RESPONSES AND ADAPTATIONS OF ROOT GROWTH AND METABOLISM TO LOW TEMPERATURE

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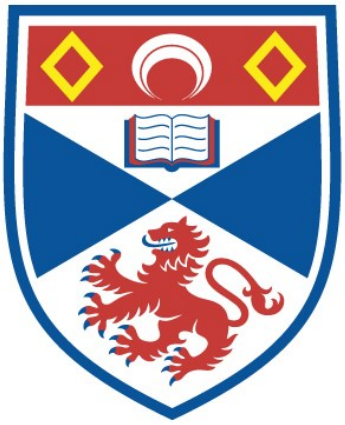
University of St Andrews



1975

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Abstract. A comparative study of the carbohydrate metabolism of roots of pea (Pisum sativum var. meteor) and maize (Zea mays var kelvedon glory) seedlings was undertaken at low temperatures (2-14°C) with the aim of demonstrating differences between these species which may be associated with the differing growth capacities of their roots over this temperature range. Pea roots displayed linear growth rates at all temperatures tested whereas maize roots ceased growth over five days at temperatures below 6°C

At the respective temperatures which were minimal for root growth of the two species, roots behaved similarly with regards to soluble sugar content; firstly, total content was maintained in the roots at the initial level, and secondly, sucrose content was at its highest value and glucose content at its lowest. With rise in temperature sucrose content declined while glucose content increased. In maize roots kept at those temperatures where growth was not sustained this relationship broke down. Total sugar content of the roots was not maintained, glucose content was abnormally high and sucrose content very low. Similarly, respiration rate of maize roots at 2°C was abnormally low.

When seedlings were grown with roots bathed in an external solution of glucose at 2°C (or of glucose or of sucrose at 6°C), the disturbances to sugar metabolism and respiration rate of maize roots were partially alleviated and this was associated with a greater amount of growth made by the roots.

Examination of the activity and Km of acid invertase extracted from the roots and partially purified, showed that the sucrose levels in roots of both species were inversely related to invertase activity. However in pea, but not in maize roots, Km values for invertase showed a lowering in value after growth of seedlings at 2°C compared with 20°C. Furthermore, in pea, after growth of seedlings at 14 or 20°C Km determined at 2°C was

significantly lower than when determined at 14°C. These properties are of adaptive significance at low temperatures since they will act to maintain an appreciable reaction rate. Shifts in Km of a homeostatic nature with respect to temperature were not recorded for invertase from maize roots and in this species the failure to control invertase activity at low temperatures with consequent depletion of sucrose may be associated with the inability of this species to show sustained growth at 2°C.

Examination of the Km of MDH likewise revealed a shift in Km value tending to buffer the effect of temperature on reaction rate for MDH from pea but not from maize roots.

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A thesis submitted for the degree of Doctor of Philosophy, University of St. Andrews, February 1975.

CAREER

I matriculated at the University of Oxford (Brasenose College) in October 1968 and graduated in July 1971 with a second class Honours degree in Botany.

In October, 1971, I matriculated at the University of St. Andrews under Ordinance General No. 12 and later as a candidate for the degree of Ph.D. under Resolution of the University Court, 1967, No. 1.

DECLARATION

I hereby declare that this thesis is based upon work done by myself, that the thesis is my own composition, and that it has not previously been presented for a higher degree. The research work was carried out in the Department of Botany, University of St. Andrews, under the supervision of Dr. R. M. M. Crawford.

CERTIFICATE

I hereby certify that Terence John Huxter has been engaged upon research work for a minimum of nine terms under my supervision, that he has fulfilled the conditions of Ordinance No. 12, and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

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I also wish to thank Professor J. A. Macdonald, in whose department the work was carried out, and all academic and technical staff of the Botany Department for freely providing much helpful advice during the course of the work for this thesis.

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From October 1971 to October 1974 I was awarded a St. Andrews University Research Scholarship. I am very grateful to have received this award.

LIST OF ABBREVIATIONS

Acetyl CoA	Acetyl Coenzyme A
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
^{o}C	degree centigrade
$C_6H_{12}O_6$	hexose
cm	centimetre
CRP	chill resistent plant
CSP	chill sensitive plant
DMSO	dimethyl sulphoxide
2,4-DNP	2,4-dinitrophenol
DW	dry weight
FW	fresh weight
g	gram
GLC	gas liquid chromatography
Hexose-P	Hexose phosphate
HMDS	hexamethyldisilazane
HMDSO	hexamethyldisiloxane
hr	hour
IU	International units of enzyme activity
Km	Michaelis constant
M	Molar solution
MDH	malate dehydrogenase
mg	millegram
min	minute
ml	millelitre
mm	millemetre
N	normal solution
NAD	nicotinamide adenine dinucleotide

NADH	nicotinamide adenine dinucleotide reduced
nm	nanometre
N.T.P.	normal temperature and pressure
OAA	oxaloacetate
PR-ATP pyrophosphorylase	Phosophoribosyl adenosine triphosphate pyrophosphorylase
Q10	temperature coefficient
R.Q.	respiratory quotient
rpm	revolutions per minute
sec	second
TMCS	trimethylchlorosilane
UDPG	uridine diphosphoglucose
UDP	uridine diphosphate
ul	microlitre
uM	micrometre
vol.	volume

CONTENTS

CHAPTER 1

INTRODUCTION

In many temperate regions of the world the successful production of certain crop plants is impossible because, early in the growing season, cold temperatures in the soil retard germination of seeds and subsequent root growth. In Britain, maize is an example of such a plant. Only a few varieties can be grown and the plants are usually harvested when young as a forage crop since mature cob-bearing plants do not have sufficient time to develop (Milbourn, 1972).

Although the British summer, at least in southern England, is favourable for the growth of maize, the cold spells in early spring at the time of planting severely retard seedling establishment. This behaviour, of course, reflects the semi-tropical ancestors of modern maize and contrasts with crop plants originating from temperate regions of the world, e.g.: cereals such as oats and wheat, and legumes, peas and beans. These crops can be planted successfully in early spring and show good germination and seedling growth.

In these field situations, the causes of a low percentage germination have been thoroughly investigated by Harper (1954, 1955, and 1956). The low temperatures by themselves may not be the prime cause. Planting is often in cold wet soils and often heavy pre-emergence mortality is then pathogen induced. A review by Wernham (1951) indicates the range of pathogens in the soil

known to be active in causing seedling blight at low temperatures.

Once seeds have germinated, however, and at least the radicle has become established, the effects of low temperatures (0-10°C) on the subsequent growth and metabolism of roots has not been extensively investigated. Yet this is an area of study which may be of considerable benefit to the production, in Britain, of crop plants originating from warmer areas of the world. Also, such a study may provide an insight into ways in which low temperatures affect root metabolism. Early root growth is a phase of development particularly suitable for the study of temperature effects since, at this early stage, the root system is small and has a definable nutrient supply, the reserves of the seed. In older plants the complexity of the root system makes a study of its growth difficult, and factors which affect the capacity of the shoot to supply nutrients to the roots (e.g.: factors which affect photosynthesis) will have an influence on root growth independent of any experimental treatment to which the roots might be subjected.

Roots of different species display a wide range of temperatures which are the minimum at which growth occurs (Table 1). It is not at first obvious why this should be so, why, that is, growth should not proceed at some positive rate at temperatures above the freezing point of water. All the biochemical reactions involved in metabolism might be expected to function at 1°C as they do at 20°C, only at a slower rate. Several reasons can, however, be suggested for different minimum growth temperatures (Levitt 1969):

1. One or more metabolic processes essential for growth may stop at a particular low temperature.
2. A minimum rate of metabolism may be necessary to produce

Table 1. The lowest temperature recorded for radicle emergence and normal seedling production in a selection of vegetable species (Kotowski 1926, Harrington 1962).

Species	Lowest temperature recorded for:			
	Radicle Emergence °C	% of sample	Normal seedlings °C	% of sample
Lactuca sativa L.	0	99	0	98
Spinacia oleracea L.	0	91	0	83
Pastinacea sativa L.	0	90	0	51
Allium cepa L.	0	98	0	90
Brassica rapa L.	0	1	0	1
Pisum sativum L.	-	-	4	84
Raphanus sativus L.	-	-	4	42
Daucus carota L.	5	48	5	48
Zea mays L.	5	2	10	47
Asparagus officinalis L.	5	52	10	61
Hibiscus esculentus L.	5	31	15	74
Beta vulgaris L.	4	-	8	100
Brassica oleracea L. var capitata	4	-	8	78
Brassica oleracea L. var botrytis	4	-	8	50
Petroselinum crispum Nym.	-	-	8	55
Lycopersicon esculentum Mill.	10	92	10	82
Capsicum frutescens L.	10	98	10	1
Cucumis sativus L.	10	3	15	95
Phaseolus vulgaris L.	11	-	18	92
Solanum melongena L.	-	-	18	21
Cucumis melo L.	-	-	18	38
Phaseolus limensis Macf.	-	-	18	60

sufficient energy for growth. The slow rate induced by low temperatures may only result in sufficient energy production for cell maintenance and not for growth (Farrell and Rose, 1967).

3. Respiration may be uncoupled from growth at low temperatures.

4. Low temperatures may cause structural changes within cells, e.g.: conformational changes in membrane lipids.

These four possible reasons illustrate the two prime effects which low temperatures have on living systems, effects on rates of reactions and effects on cellular structures. Thus 1. and 2. are concerned with the low rates of biochemical reactions induced by low temperatures, while 3. and 4. are concerned with structural changes within cells that may occur at low temperatures.

More evidence showing that low temperatures affect growth through these mechanisms has come from micro-organisms than higher plants. The available evidence for each of the possibilities is briefly discussed below.

1. Cessation of a particular reaction at low temperatures

The vast majority of biochemical reactions occuring in living organisms are catalysed by enzymes. A given low temperature may act in two ways to cause the cessation of enzyme activity. The synthesis of the enzyme may stop or the enzyme may be present in the cell but inactivated. How this may occur in higher plants can be speculated from experiments with micro-organisms.

Dealing first with enzyme synthesis, it has been shown that the sensitivity to temperature of many of the regulatory processes of enzyme synthesis is much greater than the sensitivity of the majority of other metabolic processes in micro-organisms

(Langridge and McWilliam 1967). Thus tryptophane induces tryptophanase synthesis in E. coli at 30°C but not at temperatures below 15°C (Ng and Gartner 1963). Glutamic acid decarboxylase synthesis by E. Coli requires the presence of the substrate above 30°C but below this temperature the enzyme is synthesised constitutively (Halpern, 1961). A third example from E. coli was a temperature sensitive mutant isolated by Gallant and Stapleton (1963). Alkaline phosphatase was synthesised constitutively at high temperatures but below 21°C synthesis of the enzyme was repressed by a high inorganic phosphate concentration in the cell.

In wild-type strains the synthesis of such inducible and repressible enzymes has been shown to be controlled by the interaction of specific metabolites, inducers or corepressors, with specific cytoplasmically diffused gene products termed aporepressors (Jacob and Monod, 1961). Conceivably, low temperature could alter one of at least three reactions involving the aporepressor, a) the rate of production of the aporepressor, b) the interaction of a specific aporepressor with its particular inducer or corepressor, c) the activity of the "activated aporepressor" at the site of enzyme synthesis, these alterations resulting in loss of synthesis of an enzyme critical for continued growth.

An example of a temperature-induced disturbance in the metabolism of a higher plant comes from the work of Ketallapper (1963). The physiological basis for this upset has been partially traced. His work followed the pioneer work of Bonner (1957), who proposed that climate affects specific biochemical events, and that non-optimal conditions cause a shortage of one or more metabolites. This condition could arise if a particular enzyme stopped

functioning as temperature dropped below a critical value. He further suggested that it should be possible to overcome such deficiencies by external supplements. Ketallapper demonstrated, for pea (Pisum sativum L.), that spraying plants with a sucrose solution over a four week period could offset a reduction in the dry weight of the plants caused by an unfavourable temperature regime. At the end of the experimental period the dry weight of treated plants was the same as a control group of plants grown at a temperature regime previously found to give the greatest dry weight increase over the four week period. The apparent sucrose shortage was thought to be due to either impaired photosynthesis or increased respiration. A measurement of these two factors under the two temperature regimes revealed that carbon dioxide fixation was very low at the unfavourable temperature compared with the "optimum" condition. Further experiments showed that vitamin B, ribonucleotide mixtures and vitamin C could promote growth under certain other unfavourable temperature regimes compared with no promotion of growth under "optimal" conditions. In this example the data are not sufficient to decide if a single metabolic step has been inactivated at the temperatures employed.

The second possibility, that a particular reaction may cease at a specific low temperature because of enzyme inactivation, has received attention recently; (Brandts, 1967; Levitt, 1969). They suggest that enzymes can be denatured by low temperatures as they can by high temperatures. The minimum temperature for growth of a species could reflect the temperature at which the enzyme most sensitive to low temperature undergoes denaturation. Brandts discussed the way temperature changes affect the strength of two physical bonds which are very important for enzyme conformation,

the hydrogen bond and the hydrophobic bond. The strength of hydrogen bonds is unaffected by temperatures between 0 and 30°C but the strength of hydrophobic bonds decreases with temperature over this range. The degree of unfolding of an enzyme and the temperature at which this occurs depends on the proportion of hydrophobic to hydrogen bonds for any particular enzyme. In turn, enzyme activity is known to depend on a specific folding of its component polypeptide chains, thus enzyme activity at low temperatures may be directly related to the hydrophobic: hydrogen bond ratio. If this is high the enzyme will be relatively unfolded and therefore relatively inactive and if the ratio is low relatively greater activity can be expected at low temperatures. Because of the technical difficulties involved in investigating this hypothesis no examples have yet been reported where a minimum temperature for growth is attributable to the denaturation of a of a specific enzyme.

An enzyme may be inactivated by low temperatures by means other than conformational change. Thus, where two reactions compete for the same substrate a decrease in temperature will favour the activity of the enzyme with the lower activation energy (Langridge 1963). If the difference in activation energy of the enzymes is large one reaction may cease to function, and this rate imbalance may result in growth inhibition.

Another form of enzyme inhibition at low temperature, demonstrated in E. coli, is the modification of an existing mechanism controlling rate of reaction, end product inhibition (Ingraham and Maaloe, 1967). A cold sensitive mutant required histidine for growth to occur at temperatures below 20°C. Apart from this the strain was wild-type in character. It was found in the mutant that PR-ATP pyrophosphorylase (the first enzyme in the

sequence of enzymes responsible for histidine biosynthesis) was 1000 times more sensitive to feed back inhibition by histidine than the enzyme in the wild type. Moreover, at warmer temperatures (greater than 20°C) feed-back inhibition of enzyme-activity by histidine was less marked than at colder temperatures in both the wild-type and cold-sensitive strains. In the latter the greater sensitivity to feed-back inhibition at low temperatures in combination with the much greater sensitivity to histidine concentration by the mutant enzyme reduced the histidine concentration in the cells of the cold-sensitive mutant to a value insufficient to allow growth to occur.

2. <u>A base rate for metabolism sufficient for cell maintenance but not for growth</u>.

An indication that energy production is sufficient, at a particular temperature, only for cell maintenance and not for growth, may come from experiments designed to increase energy production. Energy production, in cellular terms, means production of high-energy phosphate compounds to drive biosynthetic reactions. These compounds are produced at a rate dependent on respiration rate. Thus it is useful to find the consequences of increased respiration rate for growth, at any specific low temperature. Respiration rate may be increased if, for example, it is substrate limited (possibly because production or transport of sucrose or its conversion to hexose is limiting), by substrate feeding. If this increased respiration rate is accompanied by a resumption or increase in growth then a specific effect of temperature on metabolism has been isolated. Very few experiments have been designed to investigate this possibility but those of Rose and Evison (see

Farrell and Rose, 1967) yield interesting results. They found that the minimum temperatures for growth of a mesophilic species of Arthrobacter and Candida approximated to the respective temperatures at which the organisms were unable to respire exogenously supplied glucose. The inability of the organisms to utilise glucose may have been because, at the low temperature employed, respiration, utilising internal substrates, was unable to provide sufficient energy for the uptake of the glucose, with the consequent cessation of growth from lack of a carbon source. On returning to warmer temperatures the organisms resumed growth thus the low temperature treatment had not disrupted metabolism, in particular respiration, irreversibly, but the low rate induced was not sufficient to provide energy for uptake and growth.

3. Low temperature uncoupling of respiration.

The occurrence of respiratory uncoupling (i.e.: electron transport without oxidative phosphorylation) at low temperatures has not reveived as much investigation as at high temperatures (the latter is discussed by Ward, 1968). In both cases growth is stopped or severely retarded because of failure in the supply of high energy phosphate compounds. In one reported investigation of low temperature uncoupling Creencia and Bramlage (1971), working with flint corn (Zea mays var indurata), found that exposure of seedlings to 0.3°C caused visual injury to leaves after 36 hr. This was accompanied by a rise in oxygen uptake by chilled leaf segments compared with controls (in both cases oxygen uptake was determined at 25°C but the experimental plants had previously been kept at the chilling temperature). 2,4-DNP stimulated oxygen uptake

only in the control.

In leaf segments from chilled seedlings returned to 21°C, oxygen uptake gradually stabilised to the level of the control and 2,4-DNP was then effective in stimulating oxygen uptake both in the chilled and control material. These results indicate that the uncoupling agent was only effective on plants kept at the warm temperature. At the chilling temperature natural uncoupling was occurring but if the chilling period was relatively short the coupling of respiration was restored at the warm temperature. However, uncoupling was not detectable immediately following introduction to the chilling temperature. After 12 hr no uncoupling was apparent. It only became apparent between 12 and 36 hr and so may only be a secondary symptom of the physiological effects caused by the low temperature.

4. Structural changes in membranes at low temperatures.

Recently the possibility that structural changes within membranes, induced by low temperatures, may be responsible for different minimum growth temperatures has received much investigation (Farrell and Rose, 1967). The composition of membrane lipids is thought to be of critical importance in determining how the membrance will behave at low temperatures. Unsaturated fatty acid derivatives confer much more flexibility on membranes than saturated fatty acids, and this becomes important at colder temperatures. Thus Lyons and Raison (1970) and Lyons et al. (1964) compared mitochondria from a series of chill resistant and chill sensitive vegetables and found that the mole-fraction of unsaturated fatty acid in the mitochondria of chill resistant plants

was greater than in chill sensitive plants. This correlated with a greater capacity of the mitochondria to swell in various solutions (i.e.: with greater flexibility), and with a Q10 value for respiration which was constant over the temperature range 1.5-25^{o}C. The Q10 for the chill sensitive vegetables was constant above 10^{o}C but below this temperature the Q10 value rose considerably. Thus respiration rate dropped off abruptly at colder temperatures.

They suggest that the lipoprotein complex composing the mitochondrial membrane is on the borderline of a reversible phase transition from a liquid-crystalline structure to a coagel. As temperature falls to a critical value the hydrocarbon chains crystallise abruptly and there is then a modification of physiological function in the chill sensitive plants. A marked fall in respiration is one manifestation of this. The higher unsaturated fatty acid content of the mitochondrial membranes of the chill resistant plants is sufficient for these membranes not to undergo this crystallisation until a much lower temperature is reached.

Recently an extensive survey of the leaves of chill sensitive (CSP) and chill resistant (CRP) plants by Wilson and Crawford (1974 a and b) revealed significantly more unsaturated fatty-acid in the phopholipid fraction of leaves from the CRP as compared with the CSP and, most important, hardening of CRP at 12^{o}C was accompanied by an increase in the amount of unsaturated fatty acid in the phospholipid fraction. Their work points to a disturbance in fatty acid metabolism as the cause of chilling damage in chill sensitive plants. The percentage of linolenic acid and the total fatty acid decreases markedly in these plants when they are kept at 5^{o}C. Hardening is thought to slow these detrimental processes and thus reduce the degree of damage.

Other authors have postulated an effect of low temperature on membrane structure. Baxter and Gibbons (1962) suggested that the membrane transport systems of mesophilic and psychrophilic (cold adapted) microorganisms are affected differently by low temperature. They worked with strains of Candida (a yeast). The psychrophile respired endogenous reserves at a greater rate than the mesophile at all temperatures up to 30°C. The psychrophile oxidised exogenously supplied glucose at appreciable rates at 0°C whereas the mesophile failed to do so below 10°C. Finally, in uptake studies using glucosamine the psychrophile showed a high rate of uptake at 0 and 10°C, whereas uptake by the mesophile was not appreciable below 20°C. Thus they suggested that only the psychrophile could transport solutes at the lower temperatures and thus display a much higher growth rate under these conditions.

From the examples discussed above several physiological reasons can be suggested for the different minimum growth temperatures of roots of different species. They fall into two groupings according to whether temperature exerts its effect on rates of reactions or on cellular structures. These are tabulated in Table 2.

The aim of this thesis is to examine in detail the physiological differences, existing between the roots of species, which may be responsible for differing growth rates at low temperatures, with a view to providing an understanding of the extent to which any of the mechanisms described in Table 2 are operative. Secondly, an aim of the thesis is to determine what adaptations exist in the root physiology of species able to grow at temperatures just above zero as compared with those unable to grow at such low temperatures. In particular the growth of the radicle is examined

Table 2. Physiological mechanisms by which low temperatures may bring about the cessation of root growth.

Mechanisms affecting rates:	Reference:
1. One specific metabolic reaction stops, due to	Bonner (1957)
a) cessation of enzyme synthesis	Langridge and McWilliam (1967)
or b) enzyme present but inactive	Ingraham and Maaloe (1967)
2. Energy production insufficient to support growth, due to	
a) substrate limitation	Farrell and Rose (1967)
or b) uncoupling at low temperature	Creencia and Bramlage (1971)
Mechanisms affecting structure:	
3. Membrane lipids "solidify", with possible results of:	Lyons and Raison (1970)
a) uncoupling of respiration from ATP synthesis	
b) breakdown of transport mechanisms	Baxter and Gibbons (1962)

over the first few days of seed germination. Thus this work not only has a bearing on root growth but also on seedling establishment at low temperatures.

CHAPTER 2

GROWTH RATE OF ROOTS OF PEA AND MAIZE BETWEEN 2 AND 14°C

Introduction.

Growth is notoriously difficult to define, but probably the best working definition is "an irreversible increase in volume" (Evans, 1963). Growth of biological material can be measured in a variety of ways. In this thesis, where the growth of roots is measured, it is measured as the increase in length of the root over a number of days (Torrey 1956). This method is employed because it does not involve the destruction of the material. Since the increase in size of roots, at least in the early stages of growth, is mainly by increase in length, this method of measurement is particularly suitable.

The growth of two species was studied, pea (Pisum sativum L. var meteor) and maize (Zea mays L. var kelvedon glory - a sweet corn). These two species were chosen for the following reasons:
1. They differ markedly in their temperature requirements for growth. The minimum temperature for the growth of pea, both the root and the shoot, is around 1°C (Lang, 1965, reports the germination of peas on ice) whereas for maize it is between 5 and 10°C (2% radicle emergence was reported at 5°C, Harrington, 1965, but shoot growth requires a temperature between 10 and 12°C, Lehenbaure, 1914, Harrington, 1965). It was thought that species displaying such different minimum growth temperatures would be most likely to show differing physiological characteristics in the

range 2-14°C which would be directly due to the temperature treatments.

2. The two species could easily be grown from seed and an abundant supply of root material obtained.

3. The root system initially consists of only a single radicle, whose growth could very easily be measured.

4. Both seeds contain similar reserve materials with which to support root growth, they are predominantly "starchy" seeds (Stiles and Leach, 1933, Stiles, 1960, Toole, 1924, Table 3).

5. Both are crop plants of some importance in Britain, and the production of maize is currently being expanded (Herwin, 1973).

Selection of root material.

A standard procedure was developed for obtaining the roots used in these growth studies and all subsequent experiments involving pea and maize. Seeds of the two species were surface-sterilized by 15 min submersion in a 1% hypochlorite solution, and rinsed thoroughly in distilled water. They were then set to germinate in plastic boxes on a double layer of filter paper, moistened with distilled water. The boxes were kept in darkness at 20°C in incubators with continuous air circulation. Over the first 36 hr the seeds were watered every 12 hr, and subsequently at 24 hr intervals. Each time, sufficient distilled water was added for there to be a slight film of water over the saturated filter paper. The boxes were fitted with lids which were kept slightly displaced. To prevent the build up of anaerobic conditions around the seeds air was gently blown into the boxes at the times of watering. Also, after 48 hr, the seeds were rinsed in distilled water, the boxes cleaned

Table 3. Reserve materials in the seeds of pea and maize.

Reserve material	% DW of the seed		
	Pea[x]	Maize	
Carbohydrate	53	63[*]	83[+]
Protein	23	11	11
Fat	2	8	4

[x]Data of Stiles 1960.

[*]Data of Stiles and Leach 1933.

[+]Data of Toole 1924.

and the filter paper linings renewed. In the case of maize, this procedure was repeated again after 96 hr. Under these conditions it was found, in a preliminary trial, that the greatest number of seedlings with roots between 1 and 2 cm long was present after "germination periods" of 72 and 120 hr respectively for pea and maize.

It was at these respective times that the seedlings used in all experiments were selected. Only those with roots between 1 and 2 cm long were used so as to give initial uniformity between experiments (Brown and Broadbent, 1950, and Sexton and Sutcliffe, 1969, report that the length of the meristematic and extending zones of maize roots varies with age).

Measurement of growth.

The conditions for growth were the same as for germination, i.e.: in boxes on moist filter paper in the dark with the same schedule of watering. Twenty-five seedlings were placed in each box (Plate 1). The boxes and filter papers were renewed at 48 hr intervals. Watering was with distilled water previously equilibrated to the temperature at which growth was being measured. Growth of roots was recorded at four temperatures, 2, 6, 10, and 14^{o}C, by measuring the length of a large number of roots with a millimeter rule (the root was considered to start at the point of attachment of the cotyledons in pea and of the scutellum in maize). The first measurement was made at the end of the germination period when the seedlings were moved to the cold incubators and is referred to as time 0 hr. Thereafter, measurements were recorded at 24 hr intervals for 5 days. The roots were kept in strict order

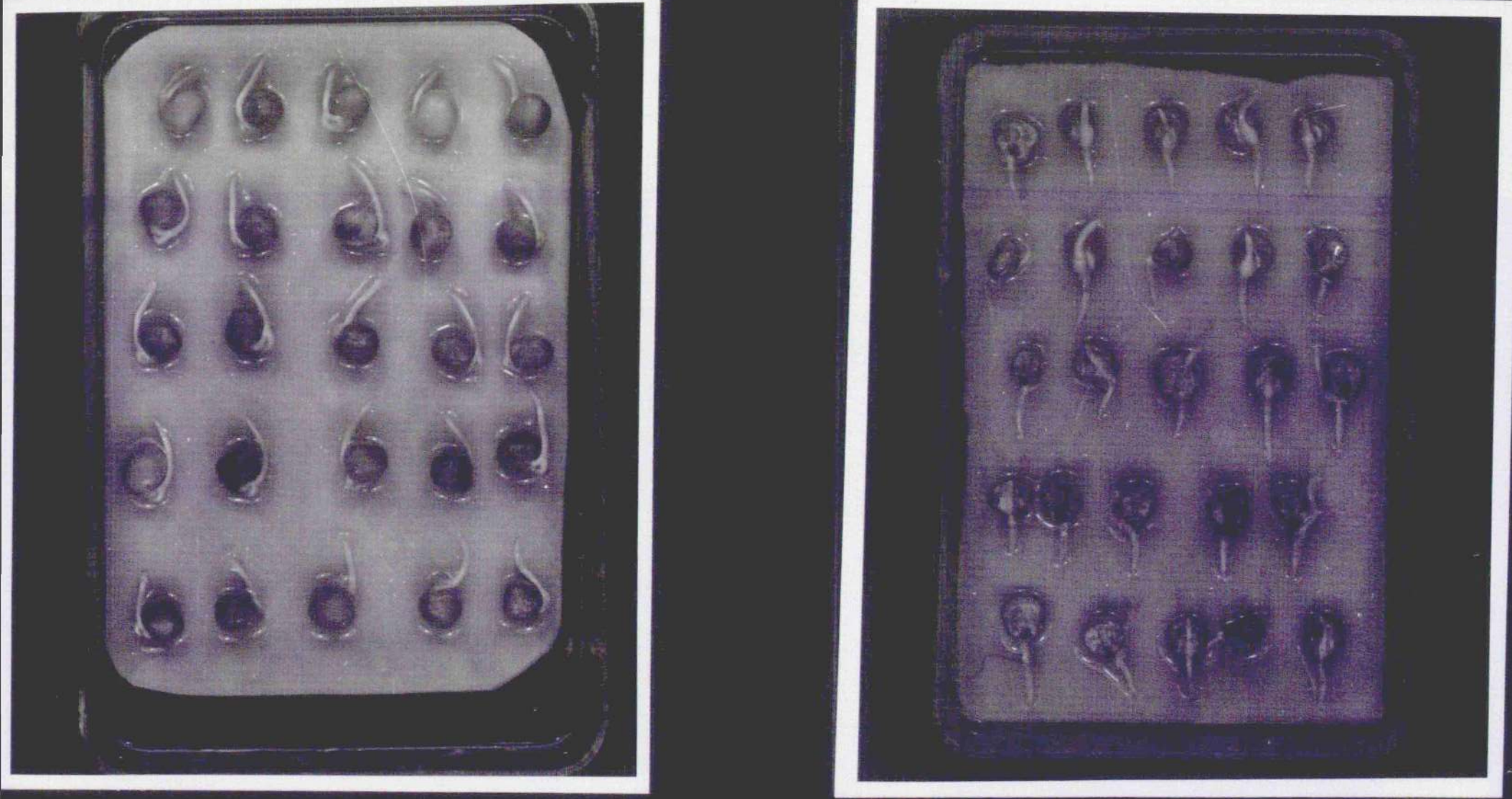


PLATE I. Seedlings of pea and maize as arranged for root growth studies.

in the boxes so that an increase in length from the initial length for each individual root could be computed daily. From these values the mean increase in length, with standard error, from time 0 hr, was calculated.

Results and discussion.

The results are presented as mean increases in root length plotted against time grown at each particular temperature, with standard error bars on the points, Figs. 1-4. Table 4 records the numbers of seedling roots measured at each temperature to obtain the values plotted in Figs. 1-4. To obtain values for mean increases in length with standard errors small in comparison with the actual values, larger numbers of roots were used at the lower temperatures. Also recorded is the rate of increase in length of the roots over the initial 100 hr period in the cold incubators.

The results show a strong contrast between pea and maize in the effect of the four temperatures on root growth. Pea maintains linear growth rates at all the temperatures, even the lowest 2°C, whereas maize roots maintain linear growth rates only at the two higher temperatures. The rates are quantitatively much slower than in pea at these two temperatures, 10 and 14°C. At the two lower temperatures, growth of the maize root slows down over the five day period studied and stops completely at 2°C and nearly so at 6°C.

Figure 5 illustrates the data for growth rate as rate for successive 24 hr intervals in the cold incubators. The continuous decline in growth rates of the maize root with successive time increments at 2 and 6°C is in contrast with the rates for pea which remain approximately constant. At the two higher temperatures the

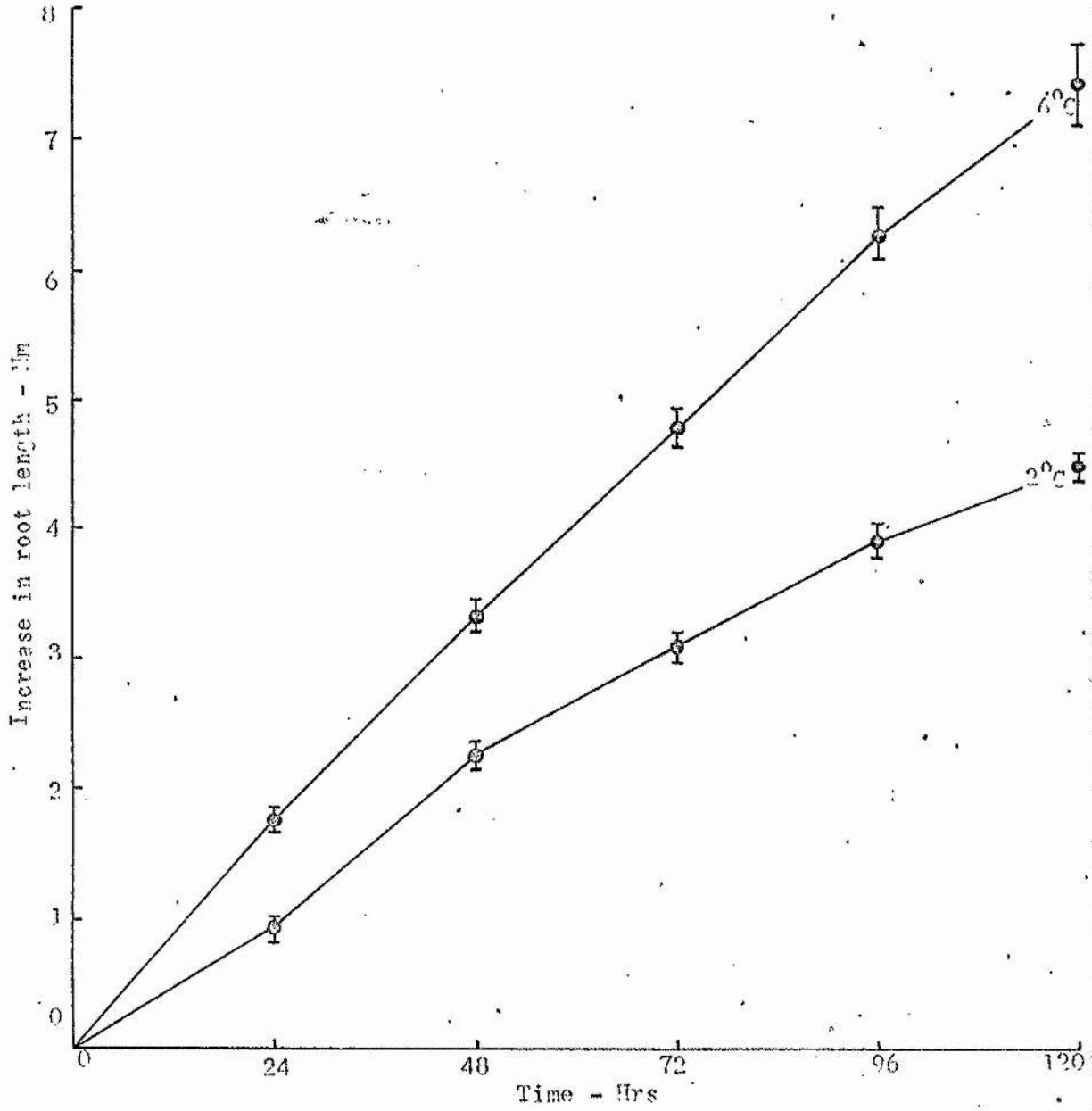


Fig. 1. Mean increase in length of pea seedling roots at the temperatures of 2 and 6°C against time. Time 0 hr was after germinating seeds 72 hr at 20°C.

