CHANGES IN ENZYME ACTIVITIES ACCOMPANYING EXTENSION GROWTH OF CULTURED ROOT SEGMENTS

By

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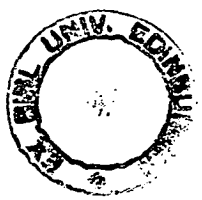

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This thesis is the original work of the author and has not been submitted previously for any other degree.

ABSTRACT

Root fragments taken from the expanding zone of primary roots and cultured in flasks on a rotary shaker have been used to investigate expansion growth. The plant material used was the garden pea, Pisum sativum, var. Feltham First. The sources of variability in growth response in the culture system were analysed and markedly reduced. The root segments were accurately and quickly measured by a 'shadow' technique which provided a permanent magnified image of the segments.

Fragments taken from the 2-4 mm sub-apical zone of the root undergo extension growth when cultured in 2% sucrose. The growth is stimulated by the addition of 6-methyl purine (6MP) at the concentration of 2×10^{-5}M and, when the sucrose medium is supplemented with IAA at the concentration of 10^{-10}M.

The activities of the enzymes glucose-6-phosphate dehydrogenase (G6PDH) and cellulose synthetase (CS) were examined during extension growth of the excised segments cultured in a range of media. Preliminary investigations were performed to establish the validity of the enzyme assays for the pea root tissue and to ensure the assays were performed under optimal conditions.

The two enzymes show different patterns of activity during the 24 hour culture period of the segments in the same culture conditions, and the patterns vary with different culture conditions. The activities are not directly related to the segment size or the amount of cell protein.

The stimulation of segment growth by the addition of 6MP (2×10^{-5}M) to the sucrose medium is brought about by an extension of the duration of the rapid phase of growth. The initial growth rate is unaffected. This mode of stimulation and the widening of the

peak in the graph of CS activity against time are interpreted as supporting the theory of Brown (1963) on cell development. Although, the G6PDH activity behaves differently, gradually declining over the culture period, after a short initial rise.

A stimulation of segment length increase, also by extending the duration of the rapid phase of growth, is observed in the presence of IAA (10^{-10}). The CS activity follows a similar pattern to that in sucrose alone; the same pattern is also observed in segments inhibited by 10^{-4}M IAA. The G6PDH activity is maintained at a level $1\frac{1}{2}$ fold the T_o activity. The results are interpreted as suggesting that IAA is maintaining the metabolic state of the cells which would otherwise be in decay in the absence of IAA in the latter half of the 24 hour culture period.

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ABBREVIATIONS

The following abbreviations are used:

CS	Cellulose synthetase
GDPG	Guanosine diphosphoglucose
G-6-P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
IAA	Indole-3-acetic acid
6MP	6-Methyl purine
NADP	Nicotinamide adenine dinucleotide
RNA	Ribonucleic acid
2-TU	2-Thiouracil
TRIS	2-Amino-2-(hydroxy-methyl)-propane 1:3-diol.
UDPG	Uridine diphosphoglucose

T_o Indication of how many hours

T_{24} segments were cultured

e.g.

T_o	Cultured zero hours, fragments taken straight from fresh seedling root
T_{24}	Cultured 24 hours

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CHAPTER ONE

CHAPTER 1

INTRODUCTION

Plants normally grow and develop in an orderly, organised way. Of the two daughter cells formed by the intricate process of division at the root apex one usually remains as a dividing cell while the other passes into the expansion phase of growth. The actual increase in the size of the plant is a direct result of the enlargement of the daughter cells and the increase in the cell size may be a hundred fold. This contrasts sharply with the situation in animals where the daughter cells expand only until they attain the original size of the mother cell.

Therefore, the expansion process is of decisive importance to the growth of the intact plant. It is also the physiological vehicle on which the phototropic, geotropic and other bending responses depend. It has been shown that the bending in response to a stimulus occurs in the expanding tissues, behind the meristematic tips. The organ bends due to the different rates of growth on the two sides of the structure. Such changes tend to be small and make these systems less suitable for the study of expansion growth. Without expansion growth there could not be the normal differentiation of cellular forms. The small, isodiametric cell typical of the meristematic region develops into the elongate form of for example the xylem tracheid through the process of expansion. It has also been suggested (Brown and Dyer 1972) that ultimately the expansion process may control the division process such that mature expanded cells may be required to provide nutrients and stimulants necessary for the meristematic cells to undergo continued divisions (Brown and Broadbent 1951).

Two major experimental approaches may be used to study cell enlargement viz. either observations with the intact growing organisms

or by the use of isolated fragments grown in culture. The former has the advantage that it reflects the true, natural process but has the particular disadvantage that it makes the system more difficult to control. The meristematic cells and mature cells will probably influence the growth of the expanding cells in an unknown and uncontrollable way. This disadvantage is largely overcome by using the second method in which the fragment under investigation is removed to a suitable culture environment, where varied treatments can be applied under controlled conditions and the resultant changes observed and noted. The main limitation in this system is that the growth response is never as extensive as in the intact plant.

A variety of tissues have been used to study expansion growth. The coleoptiles, mainly of wheat, oat and maize are popular tissues for studying cell expansion in shoots e.g. Ray (1967), Rayle et al (1970). Etiolated internodes have also been used e.g. Key (1966). However, the primary root offers a situation where the process may be readily observed in root tissue. The primary root represents a temporal developmental sequence, with the meristematic cells at the apex, the expanding cells above, and beyond are the young developing stelar tissues surrounded by cortical cells. The cells, when they are newly formed in the meristem are relatively small, isodiametric and essentially non-vacuolated. They initially increase in breadth, before commencing the much greater lengthwise expansion. With the increases in cell volume, a large central vacuole develops and the nucleus becomes situated in the peripheral coating of cytoplasm. Various changes also occur in the cell proteins and the cell wall.

