture for 5 to 23 years prior to the study and therefore the results may not be representative of the field situation.

It has been shown previously that lichens do undergo a seasonal change in their resistance to prolonged high temperature stress (Tegler and Kershaw 1981), and the CO_2 response matrix for Stereocaulon paschale also indicates a possible seasonal change in photosynthetic stability at 35°C (Kershaw and Smith 1978). In addition, acclimation to high

temperature, involving increased thermal stability of the photosynthetic apparatus, has been observed in Nerium oleander, Larrea divaricata, and Atriplex lentiformis (Pearcy et al. 1977; Armond et al. 1978; Badger et al. 1982). However, there also appears to be no seasonal change in the thermal stability of the photosynthetic FBPase from P. rufescens (Table XVII).

FBPase activity is very sensitive to in vitro denaturization at 50°C, a result which is quite surprising considering the operating environment of this lichen. Thallus temperatures in excess of 60°C have been recorded for P. rufescens by MacFarlane and Kershaw (1980), and these authors report that photosynthesis in this species is unaffected by 22 days storage of the lichen at 45°C. The high net photosynthetic rates reported at 45°C in this study (Figure 19) also suggest a much higher stability than was obtained for the in vitro denaturization. Kinbacher (1970) has observed that in vitro stability is not correlated to in vivo stability in Phaseolus sp.. In addition, a significant denaturization of PEP carboxylase extracted from the C4 plant Echinochloa crusgalli occurred at 45°C, a temperature which is near the photosynthetic optimum for this species (Robert et al. 1983).

From this, it is apparent that in vitro denaturation is not necessarily related to the in vivo process. Simon (1979b) has reported a negative correlation between thermal

stability and the degree of purification of enzyme extracts. The purification process removes various effector and cofactor molecules which help stabilize the enzyme. This is evident when the rapid denaturization of the FBPase in the extraction buffer at 50°C (Table XVII) is compared to the stable rates observed in the assay buffer at 45°C, where no loss of activity was observed over a 10 minute assay. Thus no valid ecological interpretation can be made from the *in vitro* thermal stability experiments in this study. A more valid experimental design would be the comparison of total FBPase activity extracted from thalli which have been given high temperature pretreatments for varying lengths of time.

9.2.2 Activation energy

A change in activation energy has been considered a major factor in adapting to different thermal environments (McNaughton 1974). Observations of different populations of a species have shown that E_a tends to be lower (and thus the enzyme is more temperature efficient) in cooler environments (Low *et al.* 1973; Simon 1979c; Simon *et al.* 1984a,b), but reports are often conflicting. Thus, Simon *et al.* (1984a) reported that the E_a of PEP carboxylase was lower in a northern Quebec population of *Echinochloa crusgalli* than a Mississippi population of this species, while a more detailed study involving 6 different populations found no geographic correlation with E_a (Simon *et al.* 1984b). Similarly, Simon

et al. (1983) found no differences in the E_a for MDH and glutamate-oxaloacetate transaminase from a Russian and a Libyan population of Arabidopsis thaliana, although this may be expected due to the similar thermal environment during their growing seasons. Some studies on poikilothermic animals have also failed to show a relationship between activation energy and growth temperature (Baldwin and Hochachka 1970; Barrett and Fairbairn 1971), while Smith and Platt (1985) reported that the E_a of RuBP CO_2ase was actually higher in arctic phytoplankton communities than in tropical communities.

Such conflicting reports make it difficult to generalize about the role of activation energy in enzymatic adaptation. One reason for a lack of a correlation between E_a and geographic distribution of a population may be the dependence of E_a on growth temperature. Several of the above mentioned studies (McNaughton 1972; Simon 1979c; Simon et al. 1983, 1984b) showed that the variation in E_a, related to the growth of a population at several different temperatures, was larger than the between population variation at one growth temperature. Such growth dependent variations in E_a make it difficult to observe any population trends, but suggest that seasonal changes in this parameter could be involved in the acclimation process.

In P. rufescens, the E_a determined from the V calculations, representative of substrate saturated activity,

did change on a seasonal basis at the lower assay temperatures (Table XVI), but this change appears to be counteradaptive, with E_a increasing from 93.3 to 130 $kJ \cdot mol^{-1}$ over the 5 to 20°C interval during the winter. This indicates that catalysis will be less temperature efficient. Thus a decrease in the flux through this enzyme at low temperatures in the winter would be expected, but was not observed in the gas exchange measurements (Figure 19). However, when the activation energy was calculated from the rates representative of a lower, and possibly more realistic substrate concentration of 0.020 mM FBP, this pattern was reversed (Table XVI). The E_a for the 30 to 45°C temperature interval decreased from 91.8 to 62.3 $kJ \cdot mol^{-1}$ during the summer, while no significant changes were observed at the lower temperatures. This pattern correlates with the observed seasonal change in net photosynthesis in the Muskoka population of P. rufescens (Figure 19).