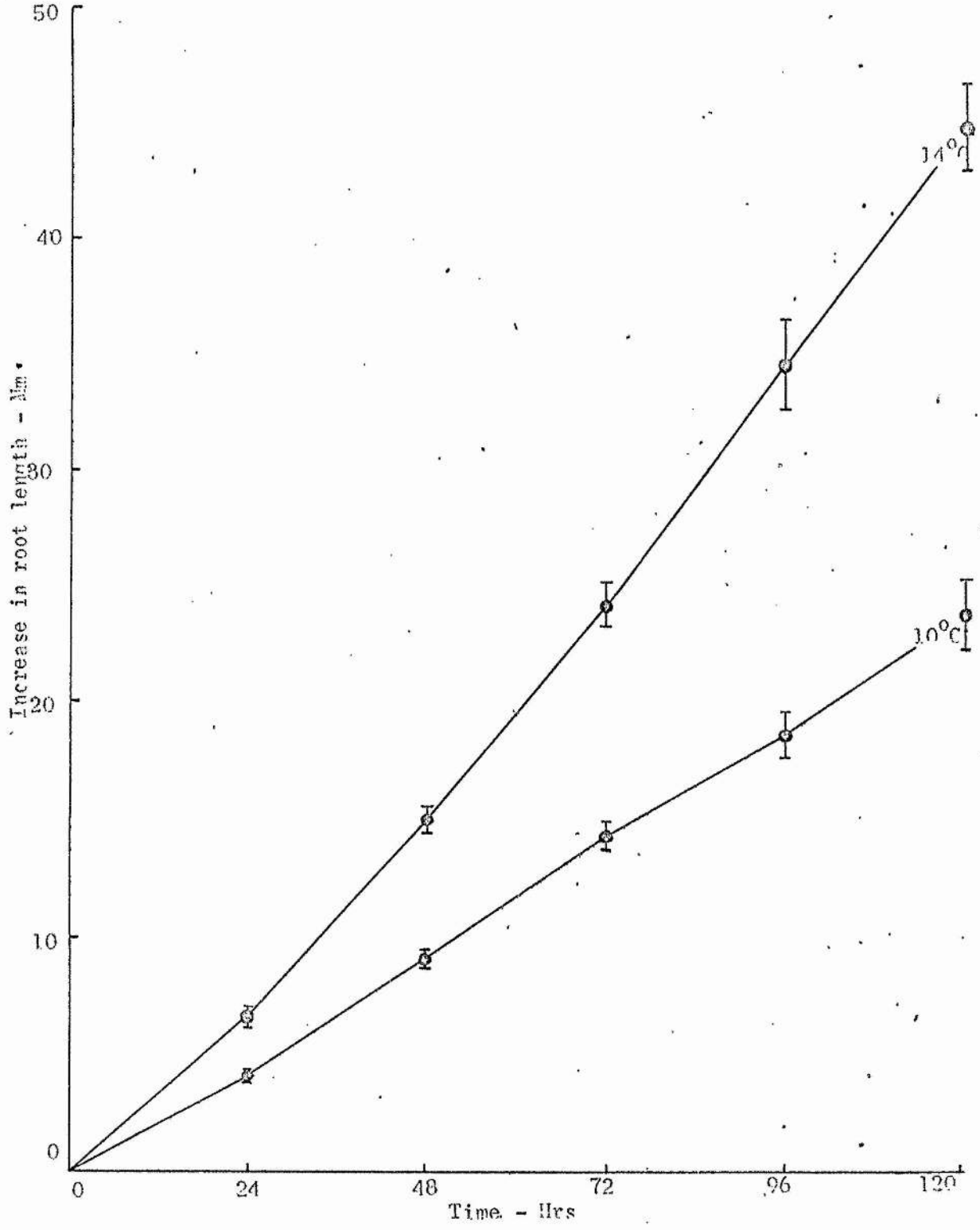


Fig. 2. Mean increase in length of pea seedling roots at the temperatures of 10 or 14°C against time. Time 0 hr was after germinating seeds 72 hrs at 20°C.

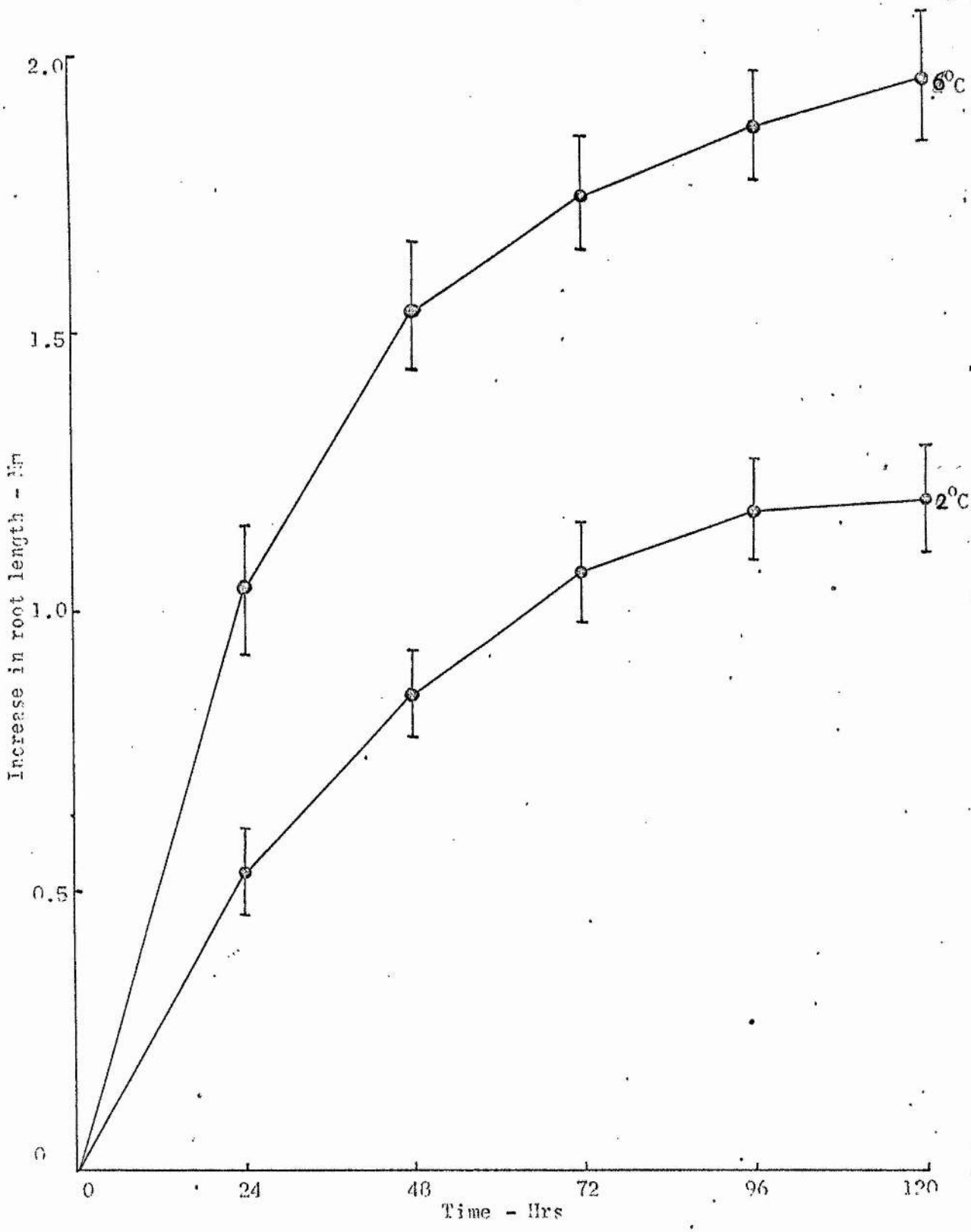


Fig. 3. Mean increase in length of maize seedling roots at the temperatures of 2 and 6°C against time. Time 0 hr was after germinating seeds 120 hrs at 20°C.

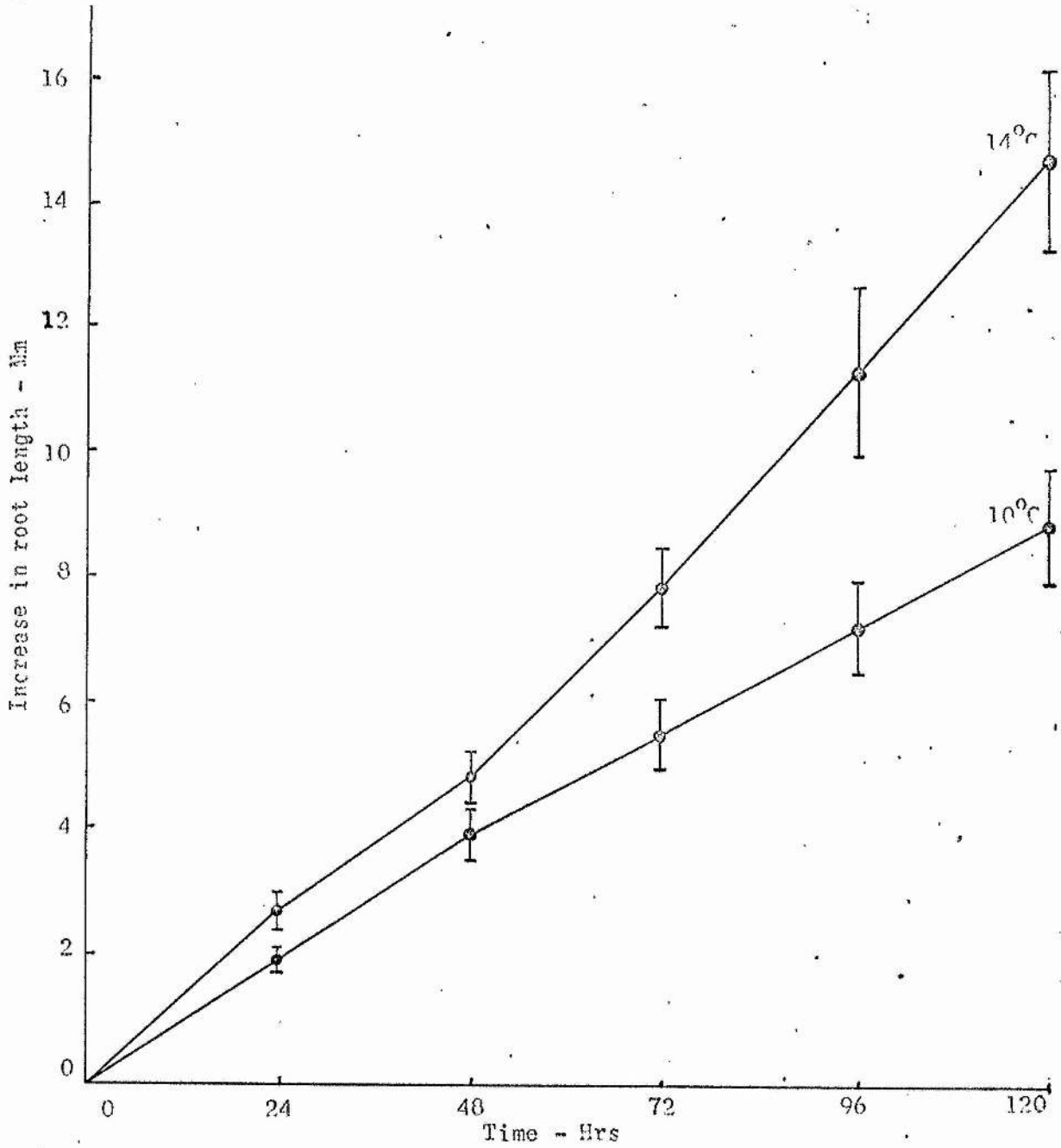


Fig. 4, Mean increase in length of maize seedling roots at the temperatures of 10 and 14°C against time. Time 0 hr was after germinating seeds 120 hr at 20°C.

Table 4. The number of seedling roots measured and their growth rates at 2, 6, 10 and 14°C. Growth rates derived from the slopes of the best straight lines through the points plotted in Figs. 1, 2, and 4, and read directly from Fig. 3 at time 100 hr where growth rate was not constant with time.

Temp. (°C)	Pea		Maize	
	No. roots measured.	Growth rate mm/100 hr/root.	No. roots measured.	Growth rate mm/100 hr/root.
2	108	4.10	55	1.2
6	78	6.15	52	1.8
10	34	19.5	37	7.7
14	32	40.5	32	11.8

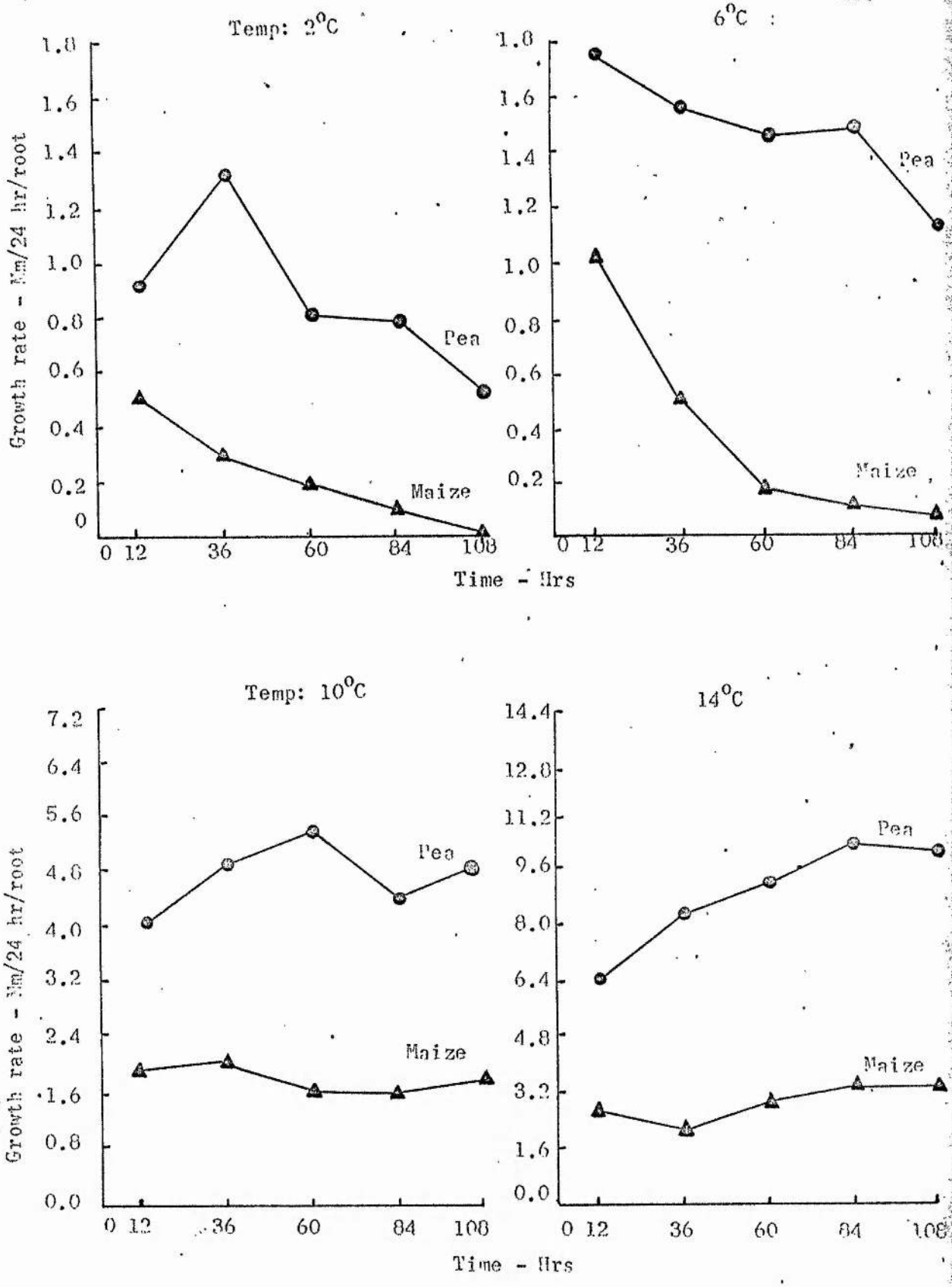


Fig. 5. Growth rates of roots of seedlings of pea and maize grown at four temperatures. Rates determined as the mean increase in length of batches of roots (see Table 4) over successive 24 hr intervals and plotted at times midway between these intervals. At time 0 hrs seedlings had been germinated at 20°C for 72 hr and 120 hr respectively for pea and maize.

rates of growth of maize roots are constant over the five day period but are much slower than the corresponding rates for pea roots.

A further expression of how growth rate of the two species is responding to rising temperature is seen in the Q10 values for the rates calculated over the four degree intervals of temperature. They are plotted in Table 5. Both species are most responsive to temperature in the 6-10°C range although with maize the very high value of Q10 = 45.8 is somewhat artificial since the growth rates being compared are not both constant. The rates are recorded as those over the time 0-100 hr in the cold incubators and during this time at 6°C the rate is declining whereas at 10°C it is constant. The high value for pea is comparable to other reports (Kotowski, 1926, gave a value of Q10 = 12.2 over the temperature range 4-8°C for rate of germination of peas) and indicates that with this rise of temperature growth rate is increasing rapidly compared to the relatively stable rate at the lowest temperatures.

Between the warmest temperatures used, 10-14°C, rate of growth of maize roots responds in a manner similar to many biological reactions where a Q10 of between 2 and 3 is typical. The value for pea, while being higher than this, is probably typical for that species where a high Q10 at all temperatures has been reported (Kotowski 1926). These Q10 values (for 10-14°C) suggest growth is responding to rising temperature in the manner expected on thermodynamic grounds.

Thus the results for growth rates of pea and maize at 2, 6, 10 and 14°C show:

1. Pea maintains linear growth rates at all the temperatures but growth is most responsive to rising temperature in the range 6-10°C.

Table 5. Q10 for rates of increase in root length of pea and maize calculated over 4^{0} intervals of temperature. (Formula for computation of Q10 was that used by Kotowski 1926).

Temperature interval (^{0}C)	Q10	
	Pea	Maize
2 - 6	2.76	3.41
6 - 10	17.9	45.8
10 - 14	6.22	2.92

2. Maize maintains linear growth rates only at the two higher temperatures. Quantitatively the rates are much slower than those for pea.

3. At the two lower temperatures growth rate of maize roots declines with time and stops completely after 5 days at $2^{o}C$.

The lines of investigation which may lead to an explanation of why the growth responses of pea and maize roots to low temperatures differ so significantly from one another, both quantitatively and qualitatively, are considered below. These considerations are presented in the context of what is currently known about the early root growth of pea and maize seedlings.

Firstly, it is important to establish how growth of the roots is occurring. In the young seedling roots there are two possibilities. Growth may be by cell elongation alone or by a combination of cell division and cell elongation. Secondly a knowledge of the source of nutrient materials to the young roots is required. At the end of the germination period the materials supporting root growth may be endogenous, i.e.: those laid down in the embryo root, or translocation from the "seed" may have started and be the major source of materials for growth of the roots. This latter process involves many enzymes systems (e.g.: those of hydrolysis, which operate in the "seed" to mobilise reserve materials, and those of translocation which are involved in transport of materials along the root), which are not involved if growth of roots is supported by endogenous reserves only. These enzymes may require a relatively high temperature for their induction but once formed may remain active at temperatures lower than those required for their formation. If this is the case then the time at which seedlings are first subjected to the low temperatures used in the present experiments

would be critically important for the ability of the roots to continue growing. Growth would only be sustained at the lower temperatures if the enzyme systems were already present by the end of the warm germination period. Presented below are the results of previous work bearing on these two problems as they affect root growth of pea and maize under the experimental conditions used here (respective germinations periods of 72 and 120 hr at 20°C).

1. How growth is achieved.

Bain and Mercer (1966 c) have studied growth of pea seedlings (var victory freezer). They distinguish three phases in seed germination, recognised by different growth characteristics.
Phase 1: Extending from the time when the dry seeds were first planted (in moist sand in a greenhouse in July, this being winter, as the work was carried out in Australia), to the end of day 5. Only the radicle made sufficient growth to emerge from the testa. This emergence occurred on day 2-3 and subsequently 10-20 mm of growth was made by cell elongation only. On day 4 cell division began and was very active on day 5 at which time the radicle was approximately 40 mm long.
Phase 2: Day 5-8. The radicle grew to 80 mm in length, developing secondary primordia. By day 8 also, the shoot had emerged above ground and the leaves greened.
Phase 3: Day 9 onwards. The growth of the pea plant rapidly became independent of the cotyledons. By the twelfth day the cotyledons had lost most of their reserves and were moribund.

In maize, cell division in the radicle begins rather sooner (Toole et al., 1956). When the dry grains are moistened

(at 25°C) the first twelve hours are a period of imbibition. From 10-20 hr elongation of the coleorhiza proceeds, it breaks through the pericarp and extends about 2 mm by cell elongation. On day 2-3 the radicle breaks through the coleorhiza and at this time cell division begins, as judged by the first appearance of significant levels of nucleic acid in the root tip and by cytological observations (Ingle et al, 1964).

Thus, at the end of the germination period in the present experiments, growth is occurring in pea and maize by both cell division and cell elongation, although in pea cell division may only just be beginning. The differing capacities of roots of pea and maize to maintain growth at 2 and 6°C and their different growth rates at 10 and 14°C reflect a fundamental difference between the two species in the response of cell division and elongation rates to temperature. The processes of cell division and elongation involve many metabolic pathways and basic to these is the demand for a high energy supply (Mayer and Poljakoff-Mayber, 1963). This is intimately involved with the second problem being discussed here, the source of materials supporting root growth of the two species.

2. Source of nutrient materials supporting root growth.

The supply of materials, required for growth, to the root tip of pea seedlings has been studied by Brown and Wightman, 1952, using root culture techniques. They found that the necessary materials come from two sources, the meristematic cells themselves and the vacuolated cells further up the root. What these different components were was not investigated. Ultimately supplies for the

young radicle of intact seedlings must come from the cotyledons. Bain and Mercer (1966 b) measured the dry weight of cotyledons of pea each day from the time of imbibition and they recorded the starch, total sugar, and reducing sugar content of these organs. Only on the sixth day did the dry weight of the cotyledons start to decrease and only then was there any transport of sucrose out of the cotyledons. At this time the radicle is over forty millimeters long and thus, it seems, is capable of making considerable initial growth on its own endogenous reserves. These reserves may be protein and fat which were seen to be laid down in the radicle of the developing embryo whilst it was on the parent plant (Bain and Mercer, 1966 a). Unfortunately, the temperature at which this experiment was performed was not specified. It was carried out in an Australian greenhouse in winter. The temperature of 20°C used in the current experiments to germinate the pea seedlings is likely to be warmer than those conditions. The faster rate of metabolism thus expected means that transport may start earlier but whether or not by the end of the germination period (72 hr) cannot be definitely determined.

In maize the embryo axis (root plus shoot) appears to draw on reserves from the scutellum and endosperm at different times dependent on the conditions of germination. Ingle et al. (1964) report that the axes of seedlings grown in the dark at 25°C first receive materials from the scutellum and endosperm around day 3 (measured from first soaking the dry seeds). They visualize the scutellum as a tissue of prime importance in synthesising sucrose from the hexoses liberated in the endosperm by starch hydrolysis, and as being responsible for transport of this sucrose to the axis.

They point out that the high energy demands of these processes may be met by the large concentration of mitochondria in the scutellum Edelman et al. (1956) have ascribed this role to the scutellum of germinating barley.

However, Oaks and Beevers (1964) emphasise the role of the scutellum itself in providing material for the growing axis. They used seedlings of hybrid maize, age 60 hr, germinated in the dark at 30°C. The scutellum has a high percentage of its dry weight as fat (Dure, 1960 a, gives a value of 27% for an inbred maize and Toole, 1924, a value of 34,8%), and they have demonstrated a very active glyoxalate cycle in this organ whereby storage lipid provides sugars for transport to the root and shoot. Experiments with seedlings where the endosperm was excised and the scutellum was supplied with acetate-2C^{14} showed a build up in the roots of radioactive glucose twelve hours after application of the acetate. Little radioactivity was accumulated in sucrose or fructose. (To account for the heavy labelling of glucose alone amongst the three sugars Oaks and Beevers put forward two alternative suggestions. The first of these was that glucose is the transport carbohydrate in young maize roots. The second suggestion was that sucrose is the transport carbohydrate, but on arrival at the root tip the sucrose is rapidly hydrolysed to glucose and fructose. The fructose is then either preferentially utilised by the roots or converted to glucose by a hexoisomerase. Either of these alternatives would account for the heavy labelling of glucose alone.) They did not observe depletion of carbohydrate from the endosperm of intact seedlings until day 5. The results of Dure (1960 b) are in agreement with the above findings although he worked with an inbred variety of maize at a temperature of 21°C.

Toole (1924) using a dent variety of maize, excised the embryos from grains and compared their germination with that of intact grains. Germination was in the dark at 21-24°C. He found that development of intact grains and excised embryos was identical until 1 cm of root growth was made. Thereafter root growth of the excised embryos was severely retarded.

Under the present conditions used for maize seeds it can be concluded that translocation of materials from the scutellum has definitely started by the end of the germination period, and probably materials from the endosperm are just beginning to be supplied to the root.

Thus with respect to supplies of materials required for growth an explanation of how low temperatures affect root growth of pea and maize in markedly different ways need only involve consideration of temperature effects on already existing hydrolytic and translocation systems in the maize seedling. In peas, the translocation system may not be in operation when the seedlings are put in the cold incubators. However, the pattern of growth rates shown by pea and maize at the temperatures 2, 6, 10 and 14°C indicates that in pea the root has an adequate supply of metabolites, even at 2°C, since approximately constant growth rates are maintained at all the temperatures. With maize the tailing off of growth with time at 2 and 6°C indicates a disturbance in metabolism. In view of the complete dependence of growth on a supply of energy, and this in turn depending on transport of substrates from the seed, an examination of the levels of the primary transport material in the root tip, sucrose, and the hexoses derived from it, glucose and fructose, was undertaken over the five day temperature treatments. This, in conjunction with measurements of

respirations rate, would reveal how significantly the temperature was affecting transport of substances to, and energy supply of, the root tip, both of these processes being essential for continued growth by cell division and elongation in the root apex. These studies are described in the following two chapters.

CHAPTER 3

STUDIES OF SOLUBLE CARBOHYDRATE LEVELS IN THE ROOT

Introduction.

The importance of a supply of soluble carbohydrates to the roots of young seedlings has been stressed in the previous chapter. They are required as substrates for respiration, and ultimately, to provide the carbon skeletons for all the biosynthetic reactants and products involved in growth. They are also required to maintain the osmotic potential of cells, which is of fundamental importance to extension growth in the zone of elongation of the roots (Hellebust and Forward, 1962, Brown and Sutcliffe, 1950).

The aim of this experiment was to determine how levels of soluble carbohydrates were affected by the four experimental temperatures, and to determine the extent to which differences in content correlated with the very different growth rates observed, both within and between species. The principle soluble carbohydrates present, and their amounts, in the roots of pea and maize, were estimated over the five day growth periods in the cold incubators.

Method.

Seedlings were germinated and grown as described previously (pp. 16-19). At time 0 hr, and after being in the cold incubators 24, 72 and 120 hr, samples of seedlings were withdrawn, and the

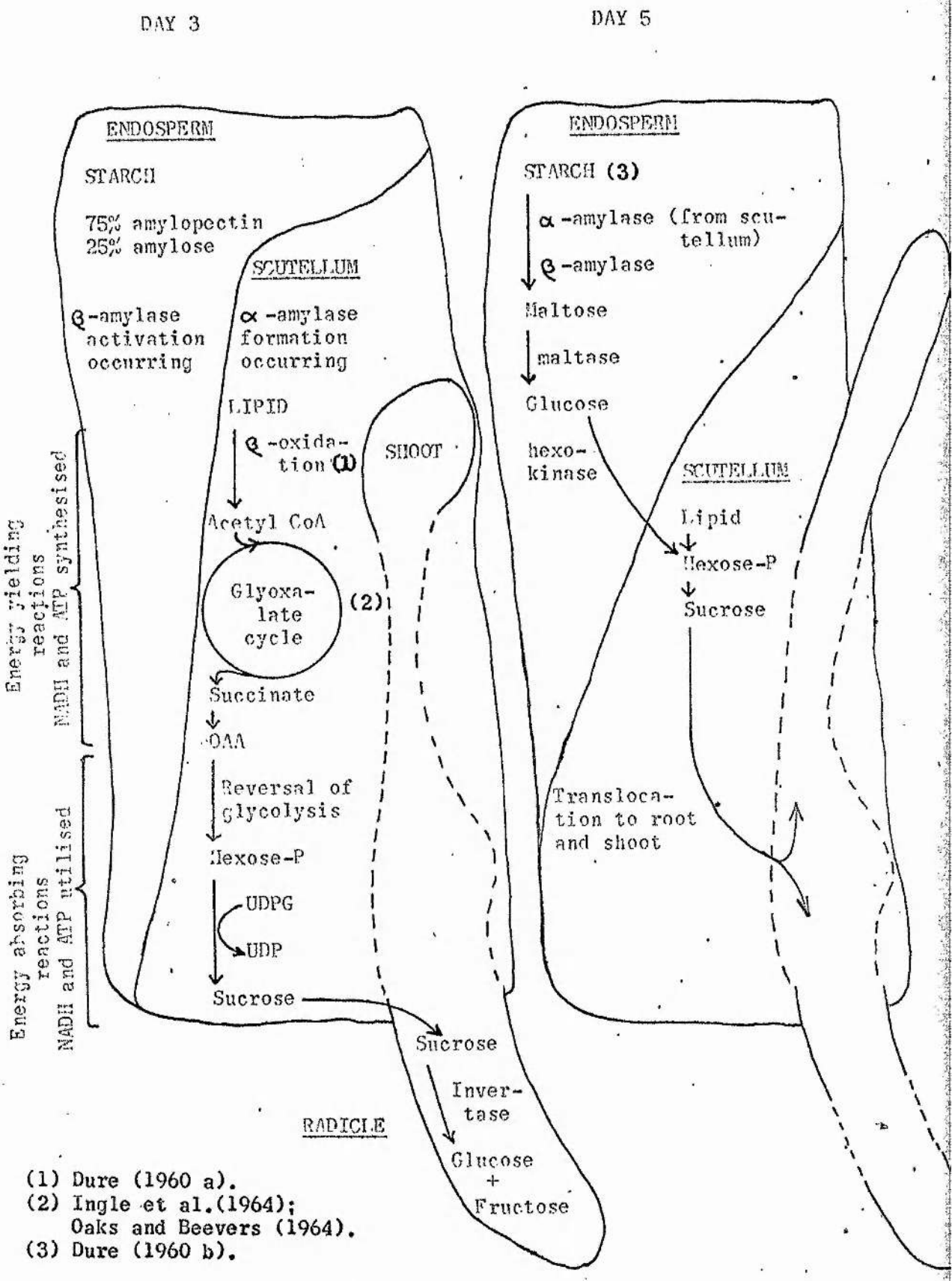
distal 1 cm of the roots excised. The soluble carbohydrates were extracted in ethanol, separated from the other soluble material and prepared for analysis by gas-liquid chromatography. Details of the procedure used are given in Appendix 1. This procedure gave good quantitative separation of the soluble carbohydrates (Plates 2 and 3). The distal 1 cm of the roots only, was analysed, because in young seedlings this segment is where almost all root growth is made (Brown and Broadbent, 1950, Hellebust and Forward, 1962). All experiments were duplicated, that is, for each of the four temperatures (2, 6, 10 and 14^{o}C) at each of the four times (0, 24, 72 and 120 hr) two batches of seedlings were grown of each species. Duplicates agreed within 5% of each other and averages of the values obtained are presented in the results.

Before presenting the results for the amounts of soluble carbohydrates in the root tip, and their changes with time at the different temperatures, a problem concerning the interpretation of these results is briefly discussed. The amounts reflect the relationship between the rates of supply and utilisation of soluble carbohydrates in the root. In young seedling roots subjected to different temperatures, two factors may be operating to alter these rates differentially.

The first of these is temperature itself. Rates of supply and utilisation are the final expression of many reactions whose rates may change by relatively different amounts with temperature. Thus lower temperatures result in a slower rate of translocation (Esau et al., 1957, Geiger, 1969). If utilisation of sugars decreases with lower temperatures at a rate different from that of translocation, changes in the levels of total soluble carbohydrate

and shifts in the proportions of the different component sugars would be expected. Also, the equilibrium position of reactions may be shifted at different temperatures and this could affect levels of sugars in the root tip. Thus Oota et al. (1956) discussed the conversions of lipid to hexose and of hexose to sucrose in storage organs of young seedlings. The formation of hexose from either lipid or sucrose is exothermic and thus is favoured at low temperatures. In maize this may be an important feature where the glyoxalate cycle in the scutellum is operating to convert storage lipid to sugars which are supplied to the axis. James (1953) has pointed out that the heat of reaction of starch to sucrose is 3.7 Kcals/mole, thus in pea cotyledons at low temperature hydrolysis is favoured. Figure 6 illustrates the stages in mobilisation of reserves in the maize scutellum and endosperm. Pea cotyledons, possessing starch reserves only, show hydrolytic processes similar to those in the maize endosperm (Young and Varner 1959).

The second factor is concerned with the time of commencement of sucrose transport from the cotyledons. In germination this soon supplements and replaces the limited reserves present in the radicle itself. To interpret changes in the amounts of soluble carbohydrate it is important to determine the time when sucrose transport to the root tip begins. Evidence is presented from the results of this experiment indicating when this time is for each species under the particular set of conditions used. The problem has already been referred to in Chapter 2 and in pea transport is expected to start between time 0-48 hr after placement of germinated seeds in the cold regimes (p. 31). With maize transport is thought to have started by the end of the germination period (p. 33).



(1) Dure (1960 a).
(2) Ingle et al.(1964); Oaks and Beevers (1964).
(3) Dure (1960 b).

Fig. 6. Mobilization of storage carbohydrate and lipid in the germinating maize grain. Day 3 and 5 refer to time after first soaking the dry grain.

Results and Discussion.