The serial section technique is suitable for studying cellular changes associated with expansion within the primary root. The

technique relies on the assumption that in seedling roots of similar lengths and grown under the same conditions, the cells at a given distance from the apex are at the same stage of development. This technique was pioneered by Brown and Broadbent (1951) and they showed that segments taken from different parts of the root will respond differently to culture, but segments taken from the same zone of roots of seedlings of similar size and age will respond similarly to culture. Also, the cell number in different regions of the same root varies but the number of cells from the corresponding region of different roots is similar (Brown and Broadbent 1951). Fragments removed from the extending zone of the root, but not the tip, continue to grow in culture when supplied with suitable media and the growth is due to cell extension only. (Brown and Sutcliffe 1950, Vaughan 1965). The cells not only increase their volume by imbibition of water, but there is also an increase in dry weight. This technique has been used to find the most suitable type of root segment to examine expansion growth in culture.

Root segments will undergo extension growth in culture but the growth response is greatly affected by the culture conditions. A relationship between nutrients and growth has been shown in expanding root segments in culture. Brown and Sutcliffe (1950) found with Zea root fragments that sucrose was the most effective sugar, with glucose not so stimulatory and fructose less still. Vaughan (1965) found similar results with pea root segments. The stimulation may be due to sucrose accumulation in the vacuole thus sustaining the osmotic pressure. The extent of elongation can be reduced by lowering the concentration of the incubation medium. With sucrose present, rapid synthesis of cellulose occurs which is linear with time, Brown and Sutcliffe (1950). Further, the rate of synthesis of cellulose increases with increased

sucrose content from 0.25% up to 2.0%, but not with higher concentrations. In the absence of sucrose, in the presence of air, the cellulose content of the segments decreases with time but with no oxygen present the cellulose stays constant. Brown and Sutcliffe (1950) also showed with Zea root segments that in the presence of sugar, potassium but not calcium ions, has a promotory effect on extension growth. The stimulation was attributed in part to the acceleration of water absorption by the K^+ ions affecting respiration. Water absorption is in part due to the active metabolic secretion of water into the vacuole (Thimann 1951).

The respiration rate of expanding root segments cultured in 2% sucrose increases by about 50% in the first 12 hours and stays constant at that level for the remaining 36 hours. (Brown and Sutcliffe 1950). Vaughan (1965) subsequently found that the addition of 2-thiouracil (2TU) stimulates oxygen uptake only in the presence of sucrose. When cultured in water, the segment respiration gradually decreases during the 12 hour culture period. There are many enzymes and co-factors directly and indirectly involved in respiration. In this investigation, glucose-6-phosphate dehydrogenase (G6PDH) has been chosen as a focus of attention. G6PDH contributes to respiration in the pentose phosphate pathway and this pathway also provides 5-carbon sugars for other metabolic pathways. G6PDH catalyses the first step of the pentose phosphate pathway, using glucose-6-phosphate as substrate and producing the reduced co-factor, nicotinanide adenine dinucleotide. The Embden-Meyerhof pathway, the alternative respiratory pathway, is important in undifferentiated tissue but as the tissue matures the pentose phosphate pathway comes to play an increasingly important role. The NADPH/NADP ratio is higher in younger tobacco leaves than in the older ones

(Yamamoto 1963). The Embden-Meyerhof or glycolysis pathway and the pentose phosphate pathway were both shown (Wong and Rees 1971 and Rees, Fowler and Wong 1972) to make substantial contributions to carbohydrate oxidation in the root stele and cortex. Wong and Rees (1971) suggested that the formation and maturation of the stele is accompanied by an increased capacity of the pentose phosphate pathway relative to that of glycolysis; the activity being about two-fold greater in extracts of stele than in cortex. But, changes in the G6PDH activity during culture need not be attributed to the differentiation of stelar tissue as Vaughan (1965) from studies on longitudinal sections of root segments cultured in 2% sucrose, found that no xylem formation is detectable after 24 hours, although some phloem is discernible. Phloem and xylem are detectable after 30 hours. Addition of 2-thiouracil to the sucrose does not change the pattern of development. Therefore, it is unlikely that there will be a significant contribution to increased pentose phosphate pathway enzymes, notably G6PDH, from the differentiation of stelar tissue during a 24 hour culture of root segments.

The pattern of enzyme activities changes significantly during extension growth, both in the intact and isolated tissues but the greatest quantitative change, second to that of fresh weight, is the change in the residual dry weight. The residual dry weight is comprised mainly of the cell wall polysaccharides, proteins and nucleic acids. The last two, show no significant quantitative change and so any increases must be attributable to the cell wall polysaccharides. Cell wall synthesis has been demonstrated in cultured root fragments (Brown and Sutcliffe 1950). The enzyme changes associated with the synthesis of the main primary wall constituent viz. cellulose were chosen as a focus of attention in this work.

The primary cell wall consists almost entirely of polysaccharides; 90% of the structural material of the wall is polysaccharide, the remaining 10% is protein (Albersheim 1975). The cellulose molecules are formed into small aggregates called micelles which lie parallel to one another so giving a crystalline structure to the microfibrils or strands of cellulose. Albersheim and his colleagues (Albersheim 1975) suggested a model from their analytical results in which the cellulose fibres have xyloglucan molecules adhering to the surface and that each xyloglucan molecule binds to a single arabinogalactan chain, which in turn binds to a single rhaminogalacturan molecule. As a result of extensive cross-linking the fibres are immobilized in a seemingly rigid matrix. The microfibrils are arranged more or less randomly in the primary wall, but usually more regularly in the secondary walls. The areas between them are filled with water, pectic substance, hemicellulose and in secondary walls with lignin and cutin (Cutter 1969). Only primary walls are capable of growth.