Behrisch (1969) has commented on the possibility that activation energy calculated from enzyme-saturated rates is not a realistic indication of the in situ efficiency, and significant changes in the enzyme's affinity for its substrate and cofactors are a better measure of acclimation. The differing response in E_a to substrate concentration (Table XVI) has been observed previously, and is the result of a change in the K_m-temperature relationship (Simon 1979a), which further stresses the importance of substrate affinity

changes to the acclimation process.

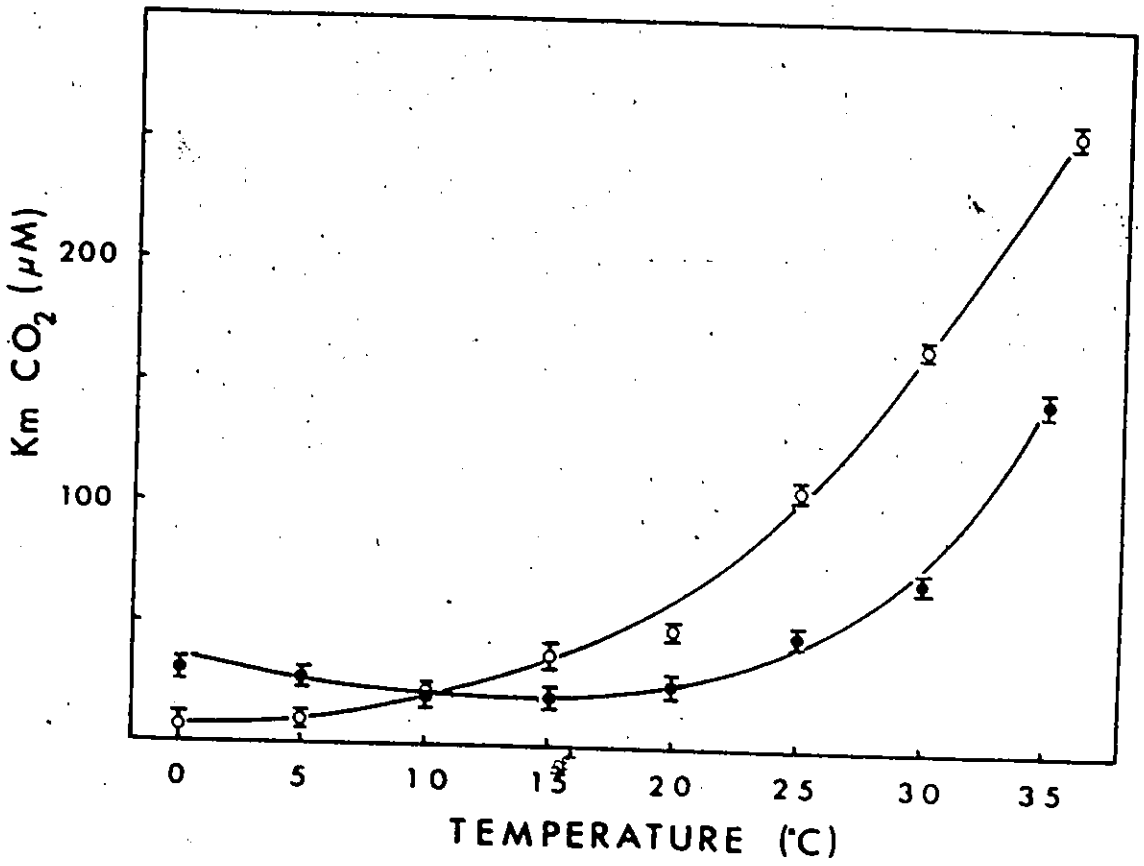
9.2.3 Substrate affinity

Seasonal adaptations in substrate affinity have been observed in fish, nematodes and several plant species (Behrisch 1969; Baldwin and Hochachka 1970; Barrett and Fairbairn 1971; Terri and Peet 1978; Huner and MacDowall 1979b; Simon 1979c) and such a process seems to be occurring in P. rufescens (Figure 49). In the winter, minimum $S_{0.5}$ values occurred at 5°C, whereas in the summer, the $S_{0.5}$ minimum had shifted to 45°C. This resulted in a large seasonal change in the substrate affinity at temperatures above 25°C, but no difference below this temperature.

This seasonal change in the $S_{0.5}$-temperature relationship is not the "classic" type of adaptation proposed by Somero and Hochachka (1971). For example, Huner and MacDowall (1979b) reported a change in the RuBP CO_2ase affinity for CO_2 during winter hardening in puma rye (Figure 51). During this process, the carboxylase underwent a conformational change (Huner et al. 1982) which resulted in a shift in the K_m-temperature response. The temperature at which the minimum K_m occurred shifted from 15°C in the enzyme from the unhardened rye to 0°C in the hardened form of the enzyme. This adaptation involved a complete shift in the K_m-temperature curve, such that at temperatures lower than 10°C, the hardened enzyme had a much lower K_m than the unhardened

FIGURE 51: Effect of temperature on the apparent K_m of RuBP CO_2ase isolated from rye grown at warm (●) and cold (O) temperature regimes.