Three soluble carbohydrates were found in the root tips of both species in much larger quantities than any others. These were the sugars sucrose, glucose and fructose. A typical chromatograph obtained using an extract from pea roots (Plate 3) compared with the one obtained from a standard mixture of sugars (Plate 2) illustrates this. It may be noted that the retention time of individual sugars in the standard was slightly shorter than in the root extract (e.g.: retention time for fructose in the standard was 4.5 min and in the extract was 5.5 min). The shorter retention time was a constant feature of chromatograms of the standard mixture of sugars. The identity of the peaks of the standard mixture of sugars and of the root extract was established by adding a known quantity of an individual sugar before performing the trimethylsilylation reaction and comparing the resulting chromatogram with that obtained for the standard mixture of sugars or the root extract alone. Where the added sugar corresponded to a sugar already present in the extract the peak area for that sugar was increased. If the added sugar was one not present in the extract, it appeared in the chromatogram as an additional separate peak. A second point emerging from Plates 2 and 3 concerns the sizes of the α- and β- glucose peaks. In the standard, β- glucose gave only a very small peak whereas in the root sample it was always the larger peak. The equilibrium between the α- and β- isomers of glucose was reached only very slowly when the sugar was dissolved in dimethylsulphoxide (DMSO). Initially, α- glucose predominated. Over a few weeks the proportion of β- glucose increased. It was found that the combined peak areas (α- plus

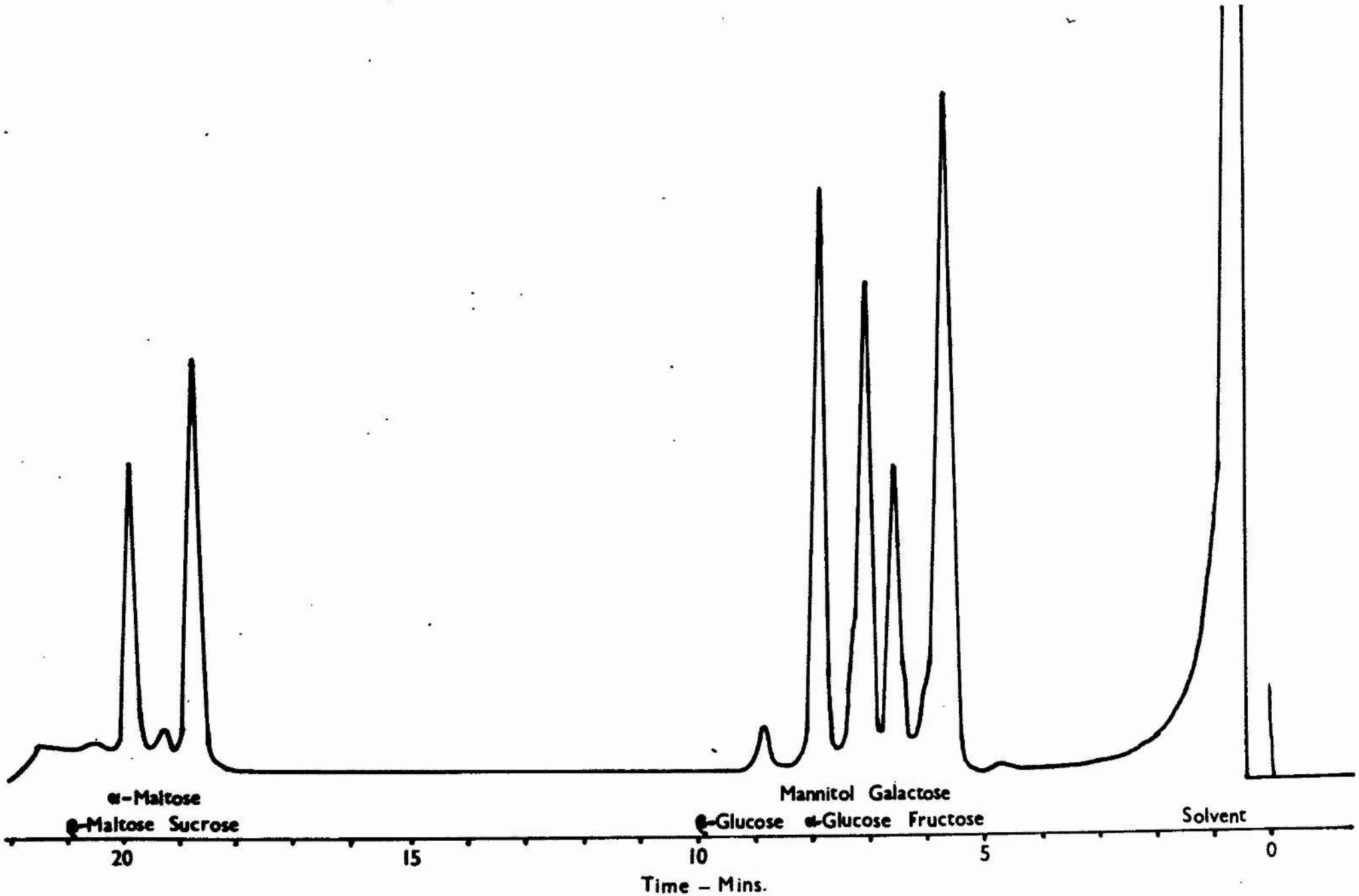


PLATE 2 GLC trace of a standard mixture of sugars – see appendix 1 for preparation of standard

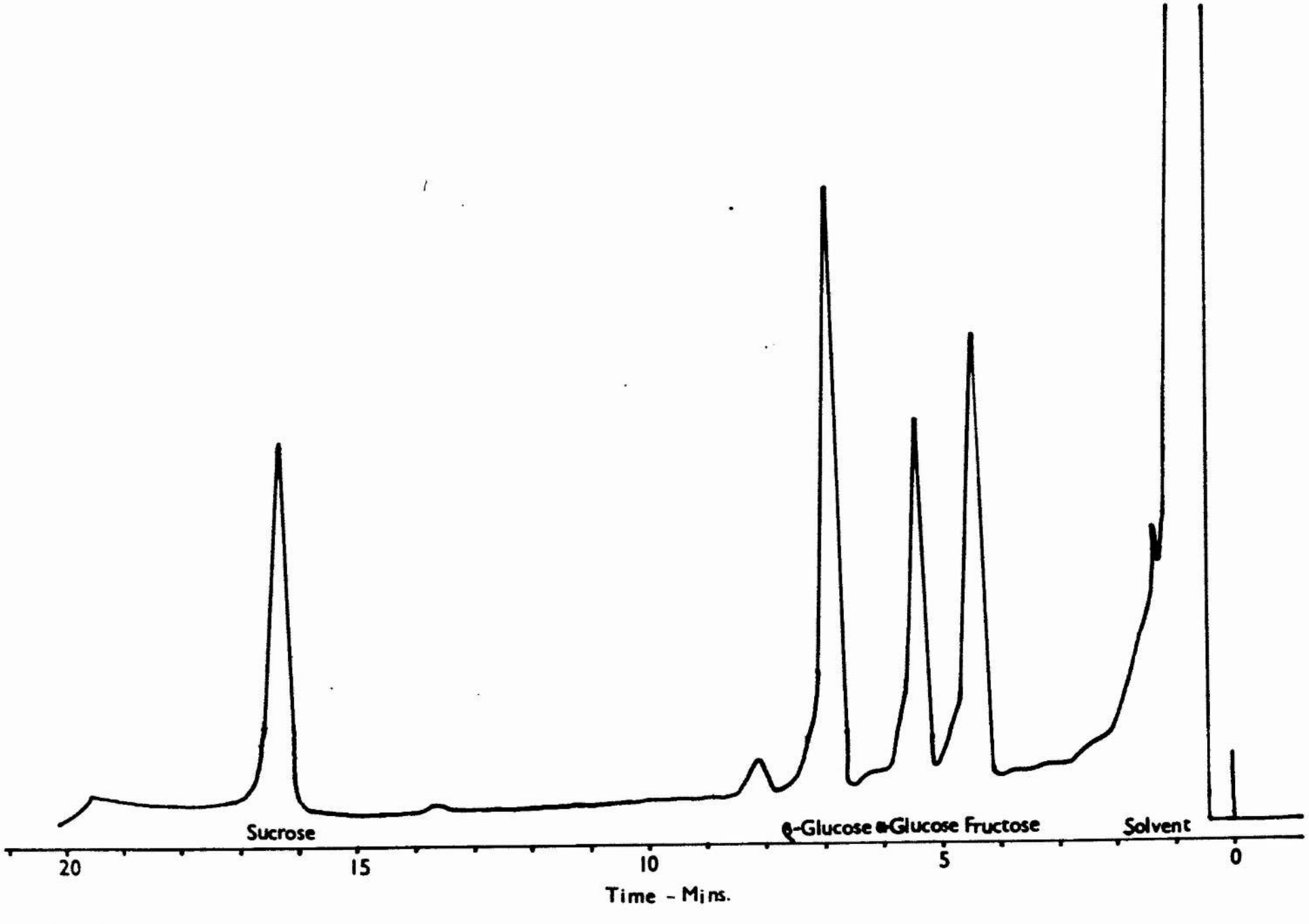


PLATE 3. GLC trace of a soluble sugars extract from root tips of pea - see appendix 1 for preparation procedure

β - glucose) remained constant for a given amount of sugar, independently of the respective sizes of the peaks, thus, in estimating glucose, combined peak areas for the sample and for the standard were always compared.

The small peak following the β - glucose peak and the suggestion of a peak between 13 and 14 min in the root sample (Plate 3), while usually being detectable, were never of any significant size. The peak following β - glucose was tentatively identified as inositol. Fructose, glucose and sucrose essentially represented the total soluble carbohydrate content of the roots. Additional peaks, if they occurred at all, possessed a combined area of less than 2% of the total area under all the peaks. The combined quantity of sucrose, glucose and fructose is referred to as total sugar content in this thesis.

Changes in total sugar content were observed in both species with respect to the growth temperatures and to the times exposed to these temperatures. These results are presented first, and are followed by the results for amounts of individual sugars.

Total sugar content.

The results for total sugar content of pea and maize are presented in Figs. 7 and 8, respectively. Those for pea are discussed first. Over the initial 24 hr period there is a decline in total sugar content at all four temperatures with this decline being greater the higher the temperature. From what has been said above this is probably because transport to the root tip has not started or is only just beginning and growth is occurring mainly at the expense of endogenous supplies, the more rapid rates at

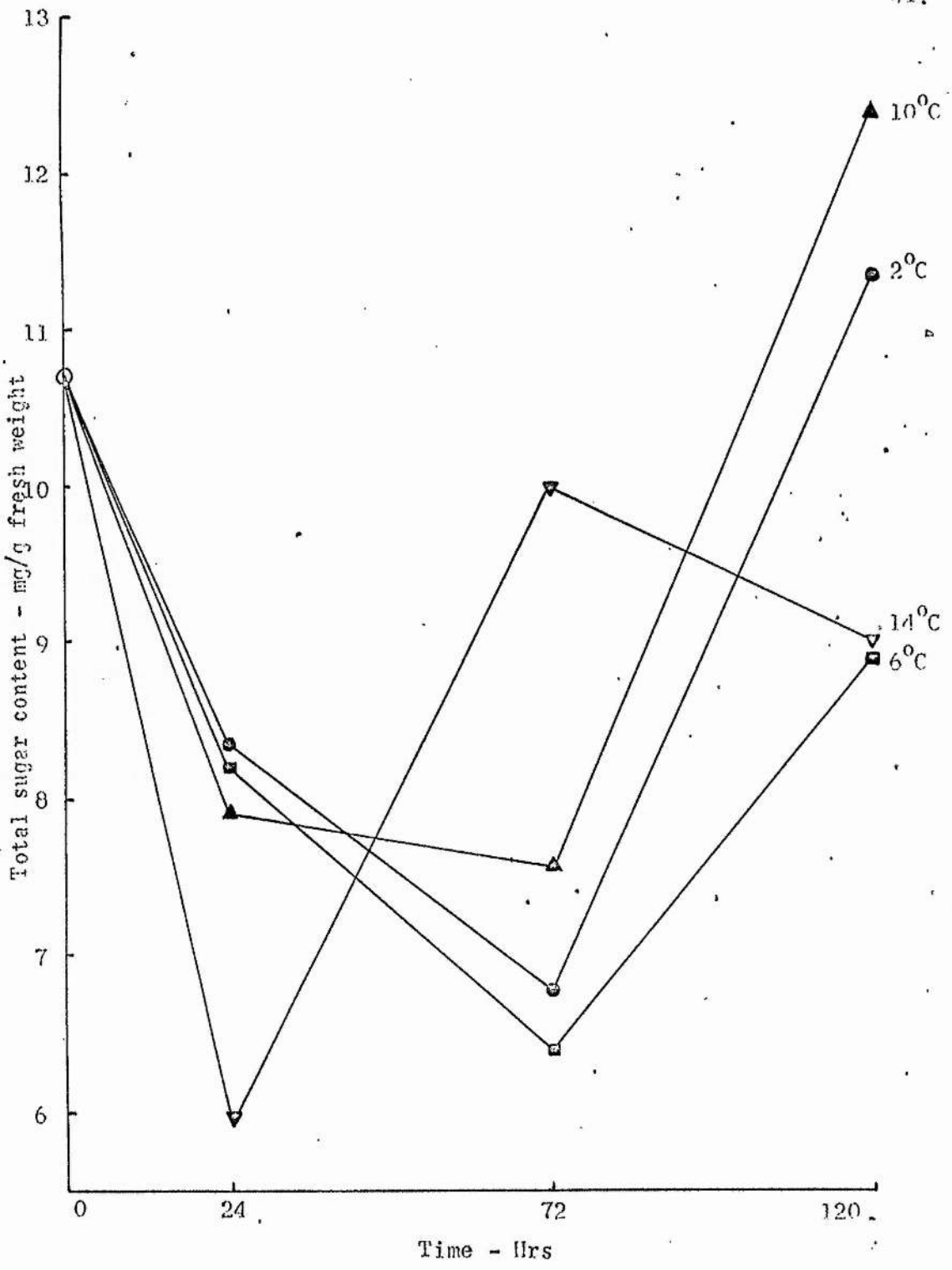


Fig. 7. Total sugar content in 1 cm root tips excised from pea seedlings grown from 0 - 120 hr at 2, 6, 10, or 14°C. Time 0 was after a germination period of 72 hr at 20°C.

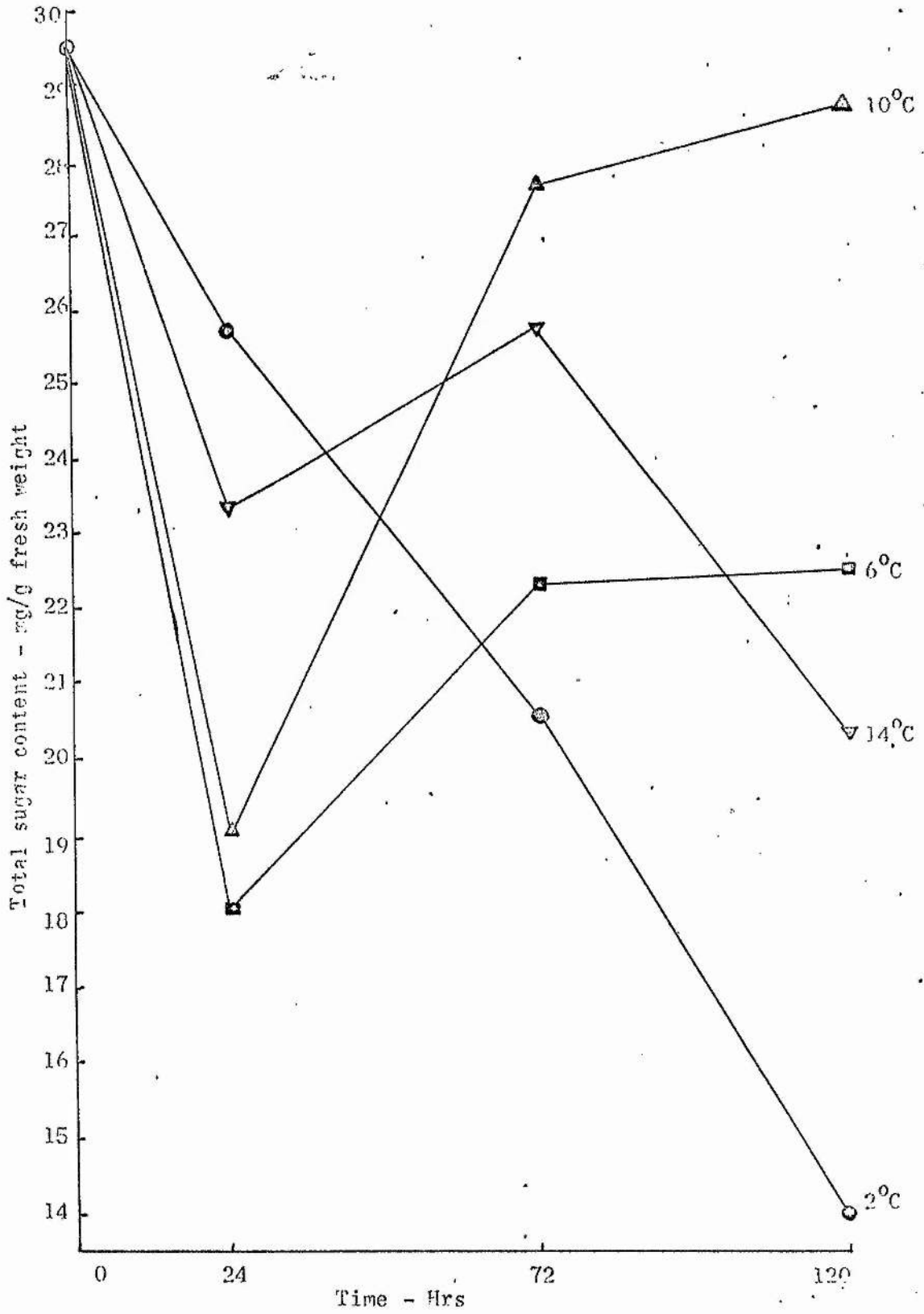


Fig. 8. Total sugar content in 1 cm root tips excised from maize seedlings grown 0 - 120 hr at 2, 6, 10 or 14°C. Time 0 was after a germination period of 120 hrs at 20°C.

higher temperatures resulting in greater depletion of soluble sugars. By the end of the period of study, 120 hr, however, a substantial recovery in the level of sugar is made at all temperatures, reflecting the start of transport from the cotyledons. The recovery is first apparent between 24 and 72 hr at 14°C, this, the warmest temperature, having the least effect on the transport process.

The comparable set of results for maize are presented in Fig. 8. Here again there is a drop in level at all temperatures over the first 24 hr in the cold incubators, but there is a contrast with pea in that the decline is smaller the higher the temperature. (Only at 2°C is there an exception to this. This significant deviation is discussed later.) This may be because transport to the root tip has already started when the seedlings were moved to the cold incubators and the effect of reducing the temperature from 20°C (the germination temperature) is to slow down transport of sucrose to the root tip more than to retard utilisation of sugars for growth and respiration.

Over the remainder of the 120 hr growth period the seedlings at 6, 10 and 14°C suffer adjustment in the sugar level which follows no clear trend. Presumably a balance between the rates of supply and utilisation is being reached which is dependent on the temperature. There is evidence for this at 6 and 10°C where the amount of sugar is fairly constant between 72 and 120 hr. However, at 14°C, the level continues to fluctuate.

Maize differs from pea in that there is two to three times the total sugar content in the root tip. This is probably a difference characteristic of the two species, since the sugar levels obtained in the present experiment are of the same order as those

reported in the literature, Table 6: (precise growing conditions, especially temperature, can affect the levels of materials in the root, and this probably accounts for the variation between results reported by different workers, Toole, 1924).

At 2°C the sugar content shows a steady decline over the entire 120 hr period. This imbalance in the root metabolism correlates with the cessation of growth at this temperature. Clearly there is utilisation of sugars at a rate much greater than they are being supplied (the decline is 50% over 5 days). This is possibly due to failure of sucrose transport to the root tip, or to failure of hydrolysis of reserves in the "seed". Growth may stop due to the amount of sugar in the root tip becoming insufficient to (1) maintain an adequate respiration rate, (2) maintain an adequate supply of carbon for biosynthetic reactions or (3) provide the osmotic potential required in cells undergoing extension.

At 6°C, where growth nearly stops, there is an indication that transport, although initially severely retarded, makes some recovery. This temperature is probably borderline for sugar transport and the continued growth of maize roots.

Individual sugars, sucrose, glucose and fructose

Since the total amounts of sugar in the roots are changing over the 120 hr period of the cold treatments, then the levels of the individual sugars will also fluctuate. Thus it is useful to look at the percentage of each sugar as a percentage of the total of all three under each set of conditions. Absolute sugar levels are of value in so far as they show how changes in the total sugar content are related to changes in the content of particular sugars.

Table 6. Quantities of sugars reported in the roots of pea and maize seedlings.

Germination conditions		Root segment analysed.	Sugar content (mg/g FW).			Reference
Temp. (°C)	Time. (hr)		Fructose	Glucose	Sucrose	
			Pea			
25	120	Distal 0-9 mm as 3 mm segments	1.4	5.8	2.6	Lyne and apRees (1971)
20	72	Distal 1 cm	3.79	3.5	3.45	Present work
			Maize			
30	60	Distal 2 cm	1	14	32	Grant and Beevers (1964)
30	70	Distal 1 cm as 2x5 mm segments	4	6	1	Hellebust and Forward (1962)
20	120	Distal 1 cm	7.91	13.4	8.18	Present work.
			Total sugar content (% DW)			
25	120	Whole seedling axis	30			Ingle et al. (1964)
20	120	Distal 1 cm of root	29.5			Present work.
			Reducing sugar content (% FW)			
30	70	Distal 1 cm	2.13			Hellebust and Forward (1962)
20	120	Distal 1 cm	2.13			Present work.

Thus the results for the determination of individual sugars are presented both as % total sugar and as mg/g F.W. (Figs. 9, 10, 11 and 12).

The results for pea are discussed first. There is additional evidence that transport of sucrose to the root tip begins about 24 hr after the seedlings are moved to the cold incubators. At this time, when total sugar at 14°C shows a large decline, the percentage present as sucrose rises markedly while that as the monosaccharides falls. This may reflect the arrival of sucrose from the cotyledons.

The levels of individual sugars are now considered in turn. The percentage of fructose at the four temperatures never shows a spread of more than 10% at any one time. Over the 120 hr period a slight fall is observed at all temperatures but otherwise temperature has little effect on the percentage amounts of fructose. The absolute levels of fructose show a similar tendency to decline, with the exception that after being at 10°C for 120 hr the initial level is restored.

However, glucose and sucrose levels, expressed on the "percent total" basis, or the absolute scale, are strongly correlated with temperature. After a growth period of 120 hr in the cold incubators glucose content, expressed on a "percent total" basis, is highest at 14 and lowest at 2°C, with 10 and 6°C occupying intermediate positions. This trend is also seen when expressed as absolute levels but the lower total sugar content at 14 as compared with 10°C has depressed the amount of glucose at 14 below that at 10°C. Sucrose behaves in a reciprocal manner to glucose. After 120 hr it is the most abundant sugar at 2°C and the least at 14°C, and again 6 and 10°C occupy intermediate positions.

Fig. 9. Percentage of the total sugar content present as fructose, glucose or sucrose in 1 cm root tip segments of pea seedlings grown 0 - 120 hr at 2, 6, 10 or 14°C. Time 0 hr was after a germination period of 72 hrs at 20°C.

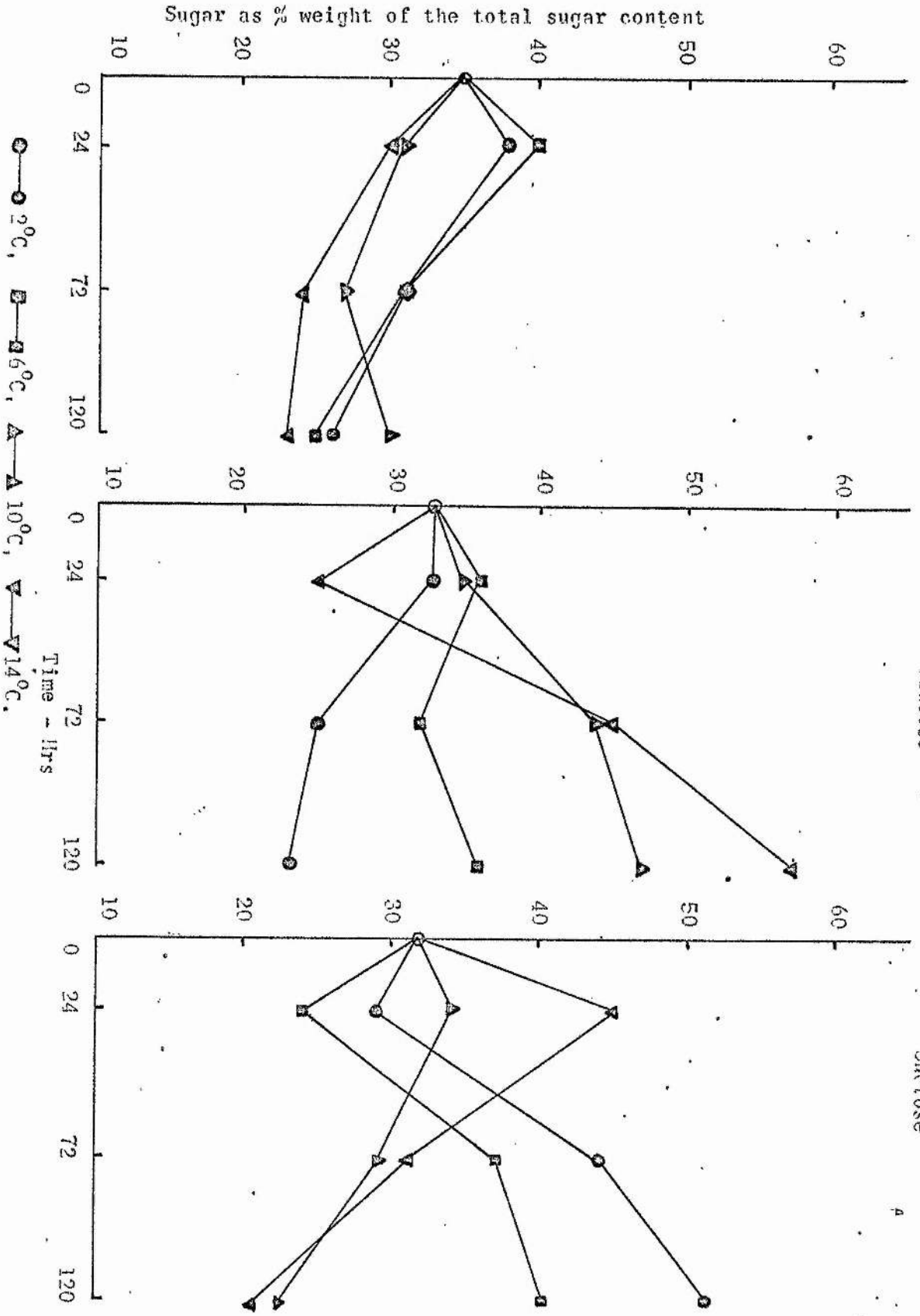

Sugar as % weight of the total sugar content
10
20
30
40
50
60
0
24
72
120
Fructose
Glucose
Sucrose
Time - Hrs
2°C,
6°C,
10°C,
14°C.

Fig. 10. Percentage of the total sugar content present as fructose, glucose or sucrose in 1 cm root tip segments of maize seedlings grown from 0 - 120 hr at 2, 6, 10, or 14°C. Time 0 hr was after germinating period of 120 hr at 20°C.

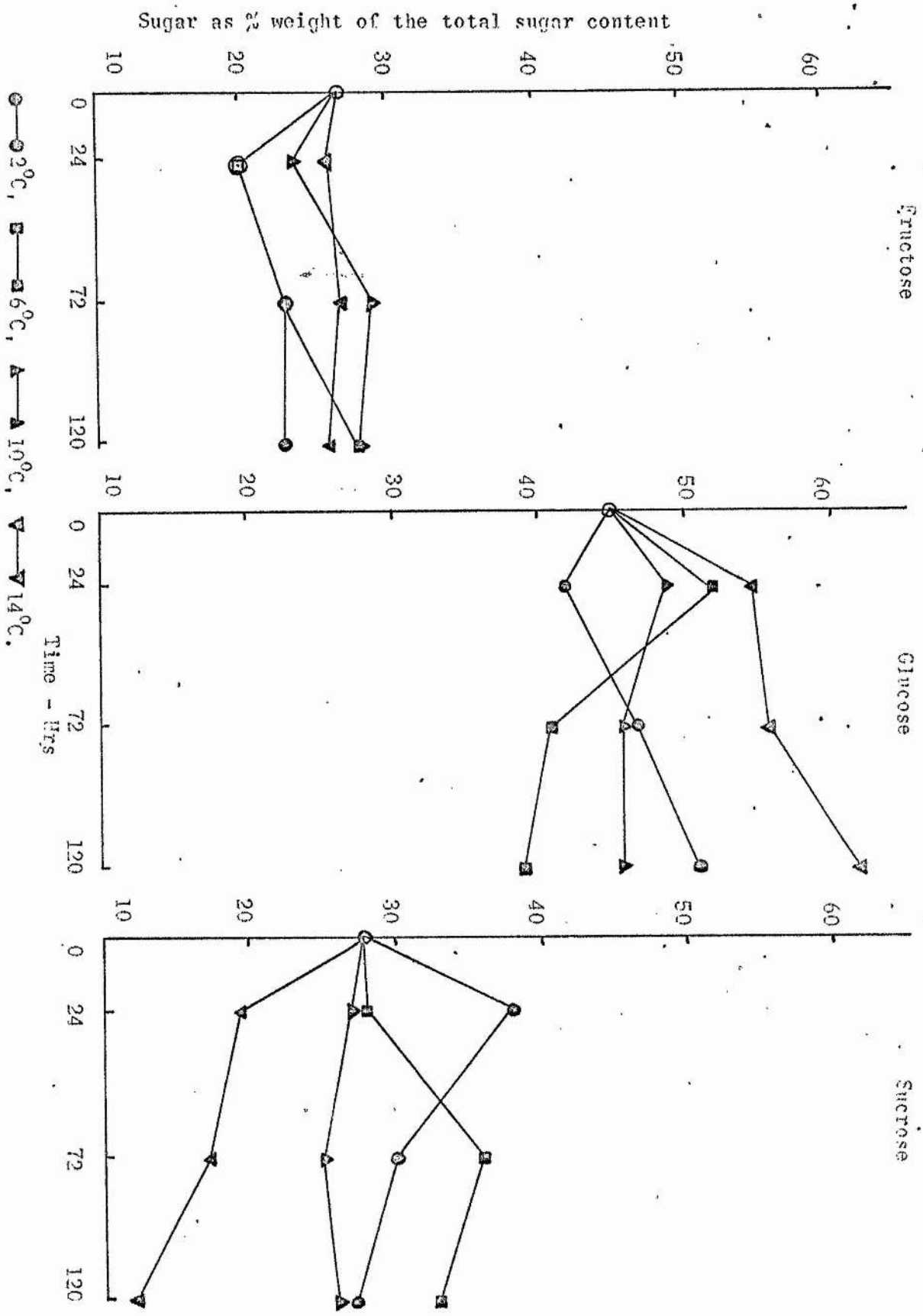

Sugar as % weight of the total sugar content
Fructose
Glucose
Sucrose
Time - Hrs
2°C,
6°C,
10°C,
14°C.

Fig. 11. Quantities of fructose, glucose and sucrose in terminal 1 cm of pea seedling roots during 120 hr growth periods at 2, 6, 10 or 14°C. At time 0 hr seedlings had been germinated 72 hrs at 20°C.

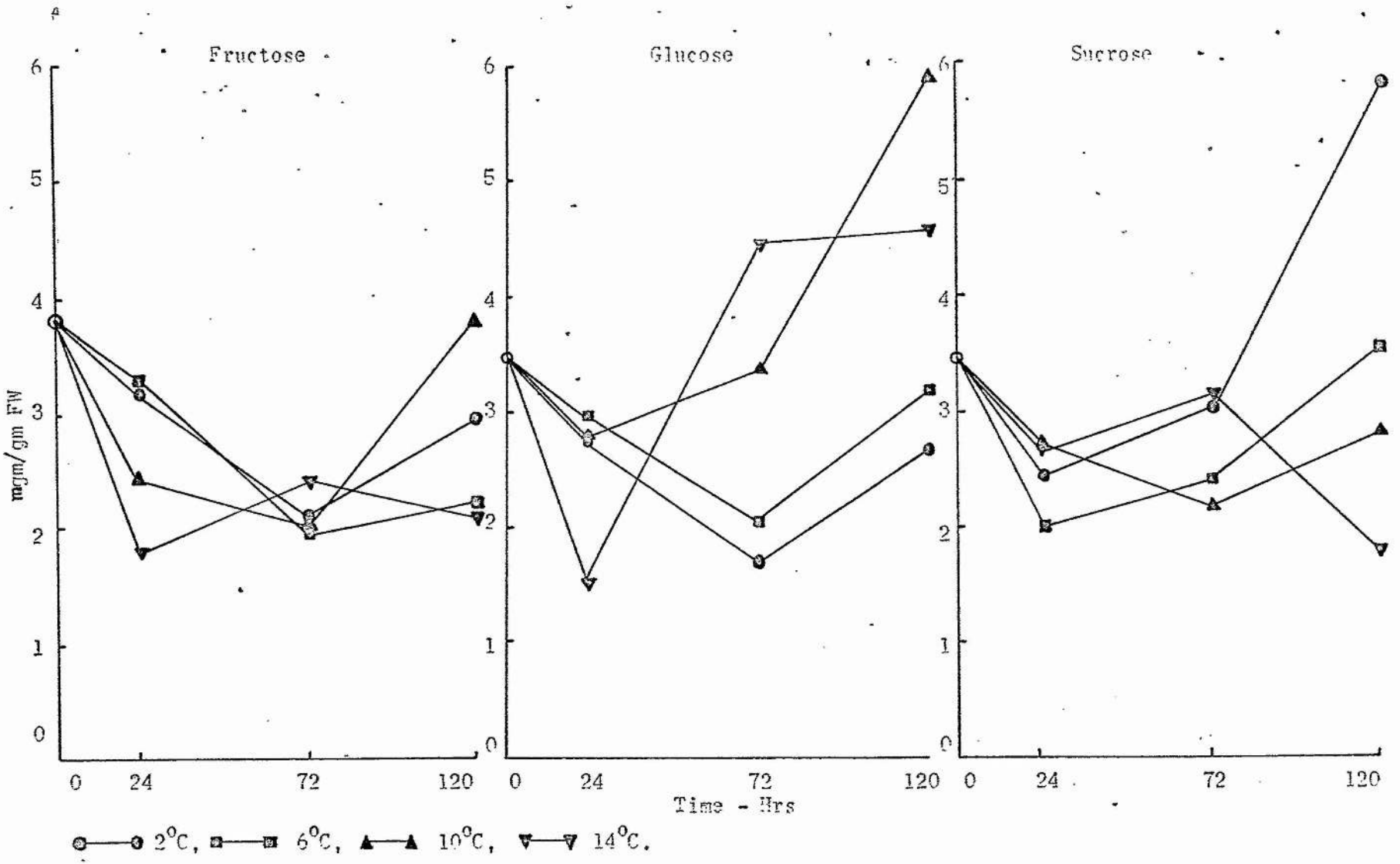
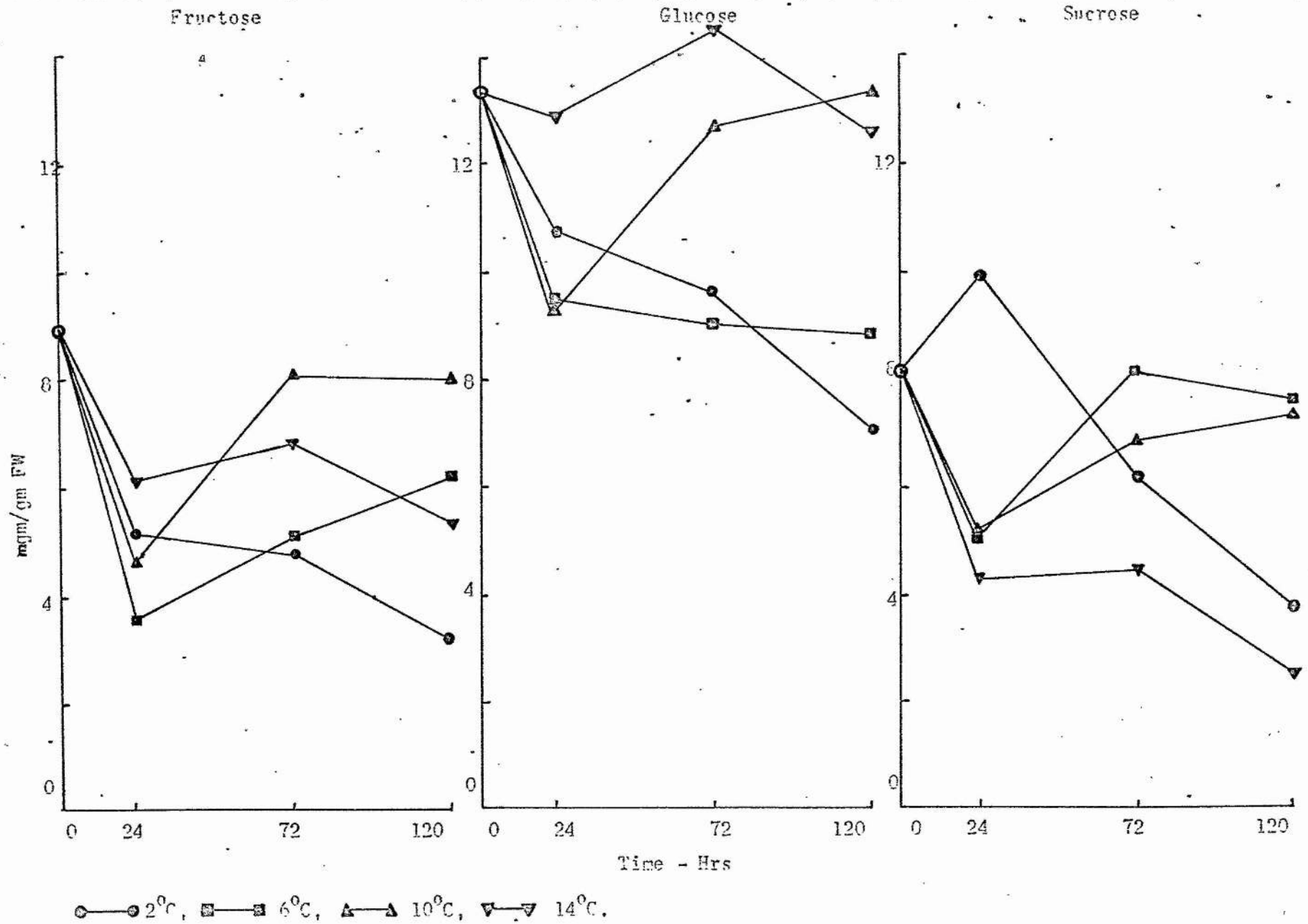

Fructose
Glucose
Sucrose
mgm/gm FW
0
1
2
3
4
5
6
0
24
72
120
Time - Hrs
2°C, 6°C, 10°C, 14°C.