Groups of proteins rich in hydroxyproline have been found in the primary walls of some tissues and have been suggested to have enzymic and structural functions. (King and Bayley 1965). These proteins, which are synthesised in the cytoplasm may play a role in orientation of the cellulose fibrils (Mühlethaler 1967) and a role in cell extension (Lamport 1965).

The chemical composition of the cell walls is not changed by elongation (Albersheim 1975), although the proportion of the constituents varies during development, and varies in different tissues. Jensen (1960) from work with onion root tips concluded that the changes in cell wall composition follow a pattern with the tissue differences being only variations in the basic pattern. The walls of the onion

root apical initials are exceptionally thin and low in all components. During the period of radial enlargement the rate of increase in wall materials is slow, but rapid during elongation. Cortical cells nearer the centre of the root develop thicker walls first and the development spreads outwards with the protoderm showing the slowest rate. In the young cortical cells, pectic substances and non-cellulose polysaccharides make up more than 10% of the wall (Jensen 1961). Recently, Kaufman (from Albersheim 1975) has demonstrated that the walls of elongated oat cells have the same thickness and the same strength per unit length as the walls of cells that have not begun to grow.

The mechanism of cellulose synthesis has been in question for many years. Glaser (1957) obtained a particulate fraction from Acetobacter which catalyses the incorporation of the glucosyl moiety of uridine diphosphoglucose (UDPG) into cellulose. Colwin (1959) later found that cellulose is formed from a precursor soluble in 80% alcohol but concluded UDPG was not the immediate precursor of bacterial cellulose. He suggested a glucolipid may be the precursor. Cell free particulate preparations from plants incorporate glucose into cellulose. Some reports (Elbein, Barber and Hassid 1964, Barber et al 1964, Chambers and Elbein 1970, Flowers et al 1970) have shown that the enzyme systems from mung beans and other species show a marked specificity for guanosine diphosphoglucose (GDPG). Barber et al (1964) could not detect the presence of UDPG pyrophosphorylase in the extracts. However, Franz (1968) could not detect any trace of GDPG in higher plants and preference was given by some workers to UDPG as the 'in vivo' precursor as it occurs in growing tissues.

Ordin and Hall (1967) found that a particulate fraction from oat coleoptile tissue could use either GDPG or UDPG. The latter is a much

more effective donor but the product after cellulase digestion yields not only cellobiose, the normal cellulase digestion product from cellulose, but also an unknown trisaccharide. The product formed from GDPG yields mainly cellobiose. Franz and Meier (1969) and the more recent work of Delmer, Beasley and Ordin (1974) suggest that GDPG and UDPG are both precursors in cellulose synthesis but are prominent at different stages of cell development.

Sites of cellulose synthesis in the cell have been recognised. Polysaccharide synthetase particles from etiolated pea seedling shoots have been identified by velocity and isopycnic density gradient centrifugation to be Golgi membranes (Ray, Shininger and Ray 1969). But, Eisinger and Ray (1972) concluded from pulse chase experiments that the polysaccharide material of the dictosomes was pectic and hemicellulose compounds and no true cellulose was present. Later Shore and MacLachan (1974) recognised a cellulose synthetase activity associated mainly with the Golgi apparatus during elongation, but during lateral swelling and termination of elongation, the intracellular location alters to include a cell fraction tentatively identified as smooth endoplasmic reticulum vesicles. These activities were interpreted as partly representing intermediates which are 'en route' to the site of action at the cell surface. They found when UDPG was provided directly to tissue slices or homogenates, the capacity to form cellulose declined markedly with increasing tissue disruption. It was concluded that a degree of integrity of cellular organisation must be retained to permit membrane-membrane or membrane-wall interaction.

Woodstock and Brown (1963) and Vaughan (1965) reported that 2-TU stimulates elongation in root cells which are in the expanding phase of growth. Another pyrimidine analogue, 5-fluorouracil (5-FU) at the concentration 2.5×10^{-3} was found by Key (1966) to inhibit

RNA synthesis up to 60% over an 8 hour period but not to inhibit growth. It appears to have a selective effect on the inhibition of protein synthesis. Key (1966) suggested that those factors essential for elongation must not have been affected. The r-RNA and t-RNA are inhibited 96% and 70% respectively but the D-RNA is unaffected and so he concluded that only the synthesis of this last type was necessary to support continued protein synthesis and cell elongation of the excised shoot tissues. This conclusion is supported by Cherry's (1962) observation that it is the type of RNA and not the amount which affects growth. Heyes (1959, 1960) has demonstrated that 8-Azoguanine (8AZ) a purine analogue stimulates extension growth in root cells. 8AZ (2.5×10^{-3}M) and 6-methyl purine (6MP) (5×10^{-4}M) were shown to inhibit RNA synthesis, protein synthesis and growth in excised soybean hypocotyls (Key 1966). Aitchison and Yeoman (1973) showed that 6MP specifically inhibits nucleoside incorporation over short-term periods but that longer exposure causes inhibition of amino-acid incorporation as well, in Jerusalem artichoke explants. In this investigation the effects of 6MP on the stimulation of growth and the pattern of enzyme activities are studied.

Auxin has a great diversity of effects on plant growth. Among the effects defined at the cellular level are those on wall plasticity and elasticity, cytoplasmic viscosity, protoplasmic streaming, respiration rate, metabolic pathways, nucleic acid content and many enzyme activities. It also has effects on cell elongation, tropisms, xylem differentiation, apical dominance, abscission processes, pollen tube growth, fruit set, fruit development, compression wood formation in conifers, tuber and bulb formation and seed germination. Extensive research has therefore been concentrated for some time on the nature

and mode of action of auxin. A very brief selection of the published work on cell extension will be considered.