(from Huner and MacDowall 1979b)


Km CO_2 (μM)
200
100
0
5
10
15
20
25
30
35
TEMPERATURE (°C)

enzyme, while at temperature above 10°C, the unhardened enzyme had the lower K_m. Similar patterns have been observed for MDH in Potentilla glandulosa and for acetylcholinesterase, PEP carboxylase, isocitrate dehydrogenase, phosphofructose kinase and citrate synthase in rainbow trout (Baldwin and Hochachka 1970; Somero and Hochachka 1971; Terri and Peet 1978).

Somero (Somero and Hochachka 1971; Somero 1975) suggested that this "classic" pattern represented the cell's attempt to maintain a fine control over its enzymatic rates. Control of a metabolic process is usually regulated via the effects that cofactors and positive and negative effector molecules have on the substrate affinity of the enzyme. These effectors can alter the K_m by only a certain amount. Thus the enzymes substrate affinity must be maintained at a fairly constant level throughout the year if its K_m is to respond adequately to a change in effector concentration. Accordingly, the K_m changes between summer and winter forms of the enzyme was to maintain a fine control on metabolism and was not an attempt to maintain homeostasis in the enzymatic rates between summer and winter (Somero 1975). A decrease in the K_m at low temperatures in the winter could help increase the catalytic rate, but on its own it cannot overcome the Q_{10} effects of the temperature change (Somero and Hochachka 1971).

The change observed in P. rufescens does not fit this

"classic" pattern (Figure 49), but was more similar to the changes observed in MDH enzyme from Arabidopsis thaliana and Ascaris lumbricoides (Barrett and Fairbairn 1971; Simon et al. 1983). In P. rufescens, the seasonal changes in FBPase substrate affinity were only statistically significant at temperatures above 25°C. The 50% reduction in the $S_{0.5}$ observed at these temperatures in the summer could result in a 1.2-fold increase in the flux through the FBPase if the substrate concentration is 0.1 mM, and a 1.4-fold difference at a FBP concentration of 0.05 mM (Figure 47). This correlated well with the changes observed on a seasonal basis (Figure 19), where the net photosynthetic capacity increased from 6 and 5 mg $CO_2 \cdot gm^{-1}h^{-1}$ in the winter to 10 and 8 mg $CO_2 \cdot gm^{-1}h^{-1}$ in the summer at 35 and 45 °C respectively, but it cannot account for the entire change in photosynthetic activity. This correlation was further strengthened by the induced winter acclimation experiments shown in Figures 49 and 50, where the decrease in substrate affinity at 35°C was paralleled by a decrease in the gas exchange rates. As the FBPase is considered to be a rate-limiting step in the Calvin cycle (Latzko and Kelly 1979; Badger et al. 1982), changes in the FBPase kinetic properties could be directly related to the observed patterns of net photosynthesis.

The winter acclimation experiment of Figure 49 provides excellent evidence that the FBPase was involved in the temperature acclimation, but the interpretation of these

results requires a note of caution. Due to the time dependence of the experiment, it was impossible to purify the Fraction B FBPase throughout the duration of this experiment. This necessitated the measurement of the $S_{0.5}$ in a crude extract which could contain several different FBPases. Although the Fraction A enzyme will not interfere with the activity at pH 8.5, it can be argued from the pH curve (Figure 34) that there may be another FBPase active above pH 7.0. However, three observations suggest that the activity of a cytosolic FBPase can be discounted. Firstly, the substrate saturation curves of the crude extract followed Michaelis-Menten kinetics. The cytosolic FBPase had a K_m of 0.003 mM FBP (Zimmermann et al. 1978; Stitt et al. 1985). If a cytosolic enzyme was active in the crude extract at pH 8.5, the substrate saturation curves would depart from Michaelis kinetics at low concentrations, contrary to my observations. Secondly, the $S_{0.5}$ changes measured during the acclimation (Figure 49a) were similar to those observed for the purified FBPase on a seasonal basis (Figure 46). Thirdly, the FBPase activity in the crude extract was not inhibited by Fr-2,6-P, a potent inhibitor of the cytosolic FBPase (Cséke et al. 1982; Herzog et al. 1984; Stitt et al. 1985). These three points thus substantiate the conclusion that the photosynthetic enzyme forms the major part of the activity measured at pH 8.5 in the crude extract.