Fig. 12. Quantities of fructose, glucose, and sucrose in the terminal 1 cm of maize seedling roots during growth periods of 120 hrs at 2, 6, 10 or 14°C. At time 0 hr seedlings had been germinated for 120 hrs at 20°C.


Fructose
Glucose
Sucrose
mgm/gm FW
0
4
8
12
0
24
72
120
Time - Hrs
2°C, 6°C, 10°C, 14°C.

Thus the sugars in the root tip of pea respond to low temperatures by establishing a high proportion of sucrose and a low proportion of glucose. With increasing temperature in the range 2-14°C the proportion of sucrose declines while that of glucose rises. This is illustrated in Fig. 13 where the glucose/sucrose ratio at each temperature is plotted for the two species, after 120 hr at the cold temperatures.

A high proportion of sucrose has been reported in other plant tissues when these were subjected to low temperatures. Arreguin-Lozano and Bonner (1949), found that potatoes sweetened when stored in the cold, as compared with warmer storage temperatures. Initially the sucrose content was 1.07% DW but after two weeks storage at 0°C this had risen to 6.65%. The other temperatures investigated were 9, 16, and 25°C and the respective sucrose contents were 1.25, 0.75 and 0.84%. Thus they declined with increasing temperature as was observed in the present experiment. Levels of glucose and fructose were however consistently low. Fructose showed the greatest fluctuations in level, rising from the initial value of 0.17% DW to 1.5% after two weeks at 0°C and to 0.34% at 9°C. Little change in level was observed at the two warmer temperatures. Thus in potato a high sucrose level is established in cold conditions but in contrast with pea roots glucose levels do not respond to temperature whereas fructose levels rise with decreasing temperature.

In potatoes the sucrose originates from the hydrolysis of starch. Its formation is thermodynamically favoured (James, 1953), but also Arreguin-Lozano and Bonner report that increased activity of the hydrolysing enzymes at the lower temperature is responsible for the sucrose accumulation.

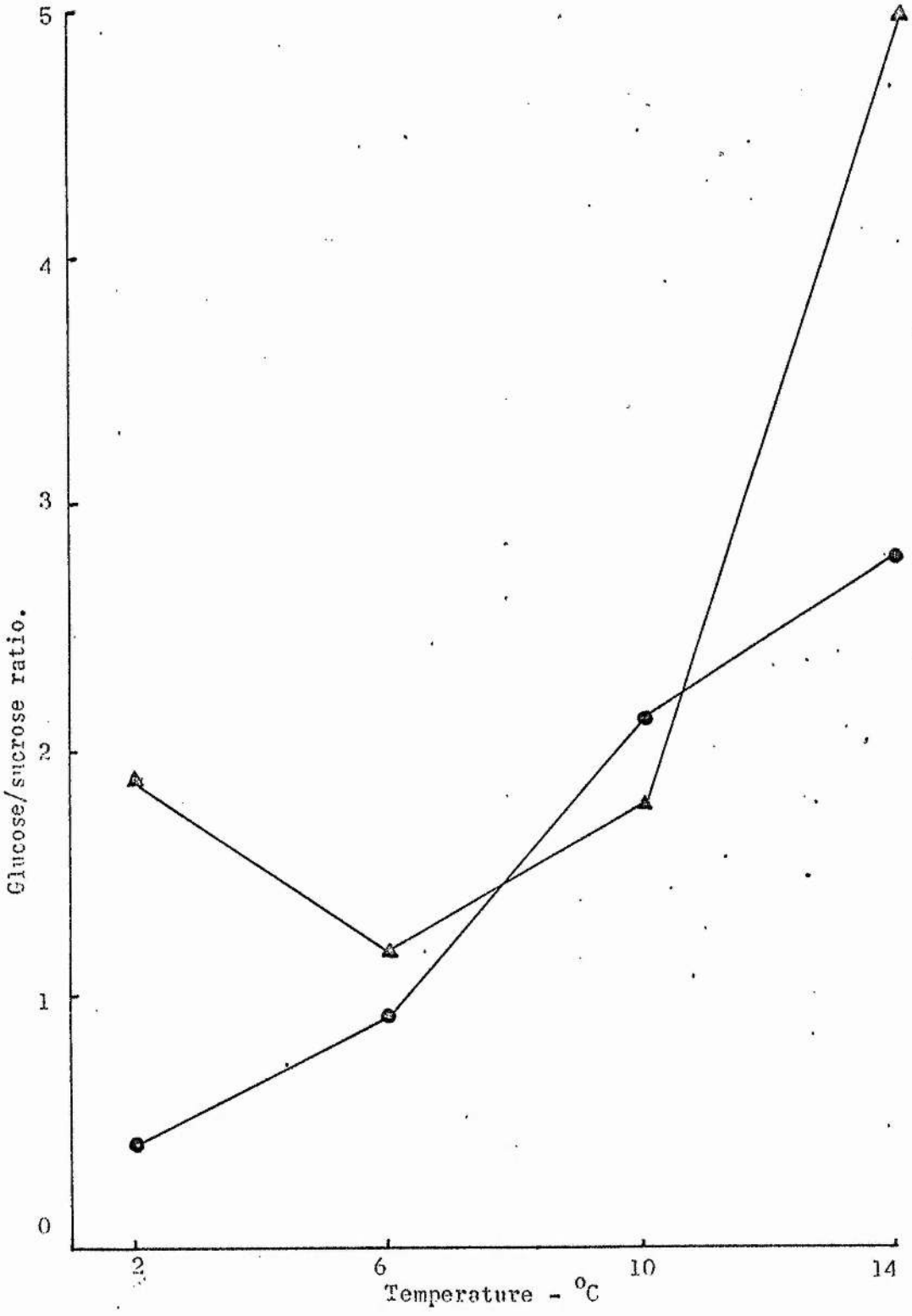


Fig. 13. Relationship between temperature and the glucose/sucrose ratio in the root tips of pea (●——●) and maize seedlings (▲——▲) grown for 120 hr at 2, 6, 10, or 14°C (after respective germination periods of 72 and 120 hr at 20°C).

"Sweetening", through whatever process, has frequently been reported to occur at low temperatures, e.g.: in the stems of grapes in winter, in the trunks of basswood and birch trees in winter, in sunflower and tobacco leaves exposed to 5°C as compared with 25°C in the dark (Forward, 1960) and in the leaves of Hedera helix in winter (Parker, 1962).

Vasil'yev (1961), examined the sugar content in leaves and in the tillering nodes of hardy strains of winter rye and wheat, and of two less hardy spring wheats, all planted outdoors at the end of August (in Russia). During September and October the temperature fluctuated but showed a general downward trend. The monosaccharide levels in the winter and spring varieties of the cereals responded to these fluctuations in a reciprocal manner. As temperature fell the levels rose and vice versa. All reported levels were between 15 and 60 mg/g FW. Sucrose, however, responded by much larger changes in level. Sucrose level rose from 60 to 220 mg/g FW but only in the winter varieties. In the spring wheats a small rise in the order of 30 mg/g FW above the initial level of 40 mg/g FW was observed. The sucrose level in the winter wheat did not rise at a uniform rate, but fluctuated with temperature in a manner similar to the monosaccharides, although showing a strong overall upward trend. A correlation of sucrose content with cold hardiness is clearly suggested by these results but further experiments showed that cold hardiness depended on more than just sucrose or sugar content. In spring wheats total sugar declined towards the end of October. This decline was due to a fall in sucrose and in monosaccharide levels. Thus in early September and late October there was a time when these plants contained equal quantities of sugar. However in September the plants were killed

by exposure to -8°C, whereas in October they survived after exposure to -14°C. Clearly the low temperatures in the intervening period had brought about more physiological changes in the plants, which conferred hardiness on them, than just an increase in sugar content during the cold period. The permanent increase in sugar content, principally sucrose in the winter varieties of wheat and rye were, however, thought to be significant in conferring greater hardiness on these plants.

The combined results of the experiments above suggest there is a relationship between high sucrose levels and low temperature tolerance in plant tissues. In the reports above sucrose build up does not correspond with glucose decline as has been seen in pea roots. Rather the reverse is the case. Glucose and/or fructose tend to increase in level also. In potato the hydrolysis of starch, and in the cerals, photosynthesis, could provide sugars and thus make possible the increases in sucrose and monosaccharides levels. In pea roots it seems the supply of material to the root tip was not sufficient to allow any increase in total sugar but nevertheless a high sucrose content in response to low temperature was established with a concomitant decline in glucose content. Thus even in this actively growing organ, low temperature tolerance appears to be associated with a relatively high sucrose content. The metabolic consequences of the high sucrose level can only be speculated on at this stage (Levitt 1972).

The results for maize will be discussed in the light of what has been found for pea, since the carbohydrate metabolism of pea is that of a plant able to grow successfully at all the temperatures studied.

At 2°C the total sugar content in maize roots showed a

continuous decline (Fig. 8). Figs. 10 and 12, where the changes in level of the individual sugars, fructose, glucose and sucrose are plotted, shows this decline is shared nearly proportionately by all three sugars. The only exception to this is the sucrose content after 24 hr of exposure to 2°C. The rise in absolute amount at this temperature may reflect the beginning of an alteration in the root metabolism such as was seen in pea, i.e.: the establishing of a high sucrose content at the cold temperature. However the continued exposure to the low temperature results in a breakdown of carbohydrate metabolism in the maize root tip as evidenced by the continuous decline in sugars over the rest of the 120 hr period.

Only at 6°C and above is the pattern seen in pea established, viz: a relatively high sucrose and low glucose content at lower temperatures and a rise in the proportion of glucose and a fall in the proportion of sucrose with rising temperature. This trend can be seen for these three temperatures from Fig. 13.

Conclusions.

1. Three soluble carbohydrates predominate in the root tips of pea and maize; they are fructose, glucose and sucrose.
2. With regard to the metabolism of these sugars both pea and maize adopt similar strategies when subjected to temperatures near their respective lower limits for growth. The proportion of sucrose is relatively high and of glucose relatively low. With rising temperature the proportion of sucrose declines while that of glucose increases. The proportion of fructose in the root tips of both species shows little change with time, at any of the four temperatures used.

3. There is an adequate supply of sugar to the roots of pea over the 120 hr period in the cold incubators as judged by the levels of sugar being approximately the same at the beginning and end of this time. Thus the altered proportions referred to above (2.) are physiological responses to temperature independent of the effect of temperature on transport. (However at time 0 hr the roots were drawing on endogenous reserves whereas at time 120 hr they were utilising reserves translocated from the "seed". The translocation of sucrose to the root tip commences in pea at or shortly after, time 24 hr in the 14°C incubator, and at the lower temperatures of 10, 6, and 2°C it starts between 72 and 120 hr. These times are 4-8 days after first soaking the dry seeds.)

4. In maize translocation has started by the time the seedlings are moved to the cold incubators, that is, by the end of the fifth day from soaking. Sugar supply is adequate at temperatures of 6°C and above. Thus as in pea the relatively high sucrose content at 6°C is a physiological response to temperature independent of sugar supply.

5. In the maize root tip exposed to 2°C the amounts of all the sugars studied declined over 120 hr to approximately 50% the initial amounts. The rate of utilisation is clearly exceeding the rate of supply.

These conclusions raise several questions:

1. How does temperature act on the seedlings to bring about the alterations in the proportions of glucose and sucrose observed in the root tips?

2. At temperatures near the minimum for growth, what is the physiological significance of relatively high sucrose and low glucose levels? Are they of significance for continued growth?

3. What is the process utilising sugars at 2°C in maize?

4. Why does the process of supply fall short of demand in maize at 2°C?

It was proposed at the end of Chapter 2 to examine the energy supply system of seedling roots with respect to temperature, to discover if temperature effects on this system account for the very different rates of growth observed between pea and maize. The level of soluble carbohydrates acting as substrates for energy supply has been examined above. In the following chapter the rate of respiration with respect to temperature is studied. The combined results and conclusions from these experiments indicate how answers to some of the above questions may be sought experimentally. A full discussion of this therefore follows the results of the experiment to study rates of respiration, presented in the following chapter.

CHAPTER 4

STUDIES OF RESPIRATION RATE IN ROOTS

Introduction.

The respiration rates of the seedling roots of pea and maize were examined at the same temperatures and over the same time periods as was carbohydrate content and growth rate. The aim of these measurements was to determine the effect of temperature on respiration rate of the two species and to determine how closely respiration rate was correlated to growth rate.

Method.

Determinations of respiration rate were made on seedlings grown under identical conditions to those used in the studies of soluble carbohydrate levels, and were made after the same time intervals at the cold temperatures (p. 35). Again, only the distal 1 cm of the seedling roots was used. These segments were excised in phosphate buffer previously equilibrated to the particular temperature at which the roots were grown, and then transferred to Warburg flasks and the oxygen uptake and carbon dioxide evolution measured on a Gilson respirometer, equipped with a refrigerated water bath. All determinations were made at the same temperature as that to which the roots had been exposed in the cold incubators, except that, at time 0 hr (after germinating at 20^{o}C) the respiration rate of the roots was determined at 2, 6, 10 and 14^{o}C. Full details of the method are given in Appendix 2.

Results and Discussion.

The results are presented in Figs. 14 and 15 respectively for pea and maize. They are expressed as oxygen uptake (µl/mg DW/hr) against time exposed to each temperature, 2, 6, 10 or 14°C. The results for carbon dioxide were similar in their general trend and are not included. Usually oxygen uptake exceeded carbon dioxide evolution, and R.Q.s in the region of 0.8 were obtained. The reason for this was not investigated, but may be due to some fixation of carbon dioxide by roots (Stolwijk and Thimann 1957).

At the lowest temperature, 2°C, both pea and maize maintain constant rates of oxygen uptake over the 120 hr period of the experiment but that of pea is approximately twice that of maize, 0.8 as compared with 0.4 µl/mg DW/hr. This low respiration rate in maize may be one of the factors contributing to the cessation of growth at this temperature. Creencia and Bramlage, 1971, reported that uncoupling of oxygen uptake from oxidative phosphorylation occurred in the leaves of maize after the plants were chilled 36 hr at 0.3°C. This uncoupling was measured at 25°C. It is possible that uncoupling in the roots also occurred but the results of the present experiment where respiration was measured at 2°C and where the rate of oxygen uptake was very low suggests that other processes involved in oxygen uptake, e.g.: the Kreb's cycle, are also disturbed by the low temperature.

The relatively high respiration rate of the pea root compared with the maize root finds a parallel in the respiration rates of plants native to arctic or alpine climates when these are compared with species from warmer areas. Frequently, it has been found at temperatures just above freezing point that the respiration rate of arctic or alpine plants is higher. Mooney and Billings (1961)

Fig. 14. Rate of O_2 uptake by excised 1 cm root tips of pea seedlings against time grown at the temperatures of 2, 6, 10 or 14°C. At time 0 hr seedlings had been germinated for 72 hr at 20°C.

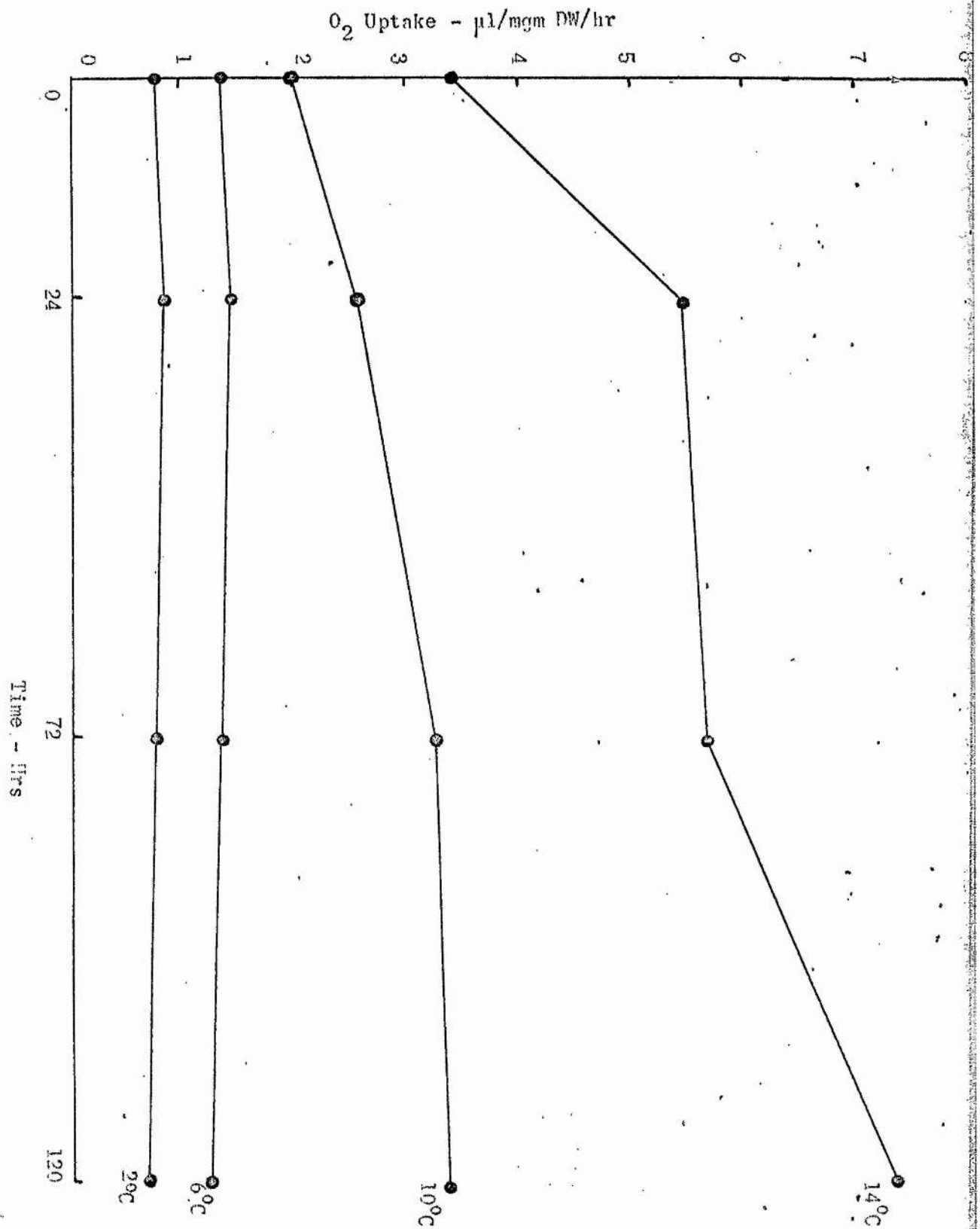
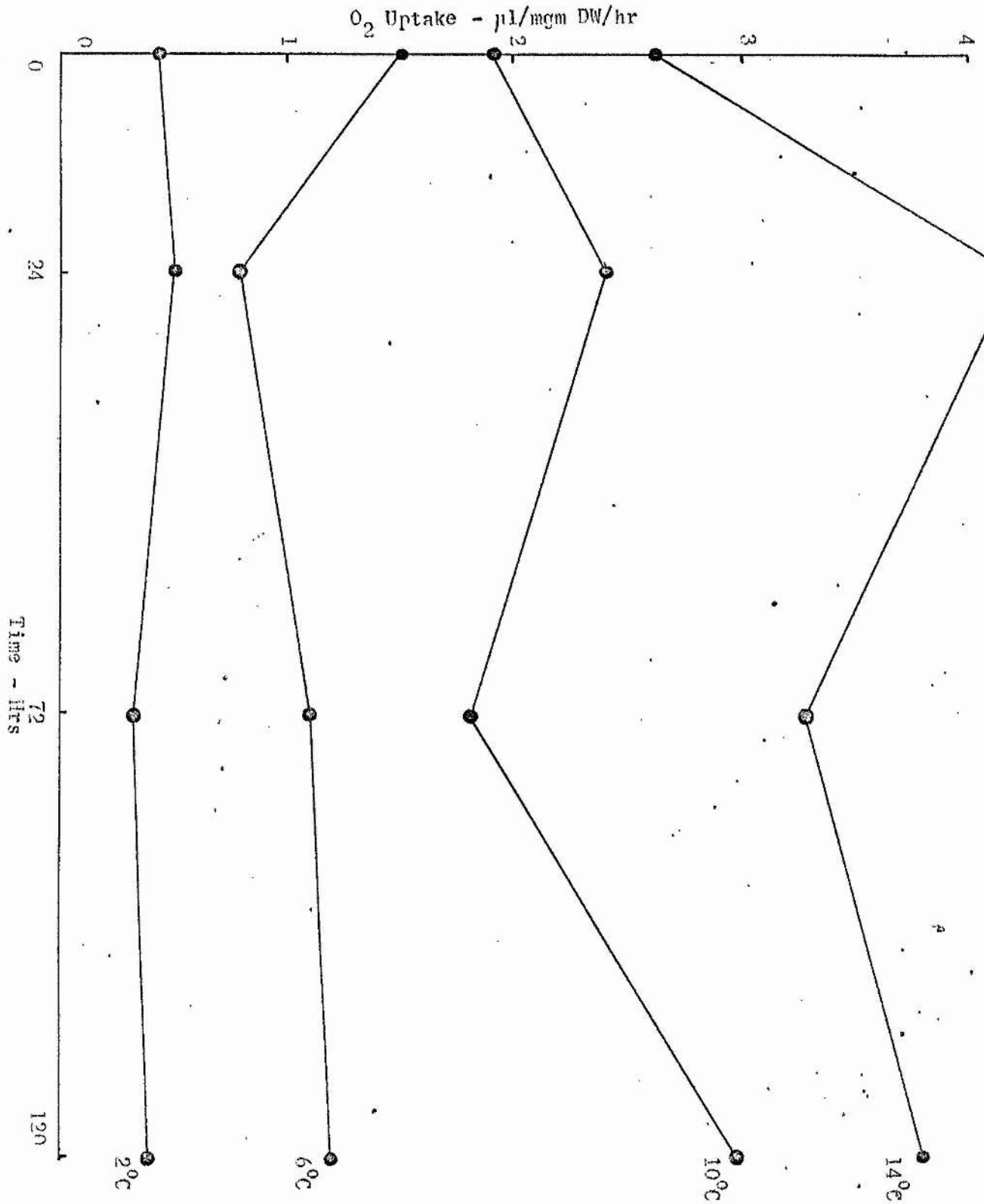

O_2 Uptake - µl/mgm DW/hr
0
1
2
3
4
5
6
7
8
Time - hrs
0
24
72
120
2°C
6°C
10°C
14°C

Fig. 15. Rate of O_2 uptake by excised 1 cm root tips of maize seedlings against time grown at the temperatures of 2, 6, 10, or 14°C. At time 0. hr seedlings had been germinated for 120 hrs at 20°C.


O_2 Uptake - µl/mgm DW/hr
0
1
2
3
4
Time - hrs
0
24
72
120
2°C
6°C
10°C
14°C

demonstrated this feature in Oxyria digyna populations from the Yukon (Northern Canada) and Colorado. The Yukon population showed a higher respiration rate than the Colorado population at all temperatures up to 20°C. Just above 0°C the higher respiration rate was believed to be important in allowing the plants to complete their life cycle in the very short growing season of the Yukon. They quote the work of Pisek and Winkler (1958) where Picea excelsa leaves were found to have a higher respiration rate at higher altitudes, and a further example comes from the work of Mooney (1963) where an examination of the respiration rates of rhizomes of Polygonum bistortoides from coastal, subalpine and alpine populations showed there was a higher respiration rate in rhizomes of plants from higher altitudes. Scholander and Kanwish (1959) examined the respiration rate of nine higher plants collected from Labrador and Massachusetts but only two species showed significantly higher respiration rates in the northern population. In lichens Scholander et al. (1952) found no evidence at all of higher respiration rates in species collected from the arctic as compared with those collected from the tropics.

The majority of evidence described above suggests that the relatively higher respiration rate of the pea roots at 2°C is a very important feature for the ability of this species to maintain active growth at this low temperature.

At 6°C, pea again maintains a steady rate, around 1.4 µl/mg DW/hr. Maize shows an initial drop in rate (from 1.5 to 0.75 over 24 hr) and then a partial recovery to 1.2 µl/mg DW/hr, over the rest of the 120 hr period. This behaviour again illustrates how the metabolism of maize is disturbed at 6°C, which is the borderline temperature for the growth of the roots. Possibly the rise in

oxygen uptake is due to progressive uncoupling of respiration but Creencia and Bramlage report under their conditions (seven day old seedlings, grown at 21°C in continuous light at the start of the chilling treatment) that no chilling damage was observed in seedlings held 8 days at 6°C.

At the two higher temperatures, 10 and 14°C, pea and maize roots show an increase in rate of oxygen uptake with increasing time exposed to these temperatures. This may be a sign of metabolic adaptation occurring. If the increasing rate is coupled to increased production of high energy compounds then biosynthesis could proceed at a faster rate. However, there is no evidence of increase in growth rate with time at these temperatures.

Two results, which emerge from the experiment, of significance for the relationship between growth and respiration rates at low temperatures are illustrated by the Q10 data and Arrhenius plots for these two processes after 120 hr in the cold incubators.

The Q10, calculated over the 4° temperature intervals, for rates of respiration and growth are presented in Table 7 and Fig. 16. In pea a close correlation exists between the values over the entire temperature range. In the range 2-6°C the root growth and respiration rates are relatively unresponsive to temperature, presumably metabolism is operating just above the minimum rate required for the root to remain viable and grow. Over the 6-10°C interval the very high Q10 indicates that growth and oxygen uptake are very sensitive to temperature and that these processes increase their rates rapidly from the low rates observed near the minimum temperature.

In maize, the actual amounts of growth made at 2 and 6°C

Table 7. Q10 for the rates of growth and of oxygen uptake of the roots of pea and maize, after 120 hr at 2, 6, 10 and 14°C (following germination at 20°C for 72 and 120 hr respectively).

Temp. interval °C	Pea		Maize	
	Q10 growth[x]	Q10 O_2 uptk.	Q10 growth[x]	Q10 O_2 uptk.
2 - 6	2.76	3.96	3.41	15.6
6 - 10	17.9	11.1	45.8	9.88
10 - 14	6.22	6.99	2.92	1.81

[x]Values calculated from data of Figs. 1, 2, 3 and 4 and Table 4.

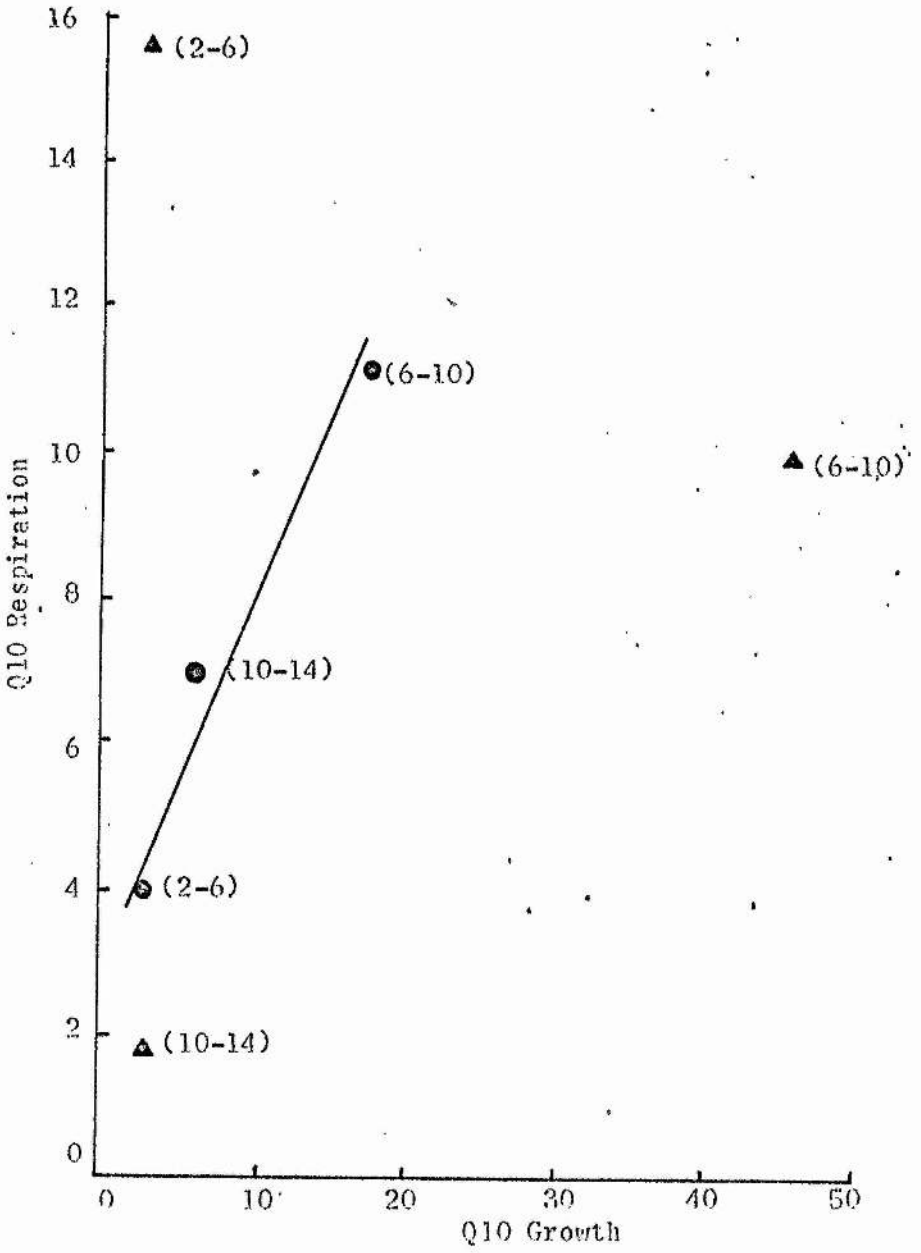


Fig. 16. Relationship between Q10 for growth and Q10 for respiration, of roots of pea (●——●) and maize (▲——▲). Figures in brackets indicate temperature ranges over which Q10 determined in °C.

are very small and are not sustained for more than approximately 120 hr (Fig. 3). The very high Q10 for growth over the range 6-10°C is due to growth continuing at a constant rate at 10°C and thus producing a much greater increase in length than was recorded at 6°C. Respiration has a lower minimum temperature, however. Thus the Q10 for this process is highest for rates measured over the lowest temperature interval and steadily declines when measured over higher intervals.

At the highest interval of temperature recorded, 10-14°C, growth and oxygen uptake of maize display a Q10 typical of many biological systems (Forward, 1960). Pea is still rather higher than might be expected but other reports in the literature indicate this species has a high Q10 for many of its growth processes (Kotowski, 1926). The close correlation between Q10 for growth and Q10 for respiration seen in the results for pea is not shown by the results for maize, Fig. 16.

The Arrhenius plots for growth and respiration are presented in Figs. 17 and 18. (The respiration rate used was that after 120 hr in the cold incubators.) For this type of graph more than four points are desirable but even with the present data a trend established in the work of Lyons and Raison (1970) can be detected. They obtained the respiration rates of mitochondria isolated from chill sensitive and chill resistant plant tissues over a range of temperatures, 0-25°C. Arrhenius plots of the data fell into two classes which were distinctive of the two groups of plants used. They found in chill sensitive plants (tomato and cucumber fruits and sweet potato roots) a linear decrease in the Arrhenius plot down to approximately 10°C. There a break in the graph occurred and below this temperature there was a marked increase in the slope. In chill resistant tissues (cauliflower buds, potato tubers, and

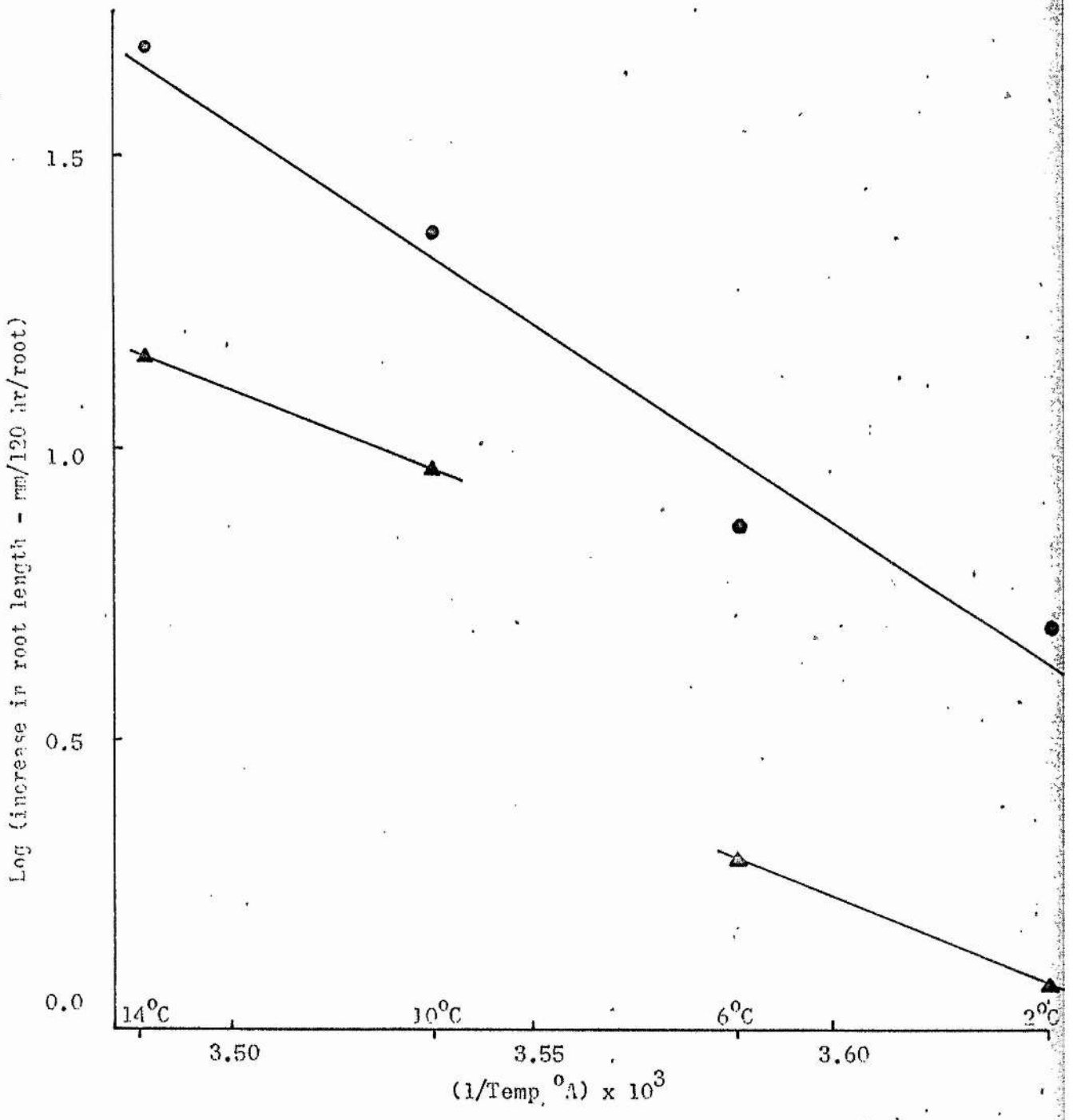


Fig. 17. Arrhenius plot for the increase in length of roots of pea (●——●) and maize (▲——▲), over 120 hr at 2, 6, 10, or 14°C (following germination at 20°C for 72 and 120 hrs respectively).