Protein and nucleic acid synthesis occurs after auxin treatment in many tissues, but can be detected only after a lag phase of up to about an hour (Hill 1972). In wheat and oat coleoptiles and in etiolated pea stem segments, growth induced by auxin is already at a maximum when such synthesis is detectable. Therefore, neither protein nor nucleic acid synthesis is required for the initial response, or the early changes are not detectable by the techniques employed. Inhibitors of protein and nucleic acid synthesis do affect auxin-induced growth in coleoptile and pea stem segments. In the pea stem segments ribosomal RNA (r-RNA) increases only slightly. (Penny and Galston 1966). The RNA synthesis can be inhibited by a low concentration of inhibitor, without affecting the auxin-induced growth. Penny and Galston (1966) suggested, from kinetic studies, that in the early stages of auxin-induced growth some previously formed substance is used and RNA protein synthesis is required only for continued response to auxin. In wheat coleoptile sections, cycloheximide inhibits over 90% of the ^{14}C-leucine incorporation into protein within 10 minutes (Pope and Black 1972). Even after a 2 hour pre-treatment with cycloheximide, IAA stimulated extension, suggesting that its growth promotory action does not directly involve protein synthesis. Kinetic experiments with cycloheximide indicate that incorporation of a structural factor, possibly a protein from a previously synthesised pool, into cell walls may be the rate-limiting process affected by IAA. A 3 hour cycloheximide pre-treatment abolishes the IAA effect. This may reflect the 'life' of a protein required for auxin action in the presence of cycloheximide. The growth rate also apparently affects the 'life' of this factor; the factor

being used up in fast growing tissues. Pope and Black (1972) concluded that auxin promotes the use of a growth limiting protein. This conclusion may be doubted as Ellis and MacDonald (1970) found that some ribosomes are insensitive to cycloheximide, but Pope and Black (1972) found no quantitative relationship between growth and protein synthesis. These results differ from those of Cleland (1971). He found that the IAA response in coleoptiles is totally abolished after 30 minutes pre-treatment in cycloheximide.

The temperature-dependent lag phase which is observed before the growth response to auxin in coleoptiles, was reduced to zero by Nissl and Zenk (1969). Barclay and Evans (1970) working with pea stem sections could only reduce the lag phase to 10 minutes. Also, Actinomycin-D does not lengthen the lag phase and so they concluded that RNA synthesis was not a pre-requisite for the initial growth response, and suggested that the primary site of action of auxin must be very accessible, possibly the cell wall. Andreae (1967) similarly concluded from work on growth inhibition of pea root segments, that the site of auxin must be outside the cell membrane in the cell wall.

Early experimental evidence indicated that the stimulatory action of auxin on cell enlargement softens the cell wall by increasing the plasticity (e.g. Heyn 1931, Tagawa and Bonner 1957, Cleland 1958). Bennet-Clark (1956) suggested that auxin removed the calcium linkages of the wall pectins, but Thimann and Takahashi (1961) and Cleland (1960) could not show any calcium loss from the wall or shift from one pectin fraction to another. Ordin et al (1955) postulated that auxin increased the incorporation of methyl groups from methionine into cell wall pectins. Methyl group availability affects the binding of calcium to the cell wall. Cleland (1960) found that such methylation is inhibited

by ethionine but there is not a similar inhibition of auxin growth promotion. The removal of methyl esters is modified by auxin (Osborne 1958). The pectin methylesterase activity is increased by auxin and Yoka (1958) showed the esterase increase closely matches the stimulation of growth by auxin treatment. Glaziou (1957) suggested the auxin affected the binding of the enzymes to the wall but Jensen et al (1960) found that auxin did not alter the association of most coleoptile esterases associated with the wall. Auxin may alter the turnover and synthesis of cell wall proteins, (Thimann and Loos 1957) and thus effect the structure of the wall.

Several reports indicate that auxin treatments increase cell wall components including cellulose and hemicelluloses in pea stems (Christiansen and Thimann 1950) and hemicellulose and pectin in oat coleoptiles (Bayley and Setterfield 1957). Albersheim and Bonner (1957) showed an increase in the incorporation of radioactivity from a glucose substrate. Some investigations have failed to show any cell wall synthesis in response to auxin treatment e.g. Bennet Clark (1956) and Ordin and Bonner (1957) in oat coleoptiles and Davison (1957) in corn coleoptile.

Permeability of the cell wall to water remains unchanged with auxin treatment, shown by Thimann and Samuel (1955) from heavy water equilibration experiments. Bonner et al (1953) suggested water uptake was a metabolic process but it is probably an osmotic mechanism as an increase in osmotic value of ambient solutions results in an experimental depression of cell enlargement (Ordin et al 1955). Dilution of the osmotic components associated with water uptake occurs as growth proceeds (Hackett 1952) and sucrose can renew the osmotic substances of the cytoplasm. (Ordin et al 1955).

In the pea root segment culture, the question of ethylene production must be considered, particularly when it is known that ethylene production can be induced by stresses such as occur during cutting (Abeles and Abeles 1972). Ethylene and 24D, inhibit the growth of etiolated soyabean seedlings, causing tissue swelling and an increase in RNA, DNA and protein content in the subapical hypocotyl tissue (Holm and Abeles 1968). Removal of ethylene by reducing the pressure stops tissue swelling but does not alter the growth rate (Abelbaum and Burg 1972). The difficulty of ethylene with culture pea root segments is probably negligible as Holm and Abeles (1968) found that the growth of excised etiolated pea sections was not affected by ethylene. Ethylene is thought mainly to act by inhibiting the polar transport of auxin.