It is important to emphasize that the changes in net

photosynthesis during the induced acclimation experiment were not correlated to changes in total FBPase activity, but only to changes in substrate affinity. Total FBPase activity showed a transient increase, (while the net photosynthetic activity decreased (Figures 49b, 50a)), and then stabilized by day 18 at a level similar to the day 0 value. This result was identical to the seasonal pattern, where no changes in total activity were observed between the summer and winter (Table XV). However, the observed shift in the $S_{0.5}$ from 0.025 mM to 0.040 mM FBP (Figure 49a) was sufficient to produce the observed change in net photosynthesis (Figure 50a) (Brown and Kershaw 1986).

9.2.4 Potential mechanisms of temperature adaptation

It can be concluded, from the activation energy and substrate affinity studies (Table XVI , Figure 46), that there was a change in the response of the FBPase between the winter and summer. It cannot be determined from this data if this change was the result of de novo enzyme synthesis or if it was a modification of the existing enzyme. Badger et al. (1982) reported that photosynthetic temperature acclimation in Nerium oleander, which involved a change in the total FBPase activity, occured over a two week period. Although this time course was similar to that observed here (Figure 49a), only a transient change in FBPase activity was observed (Figure 49c). It is likely that this slight increase was an

integral part of the acclimation process, where both summer and winter forms of the enzyme could be present, but it does not necessarily indicate synthesis of a new enzyme. The large changes in protein content during the acclimation does, however suggest that several metabolic processes are altered by the experiment (Figure 49b).

Two other molecular mechanisms are feasible and the evidence from this study is insufficient to identify which is the more probable. Winter hardening in puma rye, which involves a change in the K_m-temperature interaction of RuBP carboxylase, resulted from a conformational change in the enzyme. This change can be detected electrophoretically (Huner and MacDowall 1979a) and involved an alteration of the tertiary structure of the protein which increases its stability at low temperatures (Huner and MacDowall 1978). It has recently been shown that this change was due to an increase in the number of sulfhydryl bridges in the large subunit of the carboxylase (Huner et al. 1982).

The light activation of the photosynthetic FBPase also involves a reduction of two disulfide bridges (Pradel et al. 1981). The kinetic properties of the light activated FBPase was considerably different than those of the inactive form (Schürmann and Wolosiuk 1978; Charles and Halliwell 1980; Pradel et al. 1981). It was also possible that the seasonal changes observed in this enzyme resulted from a change in the degree of activation which the enzyme

undergoes *in vivo*. The elucidation of the actual biochemical and/or genetic basis for this seasonal change in FBPase needs to be investigated before a complete picture of the acclimation process for this enzyme is obtained.

9.2.5 Other possibilities

The role of RuBP CO_2ase was disregarded in this study because it appeared that net photosynthesis was not limited by the carboxylation reaction. This assumption was based on the analysis of the CO_2 limitation curve for a summer collection of the Muskoka population of *P. rufescens* (Figure 14). No such curve was determined for the winter lichen, therefore a seasonal change in the carboxylase cannot be ruled out and should be studied. In addition, several researchers have indicated that adaptation to high temperatures involved an increase in the stability of the photosynthetic electron transport chain (Pearcy *et al.* 1977; Armond *et al.* 1978; Badger *et al.* 1982). The importance of this to *P. rufescens* was discounted because the P-I curves suggested that no changes in quantum efficiency occurred on a seasonal basis (Table IV, Figures 21, 27, 28). However, the large variance in quantum efficiency during the acclimation experiment shown in Figure 50b,d indicates that some changes in the photosynthetic electron transport may be occurring.

Thus additional studies to determine if seasonal changes occur in RuBP CO_2ase and the electron transport chain

are necessary in the Muskoka population of P. rufescens. The lack of any observed seasonal change in the Churchill population (Figure 16) can be advantageous in these studies. A correlation observed between a seasonal change in net photosynthesis and some biochemical process in the Muskoka population can be strengthened by showing that the same process does not change on a seasonal basis in the Churchill population. Thus Churchill could be used as a control situation for the study of the biochemical basis of acclimation in the Muskoka population.

Although seasonal changes in photosynthetic capacity mediated by enzymes are so far of limited occurrence in lichens, they appear to be particularly important in some circumstances. In addition to the seasonal change in the temperature optimum for net photosynthesis in the Muskoka population of P. rufescens, Coxson and Kershaw (1984) have observed a seasonal increase in photosynthetic capacity at low temperatures for Caloplaca trachyphylla, as an adaptation to winter chinook sequences. Enzymatic studies in lichens have been hindered due to their resistance to mechanical disruption, low protein yields, and high content of secondary compounds (Fahselt 1980; Vicente and Requena 1984). The successful isolation of the Fraction B FBPase, an enzyme similar to the photosynthetic FBPases of other plants, from P. rufescens will now enable a more indepth study of the

Photosynthetic capacity changes observed in lichens.