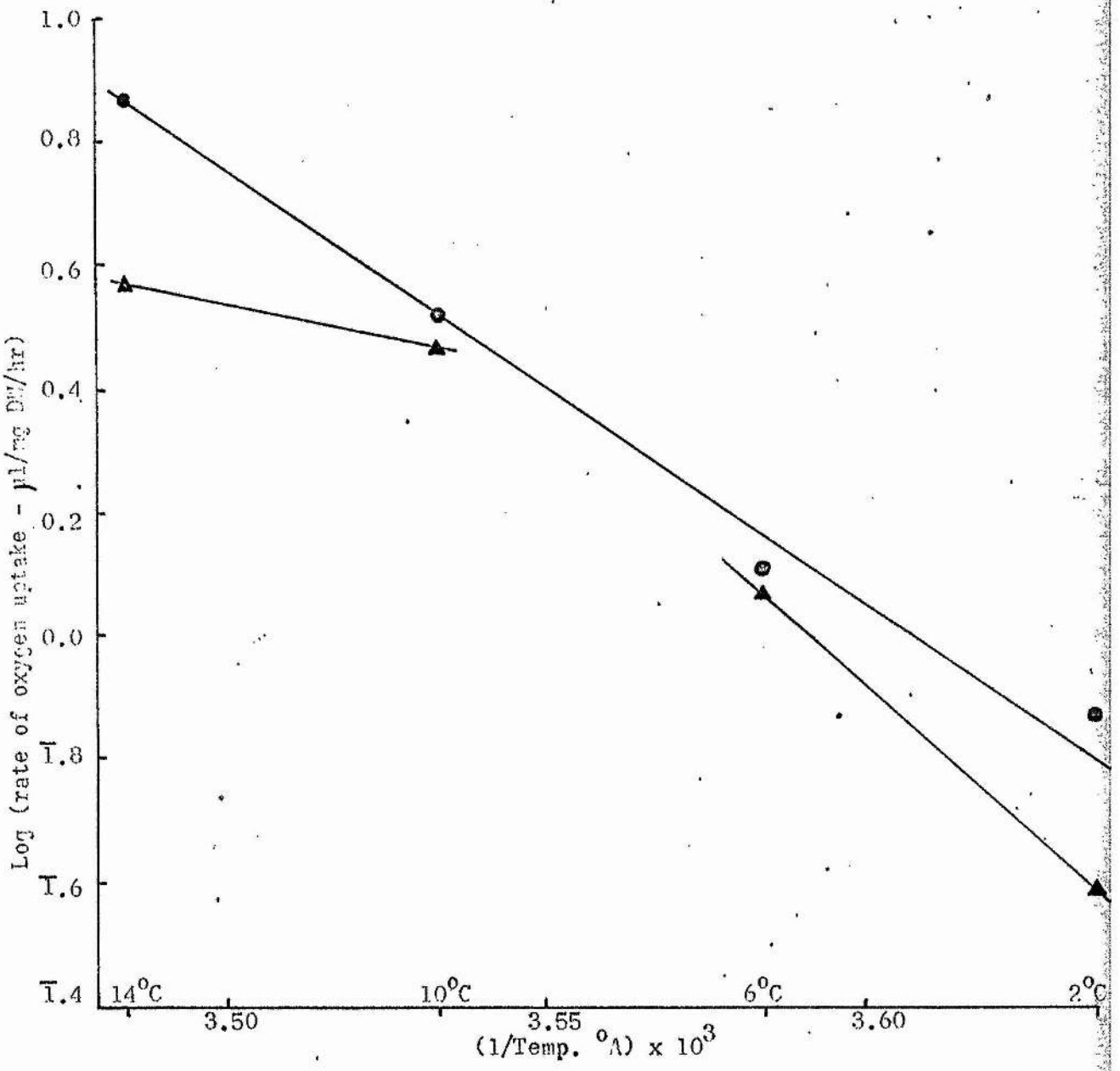


Fig. 18. Arrhenius plot for oxygen uptake by excised 1 cm root tips of pea (⊙—⊙) and maize (▲—▲) seedlings, grown 120 hr at 2, 6, 10, or 14°C (following germination at 20°C for 72 and 120 hr respectively).

beet roots) there was no evidence of any break in the graph, it decreased linearly over the range 25-1.5°C. In all tissues, phosphorylative efficiency (measured by the ADP:O ratio) remained constant at all temperatures. They concluded the response of sensitive plants to temperatures in the chilling range (0-10°C) was to depress mitochondrial respiration to an extent greater than expected fromQ10 values measured above 10°C. In the present experiment pea is behaving as a typical chill resistant plant and maize as a chill sensitive plant whether growth or respiration rate is used to construct the Arrhenius plot.

Thus the Arrhenius plot of growth or respiration rate for pea over the range 14-2°C is a straight line whereas for maize there is a distinct break between the temperatures of 6 and 10°C. (At the lower temperatures, 2 and 6°C, the slope of the line is steeper than at the higher temperatures 10 and 14°C.) As was mentioned above, the respiration rates used to construct the Arrhenius plots were those after 120 hr in the cold incubators. The question arises as to whether the break in the Arrhenius plot for maize respiration rate occurs between 6 and 10°C independently of time of exposure to these temperatures, i.e.: is respiration rate always markedly depressed by temperatures at and below 6°C compared to temperatures at and above 10°C? An Arrhenius plot for rate of respiration at the end of the germination period, i.e. when the roots had experienced only 20°C, provides an answer to this question (Fig. 19). It shows the break occurs between 2 and 6°C. This indicates that respiration suffers immediate disturbance at 2°C, but at 6°C it is only slowly disrupted. Since at 6°C, respiration rate initially declines and then partially recovers, (Fig. 15,), the disturbance

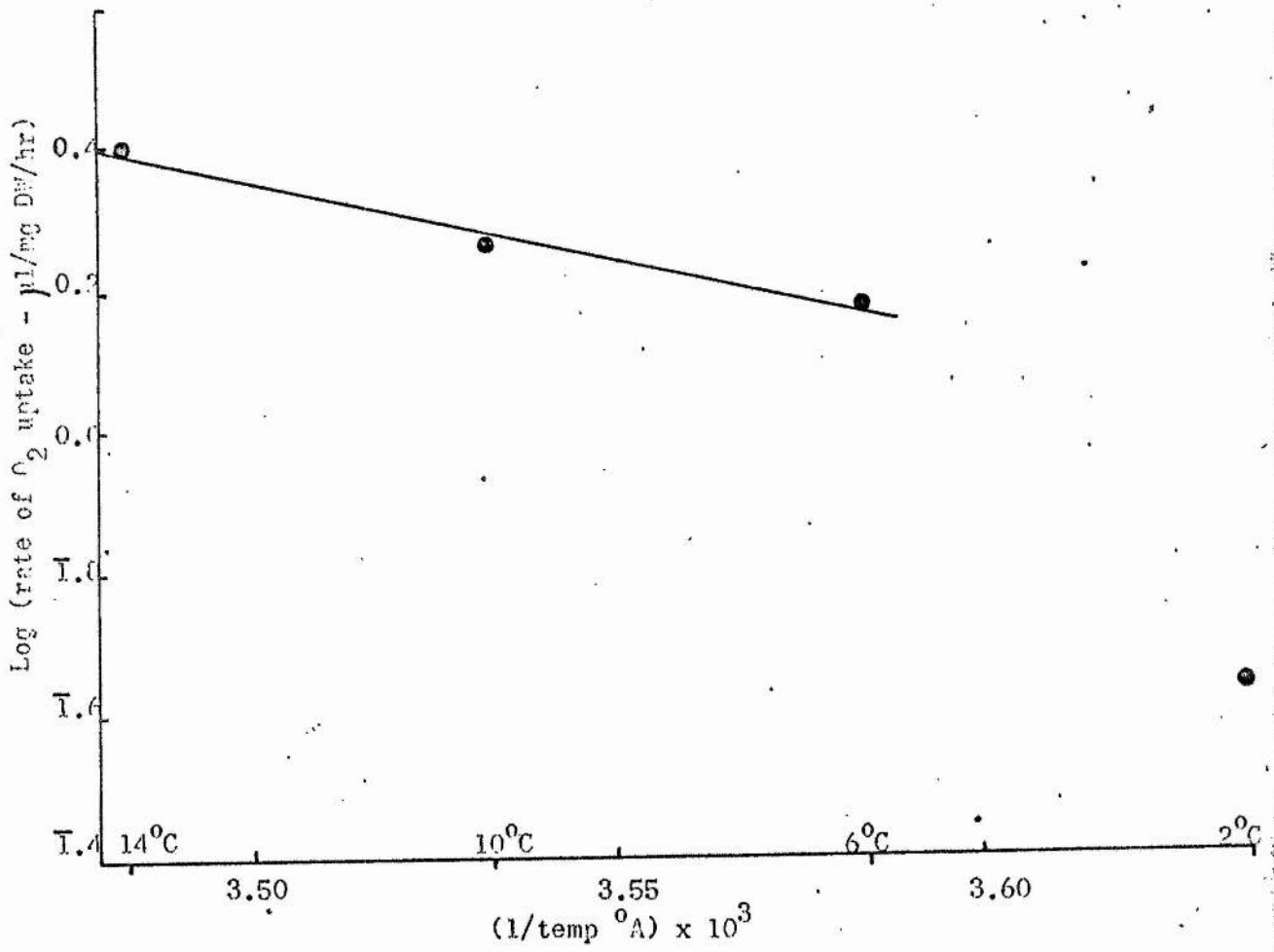


Fig. 19. Arrhenius plot for oxygen uptake by excised 1 cm root tips of maize seedlings, grown 120 hr at 20°C.

indicated by the Arrhenius plot is probably checked rather than still progressing by the end of the experimental period.

Conclusion

Two important points arise from this experiment.

1. In pea, a plant exhibiting constant growth rates at all temperatures in the range 2-14°C, there is a close correlation between the responses of respiration rate and growth rate to temperature. This correlation is not apparent in maize over the 2-6°C range. At 2°C growth ceases but respiration continues at a low rate. At 6°C growth almost stops over 5 days, whereas respiration rate is quite appreciable.
2. This lack of correlation in maize arises from a disturbance to respiration at 2 and 6°C. The roots display the physiological symptoms of chilling injury demonstrated by Lyons and Raison (1970). This is immediate at 2°C but at 6°C it only becomes apparent over a prolonged period of time at this temperature.

This thesis is concerned with the nature of the physiological differences between pea and maize which provide for the growth of pea at temperatures just above 0°C. Clearly in pea the harmony between respiration rate and growth rate is one important feature of this. From the conclusions of the previous chapter it is seen that maintenance of sugar levels in the root tip and a specific soluble carbohydrate composition are also important features in pea at 2 and 6°C. In maize, soluble carbohydrate levels are maintained only at and above 6°C and also, only then is a sugar composition comparable to pea established.

Of the four questions raised at the end of the previous

chapter (p. 60'), one can now be answered, that which asked the process utilising soluble carbohydrate in maize at 2°C. The low respiration rate is considered responsible. The observed decline in the total sugar content determined from the data in Chapter 3 is presented alongside the calculated quantity of sugar which would be consumed by the maize root tip respiring at the average rate determined in this chapter, Table 8. It is calculated that approximately 40% of the total sugar lost in maize roots at 2°C over the 120 hr period can be accounted for by respiration. The low respiration rate is presumably sufficient only for maintaining the integrity of the root cells, at least over 120 hr, and not for maintaining sugar levels or providing for root growth. The causes of the low respiration rate are now, of course, a problem in themselves requiring investigation. This problem is discussed in Chapter 6.

The remaining three questions were enquiries into how temperature acts on the root to cause the alteration of proportions of sugars; whether the particular proportions are of physiological significance for growth; and why, in maize at 2°C and to some extent at 6°C, does soluble carbohydrate supply fail. This last question is answered at least partially by the respiration rates at these two temperatures. At 2°C the very low rate may be insufficient to provide energy for sucrose transport from the "seed" down to the root meristem. At 6°C respiration rate initially declines and then recovers. The recovery is not thought to be due to progressive uncoupling occurring as the seedlings are held at 6°C (a view supported by the work of Creencia and Bramlage, 1971; Lyons and Raison 1970). It is thought that the recovery in respiration rate is coupled to oxidative phophorylation and is

Table 8. Maize seedlings incubated at 2°C for 120 hr. A comparison of the decline in sugar content in the roots with the amount of sugar consumed by respiration.

To calculate the amount of sugar consumed by respiration.

Assume respiration of the roots proceeds according to the equation:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O \qquad \text{(Equation 1.)}$$

Average rate of oxygen uptake of 1 cm root tips at 2°C is 0.4 µl/hr/mg DW (Fig. 15). Therefore, over 120 hr, 48 µl O_2 respired/mg DW

Since 1 µM O_2 occupies 22.4 µl at N.T.P.,

Then 48 µl O_2 = 2.14 µM/mg DW.

From Equation 1. 2.14 µM O_2 reacts with 0.352 µM hexose

Thus 0.352 µM of hexose utilised/mg DW in respiration of maize roots at 2°C for 120 hr.

Measured decline in sugar content in maize roots over 120 hr at 2°C.

From chapter 3 the net decline in sugar content (glucose, fructose and sucrose - considered as potentially two units of hexose) at 2°C over 120 hr is 15.48 mg/g FW (Fig. 8).

15.48 mg/g FW = 85 µM/gFW

Assume the DW of the roots = 1/10 FW

Then decline in sugar content = 0.85 µM/mg DW.

Thus respiration accounts for $\frac{0.352}{0.85} \times 100$

= 41% of the sugar loss in the roots at 2°C over 120 hr.

making more energy available for sugar transport and accumulation. This idea receives support from the observed changes in the sugar content in the root tip at 6°C in maize. The sugar content initially declines and then recovers. This parallels the changes in respiration rate and indicates the close interdependence of these processes. Thus the outstanding problems to be investigated concerning sugar metabolism are: 1) how temperature change is responsible for change in proportion of the sugars, principally glucose and sucrose, and 2) whether these proportions are of significance for respiration and growth rates or if they correlate with temperature independently of these processes.

An investigation of the second problem is described first. This problem was investigated by attempting to change the proportions of the various sugars at the lower temperatures of 2 and 6°C and observe the effect on respiration and growth rate. This experiment is described in the following chapter.

CHAPTER 5

TO DETERMINE THE EFFECT OF EXTERNAL SUGAR SOLUTIONS ON ROOT GROWTH AND RESPIRATION RATES

Introduction

At temperatures just above the minimum for the growth of pea and maize roots the proportion of sucrose (expressed as percent total sugar) has been shown to be relatively high while that of glucose was relatively low (p. 59). The experiment described below aims to discover how significant these proportions are in influencing respiration rate and for supporting growth at low temperatures. The approach used was to try and change the internal levels of particular sugars (this would alter the proportions of all sugars when expressed as percent total sugar), and observe the effects on both growth and respiration rates. This was attempted by replacing the water-saturated filter paper on which the seedling roots were grown with filter paper saturated with dilute solutions of particular sugars.

Method

Seedlings of pea and maize were germinated and grown as previously described (p. 16), but, on transference to the cold incubators, the seedlings were placed on filter paper saturated with 0.05 M fructose, glucose or sucrose solutions instead of with water. The concentration of 0.05 M was chosen because this presented no osmotic difficulties to the young roots. Every 24 hr seedlings were transferred to clean boxes with the appropriate fresh

sugar solution. This was to maintain the sugar concentration around the roots and minimise the growth of microorganisms. The boxes and solutions were previously equilibrated to the experimental temperatures. Only the two lowest temperatures were used, 2 and 6°C, and seedlings were kept in the cold incubators over the standard 120 hr period. Growth of the roots was recorded by the method described on p. 10. At the end of the 120 hr period the sugar content and the respiration rate of the root tips was determined by the methods described in Appendices 1 and 2 respectively, but with the following modifications. First, the root tips to be analysed for sugar content were rinsed thoroughly, in three changes of distilled water, to remove the external sugar solution before being surface-dried and weighed. Second, while in the Warburg flasks, the root tips used in the determination of respiration rate were bathed in phosphate buffer containing 0.05 M concentration of the same sugar as that in which the roots had been growing. Water-grown controls were treated similarly to the seedlings grown in the sugar solutions.

Results and Discussion

Pea and maize seedlings were grown for 120 hr at 2 or 6°C in the presence of either fructose, glucose or sucrose solutions. Three sets of data were obtained from the experiment:

1. The absolute levels and proportions of sugars in the seedling root tips after the 120 hr period.
2. The rate of growth of the roots during the 120 hr.
3. The rate of respiration of the root tips at the end of the 120 hr period.

The aim of growing the roots in the presence of sugar solutions was to obtain proportions of sugars in the roots different from that in water grown controls. Thus the results for the proportions of sugars in the root tips are presented first to show the instances where this change of proportions was achieved. No attempt was made to demonstrate the process of uptake of sugars from the solutions bathing the roots. Where roots were supplied with sugars and the internal levels of sugars were found to be significantly different from the water controls, it was assumed that uptake of sugars had occurred and was responsible for the differences. There are many reports of uptake of sugars from the external medium by roots (Hellebust and Bidwell, 1962; Brown and Sutcliffe, 1950). Grant and Beevers (1964), recorded the uptake of glucose, by the excised distal 2 cm segment of maize roots, from a 0.001 M solution despite a calculated minimum internal concentration of 0.05 M glucose. Furthermore, the glucose taken up mixed extensively with the endogenous pool. (Thus the specific activity of $C^{14}O_2$ evolved in respiration after feeding the roots with uniformly C^{14}-labelled glucose was, as predicted, 1/6 of the specific activity of the glucose extracted from the tissue.) White (1951), using excised roots of pea, demonstrated their ability to utilise supplied sucrose or glucose. Thus it seems certain that sugar uptake occurred in the present experiment. The low temperatures used may retard the rate of uptake (Grant and Beevers, 1964, reported that, in carrot discs, rate of uptake was ten times faster at 25°C than at 3°C), but with the long period of exposure to the sugar solutions (120 hr), it was hoped to overcome this problem and allow for entry of at least some sugar into the root cells.

1. Proportions of sugars in the root tips after growth in sugar solutions or in water.

Table 9 gives the proportions of the component sugars (as percent total sugar), in the root tips of pea and maize grown in solutions of fructose, glucose or sucrose, and in the water-grown controls. Changes in the proportions of glucose and sucrose are of particular interest, see p. 59, and are best revealed by comparing the ratio of glucose to sucrose in the experimental treatments with the ratio for the water-grown control. (The proportions of fructose in roots of both species varied little as between roots supplied with any of the three sugars and roots supplied with water. Thus changes in ratio of fructose to glucose or to sucrose are due to changes in the glucose or sucrose levels alone. For this reason the ratio of fructose to glucose or to sucrose is not considered).

From Table 9 it can be seen that, in pea roots supplied with sugar solutions, the glucose/sucrose ratio is markedly different from the water-grown control in only two cases; when grown in glucose solution at $2^{o}C$, and in sucrose solution at $6^{o}C$; whereas in maize the presence of any of the three sugar solutions externally resulted in substantial changes in the proportions of glucose and sucrose in the root tip, both at 2 and $6^{o}C$. The greater stability of the proportions of glucose and sucrose in pea roots reflects the fact that pea already possesses a metabolism suited to making growth at low temperatures and thus it may not be expected to respond to attempts to change the sugar balance of the root tip. It presumably already possesses levels of sugars which are omptimal or at least adequate for the conditions. By contrast, in maize at $6^{o}C$, and especially at $2^{o}C$, sugar metabolism has been shown to be

Table 9. Total sugar content and the proportions of component sugars in the distal 1 cm of the roots of pea and maize seedlings grown 120 hr in the presence of 0.05 M fructose, glucose or sucrose solution or in distilled water, at 2 or 6°C. (Seedlings initially germinated for 72 or 120 hr respectively at 20°C)

Solution bathing the roots	Total sugar content		Proportion of sugar in the root tip (% total sugar content)			Glucose/ sucrose ratio
	mg/g FW	% control	Fructose	glucose	Sucrose	
		Pea at 2°C.				
Water	14.08	100	24	29	47	0.62
Fructose	16.26	115	23	27	50	0.54
Glucose	24.76	176	20	40	40	1.00
Sucrose	26.26	187	20	28	52	0.54
		Pea at 6°C.				
Water	13.02	100	20	50	30	1.67
Fructose	18.40	157	26	45	29	1.55
Glucose	17.60	135	24	49	27	1.81
Sucrose	26.7	205	21	43	36	1.19
		Maize at 2°C				
Water	14.00	100	23	51	27	1.89
Fructose	16.62	119	28	34	38	0.89
Glucose	24.14	172	19	43	39	1.10
Sucrose	26.56	190	21	36	43	0.84
		Maize at 6°C				
Water	28.07	100	28	39	33	1.18
Fructose	32.50	116	32	43	25	1.72
Glucose	30.98	110	30	44	26	1.69
Sucrose	33.24	118	30	43	27	1.59

severely disturbed (the rate of utilisation is much greater than rate of supply, p. 60), and growth is not maintained for more than a short period. The external supply of sugars to the roots may provide the extra sugars needed to allow change in the internal sugar levels to those which are better suited to the low temperature conditions.

In addition to the proportions of sugars showing significant alterations under the various conditions the total sugar content also varied in both species. This is discussed below before the consequences of both these changes for growth and respiration rates are discussed.

2. Total sugar content of the root.

In all cases, with both pea and maize, root tips bathed in the sugar solutions had greater internal sugar contents than their respective water-grown controls, although in certain instances the increases over the control were small and may not have been significant. Thus in maize root tips, following growth at 6°C in the presence of any of the three sugars, the internal sugar content was not more than 20% greater than the water-grown control. Also, supplying fructose to the roots of either species usually raised the internal sugar content by not more than 20% above the control value (pea supplied with fructose at 6°C was an exception to this, sugar content of the roots was 57% greater than the control). An external supply of glucose or sucrose was responsible for the majority of large increases in total internal sugar content.

At 2°C the increased content of total sugar in maize roots grown in the presence of glucose or sucrose solution (but not

fructose solution), is sufficient to raise the total sugar content to near the level before the roots received the cold treatment, Table 10. The continuous decline in content with time seen in the water-grown control (Fig. 8 and Table 9), is largely offset, indicating that, in the root tips receiving glucose or sucrose externally, the problem of supply to the root tip is overcome.

In pea at both 2 and 6°C and in maize at 6°C the level of sugar is maintained or stabilises in roots grown in water over the 120 hr period (Figs. 7 and 8). The sugars in the external solutions serve to supplement sugars translocated from the "seed" and give the increased levels observed, Table 9.

In pea there is a very marked accumulation of sugar in the root tips supplied externally with any of the three sugars over the level of sugar in the roots of water-grown controls (except in one instance when fructose is supplied to the roots at 2°C, where there is little accumulation), Table 9. This accumulation of sugars in the roots at low temperatures, made possible by the external supply, may be compared with the accumulation seen in sweet potatoes, by starch hydrolysis, and in cereal leaves, by photosynthesis, discussed on p.55-7 (Arreguin-Lozano and Bonner, 1949; Vasil'yev, 1961). The accumulation of sugar in these two cases was in response to low temperatures as is seen for pea roots and, to a lesser extent, maize roots studied here.

3. The effects of changes in the sugar proportions on growth and respiration rates of the roots.

a) Pea at 2°C

In Fig. 20 the increase in root length is plotted for roots bathed in the sugar solutions and the water-grown controls, and in

Table 10. Total sugar content in the distal 1 cm of roots of maize seedlings before and after a period of 120 hr at 2°C in the presence of various sugar solutions or water.

Conditions of growth 120 hr at 20°C (germination period)	Solution bathing the roots	Total sugar content	
		mg/g FW	% of value at end of germination period
only	Distilled water	29.50	100
plus 120 hr 2° C	Distilled water	14.02	48
plus 120 hr 2°C	0.05 M fructose	16.62	56
plus 120 hr 2°C	0.05 M glucose	24.14	82
plus 120 hr 2°C	0.05 M sucrose	26.56	90

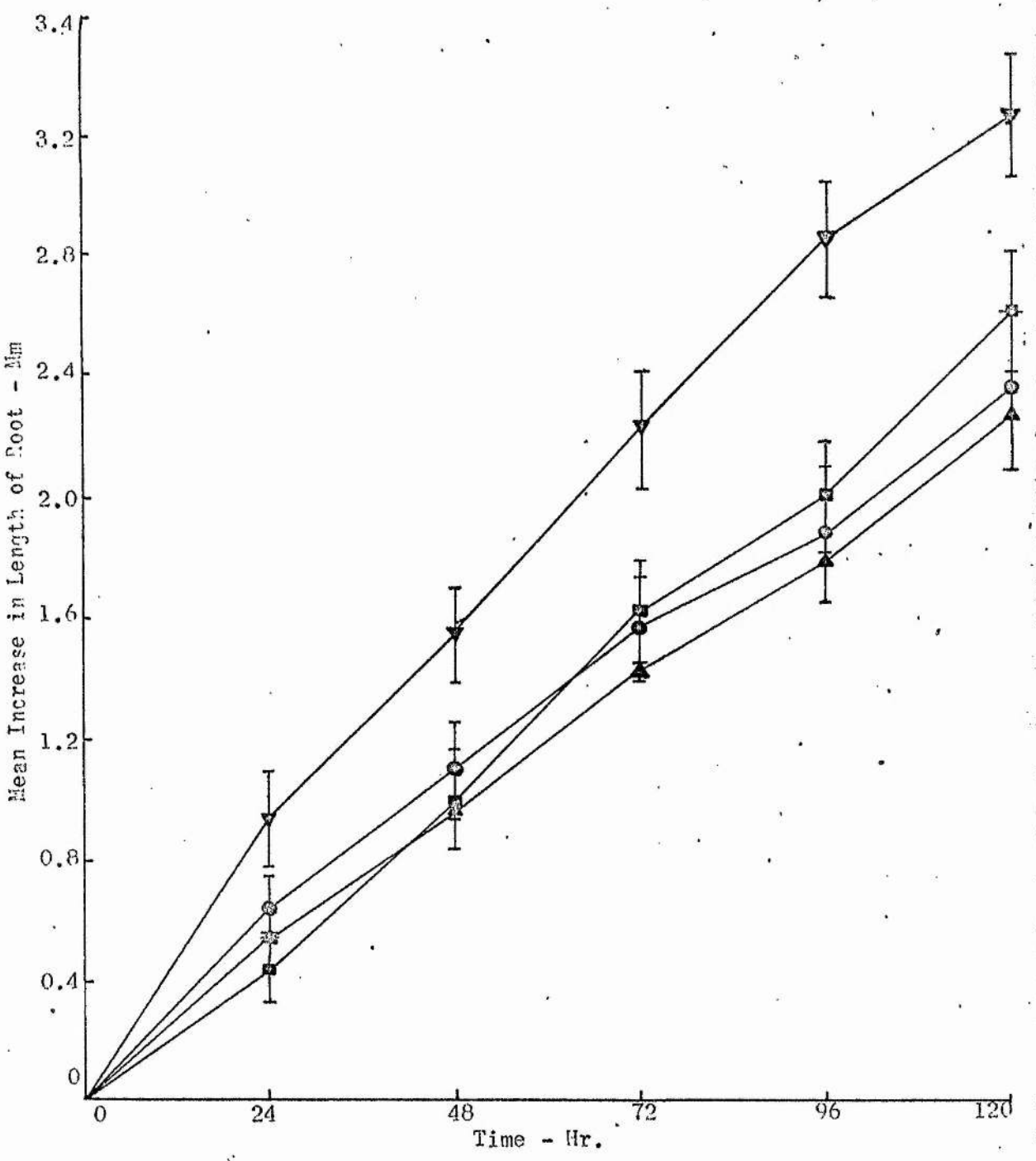


Fig. 20. Mean increase in length of pea seedling roots grown at 2°C against time. Seedlings grown in the presence of 0.05 M fructose (▲——▲), glucose (▽——▽) or sucrose solution (■——■), or distilled water (●——●). Time 0 hr. was after germinating seeds 72 hr at 20°C. (For clarity fructose error bars not shown.).

Table.11. Growth and respiration rates of the pea root after 120 hr at 2°C in the presence of 0.05 M solutions of either fructose, glucose or sucrose, or distilled water. (At the start of the experiment seedlings had been germinated 72 hr at 20°C.)

Solution bathing the roots.	Growth rate		Respiration rate	
	mm/120hr/root*	%control	$\mu l\ O_2/hr/mg\ ^{DW}$	%control
Water (control)	2.37 ± 0.27	100	0.75	100
Fructose	2.29 ± 0.21	97	0.74	98
Glucose	3.28 ± 0.20	138	0.82	109
Sucrose	2.62 ± 0.21	111	0.68	91

* Calculated from the slopes of the best straight lines through the four sets of points in Fig. 20.

Table 11 the growth rates presented are derived from the slopes of the best straight lines fitting the four sets of points on this graph.

At 2°C, the growth rate of roots supplied with glucose is significantly higher than with either of the other two sugars or the water-grown control. Respiration rate is also highest in the glucose-grown roots (9% higher than the control, Table 10). This increase, however, may not be significant, the value of 9% being the average result of replicated experiment. (In this and all cases replicates agreed within 5% of each other.) Thus in pea at 2°C, where the ratio of glucose to sucrose in the root has been raised by supplying glucose externally, growth rate has been increased substantially (39%), and this is accompanied by a smaller increase in respiration rate (9%).

By comparison, the glucose/sucrose ratio in roots supplied with fructose or sucrose was similar to the control and no significant changes in growth or respiration rates were observed.

b) Pea at 6°C

At 6°C, however, the situation is not so clear. There are no significant differences in growth rate under any of the treatments compared with the water control but the respiration rate is 19% higher in those roots bathed in glucose compared with the control, Fig. 21 and Table 12. This is despite the fact that only when the roots were grown in sucrose solution were the sugar proportions significantly different from the control. It may be that, at this temperature, which is well above the minimum for growth of pea roots, a larger change in the glucose/sucrose ratio is required for there to be a significant effect on growth rate.

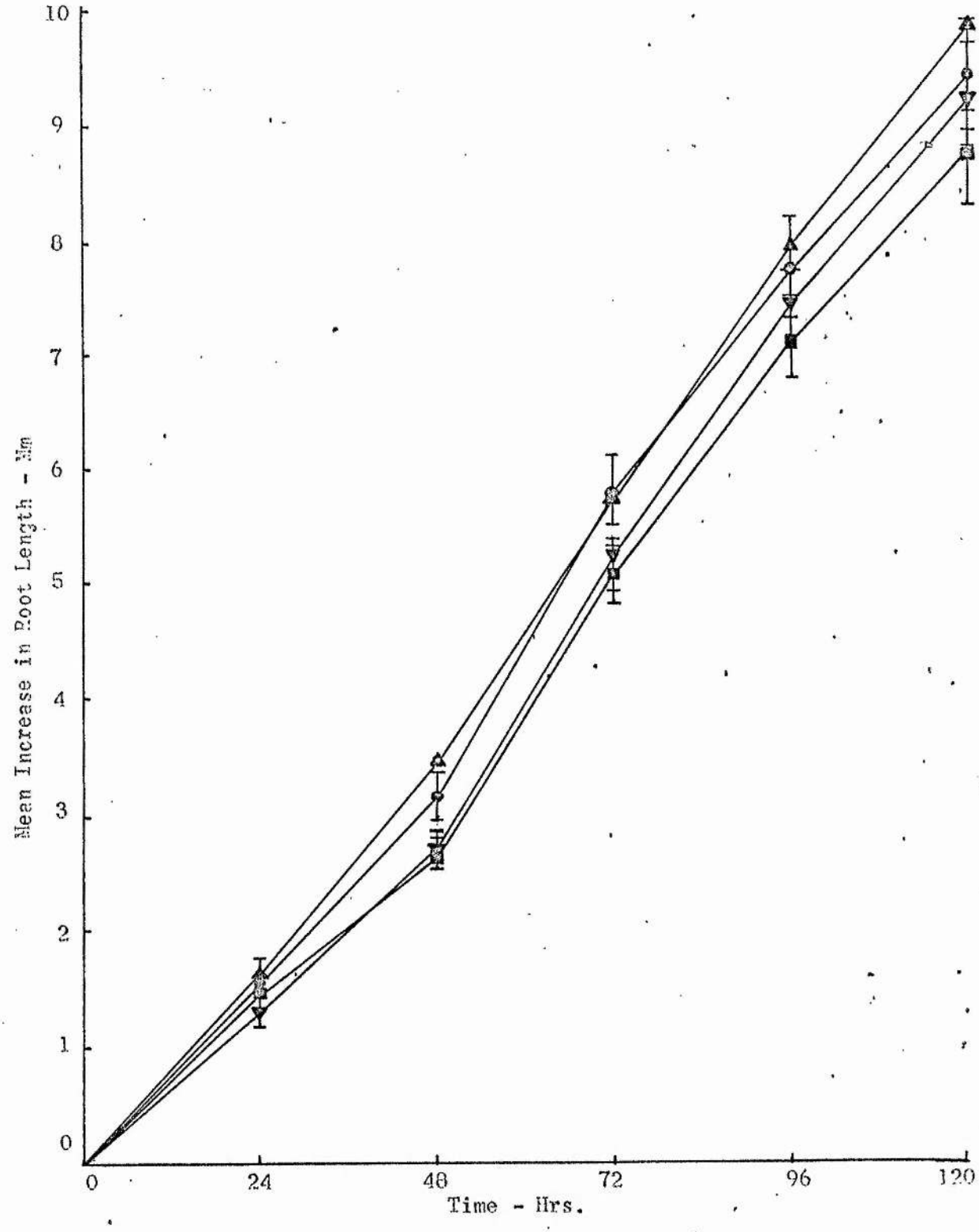


Fig. 21. Mean increase in length of pea-seedling roots at 6°C against time. Roots grown in the presence of 0.05 M fructose (▲——▲), glucose (▽——▽) or sucrose solution (■——■), or distilled water (●——●). Time 0 hr. was after germinating the seeds 72 hr at 20°C. (For clarity fructose error bars not shown.)