The importance of the protein complement in the cell expansion process has been frequently mentioned in the literature. There are large increases in the cellular nitrogenous content of the intact root, but in isolated segments there is no such increase. However, there is a redistribution in the protein content throughout the cells. The nature of the control of cell growth and differentiation which is exerted by the enzyme complex is suggested by the growth curves of successive fragments excised from the tip of broad bean roots (Heyes and Brown 1965). In this investigation, the changes in protein and selected enzyme activities will be examined and compared with the extension growth obtained.

Many aspects of the process of cell expansion in roots have been satisfactorily studied by numerous previous workers by means of the root segment culture system. In this work, a culture system has provided a model to investigate various changes which occur during

extension growth in culture. Also, the effects of the RNA inhibitor 6-methyl purine and the growth hormone IAA on extension growth were investigated. 6-methyl purine by the theory presented by Brown (1963) would be expected to extend the period of active growth. The inhibitory as well as stimulatory actions of IAA are considered.

Particular interest was paid to the change in the activities of two enzymes; glucose-6-phosphate dehydrogenase and cellulose synthetase. G6PDH is involved in the pentose phosphate pathway of respiration and may be used as an indicator of the general level of metabolism. The cell wall increase contributes the greatest quantitatively to the synthesised dry material and cellulose is the most abundant component in the expanding primary cell wall. The nature of the cellulose synthetase enzyme has been under discussion for some time. The assays for G6PDH and cellulose synthetase were developed for the pea root tissue and the kinetics of the two enzymes were examined in some detail to give confidence to the validity of the patterns of activity measured.

CHAPTER TWO

CHAPTER 2

EXPERIMENTAL MATERIALS AND METHODS

Section 1 - Plant Material

The plant material used throughout this study was the garden pea, Pisum sativum L. var. Feltham First. Seeds were obtained in 14 lb lots from Lawson Donaldson, Seed Merchants, Edinburgh and were stored in the cold room ($4^{o}C$) until required.

Section 2 - Growth of Seeds and Segments

a. Preparation of Seeds

The dry seeds were graded and individuals showing abnormalities with respect to size, colour or texture were removed. Seeds with cracked testas were also discarded. The seeds were surface sterilised in 10% sodium hypochlorite solution (equivalent to 1.4% chlorine) for 15 minutes and then washed with sterile water until the smell of chlorine was no longer detectable. When sterile material was not required unsterilised seeds were imbibed overnight in running tap water.

b. Preparation of Germination Medium

The germination medium was prepared by mixing dry vermiculite (medium grade) with distilled water in the ratio of 2:1 by volume and autoclaving at 15 psi for 20 minutes in closed Pyrex dishes. Subsequently the vermiculite was allowed to cool overnight. A 3:1 mixture of vermiculite and water was prepared immediately before planting when sterile material was not required.

c. Seed Planting and Germination

The top layer of sterilised vermiculite was placed in a pie dish lid and the washed seeds dropped evenly over the vermiculite, which had been previously loosened by stirring with a sterile glass

rod. The seeds were covered with moist vermiculite and the lid replaced. The seeds were then allowed to germinate for 70 hours in the dark at 25°C. Imbibed seeds were allowed to germinate for 48 hours.

d. Root Selection

The seedlings were carefully separated from the vermiculite by gently dropping the contents into a 5 mm mesh sieve and cautiously shaking away the vermiculite. Seedlings with straight, firm roots of average thickness 0.99 - 1.03 mm and length 2.5 - 3.5 cm were selected, washed and retained in a tray of sterile water until required.

e. Preparation of Segments

Root segments 2 mm long were cut by the method of Brown and Broadbent (1951). The cutter was modified to cut and hold upright, 20 roots. A sharp double edged razor was used, in order to produce a neat, even cut. Segments up to 10 mm in length, increasing in 1 mm steps, could be accurately produced. The variation in size of the 2 mm fragments was a maximum of $\pm$ 5%.

f. Culture of Segments

The 20 segments were placed in 1.5 ml of culture medium in 25 ml extraction flasks and the neck closed by a plug of non-absorbent cotton wool. The flasks, brushes and cutter head had previously been wrapped in aluminium foil and sterilised in an autoclave at 15 psi for 20 minutes. The medium used was a 2% sucrose solution. Vaughan (1965) found that the addition of mineral salts or buffering agents to the medium did not produce greater expansion growth in the elongating pea root segments. The medium was sterilised at 5 psi for 30 minutes to avoid the breakdown of the sugar molecules which

occurs at higher pressures. Medium supplements such as IAA or 6MP were added to the sucrose medium after autoclaving and just prior to culturing.

The flasks were agitated on a rotary shaker at 100 rpm throughout the experiment, in the dark at 25°C ± 2°C, but were exposed to periodic bursts of room light at sampling times. Brown and Sutcliffe (1950) found that intermittent bright light only slightly reduced the elongation growth of root segments. The cabinet air was kept humid by having an open flask of distilled water also being shaken during the culture period.

Section 3 - Measurement of Segments

a. Segment Size

The segments were freed from culture medium and transferred with a small paint brush on to a glass slide. They were arranged in groups and any excess fluid removed with a dry paint brush. A permanent and accurate record of the sizes and numbers of segments was quickly obtained by a 'shadow technique'. The apparatus, shown in Figure 2-1 was similar to that used by Yeoman (unpublished) to measure the friability of artichoke explants. A light was shone from above the segments and the magnified shadow images of the segments focussed on to the paper by an eye piece lens. The images were quickly traced out; a sample tracing is shown in Figure 2-2. Vaughan (1965) and earlier workers used a micrometer eye piece to measure the segments. This method is not as accurate, reliable or as quick as the present technique and does not produce a permanent graphic record of the segments. The insensitivity of the 'micrometer' method may explain the very low variability in segment length recorded by Vaughan (1965).