10. Summary

The seasonal net photosynthetic response to moisture, temperature and light has been examined in an arctic (Churchill, Manitoba) and a temperate (Muskoka Lake District, Ontario) population of the lichen Peltigera rufescens. The Churchill population shows a seasonally constant net photosynthetic rate whereas the Muskoka population exhibits a marked increase in the net photosynthetic capacity at 35 and 45°C during the summer months. This change in capacity can be experimentally manipulated by storage of air dry thalli under the appropriate combinations of temperature and daylength. It requires 10 days storage under low temperatures and short daylengths to induce a winter net photosynthetic response in the summer lichen. This capacity to change, as shown by the use of photosynthetic-illumination curves, involved changes in the light-saturated rate of net photosynthesis only. This indicated that the acclimation process was restricted to the dark reaction, presumably a seasonal enzymatic change in the Calvin cycle.

Fructose-1,6-bisphosphatase (FBPase) is an important controlling enzyme in the Calvin cycle. Enzymes exhibiting this activity were extracted from the thalli of the Muskoka

population of P. rufescens and purified via gel filtration and ion exchange chromatography into two fractions. The Fraction A enzyme had a pH optimum of 6.5, a substrate affinity of 0.2 mM and its activity was independent of the Mg^{2+} concentration in the assay. These characteristics are similar to those observed for a nonspecific acid phosphatase isolated from several plant species. In contrast, the enzyme activity of Fraction B has characteristics similar to the photosynthetic FBPase of algae and higher plants, with an absolute requirement for Mg^{2+}, a pH optimum of 8.5 (with no activity below pH 7.0), and a $S_{0.5}$ of 0.039 mM FBP at 25°C. It was therefore concluded that the Fraction B enzyme was involved in photosynthetic carbon metabolism while the Fraction A enzyme had a heterotrophic function.

The Fraction B enzyme was isolated from the summer and winter Muskoka population for a more extensive comparison of its temperature responses. It was shown that the substrate affinity was significantly higher in the summer enzyme at temperatures above 25°C. In addition, the activation energy of the summer isolate was lower than that of the winter isolate. During experimentally induced temperature acclimation, the changes in FBPase paralleled the above mentioned changes in net photosynthesis. These kinetic differences largely explain the seasonal changes in net photosynthetic capacity observed in the response matrix and support the enzymatic theory of acclimation suggested by the

photosynthetic curves of the Muskoka population.

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12. Appendix

TABLE A1. Purification of the Fraction B FBPase from Extraction 1:1 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	280			7.263		4.918	1.48
2	33			0.753		0.572	12
3	43	34	0.06	2.049		.0283	7.24

1 nmol F6P formed·µg protein^{-1}min^{-1}

2 µmol F6P formed· min^{-1}

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate

Quantitiy of lichen extracted = 3.07 gm

TABLE A2. Purification of the Fraction B FBPase from Extraction 12 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	610	660	0.06	37	0.048	32	1.16
2	180	207	0.04	8.2	0.020	4.2	1.96
3	17	34	*	*	*	*	*
4	43	13	0.39	5.16	0.038	0.50	10.3
5	18	1.44	1.43	2.06	0.06	1.05	23.8

[1] nmol F6P formed·µg protein^{-1}·min^{-1} [2] µmol formed·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

TABLE A3. Purification of the Fraction B FBPase from Extraction 13 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	745	774	0.06	43.74	0.046	33.78	1.29
2	150	505	0.07	35.39	0.026	13.2	1.77
3	25	99.5	*	*	*	*	*
4	87	37.4	0.29	10.7	0.079	2.96	3.61
5	10	3.89	0.44	1.7	0.289	1.05	1.35

1 nmol F6P formed·µg protein^{-1}min^{-1} 2 µmol F6P formed·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

Quantity of lichen extracted = 42gm

TABLE A4. Purification of the Fraction B FBPase from Extraction 14 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	850	909	0.08	73.7	0.09	85.4	0.87
2	210	594	0.07	42.1	0.02	13.5	3.2
3	15	71	*	*	*	*	*
4	63	33	1.90	6.4	0.04	1.3	4.9
5	13	1.38	8.01	1.1	0.10	0.14	7.22

1 nmol F6P formed·µg protein^{-1}min^{-1} 2 µmol·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

Quantity of lichen extracted = 51gm

TABLE A5. Purification of the Fraction B FBPase from Extraction 15 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	1030	824	0.06	52.5	0.07	58.2	0.9
2	173	524	0.05	24.3	0.02	9.6	2.53
3	13	69	*	*	*	*	*
4	38	67	0.41	27.68	0.13	8.5	3.26
5	18	2.2	1.65	3.67	0.06	0.14	25

1 nmol F6P formed·µg protein^{-1}min^{-1} 2 µmol·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