Table 12. Growth and respiration rates of the pea root after 120 hr at 6°C in the presence of 0.05 M solutions of either fructose, glucose or sucrose, or distilled water. (At the start of the experiment the seedlings had been grown 72 hr at 20°C.)

Solution bathing the roots	Growth rate		Respiration rate	
	mm/120hr/root*	%control	$\mu l \frac{O_2}{mg\ DW}/hr$	%control
Water (control)	9.48 ± 0.53	100	1.30	100
Fructose	9.56 ± 0.25	101	1.37	105
Glucose	9.48 ± 0.44	100	1.55	119
Sucrose	9.42 ± 0.41	100	1.34	103

*Calculated from the slopes of the best straight lines through the four sets of points on Fig. 21.

The different responses of the roots to glucose versus fructose in the one case, and to the monosaccharides versus sucrose in the other have been reported in other studies, using root cultures. White (1951) reported that sucrose was superior to glucose as an energy source in culturing pea roots (growth was faster and the roots more robust, with a larger, more extensively lignified stele and more cell layers in the cortex). Butcher and Street (1964), working with tomato root cultures, reported similar findings. They described the glucose-cultured roots as "carbohydrate deficient". This was despite the fact that both sucrose and glucose were rapidly absorbed by starved mature root cells and a similar pattern of C^{14} incorporation into cellular fractions was found after feeding uniformly labelled glucose or sucrose. Also, these starved cultures showed a stimulation of respiration rate when glucose, fructose or sucrose was supplied. This paradox was resolved when the root apex (the region where root growth occurs), was analysed separately from the whole root culture. Then it was found after sucrose feeding that higher levels of sucrose, fructose and glucose were established in this zone. After glucose feeding these higher levels were not established in the growing zone of the root apex. The mature root cultures responded only to sucrose since only sucrose was translocated to the root apex from mature cells.

In the present experiment the distal 1 cm of the roots was analysed. This unit is larger than that referred to by Butcher and Street (they were concerned with the distal 5 mm), but nevertheless, in intact pea roots as in cultured tomato roots, supplying sucrose raises the internal sugar content to a higher level than when supplying glucose or fructose, Table 9. However, in pea, the

increased sugar content of the roots after supplying sucrose is not associated with an increase in root growth. This may be because the sucrose supply does not stimulate respiration rate of the intact root.

c) Maize at 2°C

Table 9 shows that at 2°C the proportions of glucose and sucrose in the root tips of maize are altered, relative to the control, by supplying any of the three sugar solutions externally. The glucose/sucrose ratio is lowered and reaches values which are the lowest recorded for maize at any temperature, Table 13. Thus the trend established in pea over the temperature range 2-14°C now becomes evident in maize even at 2°C. This trend in pea was towards establishing a relatively higher level of sucrose and lower level of glucose (i.e.: a relatively low glucose/sucrose ratio) with lower temperature. In maize roots, supplied only with water, this trend was observed only over the temperature range 6-14°C (see p. 59). Now, following incubation of maize roots at 2°C in the presence of fructose, glucose or sucrose, the trend is extended to include this temperature. Since the metabolism of pea is able to support linear growth rates over the range 14 down to 2°C, an alteration in the metabolism of maize which results in closer resemblance to the metabolism of pea is regarded as an alteration better suiting the maize root to grow at this temperature, 2°C.

It is, in fact, found that the maize root is able to make significantly more growth in the presence of glucose solution compared with the control, Fig. 22 and Table 14. Fructose and sucrose solutions, however, do not affect growth of the maize root significantly. Growth rates over successive 24 hr intervals at 2°

Table 13. Proportions of glucose and sucrose in the distal 1 cm of roots of maize grown 120 hr at various temperatures and in different solutions. (At the start of the experiment seedlings had been germinated 120 hr at 20°C.)

Conditions of growth	% total sugar as:		Glucose/sucrose ratio
	glucose	sucrose	
14°C in water	62	12[x]	5.17
10°C in water	46	26[x]	1.77
6°C in water	39	33[x]	1.18
2°C in water	51	27[x]	1.89
2°C in 0.05 M fructose	34	38	0.89
2°C in 0.05 M glucose	43	39	1.10
2°C in 0.05 M sucrose	36	43	0.84

[x]Values obtained from Fig. 10.

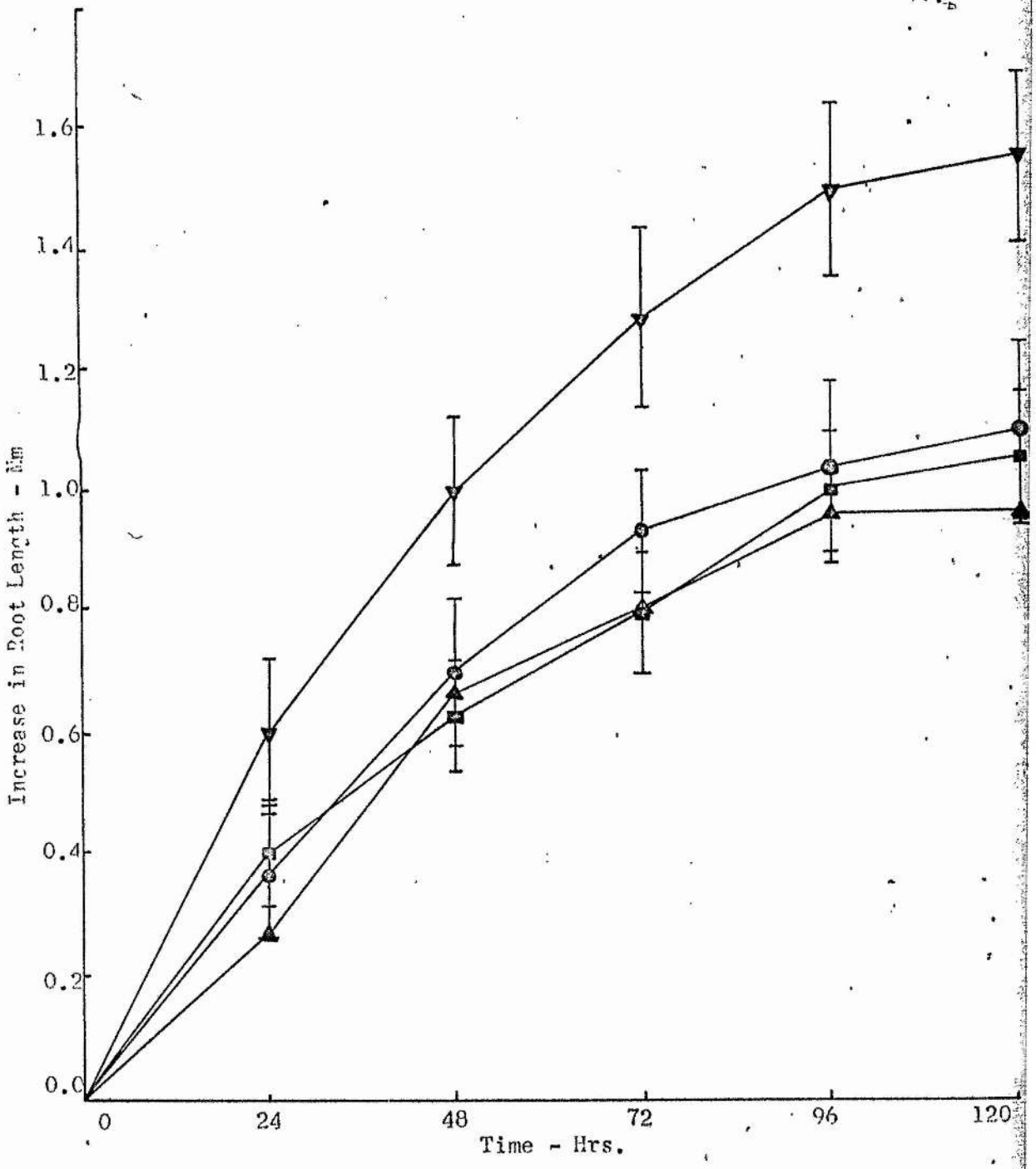


Fig. 22. Mean increase in length of maize-seedling roots at 2°C against time. Roots grown in the presence of 0.05 M fructose (▲——▲), glucose (▽——▽) or sucrose solutions (■——■), or water (●——●). Time 0 hr was after germinating the seeds 120 hr at 20°C. (For clarity fructose error bars not shown.)

Table 14. Growth and respiration rates of the maize root after 120 hr at 2°C in the presence of 0.05 M solutions of either fructose, glucose or sucrose, or distilled water. (At the start of the experiment seedlings had been germinated 120 hr at 20°C.)

Solution bathing the roots	Growth made over first 120 hr at 2°C		Respiration rate	
	mm/root	%control	$\mu l\ O_2/hr/mg\ DW$	%control
Water (control)	1.10 ± 0.15	100	0.39	100
Fructose	1.29 ± 0.14	117	0.72	185
Glucose	1.55 ± 0.14	139	0.55	140
Sucrose	1.05 ± 0.11	95	0.45	116

decline at approximately equal rates in water-grown (control) roots and in the roots grown in the three sugar solutions but where glucose is the sugar supplied externally the effect of the low temperature on growth rate is less marked over the initial 24 hr period at 2°C, Fig. 23. This results in the observed stimulation of growth at 2°C when roots are bathed by glucose solution. In this connection it is notable that in root culture of monocotyledons, glucose has repeatedly been found superior to sucrose in supporting growth, (Butcher and Street, 1964; Dure, 1960 a and b; White, 1951).

Respiration rate of roots grown in fructose or glucose solution is markedly higher than the rate in the control. The rate for roots in fructose solution is especially high. Sucrose produces only a small increase in rate (Table 14).

Thus in maize at 2°C the effects of supplying different sugars to the roots can be summarised as follows:

1. Supplying fructose, glucose or sucrose results in the establishment in the roots of a glucose/sucrose ratio lower than that recorded in roots supplied only with water, either at 2°C, or at any other temperature.
2. Supplying glucose or sucrose results in maintenance of the internal sugar content of the roots over the five day period at 2°C (compared with the decline in content observed with fructose or water, to values of 56 and 48% respectively of the initial level), Table 10.
3. Supplying fructose or glucose stimulates the respiration rate over the value observed both for the water-grown control and for roots supplied with sucrose.
4. Only a supply of glucose to the roots of maize at 2°C resulted in any stimulation of root growth and only glucose produced a positive effect on the three features of carbohydrate metabolism being studied over the five day period at 2°C, that is on the establishment of a low glucose/sucrose ratio, on the maintenance

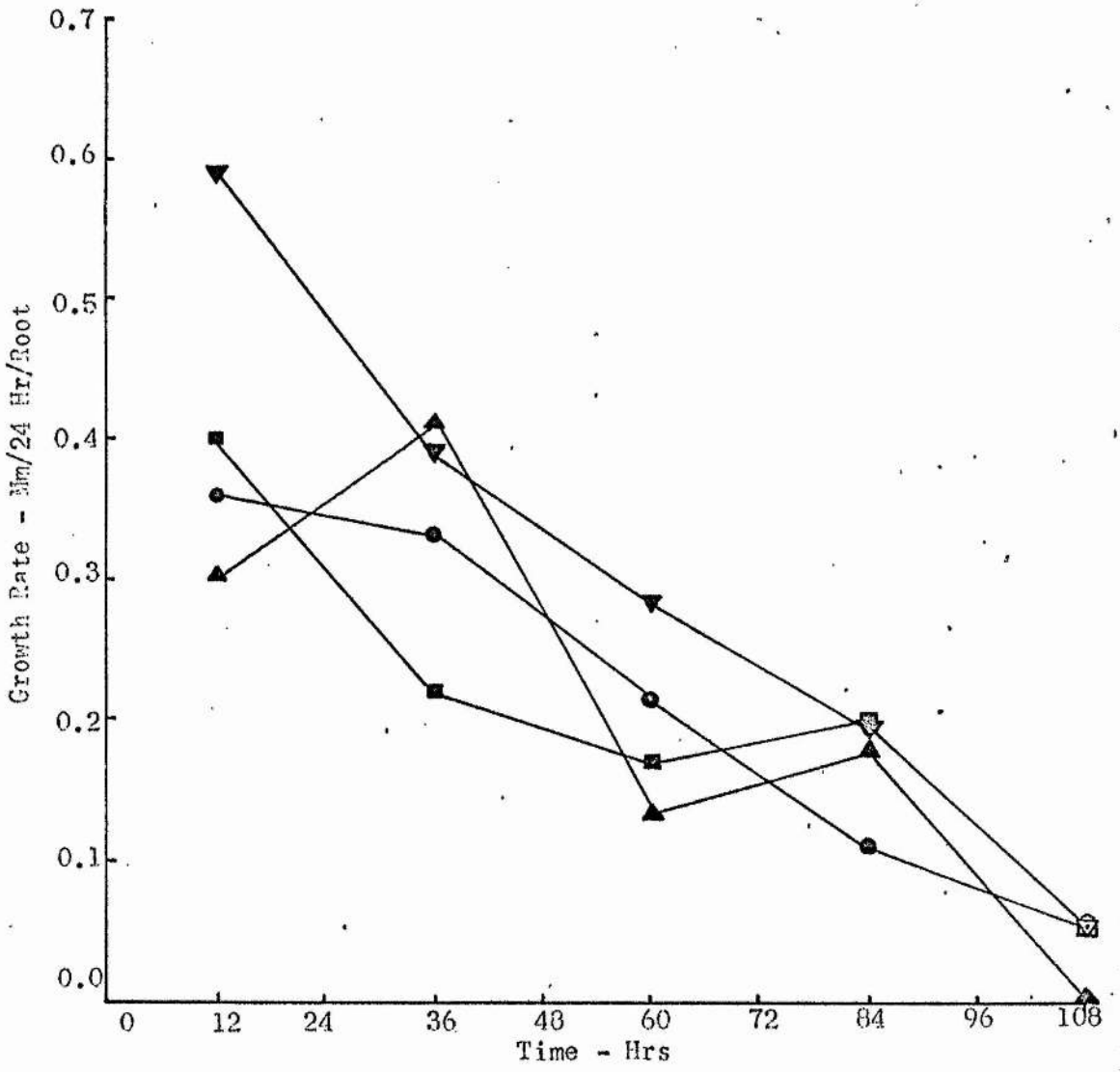


Fig. 23. Growth rates of roots of seedlings of maize grown at 20°C in either distilled water (●——●) or 0.05 M solution of glucose (▼——▼), fructose (▲——▲) or sucrose (■——■). Rates determined as the mean increase in length of roots over successive 24 hr intervals and plotted at times midway between these intervals. At time 0 hr seedlings had been germinated 120 hr at 20°C.

of the internal sugar content, and on the maintenance of an adequate respiration rate.

d) Maize at 6°C

At 6°C the glucose/sucrose ratio is higher in root tips grown in sugar solutions than the control, Table 9. However, growth rates in the presence of any of the three sugar solutions are not significantly different from the control (Figs. 24-25), although with glucose and sucrose solutions bathing the roots the growth rates are higher than the control by 11 and 9% respectively, Table 15. Also, growth rate shows less tendency to decline over the 120 hr period when sucrose or glucose solution is supplied, than when water alone is supplied to the roots. This is especially clear between time 72-120 hr. Between each 24 hr interval within this time there is a significant increase in the length of the roots grown in glucose or sucrose solutions but in the water-grown control and in roots bathed with fructose solution the error bars of successive points on the graph overlap. Approximately 100 roots were used for each determination of growth rate. It is possible that if more were used a significant increase in growth in the presence of glucose and sucrose solutions over the control may be found. The increase in respiration rate of roots supplied with glucose or sucrose favours this possibility (Table 15).

Summary of experiment

The aim of the experiment was to establish whether or not the proportions of sugars in the roots of pea and maize seedlings directly affected growth rate and respiration rate of roots. The method adopted was to try and change the proportions of sugars in the roots, by bathing the roots in 0.05 M solutions of fructose,

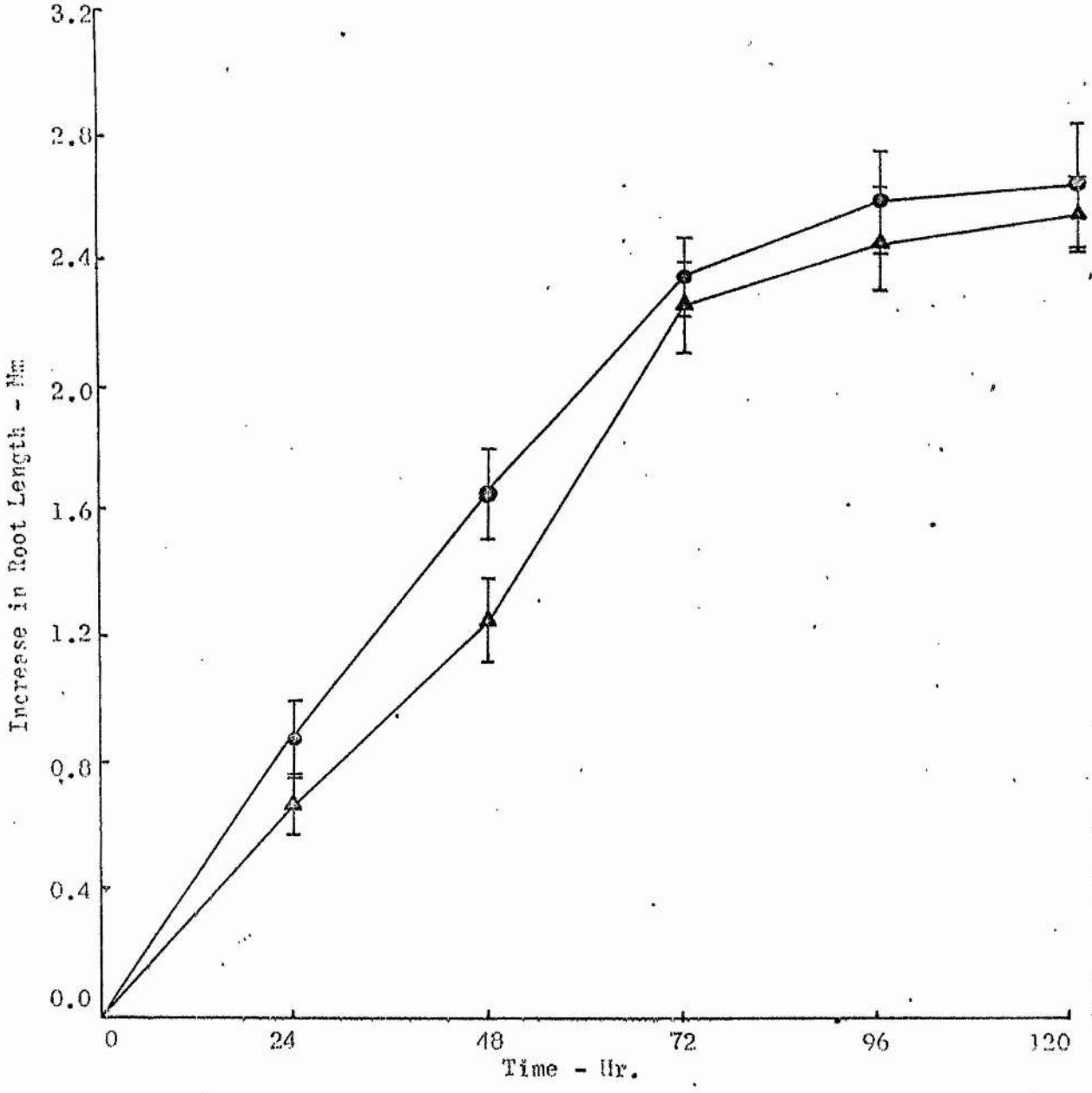


Fig. 24. Mean increase in length of maize seedling roots at 6°C against time. Roots grown in the presence of 0.05 M fructose solution (▲——▲) or water (●——●). Time 0 hr was after germinating the seeds 120 hr at 20°C.

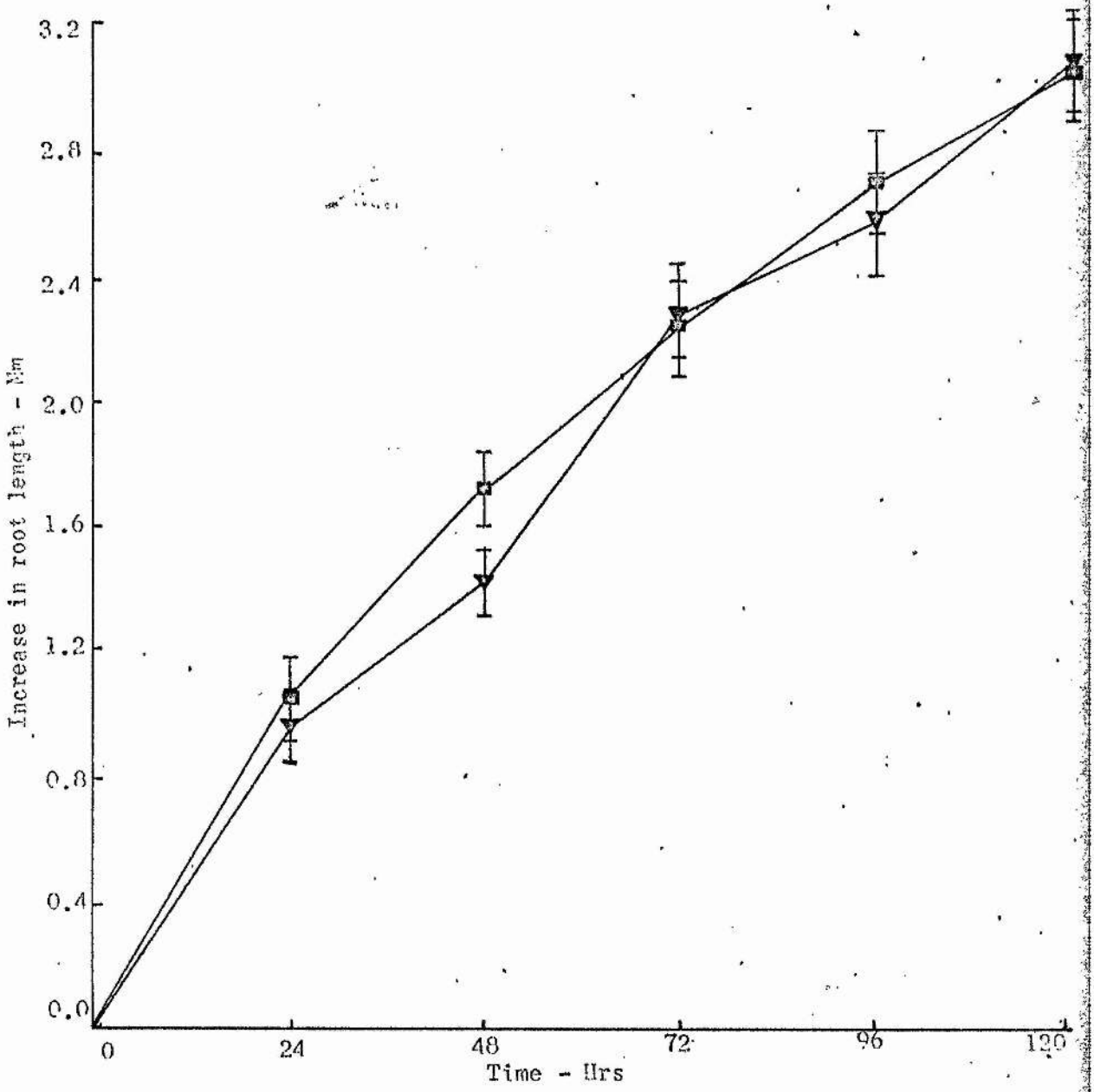


Fig. 25. Mean increase in length of maize seedling roots at 6°C against time. Roots grown in the presence of 0.05 M glucose (▽——▽) or sucrose (■——■) solution. Time 0 hr was after germinating the seeds 120 hr at 20°C.

Table 15. Growth and respiration rates of the maize root at 6°C in the presence of 0.05 M solutions of either fructose, glucose or sucrose, or distilled water. (At the start of the experiment seedlings had been germinated 120 hr at 20°C.)

Solution bathing the roots	Growth made over first 120 hr at 6°C		Respiration rate	
	mm/root	%control	ul O_2/hr mg $^{-1}$DW	%control
Water (control)	2.80 ± 0.17	100	1.04	100
Fructose	2.64 ± 0.20	94	1.06	102
Glucose	3.10 ± 0.16	111	1.54	148
Sucrose	3.06 ± 0.16	109	1.31	126

glucose or sucrose, and observe the consequences for growth and respiration rates. The investigation was at two temperatures, 2 and 6°C, and all measurements of the sugar proportions, growth rates and respiration rates were referred to the values for roots of water-grown seedlings (the control). In the course of the experiment a further feature of sugar metabolism, found to be of significance for growth rate, was the total sugar content of roots.

Conditions where the external supply of sugars resulted in shifts in the internal proportions of sugars in the roots of pea and maize relative to the control were when:

pea roots were bathed by { glucose solution at 2°C / sucrose solution at 6°C

maize roots were bathed by { fructose, glucose or sucrose solution at both 2 and 6°C

The changes in two features of metabolism associated with these new sugar proportions, were examined, 1) the total internal sugar content and 2) the respiration rate, along with the consequences for growth rate of the seedling roots.

Conclusions

1. Pea roots at 2°C

At 2°C, only a supply of glucose to pea roots altered the proportions of glucose and sucrose in the roots. The glucose/sucrose ratio was increased. An increase in the growth rate and the respiration rate of the roots accompanied this increase in the glucose/ sucrose ratio.

Previously it has been shown that the glucose/sucrose ratio in pea roots was increased as the temperature at which the seedlings

were grown increased (Chapter 3). Also correlating with an increase in temperature was a rise in growth rate and respiration rate of the roots (Chapter 2 and 4 respectively).

Thus whether temperature or substrate feeding increases the glucose/sucrose ratio, there is a concomitant increase in the growth rate and the respiration rate of the roots. It is concluded that the glucose/sucrose ratio is directly influencing growth rate and respiration rate of pea roots.

Supplying sucrose or fructose solution to the roots did not alter the glucose/sucrose ratio, and the external supply of these solutions had no effect on growth or respiration rate of the roots.

2. Pea roots at $6^{o}C$

At $6^{o}C$, feeding only with sucrose solution resulted in a shift in the glucose/sucrose ratio in pea roots. The ratio was lower than in the control but there was not the expected corresponding decrease in growth rate. This may be because of the high absolute levels of the sugars in the roots under these conditions (Table 9), or because a larger change in the value of the ratio is required before any effect on the growth rate is observed at $6^{o}C$, this being a temperature well above the minimum for growth of pea roots. Evidence supporting this latter possibility is furnished by comparing the value for the glucose/sucrose ratio obtained in Chapter 3, where the roots were grown in water at $6^{o}C$, with the value obtained for the water-grown control in the present experiment. In Chapter 3 the value of the ratio at $6^{o}C$ was determined as 0.95 (Fig. 13), whereas in the present experiment the value was determined as 1.67 (Table 9).

This variability between different batches of pea seedlings

may account for the lower [illegible] of the ratio, without the expected lower growth rate, which was recorded in the sucrose-grown roots in the present experiment. Respiration rate at 6°C altered little in the presence of any of the sugars compared with the control. This may be an additional factor contributing to the similar growth rates of the roots grown in any of the sugar solutions.

Thus in pea at 6°C the supply of sugars to the roots has not produced a sufficiently large shift in the glucose/sucrose ratio to demonstrate whether or not growth and respiration rate are dependent on this ratio.

However, on the basis of two lines of evidence it can be concluded that the glucose/sucrose ratio in the roots of pea determine growth rate and respiration rate of the roots. These two lines of evidence are:

1. At 6°C the glucose/sucrose ratio in water-grown roots of pea is higher than the ratio for roots grown at 2°C, and likewise growth and respiration rates are higher. This relationship of a higher glucose/sucrose ratio correlating with higher growth and respiration rates continues for higher growth temperatures (Fig. 13 compared with Figs. 1, 2 and 14).
2. In roots grown at 2°C the glucose/sucrose ratio is increased by supplying roots with glucose solution compared with the value of the ratio for water-grown roots. Again growth rate and respiration rate are higher in the glucose-grown roots compared with the water-grown control. (Supplying sucrose or fructose solution does not alter the ratio from the control value and neither do the two sugars alter growth or respiration rates of the roots.)
3. Maize roots at 2°C

The total sugar content in the roots of maize, maintained

at 2°C and supplied with distilled water, declines over 120 hr to a level 50% of the initial level (Chapter 3). Sugar metabolism of this species is clearly disrupted by the low temperature treatment.

A very important effect of supplying glucose or sucrose to the roots was that total sugar content at 2°C was maintained at the initial level. The breakdown in supply of sugars to the root tip of maize at 2°C is thus remedied almost entirely by the external supply of glucose or sucrose to the roots. (Fructose however did not prevent the decline in sugar content but behaved similarly to the control.)

At 2°C all three sugars, when supplied to the roots of maize seedlings, led to a significant decrease in the glucose/sucrose ratio, and the values reached were the lowest recorded for maize. By comparison with pea this is to be expected at 2°C, the lowest experimental temperature used. Previously, with the roots bathed only in water, 6°C was the temperature at which maize showed the minimal glucose/sucrose ratio, Fig. 13.

However, the only sugar supplied externally to the root at 2°C which significantly affects root growth is glucose. The growth made over 120 hr is significantly greater than in the water-grown control. Along with this stimulation of growth there is a stimulation of respiration rate.

The relationship between growth and each of the factors, respiration rate, glucose/sucrose ratio and total internal sugar content of the root, for each of the sugars (fructose, glucose and sucrose), supplied externally to the root, is shown in Fig. 26. The shaded areas of the graphs are those where response of each of the factors is less than 20% different from the control value. It can immediately be seen that only glucose supplied to the roots is effective in substantially affecting respiration rate, glucose/sucrose

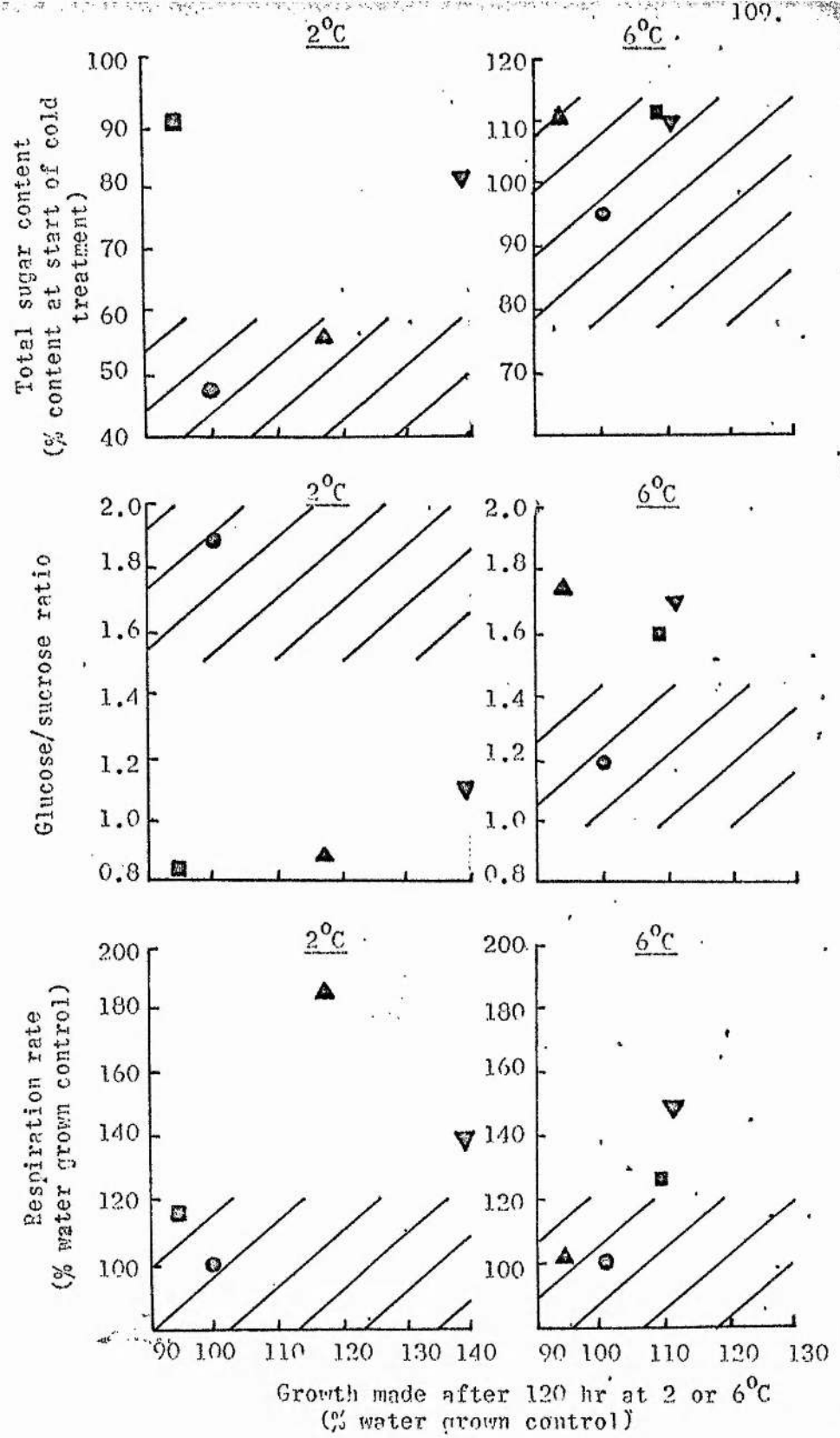


Fig. 26. Relationship between amount of growth made by roots of maize seedlings bathed by distilled water (●), fructose (▲), glucose (▼), or sucrose (■) solution (0.05 M) and (a) total sugar content, (b) glucose/sucrose ratio, (c) respiration rate, after 120 hrs, at either 2 or 6°C. Seedlings initially germinated 120 hr at 20°C. (The areas shaded include values less than 20% different from the distilled water control).

ratio and the total sugar content, and only this sugar is effective in promoting the amount of growth made over 120 hr at $2^{o}C$.

The other two sugars supplied to the roots did not increase growth. Fructose supplied externally failed to stimulate growth because the internal sugar content was not maintained at an adequate level and sucrose failed to stimulate growth because respiration rate was not maintained at an adequate level.

4. Maize roots at $6^{o}C$

At $6^{o}C$, roots bathed by any of the sugar solutions gave values for the glucose/sucrose ratios in the root tip higher than the control.

With glucose and sucrose solutions there was some increase in growth made over the 120 hr period, respectively 11 and 9% above the control (but with the number of roots measured these increases were not significantly different from the water-grown control and so there is some doubt as to whether or not they indicate actual stimulation of growth). However, in addition to the increase in growth, glucose and sucrose solution were effective in preventing the decline in growth rate seen in the water-grown control roots over the 120 hr period at $6^{o}C$. Furthermore supplying the roots with glucose or sucrose solution raised the respiration rate of the roots by 48 and 26% respectively. (With fructose solution bathing the roots there was no effect on growth rate. However this sugar did not alter respiration rate of the roots, relative to the control and this is considered to be the reason that growth rate in the fructose-grown roots was the same as in the control.)