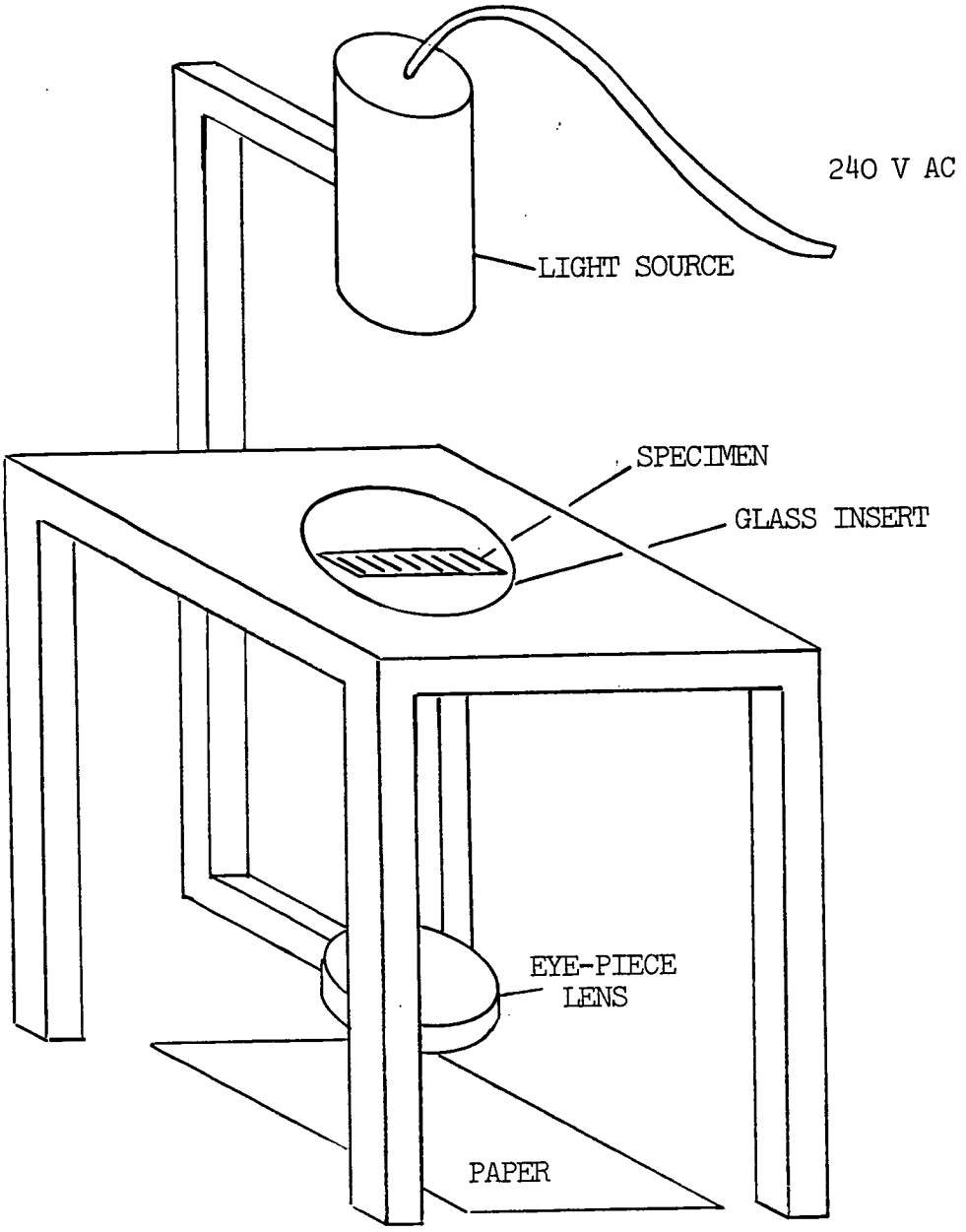


FIGURE 2.1

Apparatus used in the 'shadow technique' for measuring segments.

Segments are placed on a glass microscope slide on the platform and a light is shone from above. A magnified shadow image of the segments is focused on to the paper by a microscope eye-piece lens.