Quantity of lichen extracted = 60gm

TABLE A6. Purification of the Fraction B FBPase from Extraction 16 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	1260	1294	0.031	40	0.056	72.6	0.55
2	300	697	0.066	46	0.021	14.41	3.29
3	15.5	88	*	*	*	*	*
4	43	24	0.55	13.6	0.019	0.47	29
5	20	1.0	1.91	1.92	0.036	0.06	48

1 nmol F6P formed·µg protein^{-1}min^{-1} 2 µmol·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

Quantity of lichen extracted = 83gm

TABLE A7. Purification of the Fraction B FBPase from Extraction 17 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	1020	747	0.16	121	0.06	44.4	2.73
2	180	363	0.09	34	0.05	17.9	1.91
3	15	45	*	*	*	*	*
4	61	19.9	1.11	22	0.06	1.25	18
5	22	1.21	8.67	11	0.01	0.1	114

1 nmol F6P formed·µg protein^{-1}·min^{-1} 2 µmol·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

Quantity of lichen extracted = 55gm

TABLE A8. Purification of the Fraction B FBPase from Extraction 18 of the Muskoka population of P. rufescens.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	700	750	0.05	46.6	0.07	50	0.8
2	180	360	0.06	20.2	0.06	21.4	1.9
3	7	38	*	*	*	*	*
4	51	14	0.48	6.68	0.12	1.6	4.1
5	11	1	3.7	3.6	0.15	.02	25

1 nmol F6P formed·µg protein^{-1}·min^{-1} 2 µmol·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

Quantity of lichen extracted = 37gm

TABLE A9. Purification of the Fraction B FBPase from Extraction 19 of the Muskoka population of P. rufescens.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	710	844	0.01	11.1	0.02	18	0.6
2	175	434	0.14	61.5	0.02	9.9	6.12
3	7		*	*	*	*	*
4	54	7	3.56	24.9	0.19	1.35	18.5
5	8	0.14	8.36	9.3	0.13	0.15	55.7

1 nmol F6P formed·µg protein^{-1}min^{-1} 2 µmol·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4= Sephadex G-100; 5= DEAE Sephacel

Quantity of lichen extracted = 38gm

TABLE A10. Purification of the Fraction B FBPase from Extraction 20 of the Muskoka population of *P. rufescens*.

Step	Volume (ml)	Total Protein (mg)	FBPase Activity pH 8.5 Specific[1]	pH 8.5 Total[2]	pH 6.5 Specific[1]	pH 6.5 Total[2]	Ratio
1	790	616	0.05	29	0.08	49	0.6
2	140	540	0.03	14	0.03	15.5	0.9
3	8	60	*	*	*	*	*
4a	3	3.7	0.01	0.04	0.42	1.4	0.1
4b	75	31	0.21	6.45	0.16	5.0	1.3
5	16	2.8	1.74	1.74	0.39	0.2	7.2

1 nmol F6P formed·µg protein^{-1}min^{-1} 2 µmol·min^{-1}

* FBPase activity was strongly inhibited by ammonium sulfate, therefore activity could not be determined at this step.

STEPS

1=crude extract; 2=pH 4.5 pellate; 3= 40-80% ammonium sulfate pellate; 4a= Sephadex G-100, Fraction A; 4b= Sephadex G-100, Fraction B; 5= DEAE Sephacel, Fraction B

Quantity of lichen extracted = 40gm

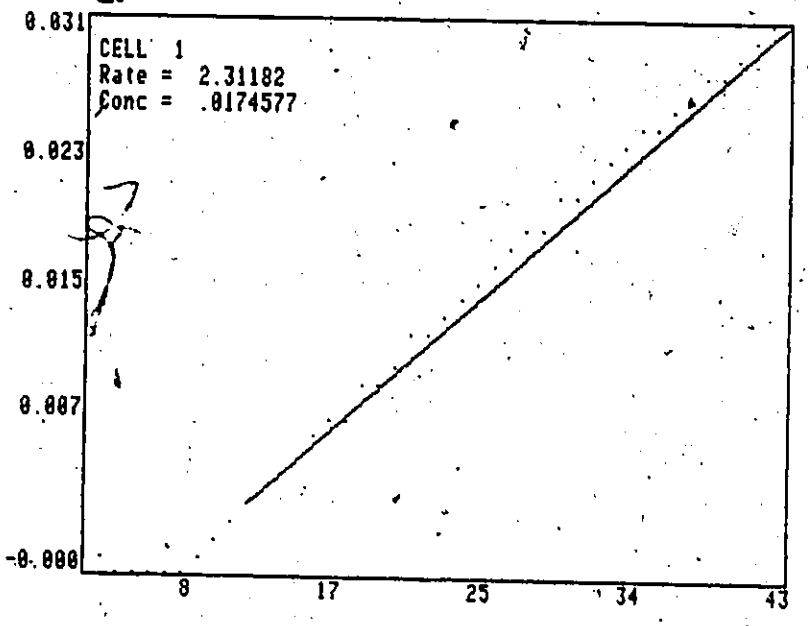
Appendix 2.