At $6^{o}C$, in contrast to $2^{o}C$, growth rate is stimulated by a rise in the glucose/sucrose ratio because this temperature ($6^{o}C$)

is in the range where the glucose/sucrose ratio is directly related to growth rate. It is at the extreme lower end of the range of temperatures where this relationship is observed. Whether a higher growth temperature or "feeding" with sugar solution raises the glucose/sucrose ratio in the roots growth rate of the roots increases (providing, in the case of "feeding", that the sugar supplied also increases the respiration rate).

Water-grown roots at 2°C lie outside the range of this relationship. The disruptive effect of the low temperature on the total sugar content of the roots is associated with a glucose/sucrose ratio in the roots higher than at 6°C. To adjust the ratio at 2°C to be in accordance with the trend observed at 14, 10 and 6°C a lowering of the glucose/sucrose ratio at 2°C is required. It is for this reason that the growth of maize roots is potentially stimulated, at 2°C, by a lowering of the glucose/sucrose ratio, and, at 6°C, by an increase in the glucose/sucrose ratio.

Thus it is concluded, in maize roots, as in pea roots, the glucose/sucrose ratio is intimately associated with growth and respiration rates. However, when sugar solutions are supplied to maize roots, a demonstration that the shift in the glucose/sucrose ratio at 2 or 6°C is associated with a change in growth rate, is subject to the condition that the external sugar supply also corrects other features of root metabolism, disturbed by these low temperatures. These disturbances are, at 2°C, a very low respiration rate and a continuously declining sugar content in the roots, and at 6°C, an inadequate rate of respiration (the sugar content is maintained in the water-grown control at 6°C).

With these findings the results obtained in Chapters 3 and 4 can be reviewed. The correlation of both growth rate and the

glucose/sucrose ratio with temperature, observed over the wider range of temperatures studied in Chapter 3, is now seen to be due to the dependence of growth rate on the glucose/sucrose ratio. The rate of respiration is also dependent on the glucose/sucrose ratio to the extent that where changes in this ratio are associated with a change in growth rate, then there is a concomitant change in the respiration rate.

Thus, of the two problems raised at the end of Chapter 4 concerning sugar metabolism, one has been answered above, that which asked whether or not growth rate correlated with temperature independently of the glucose/sucrose ratio. Growth rate and the glucose/sucrose ratio have been shown to be intimately related. The results of the experiment in the present chapter lead to the conclusion that the extent of the adaptability of the glucose/sucrose ratio in roots of both species subjected to low temperatures strongly influences the minimum temperature at which the roots are able to maintain growth. In pea roots, bathed in distilled water, the ratio declines with temperature down to 2°C and growth rate of the roots similarly declines but is maintained at a constant rate even at this temperature. In maize roots, likewise provided only with distilled water, the glucose/sucrose ratio declines with temperature down to 6°C and this temperature is just below that at which a constant growth rate is achieved (growth rate at 6°C decreases over the 120 hr period of measurement).

The remaining question from Chapter 4 was an enquiry as to how different temperatures are responsible for the establishment of different proportions of glucose and sucrose in the root. In pea roots the system responding to temperature and leading to the establishment of specific glucose/sucrose ratios at specific

temperatures clearly operates efficiently at temperatures lower than the corresponding system in maize roots.

A further problem suggested from the results of Chapter 4, and confirmed by the results of the experiment in the present chapter, concerns respiration rate of maize roots at 2°C. In Chapter 4 the respiration rate of maize roots at 2°C was only 50% of the rate in pea roots. It was suggested this low rate was inadequate to support growth. In the present chapter it has been found that growth of maize roots at 2°C was stimulated by external sugar supplies only when respiration rate also increased (in addition to when the internal sugar content of the root was maintained at an adequate level). The conclusion is drawn that at 2°C respiration rate of roots of maize seedlings supplied only with water is inadequate to support growth.

Thus two metabolic systems which show marked difference between pea and maize roots at low temperatures are those involved with respiration rate and with sugar metabolism, the glucose/sucrose ratio. Differences in growth rate between the two species at low temperatures have been shown to be intimately associated with the differences in these metabolic systems. Ultimately, differences in metabolism must be based on differences in enzyme characteristics in the two species. The following experiments describe examinations of two enzymes from the roots of pea and maize involved respectively with respiration and with sugar metabolism. The enzymes investigated are malate dehydrogenase and invertase. The Michaelis constants and activities are examined for these enzymes extracted from roots previously subjected to various temperatures regimes to determine if differences in their

kinetic properties could be responsible for the differences between the two species with respect to observed respiration rates and to sugar proportions.

CHAPTER 6

KINETIC PROPERTIES OF MALATE DEHYDROGENASE EXTRACTED FROM THE ROOT TIPS OF PEA AND MAIZE SEEDLINGS

Introduction

It has been shown that the respiration rate, measured as oxygen uptake (µl/mg DW/hr), of root tips of pea seedlings is twice the corresponding rate for root tips of maize seedlings, when determined at 2°C (see Chapter 4). The inability of the maize root to maintain growth at 2°C is considered to be partially due to this low respiration rate. Thus where growth is increased at 2°C by externally supplying glucose to the maize roots then respiration rate is markedly increased (growth made by roots over 120 hr was increased 40% in the presence of glucose solution and then respiration rate was 39% greater than in the water-grown controls, Table 14). Conversely, the ability of the pea root to maintain growth at a constant rate at 2°C is considered to be due to the relatively high respiration rate of the root at this temperature. Furthermore, for a given rise in temperature the response of growth rate closely parallels the response of respiration rate (Fig. 16). This correlation of growth rate and respiration rate in pea roots is also seen at any one temperature (e.g.: 2°C), where supplying glucose to the roots increases the rate of both processes, Table 11.

At warmer temperatures, 10 and 14°C, both pea and maize roots display appreciable respiration rates (measured as oxygen uptake) and both species maintain constant growth rates at these temperatures. It is only low temperatures, in the region of 2°C, which affect the respiratory processes of pea and maize roots to differing extents (see Chapter 4). Of central importance for the functioning of the respiratory processes

associated with oxygen uptake is the operation of the Kreb's cycle. This cycle involves several enzymic transformations of organic acids with the net effect of oxidising pyruvate to carbon dioxide and water. The temperature characteristics of enzymes in this cycle will have a major influence on the overall temperature characteristics of the respiratory process. For example, if the enzymes are inactivated by low temperature then the operation of the Kreb's cycle will cease and respiration (oxygen uptake) will no longer occur. Since oxygen uptake is directly dependent on the activity of the dehydrogenase enzymes of the Kreb's cycle (in conjunction with the enzymes of the electron transport chain, located in the mitochondria with the Kreb's cycle enzymes), the temperature characteristics of one of the dehydrogenases of the cycle was studied to discover if differences in the properties of the Kreb's cycle dehydrogenase enzymes from the two species may be associated with the differences in respiration rate of pea and maize roots at 2°C.

Malate dehydrogenase (MDH) was chosen since this enzyme has previously been found to have markedly different properties in organisms able to grow at very different temperatures. Burton and Morita (1965) have demonstrated that MDH from the micro-organism Vibrio marinus (a cold tolerant marine bacterium) has an activation energy only half that of a mesophilic strain of E. coli.

A study of the Michaelis constant (Km) and activity (IU/mg protein) of the enzyme was undertaken for MDH extracted from roots of pea and maize seedlings grown at 20, 14 or 2°C. Km and specific activity were determined at two temperatures, 2 and 14°C. These enzyme characteristics were measured since together they describe the rate of reaction under a range of substrate concentrations; Km is a measure of the substrate concentration giving half maximal rate of reaction, and the specific activity is directly proportional to the maximal rate of reaction when

measured at zero order kinetics. The results of this study bear on three important questions concerning the Km and activity of MDH, and any difference in these characteristics as between pea and maize may provide an explanation for the observed differences in rate of oxygen uptake between the two species at 2°C. The three questions are:

1. What differences exist both in the Km and in the specific activity of MDH extracted from pea and maize roots?

2. Within each species, are the values of Km the same at the two temperatures, 2 and 14°C? (The Km of an enzyme may vary with temperature if, for example, different temperatures induce the formation of different physical bonds between and within the polypeptide chains composing the enzyme molecule. If such conformational changes involve a change in the active site of the enzyme then this could be recorded as a change in the affinity of the enzyme for the substrate, i.e.: as a change in Km).

3. Do the kinetic properties of the enzyme (Km and specific activity) alter following exposure of the roots to different temperatures regimes? (Thus different species of an enzyme may be synthesised at different temperatures such that at any one temperature the species of enzyme synthesised displays a greater rate of reaction than any other species of the enzyme active at that temperature. Alternatively, a range of species of an enzyme may be synthesised in the root irrespective of the growth temperature but there is then selective elimination of those species least suited to the particular temperature to which the root is subjected.)

In summary, the aim of the experiment described below was to examine the kinetic properties of MDH, extracted from the roots of pea and maize, to determine whether differences in these properties between the two species existed, and could be related to the differences in oxygen uptake recorded for the two species at 2°C. By incubating the

seedlings at three temperatures, 2, 14 and 20°C and examining the Km and specific activity of the enzyme at two temperatures, 2 and 14°C, it was possible both to determine how the enzyme behaves at a low temperature, 2°C, compared with a moderate temperature, 14°C, and to determine if the temperature at which the roots are incubated affects the properties of the MDH synthesised in the roots.

Method

Seeds of pea and maize were germinated and selected for the cold incubators as described on p. 16. At the end of the respective germination periods for pea and maize and also after seedlings were grown in the cold incubators at 2 and 14°C for 120 hr, MDH was extracted from the distal 1 cm segment of the roots, partially purified, and the Km and specific activity determined by the procedure described in Appendix 3. The conditions of growth in the incubators were as described on p. 18. Batches of roots of both species were also grown at 20°C for 120 hr following the germination period and the enzyme extracted, partially purified and assayed as above. All enzyme assays were performed at two temperatures, 2 and 14°C. Duplicate experiments were performed in some cases, and in these cases the two values for Km and for specific activity are presented in the results.

Results and discussion

Michaelis constant (Km)

All values for the Km of MDH in the two species are recorded in Table 16.

The results at the end of the germination period are considered

Table 16. Km of MDH extracted from the distal 1 cm of the roots of pea and maize grown under four temperature regimes. Km was determined at two temperatures, 2 and 14°C, units of Km, x 10^{-4} M.

Conditions of growth.	Km (x 10^{-4}M) determined at: 2°C	14°C
Germination for 72 hr at 20°C	Pea	
ONLY	1.60 ± 0.33	0.93 ± 0.05
	1.59 ± 0.33	1.04 ± 0.17
plus 120 hr at 20°C	0.77 ± 0.09	0.80 ± 0.07
	0.99 ± 0.19	-
plus 120 hr at 14°C	0.88 ± 0.19	1.24 ± 0.27
	0.73 ± 0.07	1.59 ± 0.17
plus 120 hr at 2°C	0.97 ± 0.30	1.52 ± 0.11
	0.82 ± 0.08	-
Germination for 120 hr at 20°C	Maize	
ONLY	0.94 ± 0.10	0.94 ± 0.16
	0.80 ± 0.03	1.13 ± 0.10
plus 120 hr at 20°C	0.55 ± 0.10	0.44 ± 0.05
plus 120 hr at 14°C	1.27 ± 0.13	1.15 ± 0.15
	-	0.99 ± 0.12
plus 120 hr at 2°C	0.97 ± 0.11	0.92 ± 0.08
	-	0.99 ± 0.09

first, since this is the natural starting point from which to observe any changes in Km following further incubation of the roots at the different temperature regimes. At the end of the germination period the roots of both species have experienced only 20°C. The Km is determined at 2 and 14°C. The results for maize indicate that the enzyme has the same affinity for its substrate at both these temperatures - the Km is the same at 2 as at 14°C. In pea however, while the value of Km determined at 14 is similar to that in maize, the Km determined at 2°C has a considerably higher value (approx. 60% higher), indicating the enzyme has a lower affinity for its substrate at this temperature.

Following growth for a further 120 hr at 20°C, then, in pea seedlings, the Km determined at 2°C is similar to that determined at 14°C and both values are close to that previously determined at 14°C (at the end of the germination period). In maize seedlings however, the Km shows a marked lowering in value whether determined at 2 or 14°C.

Thus in young seedlings of both pea and maize, germinated and grown at 20°, MDH in the roots possesses different properties in seedlings of different ages. In pea, the enzyme initially has a relatively low affinity for its substrate at 2°C (after 72 hr, Km=1.6 x 10^{-4}M), but after 192 hr the affinity is equal to that at 14°C (Km is approximately 0.8 x 10^{-4}M). In maize the value of Km, while being similar when determined at 2 or 14°C, declines markedly in roots of older seedlings. The value at 240 hr is approximately 0.5 x 10^{-4}M, while after only 120 hr it is 0.94 x 10^{-4}M.

When the seedlings of both species are kept at the lower temperatures of 14 or 2°C the changes seen in Km are different, in certain instances, from those reported above for growth at 20°C. In pea, maintained at either 2 or 14°C, the Km, determined at 2°C, is lower than at the end of the germination period, just as was recorded following

continued growth at 20°C. But Km determined at 14°C is higher either than at the end of the germination period or than after continued growth at 20°C. That is, in pea, MDH has a Km approximately 0.8 x 10^{-4}M when determined at 2°C in seedlings aged 192 hr (the germination period of 72 hr plus 120 hr in the incubators), irrespective of the growth temperature to which the seedling roots are subjected. When determined at 14°C Km is relatively high, approximately 1.4 x 10^{-4}M following growth of the seedlings at all but the warmest temperature (20°C).

In summary, MDH of pea roots generally has a greater affinity for its substrate at 2°C compared with 14° irrespective of the temperature at which the roots are grown (only very young roots and those grown at the warmest temperature, 20°C, also display a relatively higher affinity for the substrate at 14°C). Under conditions where substrate concentration is not excessive (i.e.: either where it is in the range of, or below, the Km concentration), the effect of the low temperature, 2°C, on slowing down rate of reaction, catalysed by MDH must, to some extent, be offset by the greater sensitivity of the enzyme to its substrate at the low temperature, 2°C, compared with 14°C. This fact may provide an explanation of the ability of the pea roots to respire at a relatively high rate at low temperatures.

The results for maize following incubation of the seedlings for 120 hr at 14 or 2°C contrasts with those for pea. The value of Km at 2 and 14°C are similar to those recorded at the end of the germination period. The marked lowering in Km, seen following continued growth of the roots at 20°C, is not observed, despite the fact that a lower Km value at the lower growth temperature would be most beneficial for the ability of the roots to maintain a relatively active metabolism at the lower growth temperatures of 2 and 14°C. This failure in modifying the Km in maize roots grown at 14 and 2°C may be a factor involved in the low

respiration rate at these temperatures.

The absolute values of Km determined at 2°C in maize are very similar to the values of Km determined in pea. This does not necessarily imply similar kinetic behaviour for the enzyme in vivo between the two species. Of much greater significance is the shift, seen in older compared with younger pea roots, of Km value determined at 2°C. (MDH from roots of eight day old seedlings has a lower Km determined at 2°C than MDH from 3 day old seedlings.) This lowering of Km may allow pea roots to maintain a relatively high metabolic rate (rate of oxygen uptake and the capacity to continue growth), at low temperatures. In maize this adaptability in Km is seen only at the growth temperatures of 20°C, and not at 14 or 2°C, and this species shows a very low rate of oxygen uptake and is not able to maintain growth at low temperatures.

Activity of MDH

The second factor, measured in the experiment, which may affect rate of reaction (principally under conditions where the substrate concentration is much greater than the Km) is specific activity of the enzyme (IU/mg protein), Table 17. These values are directly related to the maximum rate of reaction possible when the enzyme is saturated by the substrate.

In pea the specific activity at the end of the germination period, (72 hr), is higher than after any of the subsequent 120 hr growth periods at 20, 14 or 2°C. The values of activity determined at both 2 and 14°C after these growth periods are very similar between the temperature treatments, and thus growth temperature appears to have no effect on specific activity of the MDH.

In maize there is an increase in enzyme specific activity with time at the warmest temperature, 20°C. The values for specific activity determined at both 2 and 14°C are higher after 240 hr at 20°C than after

Table 17. Activity of MDH extracted from the distal 1 cm of the roots of pea and maize grown under four temperature regimes. Activity determined at two temperatures, 2 and 14°C.

Conditions of growth	Activity (IU/mg protein) determined at: 2°C	14°C	Q10 (for temp. range 2-14°C)
Germination for 72 hr at 20°C		Pea	
ONLY	4.98	7.49	1.4
plus 120 hr at 20°C	2.42	5.35	1.9
plus 120 hr at 14°C	2.00	5.11	2.2
plus 120 hr at 2°C	2.43	5.82	2.0
Germination for 120 hr at 20°C		Maize	
ONLY	2.45	7.04	2.4
plus 120 hr at 20°C	3.41	11.41	2.7
plus 120 hr at 14°C	2.56	7.45	2.5
plus 120 hr at 2°C	2.57	7.13	2.3

only 120 hr. This increase in specific activity with time is not seen when the seedlings are transferred to 14 or 2°C after the germination period.

The increase in specific activity with time at 20°C will increase rate of reaction under conditions of substrate saturation and this will serve to enhance the already existing difference in activity between roots grown at 20°C and those grown at 14 or 2°C, which occurs by virtue of these different growth temperatures (the higher the temperature the higher will be the enzyme activity).

Calculation of the Q10 for specific activity of MDH in the two species over the temperature range 2-14°C reveals a lower Q10 in pea than in maize (Table 17). This indicates that specific activity in pea is less influenced by temperature than specific activity in maize . Under low temperature conditions this could be important for maintaining a higher metabolic rate in pea compared with maize roots, since the level of enzyme activity in pea will be relatively higher (closer to the maximum specific activity recorded at the temperature optimum for the enzyme) than in maize.

Conclusions

In summary, the changes which were observed in the kinetic properties of the enzyme in the two species were such that in pea, but not in maize, they were towards favouring a relatively high rate of reaction at low temperatures. Firstly in pea roots there was a decline in Km value, determined at 2°C, between seedlings aged 72 and 192 hr (from first soaking the dry seeds), independently of the temperature regime which the roots experienced (2, 14 or 20°C were the growth temperatures tested). Meanwhile, Km determined at 14°C at the above respective times, increased in value

(except after 192 hr at the warmest temperatures, 20°C, where Km was maintained at the value recorded after 72 hr). As a consequence of these Km changes, as the seedlings age, then, for a given substrate concentration, rate of reaction at 2°C compared with 14°C is relatively greater in the older seedlings. The full importance of this shift in vivo could only be established after a study of substrate concentration in the cell or more precisely in the vicinity of the enzyme itself. It is however potentially a very important mechanism for maintaining reaction rate in the face of low temperatures.

In maize grown at 20°C the enzyme showed a decline in the Km (determined at both 2 and 14°C), between day 5 and 10 from first soaking the seeds. But after subjecting the seedlings from day 5-10 to either 14 or 2°C this decline in Km was not observed, yet these growth temperatures are precisely those where a lower Km could be beneficial in promoting a relatively rapid rate of reaction.

At growth temperatures of 2 and 14°C the failure to modify the Km in the manner seen at a growth temperature of 20°C may be a very important factor associated with the inability of maize seedlings roots to continue growing at low temperatures, specifically those at and below 6°C (see Chapter 2).

Secondly, with respect to specific activity, that of pea was relatively higher than that of maize at low temperatures, again a feature better adapting pea roots to cold conditions than maize roots.

A range of other vegetable species, some low temperature tolerant and others low temperature sensitive, were assayed for the Km of MDH in the root system to determine if, in other species, the trends seen in pea and maize were evident, that is, to determine if, in cold tolerant

species, the value of Km determined at 2°C declines in older seedlings (particularly when subjected to a period of time at 2°C), whereas in cold sensitive species this is not to be observed after a low temperature treatment.

The species of vegetables were chosen to cover a range of vegetables grown in this country. To obtain sufficient root material large seeded species only were used. The species studied were, oats (Avena sativa L.), barley (Hordeum vulgare L., var golden promise), broad bean (Vicia faba L. var. early long pod), marrow (Cucurbita pepo L. var medullosa), cucmber (Cucumis sativus L. var. telegraph) and kidney bean (Phaseolus vulgaris L. var. canadian wonder).

The general form of the experiment for each species was to germinate seed at a warm temperature for the species, 20 or 26.5°C, and select seedlings from each batch for further growth at a time when a majority of seedlings had made between 1 and 2 cm of root growth. Only the seedlings with roots in this length range were used. At this time and after further periods of growth either at the germination temperature or at 2°C, the roots were extracted and assayed for MDH by the method described for pea and maize, appendix 3. The Km was determined at 2 and 14°C.

Precise temperatures and times for each stage of germination and growth for all the species are given in Table 18. This table also gives the medium in which the seedlings were grown.

Results and Discussion.

The values for the Km MDH determined at 2 and 14°C for the roots of the six species of vegetable seedlings are presented in Table 19.

Four of the species are cold tolerant, oats, barley, broad bean

Table 18. Conditions for germination and growth of six species of vegetables used in the assay of MDH from the roots.

Species	Germination medium	Germination conditions	Time period for further growth at germination temp. or at 2°C (hr)	Portion of the root harvested for MDH assay
Oats	Saturated filter paper	96 hr at 20°C	144	Whole root system
Barley	Saturated filter paper	54 hr at 20°C	120	Whole root system
Broad bean	Peat and sand mixture	168 hr at 20°C	168	1 cm root tip
Marrow	Saturated filter paper	72 hr at 26.5°C	168	Whole root system
Cucumber	Saturated filter paper	36 hr at 26.5°C	120	Whole root system
Kidney bean	Peat and sand mixture	72 hr at 26.5°C	96	1 cm root tip

Table 19. Km of MDH from the roots of six species of vegetable. Km determined at 2 and 14°C. Units x 10^{-4} M.

Species	Temp. at which KM determined	KM determined at the end of the germination period:		
		Only	Plus growth[x] at germination temp.	Plus growth[x] at 2°C
Oats	2	0.65±0.09	0.63±0.12	0.72±0.09
	14	0.91±0.11	0.74±0.01	0.99±0.13
Barley	2	0.68±0.11	0.98±0.10	0.67±0.04
	14	-	1.06±0.21	0.81±0.13
Broad bean	2	0.75±0.04	0.65±0.06	0.88±0.09
	14	0.94±0.07	0.67±0.04	0.94±0.15
Marrow	2	0.83±0.03	0.97±0.07	0.70±0.04
	14	0.60±0.04	1.06±0.05	0.73±0.14
Cucumber	2	0.63±0.06	0.86±0.12	0.78±0.24
	14	0.69±0.15	0.96±0.14	1.18±0.32
Kidney bean	2	0.82±0.07	-	1.15±0.09
	14	0.81±0.07	-	0.98±-.08

x See table 18 for details of these temperatures and times

and marrow and the other two cold intolerant, cucumber and kidney bean. Thus the former four species were thought to be the ones most likely to show changes in the value of Km of an adaptive nature to cold temperatures. However all four species showed only small variations in the value of Km following growth at the different temperature regimes, but in all cases except one (that of marrow at the end of the germination period), the Km determined at 2 was less than that at 14°C. Thus, in these species (oats, barley, broad bean and marrow) the enzyme appears to possess the inherent property of being more sensitive to its substrate at lower temperatures.

The Km of MDH from oats, when determined at 2°C, varied little between the different growth-temperature regimes used, but Km of barley and marrow was lower after growth at 2°C than at 20°C. However with broad bean the reverse was found. Km determined at 2°C was higher after growth at 2 than at 20°C. No species showed the initial high Km at 2°C at the end of the germination period that was seen in pea, and also unlike pea, following growth at 2°C the Km determined at 2°C is not markedly lower than the value at 14°C, although in the four species it is invariably somewhat lower.

The two species intolerant of low temperature, cucumber and kidney bean, both show higher values of Km following growth at 2°C compared with the values at the end of the germination period. This is comparable to what was found for maize. But in contrast with maize, following further growth at the germination temperature Km did not show a decline in value, rather the reverse was found. The data for cucumber shows that in this species after growth at 20°C Km value rose to a value higher when determined at 2°C than that following growth at 2°C.

In summary, none of the six vegetable species studied above

(four cold tolerant and two cold sensitive) showed distinctive properties of Km for MDH which could be related to their cold tolerance. There was, however, a tendency in the cold tolerant species for Km to be lower when determined at a low temperature, 2°C, compared with 14°C. This will favour maintenance of reaction rate at low temperatures and thus may be a mechanism whereby cold tolerant species maintain metabolism at low temperatures. Growth temperatures (2 or 20°C) had little influence on Km values.

By contrast, in cold sensitive species the low growth temperature, 2°C, following the germination periodat 26.5°C, resulted in Km values for MDH, at both 2 and 14°C, increasing , thus effectively reducing the reaction rate for substrate concentrations in the region of, or lower than, the Km.

CHAPTER 7

Km AND ACTIVITY OF INVERTASE EXTRACTED FROM THE ROOT TIPS OF PEA AND MAIZE

Introduction

The levels and proportions of fructose, glucose and sucrose in the root tip of pea and maize show large differences when compared for roots grown at different temperatures. Growth rate is related to the quantities and proportions of these sugars, and to respiration rate (Chapter 5). (Specifically, in pea, growth rate is directly related to the ratio glucose content/sucrose content of the root, and to respiration rate, p.107; in maize, at temperatures above 6°C, the same relationships are observed. At 2°C, a third factor influencing growth of maize roots is the total sugar content of the root, p. 108.) In the experiment described below a study of the kinetic properties of invertase extracted from roots grown under different temperature regimes is undertaken, to determine to what extent the effects of temperature on levels of sugars in the root are exerted through temperature effects on the kinetic properties of invertase. As for MDH, in the previous chapter, Km and activity of invertase are determined at two temperature (2 and 14°C), after growth periods at three temperatures (2, 14 or 20°C), with similar aims, i.e.: to determine whether, between the two species, there are differences in the properties of the enzyme, and, within each species, there are changes in the properties of the enzyme dependent on the temperature regime the seedlings experience.

Method

Seeds of pea and maize were germinated and selected for the cold incubators as described on page 16. At the end of the respective germination periods for pea and maize, and also after seedlings were grown in the cold incubators at 2 and 14°C for 120 hr, a crude homogenate displaying invertase activity was prepared by grinding the distal 1 cm segment of the roots in McIlvaine's buffer. This homogenate was partially purified by fractional precipitation of the proteins, using increasing concentrations of ammonium sulphate. The fraction containing invertase activity was resuspended, dialysed, and the Km of invertase determined, using Sumner's colour reaction to measure the amount of hexose produced following incubation of sucrose with the invertase resuspension. All determinations of Km were made at two temperatures, 2 and 14°C. Duplicate experiments were performed in some cases. The full procedures used are given in Appendix 4.

The conditions of growth of the seedlings in the incubators were as described on p. 18. Batches of roots of both species were also grown at 20°C for 120 hr following the germination period and the enzyme extracted, partially purified and assayed, as above.

Results and Discussion.

Invertase from pea roots.

Lyne and apRees (1971), have recently demonstrated the existence of two different invertases, in the roots of pea, which have different pH optima. They refer to them as acid and alkaline invertase, pH optima 5.1 and 7.3 respectively, for the hydrolysis of sucrose. The extraction and partial purification procedure used by Lyne and apRees was followed in the present experiment but only sufficient material was

obtainable to measure acid invertase activity. The value obtained by Lyne and apRees for the Km of sucrose hydrolysis by invertase was 5.3 x 10^{-3}M, using roots of pea germinated 130 hr at 25°C and determining Km at 30°C.

The value obtained in the present experiment, for pea root tips excised from seedlings grown 72 hr at 20°C and Km determined at 14°C, was 4.68 x 10^{-3}M (average of the values given in Table 20). At 2°C the value was 4.62 x 10^{-3}M. These values are not significantly different from each other or from the value obtained by Lyne and apRees. After a further period of 120 hr growth at 20°C the Km determined at 2°C was slightly higher and Km determined at 14°C was somewhat lower, though in both cases the errors attached to the values overlap those for the values at the end of the germination period. Thus Km for invertase remains at a constant value whether determined at 2 or 14°C for the enzyme extracted from seedlings grown 120 or 240 hr at 20°C.

However marked changes in the Km are observed following growth at the temperatures of 14 and especially at 2°C. In both these cases Km determined at 2°C is significantly lower than that determined at 14°C.

The important feature of these results is that invertase extracted from the roots of seedlings which have only experienced the warm temperature of 20°C display a single value for Km independently of the temperature at which Km is determined, wherease seedlings which have experienced the lower growth temperatures of 14 and 2°C display: 1) a lower Km value when this is determined at 2 compared with 14°C and and 2) a Km value determined at 2°C lower than that for invertase from seedlings grown at 20°C. This has important consequences for rate of reaction under conditions where substrate concentration is in the range of or lower than the Km. Thus roots of seedlings grown at 20°C will

Table 20. Km of invertase extracted from the distal 1 cm of pea or maize roots grown under different temperature regimes. Km was determined at two temperatures, 2 and 14°C. Units for the values of Km are mM.

Conditions of growth	Km (mM) determined at: 2°C	14°C
Germination for 72 hr at 20°C	Pea	
ONLY	- 4.62±0.91	5.44±1.30 3.93±0.25
plus 120 hr at 20°C	5.32±1.42	3.72±0.69
plus 120 hr at 14°C	3.10±0.15	5.56±0.16
plus 120 hr at 2°C	1.56±0.18 2.08±0.33	6.19±0.20 4.03±0.19
Germination for 120 hr at 20°C	Maize	
ONLY	8.26±0.61	7.10±0.47
plus 120 hr at 20°C	8.18±0.69	9.37±0.69
plus 120 hr at 2°C	5.13±0.90	4.54±0.75

show a greater decline in rate of reaction catalysed by invertase over the temperature range 14 down to 2°C than will roots of seedlings grown at these two respective temperatures. This is especially true for roots grown at 2°C. In these roots the lower Km determined at 2°C indicates a greater affinity of the enzyme for its substrate and thus a relatively faster rate of reaction than would be expected on the basis of the Km determined at 14°C. The lower Km at 2°C represents a specific adaptation to low temperatures, being developed only after growth of the seedlings at 2 or 14°C, and suiting the roots to low temperature conditions by increasing enzyme affinity for its substrate.

The implications of this shift in Km for sucrose hydrolysis in intact roots could only by fully assessed if a careful study of the cellular localisation of the enzyme, the availability of substrate in the vicinity of the enzyme, cellular pH and enzyme inhibitors in vivo were undertaken. However even without such a study, the shift in Km in pea following growth at 2°C can be seen to be of adaptive significance. The increased affinity of the enzyme for its substrate at the lower temperature, 2°C favours maintenance of a relatively higher rate of reaction for any particular substrate concentration. This may be of importance for maintaining the general rate of metabolism at the low temperature and thus ultimately for maintaining growth of the roots. Potentially, change of the type seen in invertase in the kinetic properties of any enzyme could be of great adaptive significance in low temperature conditions.

Lyne and apRees (1971), Ricardo and apRees (1970), and Ricardo (1974) believe invertase to be a very important enzyme in pea roots and in carrot root tissues in controlling sugar metabolism. The existence of two invertases, alkaline and acid invertase, which they believe to be located in two different parts of the cell, the cytoplasm and the tonoplast

and/or cell wall respectively, allow a considerable degree of control over whether sugar is stored or accumulated in the cell vacuole as sucrose, or whether rapid inversion occurs to provide hexoses. The levels of the two invertases have been found to vary independently of each other both at different stages of growth in carrot and at different positions along the roots of pea. Bradshaw et al (1969), and Bradshaw and Edelman (1969), using discs of Jerusalem artichoke tubers, have shown that levels of invertase in this tissue are controlled by gibberellin and in sugar cane it is possible that glucose has a similar controlling influence (Sacher et al. 1963). These findings together provide strong evidence that invertase is a key enzyme in regulating sugar metabolism in plant tissues. The change in Km of invertase observed in the present experiment may represent another method whereby certain plant tissues, e.g.: pea roots, control their sugar metabolism at low temperatures. How this alteration in Km may be achieved has been discussed in the previous chapter (p.117). The possibilities are as follows.

1. An alteration in the tertiary or quaternary structure of the enzyme may be induced by temperature changes. Thus exposure of the invertase protein molecule to different temperatures may results in a different molecular conformation which in turn causes a different affinity of the enzyme for the substrate.

2. Different species of the invertase enzyme may be synthesised at at different temperatures; that at the lower temperature, in the present case, having a greater affinity for the substrate.

3. At all temperatures, a range of enzyme species may be synthesised. There is then selective degradation of those species having inferior catalytic properties at the particular temperature to which the roots are exposed.

Of immediate concern is another finding of Lyne and apRees (1971). In addition to there being two invertases in pea roots they showed that the activities of the enzymes varied both along and across the root. Thus acid invertase activity in 3 mm segments of the root taken successively back from the tip was inversely related to sucrose content in the segments. In the zone of cell division and elongation (3-9 mm) the acid invertase activity per mg protein was four times greater than in segments of the root taken beyond 15 mm from the root tip. Sucrose content behaved reciprocally. In the zone of elongation the content was only 50% of that recorded further from the root tip. Across the root they found that acid invertase predominated in the cortex and alkaline invertase in the stele (although the activity of the latter was much lower in both tissues). The cortex was where sucrose concentration was relatively lower. Thus, here again acid invertase activity is inversely related to sucrose content.

The data obtained in the present experiment for levels of invertase activity further support the idea that acid invertase activity and sucrose content are inversely related when expressed on a fresh weight basis but here temperature is the factor associated with the differences in sucrose content (Table 21) (see Chapter 3). After 120 hr in the cold incubators sucrose levels reach the highest values in seedling roots kept at the lowest temperature (Fig. 11). The activity of the acid invertase in root tips after 120 hr at $2^{o}C$ and at $14^{o}C$ can be seen to be inversely related to these sugar levels.

However an exception to this relationship occurs at the end of the germination period. Sucrose concentration is intermediate compared with the values after 120 hr growth at 2 and $14^{o}C$ but the acid invertase level is not intermediate as would be expected but is rather low (similar to that recorded for roots after 120 hr at $2^{o}C$). This is

Table 21. Activity of invertase, and sucrose content, extracted from the distal 1 cm of pea or maize roots grown under different temperature regimes.

Conditions of growth	Invertase activity IU/mg FW		Sucrose content* mg/g FW
	2°C	14°C	
Germination for 72 hr at 20°C		Pea	
ONLY	0.049	0.142	3.45
plus 120 hr at 14°C	0.091	0.258	1.81
plus 120 hr at 2°C	0.057	0.136	5.83
Germination for 120 hr at 20°C		Maize	
ONLY	0.036	0.085	8.18
plus 120 hr at 2°C	0.056	0.113	3.72

*Values obtained in Chapter 3.

probably because this enzyme is in the process of de novo synthesis by the young roots, and has not achieved its final level. The lower sucrose level 24 hr after transference to the cold incubators (a time at which sucrose translocation from the cotyledons has not started, see p. 33) supports this interpretation, Fig. 11. The lower level is considered to be due to the continued build up of acid invertase activity by de novo synthesis.

The correlation of acid invertase activity and sucrose content may be extended further to include growth rate. In pea roots the low acid invertase activity and the high sucrose content are both present under low temperature conditions, where growth was at a low rate. Hatch and Glaziou (1963), working with the immature internodes of sugar cane also observed this same correlation. In their case low growth rate, whether induced by low temperatures or by drought was associated with a relatively high sucrose content and a low acid invertase content, in the tissue.