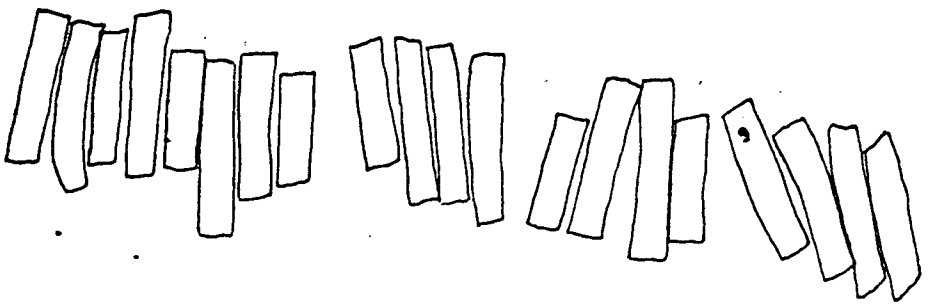
FIGURE 2-2

SAMPLE OF GRAPHIC RECORD OF SEGMENTS,

OBTAINED BY 'SHADOW' TECHNIQUE OF MEASUREMENT

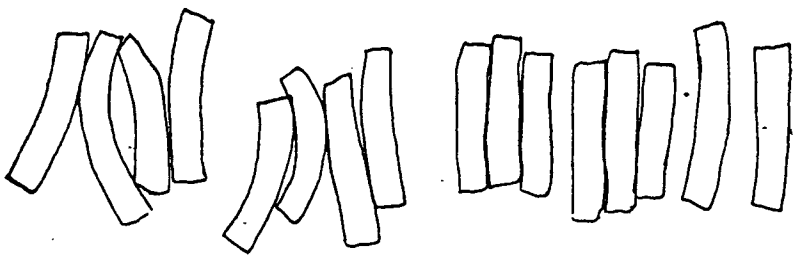
EXPERIMENT PERFORMED 8-9/9/74

Culture Time and Medium



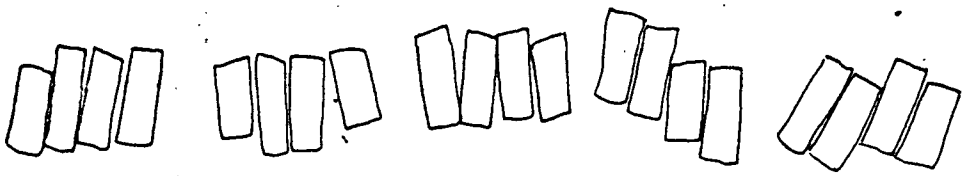
T_{24}
2% sucrose

n = 20 l = 4.95 ± 0.69 mm w = 1.06 mm



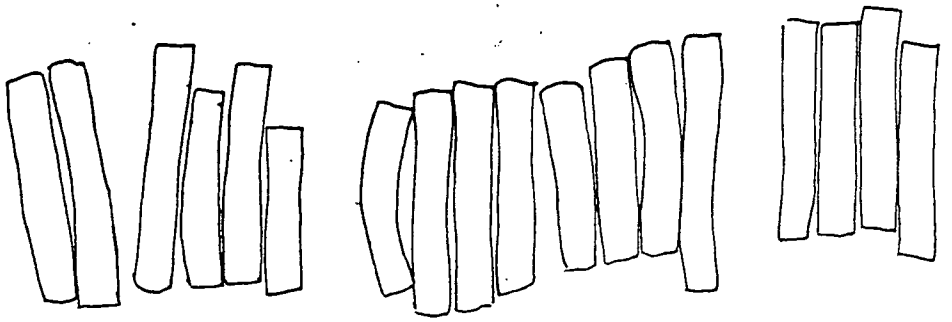
T_{24}
2% sucrose + 6MP (2×10^{-5} M)

n = 16 l = 5.24 ± 0.39 mm w = 1.00 mm



T_{24}
2% sucrose + IAA (10^{-4} M)

n = 20 l = 2.98 ± 0.22 mm w = 1.14 mm



T_{24}
2% sucrose + IAA (10^{-10} M)

n = 18 l = 7.06 ± 0.69 mm w = 1.22 mm

l = length w = width

The segments were moistened with a little distilled water and stored for a short time in aluminium foil squares on ice prior to the extraction procedure or other measurements.

b. Measurement of Segment Cell Number

The number of cells per segment was determined by the method of Brown and Rickless (1949) as modified by Brown and Broadbent (1951). Five segments were left in 1.0 ml of a 5% solution of chromium trioxide in water for 24 hours at room temperature. The tissue was then macerated by gently breaking it with the tip of a Pasteur pipette and then forcing the fragments in and out of the glass pipette.

After diluting to 5.0 ml with water, the number of cells in a known volume of suspension was counted under a microscope using a Fuchs-Rosenthal bright line haemocytometer slide with a counting chamber depth of 0.2 mm. The number of cells per segment was calculated from the average value of counts made on 3 samples of each suspension.

Certain precautions were observed when using this technique:-

a) The slides and cover slips were kept in alcohol between counts to ensure they were grease free.

b) The macerate must flow smoothly under the cover slip.

c) Any overflows of macerate from the counting chamber and the preparation was discarded.

d) Aliquots of macerates with clumps of over 10 cells were rejected.

c. Segment Fresh Weight

Twenty segments were carefully blotted dry on filter paper and tared in a specimen tube of known weight on a balance to $\pm$ 0.0001 g.

d. Segment Dry Weight

After the fresh weights had been determined, the segments were heated to 90°C in a specimen tube for not less than 8 hours, cooled to room temperature in a desiccator and then weighed on a balance to ± 0.0001 g.

e. Residual Dry Weight

Forty segments were boiled for 10 minutes in 80% ethanol, filtered off, washed in water, dried overnight at 90°C and weighed to ± 0.0001 g.

f. Protein Determination

The protein content was determined by the method of Lowry, et al (1951) 0.2 ml of extract (20 segments/1.5 ml buffer) were made up to 1 ml with 0.1 N NaOH. 0.5 ml aliquots were mixed with 5 mls of freshly prepared copper sulphate solution (in 1% sodium tartrate) and allowed to stand for at least 10 minutes. 0.5 ml of a freshly prepared Folin solution was then added to each sample and mixed well. The density of colour which developed after 20 minutes was measured in an EEL colorimeter with the 608 filter inserted. The OD values obtained were compared with a standard curve obtained from a series of solutions of bovine serum albumin.