Scatterplots of the progress curves from representative assays demonstrating the application of the chord analysis in determining the velocity and substrate concentration used n calculatiog the $S_{0.8}$ values. The ordinate is cycle number and the abscissa is absorbance.

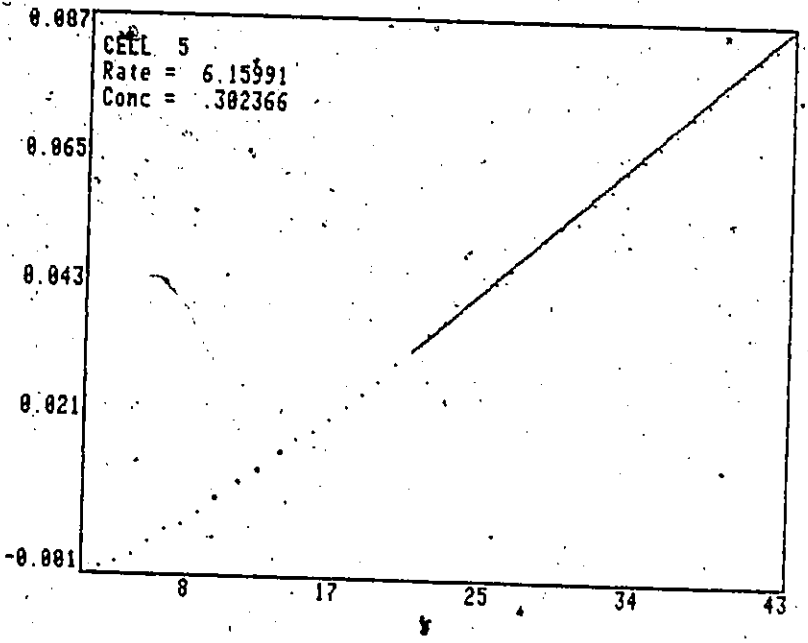
The examples shown are:

	Extraction	Temperature °C	Initial Concentration mM FBP
a	12	5	0.020
b	12	5	0.313
c	17	30	0.039
d	19	30	0.010
e	19	30	0.020
f	19	30	0.039

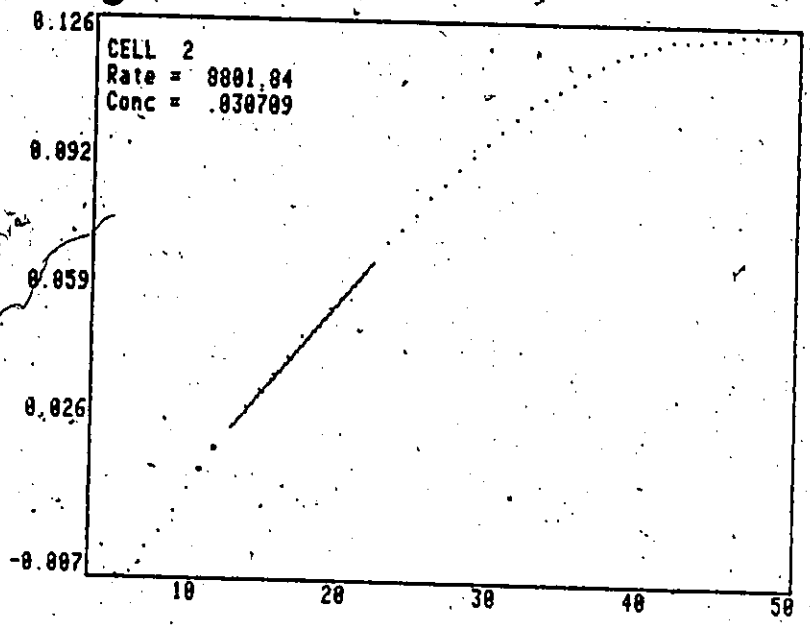
a


0.031
0.023
0.015
0.007
-0.000
CELL 1
Rate = 2.31182
Conc = .0174577
8
17
25
34
43

b


0.087
0.065
0.043
0.021
-0.001
CELL 5
Rate = 6.15991
Conc = .302366
8
17
25
34
43