Invertase from maize roots

Hellebust and Forward (1962), have reported Km values for invertase from the roots of maize of 6.2, 7.9 and 6.10 x 10^{-3}M (determined on root segments 0-2, 2.5-5.0 and 0-10 mm respectively from the tip). They used roots of seedlings grown at 30°C for 70 hr. At the end of the germination period in the present experiment the roots, aged 120 hr at 20°C, possessed an acid invertase with a Km of 7.1 x 10^{-3}M at 14°C (Table 20) which is similar to the values quoted above. At 2°C the Km was higher, 8.26 x 10^{-3}M, but the difference between these values may not be significant. Continued growth at 20°C resulted in no significant change in Km determined at 2°C but a rise in Km determined at 14°C. However values for Km at these two temperatures possessed overlapping errors and these are not therefore considered

significantly different. Following growth at 2°C the trend seen in pea is not followed by maize to any marked degree. There is a downward shift in Km but unlike pea this is not confined to the Km value determined at 2°C. The value determined at 14°C is also lower and actually showed the larger decrease. A lower value determined at the temperature of 2°C but an unchanged value at 14°C (compared with the values at these respective temperatures after growth of the roots at 20°C), as was found in pea, is not apparent in maize. Thus maize does not show a shift, in the Km of invertase, related to low growth-temperature, which may be of adaptive significance for low temperature conditions.

A further point is that, in absolute value, the Km of invertase from pea roots is lower than that from maize roots and this greater affinity of the enzyme for its substrate in pea roots will be advantageous for maintaining an appreciable rate of reaction at lower temperatures.

With respect to the activity of acid invertase in maize roots, it is found that after exposure of the seedlings to 2°C, the activity of the enzyme is greater than after exposure to 20°C, Table 21. This is the reverse of what is expected from previous work (see discussion above for pea, and Hatch and Glasziou 1963; where growth rate is low - that is at the low temperature of 2°C in the present example - acid invertase activity is normally low). The result is very interesting in that 2°C is the temperature at which sugar metabolism in maize roots has been shown to be disturbed (p. 59). The glucose/sucrose ratio was abnormally high, due mainly to a low level of sucrose, and the total sugar content of the roots declined continuously with time exposed to 2°C. The high acid invertase activity demonstrated in the present experiment is considered responsible for these disturbances.

It is suggested that, in the maize root exposed to low temperatures (e.g.; 2°C) there occurs a breakdown in the system controlling acid invertase activity, and therfore a breakdown in control of enzyme production by the roots, since activity was measured in partially purified homogenates, and reflects quantity of enzyme present in the root. This may be a very important cause of breakdown in sugar metabolism and cessation of root growth seen in maize seedlings at low temperatures.

Conclusions

1. Pea and maize roots show a difference in their capacities for modifying the kinetic properties of invertase at low temperatures.
2. In pea, modifications occur which may have adaptive significance.

a) First, after exposure of seedlings for 120 hr to growth temperatures of either 2 or 14°C, Km determined at 2°C is lower than Km determined at 14°C. This lower value at 2°C is not seen either before seedlings have experienced these growth temperatures or after experiencing 120 hr at 20°C. The lowering of Km is a specific response to low temperatures, 14°C and below. The lower Km, determined at 2°C, and developed after growth at 2°C will enhance rate of reaction, for substrate concentrations in the range of or lower than the Km, relative to the rate at 2°C for seedlings which have experienced only 20°C. After growth at 14°C the same potential is present as after growth at 2°C (enhanced rate of reaction at 2°C), but has not been realised since seedlings have not experienced the temperature of 2°C. The mechanism resulting in production of invertase with Km lower when determined at 2°C than at 14°C, thus comes into operation between the growth temperatures of 14 and 20°C.

The importance of lower Km at the temperature of 2°C is due to the relatively greater reaction rate that can be maintained with low substrate concentrations (those lower than the Km), thus counteracting the usual effect of low temperature to slow down reaction rate. Potentially the general rate of metabolism may thus be maintained at an appreciable rate at low temperature and be sufficient to maintain root growth.

b) Second, specific activity of acid invertase in pea is directly correlated with temperature and root growth rate, and inversely related to sucrose content of the root tissue. At low temperatures the high sucrose content associated with low acid invertase activity is probably of importance for conferring cold tolerance on the roots (see discussion pp.55-8).

The differences in Km and specific activity of invertase, determined at 2°C, between roots grown at 2 and at 20°C, are such as to affect rate of reaction in opposite ways. The Km after growth at 2°C relative to growth at 20°C shifts in a manner enhancing rate of reaction (it becomes lower thereby increasing affinity of the enzyme for its substrate) while activity acts in a way which will decrease rate of reaction (activity is lower - determined at 2°C after growth at 2°C compared with growth at 20°C - giving a slower rate of reaction for any substrate concentration because there is less enzyme present in the tissue). At the same time and as a consequence of the lower invertase activity, sucrose concentration is higher in roots grown at 2°C. This higher sucrose concentration in combination with the lower Km shown by invertase presumably acts to maintain an appreciable but controlled rate of sucrose hydrolysis at 2°C providing hexoses essential for respiration and the synthetic processes involved in

growth of the roots.

3. In maize roots, invertase does not show changes in its kinetic properties, after growth at 2°C, of adaptive significance for low temperature conditions.

a) In maize, a modification in the Km of the type seen in pea is present but to a much smaller extent. After growth at 2°C the Km determined at 2°C is reduced by less than half of the value at the end of the germination period (whereas in pea Km was three times lower after growth of the seedlings at 2°C). This reduction in the Km value determined at 2°C is less than that observed when determined at 14°C (thus affinity of the enzyme for the substrate is actually greater at 14°C), and therefore cannot be considered effective in maintaining reaction rate at low temperatures and low substrate concentrations.

b) The activity of invertase from maize kept at 2°C (when growth ceases over 5 days, Fig. 3) is unexpectedly high. Low growth rate is normally associated with low acid invertase activity (see pea above and Hatch and Glasziou, 1963) but in maize, activity of invertase from roots grown at 2°C is higher than from roots grown at 20°C. A breakdown in the system controlling acid invertase synthesis is thought to occur. The enzyme is over-produced, resulting in rapid hydrolysis of sucrose in the root, and depletion of the total sugar content (Fig. 7) since the hexoses produced from sucrose hydrolysis are utilised, presumably in respiration and biosynthesis (Table 8), faster than they are replaced by transport from the maize grain.

Thus the differences in invertase behaviour at low growth temperatures between pea and maize roots result in different patterns of sugar accumulation and utilization by the roots, which in turn have a direct effect on growth rate, or the capacity of the roots to exhibit growth, at 2°C. A differential effect of low temperature on the behaviour

of a specific enzyme in the roots of pea and maize has thus been implicated as the basis for the differential effect of low temperature on growth of the roots of these species.

FINAL SUMMARY AND CONCLUSIONS

A study of the carbohydrate metabolism of pea and maize roots was undertaken to determine what metabolic differences underlay the differing capacities of roots of the two species for growth at low temperatures.

(1) Growth and temperature Growth of pea and maize roots was studied at four temperatures, 2, 6, 10 and 14°C. Pea roots showed constant rates of growth at all these temperatures, even the lowest, whereas growth rate of maize roots at 2 and 6°C declined over five days of exposure, ceasing entirely at 2°C. The temperature of 6°C was on the borderline for continued growth of maize roots.

(2) Sugar metabolism and temperature At the minimum temperatures which were capable of sustaining growth (2°C for pea; 6°C for maize) there was a similar situation with regard to soluble sugars, with the sucrose content of the roots attaining its highest value and glucose content its lowest value. With rise in temperature above the absolute minima for growth there was a decline in sucrose and an increase in glucose content in the roots.

At temperatures below which there was no sustained root growth (less than 6°C in maize), all sugars fell dramatically (to 50% the initial content, after five days at 2°C). This condition was never produced in pea. The decline in sucrose content was particularly significant for the differing capacities for root growth in the two species since a high sucrose content (as was recorded in pea roots) is involved in tolerance of plant tissues to low temperatures.

(3) Respiration and temperature The respiratory response of pea roots to temperature was typical of a chill tolerant species - the Arrhenius

plot for rate of oxygen uptake was linear. The comparable plot for maize roots, after they were exposed for five days to the four experimental temperatures, showed a break in the slope of the graph between 10 and 6°C - a feature found in many chill sensitive species. This break was due to a very low respiration rate at 2 and 6°C. In particular, the respiration at 2°C was only 50% of the rate displayed by pea roots. The higher rate in pea roots is important for sustaining root growth at 2°C.

(4) Sugar feeding experiments It was shown that the disturbed sugar metabolism of the maize roots at 2°C was directly influencing growth and respiration rates of the roots. In experiments where sugars were fed to the roots, then on supplying glucose the disturbance observed in carbohydrate metabolism of maize roots was alleviated. Total sugar content of the roots was maintained, sucrose content of the root increased to the highest levels recorded in roots of that species, and respiration rate was stimulated: this was associated with an increase of growth made by the roots.

(5) Enzyme mechanisms underlying metabolic differences in pea and maize roots (a) Kinetic properties of MDH The kinetic properties of MDH extracted from pea roots altered in roots of older seedlings such that the enzyme had increased affinity for its substrate at low temperatures. This was not found for MDH extracted from maize roots. A second difference between the two species concerned the specific activity of MDH. In pea, the decline in specific activity with temperature (14°C was compared with 2°C), was smaller than that recorded for MDH from maize roots. With regard to both these properties of MDH greater reaction rate in pea as compared with maize roots is favoured at low temperatures.

(b). Differential response of invertase to low temperatures in pea and maize It was possible to relate the sugar levels found in the roots of either species at 2°C to the properties of acid invertase extracted from the roots.

In pea, specific adaptations of the kinetic properties of acid invertase to low temperature conditions occurred following exposure of the roots to low temperatures whereas in maize the kinetic properties of this enzyme were not adaptable to cold conditions and this could be traced as the cause of the disturbance in sugar metabolism noted above.

Specifically, in pea roots, at 2°C, invertase levels were lower than in roots grown at higher temperatures, accounting for the higher sucrose content recorded in roots at 2°C. Km showed a growth-temperature related shift in value which could be interpreted as adapting the root to low temperatures - following growth at 2°C the Km determined at this temperature was lower than that for roots grown at higher temperatures, thus (1) increasing sensitivity of the enzyme for its substrate, and (2) providing for a relatively faster rate of reaction, and so opposing the decelerating effect of low temperature on reaction rate and maintaining active metabolism in the cold conditions.

In maize roots invertase levels were higher in roots grown at 2°C compared with 20°C, accounting for the depletion of sucrose in the root after five days exposure to 2°C (and associated with this the lack of cold tolerance of roots of this species). Km did not show a growth-temperature related decline in value as was foumd in pea and thus does not possess the adaptive features shown by this enzyme in pea.

Control of invertase synthesis and alteration of its kinetic properties are thus seen in pea to be important factors for growth of

pea roots at 2°C, via the consequences of this enzyme on sugar metabolism. Conversely, in maize, the inability of this species to control enzyme activity at low temperatures and failure to adapt Km are associated with a disturbance to sugar metabolism at low temperatures, and, arising from this, a failure of root growth.

Appendix 1. Method for the quantitative extraction and analysis of the soluble carbohydrates in the distal 1 cm of roots of pea and maize.

Principle.

The method employed has been developed from that of Sweeley et al., 1963, and Ellis, 1969. Mono- and disaccharides were extracted from the roots of pea and maize in boiling ethanol and then all ethanol and water was removed. The dry sugars were converted to volatile derivatives, suitable for analysis by GLC. They were resuspended in DMSO and converted to their trimethylsilyl (TMS) derivatives by reaction with excess HMDS plus TMCS. Thorough shaking was required for this reaction to occur since DMSO and HMDSO (the reaction product of excess HMDS and TMCS) are immiscible. Completely dry reagents and sugars were required since HMDS and TMCS react violently with water.

The TMS-derivatives of the sugars have a strong affinity for the HMDSO phase (the upper phase). A sample of this phase was injected into the GLC, where it was vaporised, and swept through a column containing methyl-phenyl-silicone gum, by a flow of dry nitrogen. The TMS-derivatives were retained in the column for different characteristic lengths of time. Their emergence from the column was recorded by a flame ionisation detector coupled to a chart recorder.

Method

The distal 1 cm of the seedling roots were excised in a plastic tray, on the base of which was etched parallel lines 1 cm

apart. During excision the roots were bathed in chilled distilled water. Batches of approximately 30 root tips were used (approximately 500 mg FW). Each batch was then put through the following procedure:

1. Surface dry root tips and obtain the FW.
2. Boil gently in 2-3 ml 80% ethanol. Pour off the ethanol into a rotary evaporator flask. Repeat 3 times in all.
3. Repeat stage 2, but using 60% ethanol and performing the operation only twice.
4. Evaporate the combined extracts to dryness on a rotary evaporator at $70^{o}C$.
5. Store the extract over phophorus pentoxide 3-4 days until completely dry, and continue this form of storage between all subsequent stages.
6. Resuspend in 1 ml DMSO by swirling occasionally over 24 hr.
7. Using an Oxford Sampler micropipette, pipette 0.2 ml resuspension into a "cherry bottle" (a round-bottomed flask with graduated neck, vol. of bulb approx. 1.0 ml, vol. of neck approx. 0.2 ml).
8. Add 0.1 ml HMDS and shake manually.
9. Add 0.05 ml TMCS and immediately seal with parafilm and shake vigorously on a mechanical shaker for 90 secs.
10. Stand overnight in a phosphorus pentoxide desiccator. Two phases separate out. The upper phase is HMDSO. The TMS-derivatives have a strong affinity for this phase.
11. Using a 2 ml syringe inject DMSO into the lower phase in the bulb of the "cherry bottle" to displace the upper phase into the graduated neck of the flask. (The volume of the upper phase depends on the volume of trimethylsilylation reagents used, Ellis 1969.)

12. Immediately a measured volume (0.5-1.0 µl) is withdrawn from the upper phase for injection into the GLC. This sample must be withdrawn quickly because some repartitioning of the sugar derivatives will occur over the next few hours.

13. Measure the volume of the upper phase.

14. Results were obtained from the GLC as a line trace. Standard solutions of sugars, treated similarly to root tip extracts, gave sharp individual symmetrical peaks and the areas under these peaks, calculated by multiplying the height by half the base length, was found to be proportional to the quantity of sugar, Plates 2-3, Fig. 27.

15. The GLC used was a Pye series 104 with a temperature programmer and a flame ionisation detector, linked to a Philips PM 8000 recorder. The operating conditions were:

Carrier gas	Nitrogen: Flow rate 30-35 ml/min
Flame ionisation detector	Hydrogen: Flow rate 30-35 ml/min
	Air: Flow rate 25-30 ml/min

Injection heater 2.25

Temperature programme 130-250°C at 6°C/min.

Column - glass (5 ft x 1/4 in) packed with 1% E52 diatomite CQ

Attenuator 20-50 x 10^2

Detector oven 250°C

Paper speed of recorder 1 cm/min.

16. To prepare the column used for separating TMS-derivatives of mono- and di-saccharides:

15-20 g diatomite CQ weighed out absolutely dry. 1% of this weight of E52 (methylphenylsilicone gum) then dissolved in chloroform. This is added to the CQ to give a slurry. Evaporate to dryness under reduced pressure at room temperature. Pack into the GLC glass column evenly and "age" (subject to a temperature,

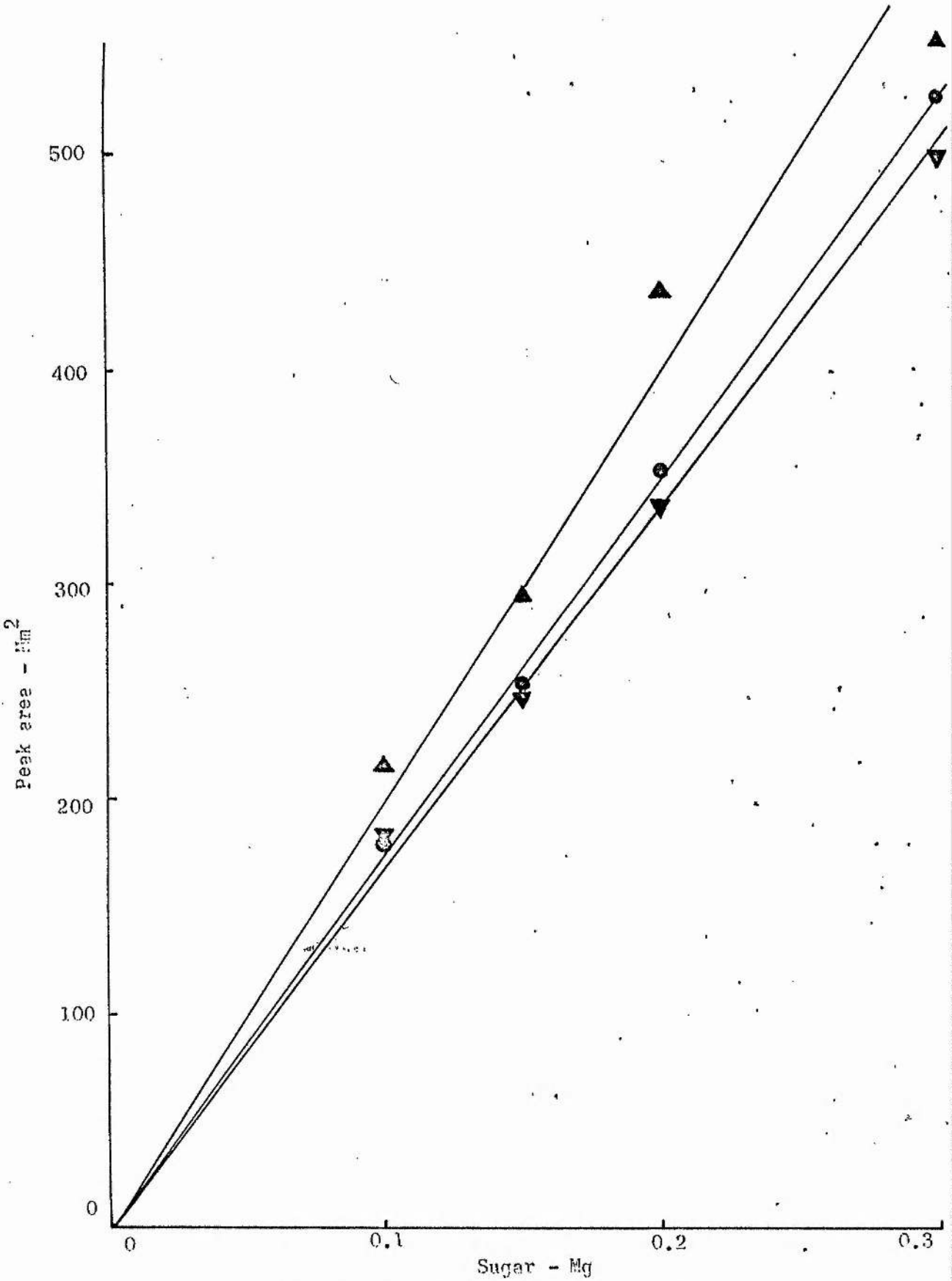


Fig. 27. Relationship of peak area of GLC trace to quantity of sugar (●—● fructose; ▲—▲ Glucose; ▼—▼ Sucrose) reacted in the trimethylsilyation reaction. (The sugars were dissolved in 0.2 ml DMSO. They were reacted with 0.1 ml HMDS plus 0.05 ml TMCS. A 1µl sample was injected into the GLC, Attenuation $50x10^2$, paper speed 1 cm/min. Full experimental details given in Appendix 1.)

near the maximum operating temperature to be used, for several hours).

17. The syringe used for injecting the samples into the GLC was 1.0 µl SGE syringe and was rinsed out between injections with pyridine.

Appendix 2. Method for determining the respiration rate of the distal 1 cm of the roots of pea and maize seedlings.

Batches of roots were excised as in Appendix 1 but with the bathing solution being 0.05 M phosphate buffer pH 5.5, equilibrated to the particular temperature to which the roots had been exposed. 20-60 root tips per flask were found to be sufficient, depending on the temperature. Individual batches of roots were divided equally between two Warburg flasks, each containing 3 ml phosphate buffer in the outer well, but one containing 0.2 ml water in the centre well (this flask measures net gas volume change) and the other 0.2 ml 5% potassium hydroxide solution (this flask measures oxygen uptake). Flasks were attached to a Gilson respirometer and immersed in a waterbath set at the temperature to which the roots had previously been exposed (2, 6, 10 or 14^{o}C as appropriate). The exception to this was at time 0 hr. Here the roots had experienced only the germination temperature of 20^{o}C. The respiration rate of these roots was determined at the above four temperatures. The apparatus was equilibrated at least 30 min. Then the valves isolating each flask were closed to start the experiment. At this time a check for leaks was made by briefly lifting each flask in turn out of the water bath. A rapid change in the manometer fluid level occurred only if the system was gas tight. The maximum time which elapsed from excising the first root to making the first reading was less than 2 hr. Readings of the volume change in each flask were made, usually every 10 min, over a period of at least 1 hr. The apparatus used was calibrated to give readings directly in μl gas consumed or evolved

per flask. These figures were multiplied by a correction factor dependent on the atmospheric pressure and water vapour pressure to obtain the values of oxygen uptake or net gas volume change, as appropriate, for each flask. Carbon dioxide evolution was calculated by subtracting the net gas volume from the oxygen uptake for a pair of flasks.

At the end of the experiment the root tips were recovered from the flasks, surface dried and then ovendried at 80°C to constant weight thus obtaining the dry weight. Results were expressed as µl gas produced or consumed/mg DW/hr. All experiments were duplicated and the average of the results obtained is presented. Duplicates agreed within 5%.

Appendix 3. To extract, partially purify, and assay malate dehydrogenase from the roots of pea and maize seedlings.

Only the distal 1 cm of the roots was used. These segments were excised in chilled phosphate buffer 0.1 M pH 6.5 (containing KH_2PO_4 and K_2HPO_4). An acetone powder of this material was prepared and proved to contain MDH activity which was very stable, maintaining full activity, when resuspended, after at least 10 weeks storage at 0^oC. This powder was prepared as follows:

The root tips were surface dried and weighed (at least 2 g were used) and then ground up in acetone using a mortar and pestle, all these materials being at -11^oC. The suspension was filtered through a Buchner funnel and the residue on the filter paper was spread on a fresh filter paper tray and the acetone allowed to evaporate completely at room temperature (approximately 24 hr). The resulting powder was stored at 0^oC until assayed for malate dehydrogenase activity by the following procedure:

A quantity of the powder was weighed (approximately 200 mg) and resuspended in phosphate buffer (approximately 5 ml) by stirring very slowly with a magnetic stirrer at room temperature for 4 hr. The resuspension was filtered through muslin and then heated for 10 min at 55^oC. Cellular debris was removed by centrifuging at 12,000 r.p.m. for 10 min, at 2^oC. This resuspension maintained its MDH activity at the initial level for at least 4 days when stored at 0^oC.

The K_m was determined spectrophotometrically by following the decline in absorbance of 340 nm, due to the conversion of NADH to NAD as the enzyme converts OAA to malic acid.

Cuvettes were prepared containing 0.01 ml NADH (40 mg/ml), a volume of the enzyme resuspension found to give a suitable rate of

reaction over the range of substrate concentrations used (usually 0.05-0.1 ml enzyme resuspension was required), and a volume of buffer to bring the final volume to 2.9 ml. The cuvettes were placed in a Unicam SP1800 Spectrophotometer in a temperature controlled jacket and allowed to equilibrate. The reaction was started by adding 0.1 ml of OAA. A range of concentrations was used to give a final concentration range in the cuvette between 0.0286 and 0.333 mM. Small volumes were introduced into the cuvettes on glass spatulas which also served as stirrers, as the volumes were introduced.

The decline in absorbance was traced on a Unicam AR25 Linear recorder paper speed 1 cm/min. The initial rate of reaction was measured from the slope of this trace. The Km was calculated from a Lineweaver-Burke plot by plotting the reciprocal of OAA concentration against the reciprocal of rate of reaction. This was performed by computer as described in Appendix 5. The error quoted on the Km values was that due to the scatter of the data points.

The protein content of the enzyme resuspension was determined by the Folin-Lowry method (Plummer 1971). Alkaline solution was prepared by mixing 50 ml of 2% $Na_2C)_3$ in 0.1N NaOH with 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% NaKTartrate. Folin-Ciocalteau's reagent was prepared by mixing 1 part of commercial reagent (BDH Folin-Ciocalteau's phenol reagent) with 1 part of water.

5 ml of the alkaline solution was added to an appropriate volume of the MDH resuspension (usually 0.1-0.2 ml), and the volume made up to 7 ml with distilled water. The mixture was stirred and left to stand 10 min. Then, while stirring vigorously, 0.5 ml of the Folin-Ciocalteau reagent was added. After standing 40 min the absorbance at 750 nm was measured and referred to a standard graph of absorbance prepared at the same time using 0-0.6 mg of crystallised bovine albumin.

Appendix 4. To extract, partially purify and assay invertase from the roots of pea and maize.

The procedure described is that for pea. Modifications of this procedure for maize are given at the end of this appendix.

Only the distal 1 cm. of the roots was used. These segments were excised in chilled McIlvaine's buffer pH 7.0, (0.2M $Na_2HPO_4.2H_2O$ and 0.1N Citric acid.H_2O). After surface drying the fresh weight was obtained (sufficient roots were used to yield approximately 10 g) and the root tips were then subjected to the extraction procedure below. The basis of the technique was to obtain a partially purified preparation of invertase by $(NH_4)_2SO_4$ precipitation of the protein from an aqueous root homogenate.

1. Grind up root tips in chilled buffer, pH 7.0, using a mortar and pestle stood on ice. All subsequent stages are performed on ice to prevent denaturation of the enzyme.
2. Centrifuge the suspension at 12,000rpm for 20 min at $2^{o}C$.
3. The precipitate, insoluble cellular material, is discarded. The supernatant is poured off, its volume recorded and then solid $(NH_4)So_4$ added to bring to 30% saturation. The $(NH_4)SO_4$ is added slowly, stirring the supernatant continually.
4. Allow to stand 40 min during which a fraction of the protein precipitated out.
5. Repeat step 2 on the fraction above.
6. Discard the precipitate. Pour off the supernatant and repeat step 3, but bringing to 40% saturation.
7. Stand 40 min. The fraction of protein which precipitates out contains the invertase activity.
8. Repeat step 2 on the above fraction.

9. Discard the supernatant. The precipitate is resuspended in approximately 50 ml McIlvaine's buffer, pH 5.1, of one tenth the strength given above. The volume of the resuspension is measured accurately.

10. The resuspension is dialysed overnight against the 1/10 strength buffer at 2°C. The dialysis solution is renewed after 6 hr. The volume of the resuspension after dialysis is recorded.

The partially purified preparation of invertase obtained keeps its full activity for at least three days stored at 0°C. The protein content of this preparation was determined as in Appendix 3.

The Km of invertase was determined by incubating the enzyme in a range of sucrose concentrations and estimating the rates of hexose formation, using Sumner's reagent (Sumner 1925). The procedure is described below:

A series of incubation tubes was set up, usually 8, in a waterbath at constant temperature. Each tube contained 0.5 ml glucose solution (1 mg/ml) in McIlvaine's buffer, pH 5.1 1/10 strength, and 3.5 ml of the same buffer containing dissolved sucrose. A range of sucrose concentrations was used from 1.3 - 40 mM giving a final range of concentrations in the tubes (after the invertase solution was added) from 0.66 - 20 mM. The glucose solution was added as it was found to remove a lag phase in glucose production by the enzyme (Lyne and apRees 1971).

The reaction was started by adding 3.0 ml enzyme solution to each tube. At this time and at appropriate time intervals for each concentration thereafter 1.0 ml samples of the incubation mixture

were pipetted out, and run into 1.0 ml aliquots of Sumner reagent (see preparation below). This stopped the reaction. Immediately the samples in the Sumner reagent were stood in a boiling water bath for 5 min. The samples were then cooled in the incubating bath. 1.0 ml distilled water was added to each sample and the absorbance of the orange-red colour developed was measured on a Unicam SP1800 UV Spectrophotometer at 510 mm using a 3 ml cuvette with 1.0 cm light path. The absorbance is very temperature sensitive and the cuvette cells in the spectrophotometer were cooled with water from the incubating bath. The absorbance is proportional to hexose concentration, and hexose standards were prepared under identical conditions to calibrate the machine for each experiment.

Sumner reagent was prepared as follows:
10 g crystallised phenol and 22 ml 10% NaOH were dissolved in a little water and diluted to 100 ml. 69 ml of this solution were used to dissolve 6.9 g sodium bisulphite, and this was then mixed with a solution containing 300 ml 4.5% NaOH, 255 g Rochelles' salt and 800 ml 1% dinitrosalicylic acid. The reagent is kept tightly stoppered in well filled bottles.

For each incubation tube (total volume 6.5 ml) six 1.0 ml samples were withdrawn at increasing times from time 0 min, when the invertase was added. After correcting the quantity of glucose in each sample for that added initially, rate of glucose production was calculated by plotting glucose production against time. This plot was performed by computer using a least-squares program to obtain the best straight line through the points. The slope of this line is rate of glucose production. The program is presented in Appendix 5. Over the time courses used, 15 min for the highest sucrose concentrations

and 4 hr for the lowest, the rate of glucose production remained constant, i.e.: straight line plots were obtained.

The Km was calculated from a Lineweaver-Burke plot by plotting the reciprocal of sucrose concentration (1/s) against reciprocal of rate of reaction (rate of glucose production, 1/v) and dividing the slope of the best straight line through the points by the intercept on the 1/s axis. This calculation was also performed by the above computer programme. The errors of the Km values were calculated from the scatter of the data points about the best straight lines.

The following modifications to the above extraction and assay procedure for pea were made for maize (since the pH optimum and Km of invertase in this species are different from pea).

1. McIlvaine's buffer pH 4.6 replaced buffer at pH 5.1.
2. The range of final sucrose concentrations in the incubation tubes was 1-50 mM.
3. Samples withdrawn from the incubating tubes were run into 1.0 ml Sumner reagent plus 0.1 ml 10% NaOH. This was required to enhance colour development which was otherwise poor. After boiling the samples for 5 min and then cooling, 0.9 ml distilled water was added to yield the same final volume of 3.0 ml as in pea.

The extraction and assay procedures were developed from those used by Hellebust and Forward (1962) and Lyne and apRees (1971), and Arnold (1965).

Appendix 5. Computer programme used to fit the best straight line through a series of data points by the method of least squares.

```
FORTRAN IV G LEVEL 44PS V 1.0          GMAIN44          DATE = 75069  TIME = 18.59.38

C
C     T.J.  HUXTER  DEPARTMENT OF BOTANY
C
C     UNWEIGHTED LINEAR LEAST SQUARES SOLUTION FOR THE EQUATION :
C
C                  (1/V)=(KM/VM)(1/S) + (1/VM)
C                  B=(KM/VM)          A=(1/VM)
C
C
C     AT PRESENT THE PROGRAMME PERMITS A SOLUTION FOR UP TO 50 DATA POINTS.
C     TO INCREASE THIS NUMBER IT IS ONLY NECESSARY TO ALTER THE DIMENSION
C     OF ALL THE VARIABLES IN THE DIMENSION STATEMENT.
C
C
C     INPUT
C     *****
C
C     FOR EACH REGRESSION:FIRST CARD STATES  NO. OF DATA CARDS TO FOLLOW,COL1-3
C       DATA CARDS,  COLS. 10-19 1/S, COL. 20-29 1/V
C
C     OUTPUT
C     ******
C
C     A LIST OF DATA POINTS IS OUTPUT + THE CORRECT VALUE FOR 1/S GIVEN DATA
C     VALUE FOR 1/V AND THE DISTANCE OF THE DATA 1/S VALUE FROM THE CORRECT
C     VALUE. VALUES FOR B (THE GRADIENT) AND A (THE 'ZERO POINT') OF THE
C     LINEAR TRANSFORMATION ARE ALSO GIVEN. ALSO THE VALUE OF KM IS OUTPUT
C     WITH ITS STANDARD ERROR.
C
C
C
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION HR(50),BS(50),BI(50),DD(50),D(50),BSCALC(50)
      DIMENSION RINFO(8)
  100 A=0.0D0
      B=0.0D0
      SUMBI =0.0D0
      SUMBS =0.0D0
```

```
0008                SUMBBS=0.0D0
0009                SUMBIS=0.0D0
0010                SUMPRD=0.0D0
                C
                C       READ AND WRITE OUTPUT HEADINGS
                C
0011                READ(5,120) N,IDAY,JDAY,MONTH,IYEAR,RINFO
0012              120 FORMAT(I3,3A3,I5,A4,7A8)
0013                IF(N.EQ.999) GO TO 998
0014                WRITE(6,140) IDAY,JDAY,MONTH,IYEAR,RINFO
0015              140 FORMAT('1',2X,'LINEAR LEAST SQUARES SOLUTION FOR',3A3,I5//
                   11X,A4,7A8//)
                C
                C
0016              150 FORMAT('UNWEIGHTED LINEAR TRANSFORMATION FOR ',3A3,I5)
                C
                C       LOOP FOR SUMATION OF SQUARES
                C
0017                DO 200 I=1,N
0018                READ (5,160) HR(I),BS(I),BI(I)
0019              160 FORMAT(2X,A8,2F10.3)
0020                SUMBS=SUMBS+BS(I)
0021                SUMBI=SUMBI+BI(I)
0022                SUMBBS=SUMBBS+BS(I)*BS(I)
0023                SUMBIS=SUMBIS+BI(I)*BI(I)
0024                SUMPRD=SUMPRD+BI(I)*BS(I)
0025                X=BS(I)
0026                Y=BI(I)
0027              200 CONTINUE
                C
                C       COMPUTATION OF A AND B
                C
0028                XN=DFLOAT(N)
0029                P=XN*SUMPRD
0030                Q=SUMBS*SUMBI
0031                R=SUMBS*SUMBS
0032                S=XN*SUMBBS
0033                T=SUMBIS*XN
```



```
0034                U=SUMBI**2
                C
0035                DELTA=(S-R)
0036                B=(P-Q)/DELTA
0037                A=((SUMBBS*SUMBI)-(SUMBS*SUMPRD))/DELTA
                C
                C       COMPUTATION OF STANDARD ERROR IN A AND B
                C
0038                DO 250 J=1,N
0039                BSCALC(J)=(BI(J)-A)/B
0040                D(J)=BSCALC(J)-BS(J)
0041                WRITE(6,240) HR(J),BS(J),BI(J),BSCALC(J),D(J)
0042              240 FORMAT(3X,A8,4(3X,F10.3))
0043              250 CONTINUE
0044                SIGMA=(T-U-(P-Q)**2/DELTA)
0045                RB=DSQRT(SIGMA/((XN-2.0D0)*DELTA))
0046                RA=DSQRT(SUMBBS/XN)*RB
                C
                C
                C
                C       OUTPUT
                C
0047              290 WRITE(6,300) A,RA,B,RB
0048              300 FORMAT('0',//,3X,'A = ',F10.4,10X,F10.4,'     (STANDARD ERROR IN A)
                   *',
                   *//,3X,'B = ',F10.4,10X,F10.4,'     STANDARD ERROR IN B)',////)
0049                ZKM=B/A
0050                RZKM=(B*RA+A*RB)/(A**2)
0051                WRITE(6,400)ZKM,RZKM
0052              400 FORMAT(1X,//,20X'KM=',F10.7,10X,'ERROR IN KM =',F10.7)
0053                GO TO 100
0054              998 WRITE(6,999)
0055              999 FORMAT('1')
0056                STOP
0057                END
```

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