c


CELL 2
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Conc = .030709
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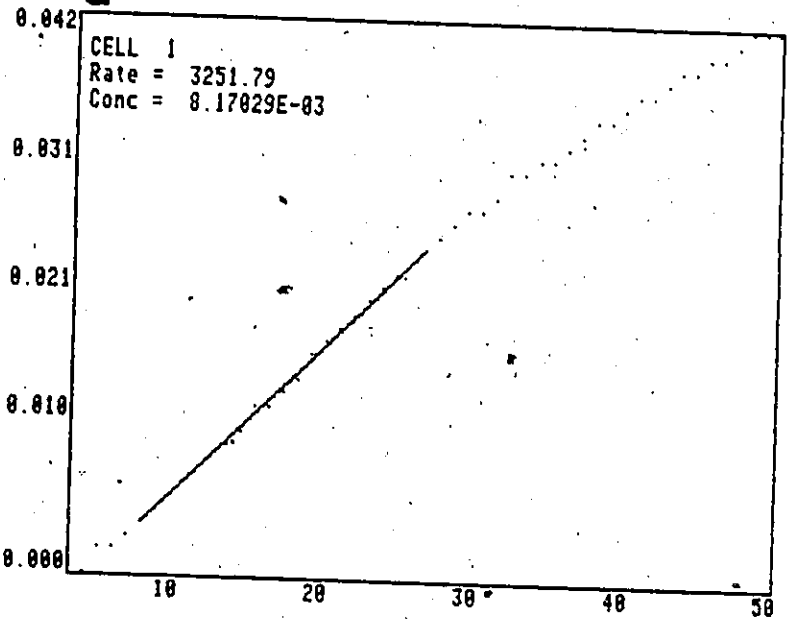
0.092
0.059
0.026
-0.007
10
20
30
40
50

d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Rate = 3251.79
Conc = 8.17029E-03
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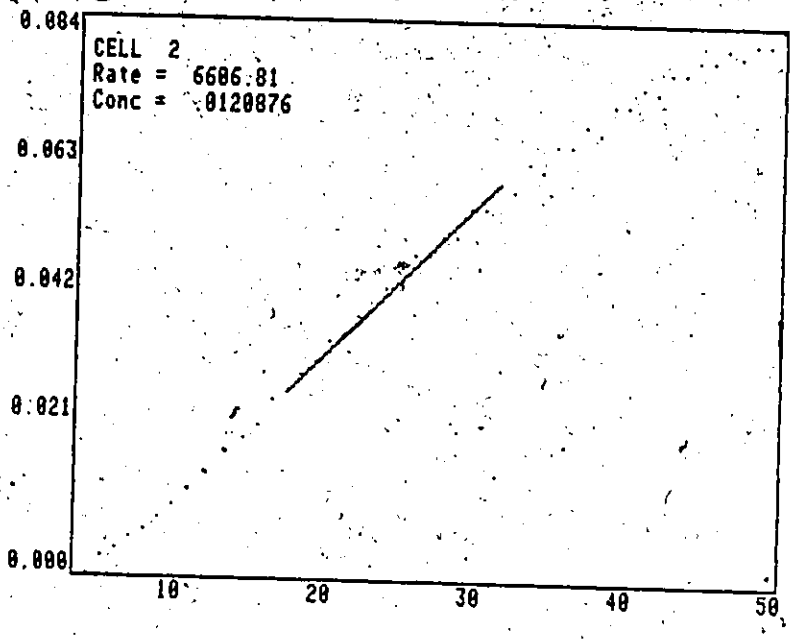
0.031
0.021
0.010
0.000
10
20
30
40
50

e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Conc = .0120876
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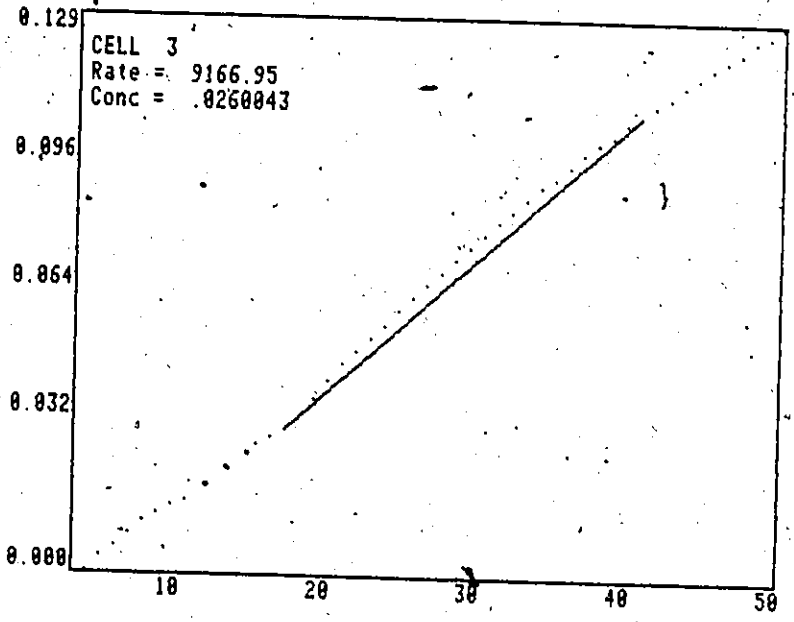
0.063
0.042
0.021
0.000
10
20
30
40
50

f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Conc = .0260043
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0.064
0.032
0.000
10
20
30
40
50