Section 4 - Measurement of Enzyme Activities in Segments

A major part of the work presented in this thesis involved the development of assay methods for the enzymes glucose-6-phosphate dehydrogenase and cellulose synthetase using pea root tissue. In this section only the optimal conditions for the assays, determined from a series of experiments, are presented. Both enzymes have been assayed in crude extracts. It was considered important to assay both enzymes in the same segment macerate and so the assay and kinetics

were examined with this in consideration. G6PDH was assayed in the supernatant from a low speed spin, thus all cell components except the walls were present. CS was assayed in a particulate fraction obtained from a high speed centrifugation of the first spin supernatant. The values obtained from the enzyme assays represent the extractable enzyme activities. It is recognised that these values do not necessarily represent the total amount of enzyme protein present. Therefore any discrepancy may be accounted for by the inhibition, activation or failure of extraction of the enzyme protein molecules. Activation or inhibition of the enzyme molecules may occur by molecules coming into contact during the physical disruption which occurs during the extraction process. Whereas in the undisturbed cell, these molecules would not be in contact. The reliability of the extraction and assay procedures is reflected by the repeatability of the results. The presence of enzyme activators or inhibitors can be tested by adding known amounts of pure enzyme to the test extract and checking the observed activity with the expected total activity. It is the change in enzyme activities which is of particular interest in this work.

a. Preparation of Extracts

The twenty segments of each sample were transferred to an ice cold glass homogenising tube containing 0.5 ml of extraction medium (50 mM TRIS, 50 mM Mercaptoethanol, B.S.A. 40 mg/100 ml pH 7.8) and the tissue quickly homogenised. The extract was then transferred with two 0.5 ml washings of medium to an ice cold 15 ml plastic ultra-centrifuge tube. The extract was given a low speed spin (1×10^3 g) for 5 minutes in a refrigerated centrifuge (MSE 18) at $5^{o}C$. Aliquots of the supernatant were removed for the glucose-

6-phosphate dehydrogenase assay and the remainder spun at 25 x 10^3 g for 30 minutes. The pellet from this spin was resuspended in 0.10 ml of buffer (50 mM TRIS, 50 mM Mercaptoethanol pH 8.0) and used for the cellulose synthetase assay. All procedures were carried out rapidly and both assays were performed immediately.

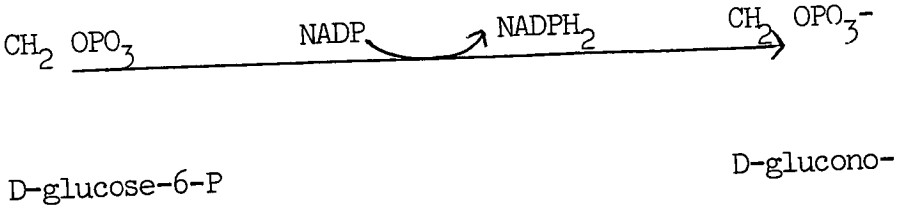
b. Assay of Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase is a component of one of the major pathways, the pentose-phosphate pathway, so called as pentoses participate catalytically. It is also known as the Phosphogluconate oxidative pathway and the Hexose monophosphate shunt. The constituent enzymes are generally localised in the cytoplasm. The importance of this pathway was discussed in Chapter 1.

The first oxidative step is catalysed by glucose-6-phosphate dehydrogenase, an important enzyme of very wide distribution which was discovered and studied by Warburg. In general terms, the reaction catalysed is the reversible dehydrogenation.

$$R_1\ R_2\ CHOH \rightleftharpoons R_1\ R_2\ C = O\ ,$$

catalysed by a pyridine nucleotide requiring dehydrogenase. The equation for the reaction being monitored in the assay is



Glucose-6-phosphate dehydrogenase is detected analytically by following the reduction of the obligatory coenzyme NADP to $NADPH_2$. This reduction is assayed spectrophotometrically. Spectrophotometry is one of the most widely used and versatile of the biochemical analytical tools. The method of analysis is non-destructive,

selective and enables one to follow the details of a fast reaction. It is selective as each compound has a characteristic spectrum and so can be singled out in a solution. $NADPH_2$ has a peak of absorbence at 340 nm. NADP reduction is also linked to the oxidation of 6-phosphogluconic acid (6-PG) which is formed from D-glucono-δ-lactone-6-P, and could possibly result in an overestimation of the G6PDH activity. However, Aitchison and Yeoman (1973) found in artichoke tissue that this effect was negligible as the concentration of 6-PG attained was so low.

The G6PDH activity was assayed by following the rate of reduction of NADP at 340 nm in a spectrophotometer (UNICAM SP800) at $37^{o}C$.

The aliquots removed from the supernatant from the first centrifugation were assayed immediately. The reaction mixture consisted of:

0.15 ml extract - 18×10^{-5} g protein

NADP 0.5 μM

G6P 0.2 μM

TRIS 100 μM

Total volume 2.05 ml pH 7.6

The balance cuvette contained water in place of the NADP and G6P.

All the reactants, except the G6P, were added to the cuvettes and the OD monitored for several minutes until it was constant. The G6P (0.2 μM) was then added to the reaction cuvette and the OD recorded at intervals of about 30 seconds for 5 - 10 minutes.

The G6PDH activity was expressed as OD increase per minute per segment.

c. Assay of Cellulose Synthetase

Cellulose is a very large molecule composed of repeating glucose units. The synthesis of cellulose may be represented by the general equation

NuDP-sugar + R-OH ⟶ R-O-C-1-sugar + NuDP

where NuDP is a nucleotide diphosphate and

ROH is an acceptor or cellulose 'tail'

In the present work, a value for the cellulose synthesising capacity of the tissue, or the cellulose synthetase activity, is obtained using a radiochemical technique. The sugar moiety of uridine diphosphoglucose (UDPG) is uniformly labelled with either ^{14}C or ^{3}H. Guanosine diphosphoglucose uniformly labelled in the glucose by ^{3}H was also used. A measure of the activity of the enzyme system is given by the amount of label which results in a cellulose fraction under standardised conditions. The labelled sugar present is determined by scintillation counting.

The 25,000 g pellet from the low speed spin supernatant was resuspended in 100 µl of buffer (50 mM TRIS, 50 mM Mercaptoethanol) pH 8.0. The extract was assayed immediately.

The reaction mixture contained:

Extract - 100 µl	-	Protein -	11×10^{-4} g
UDPG	5.2×10^{-3} µM		3×10^{4} cpm
$MgCl_2$	4 µM		
TRIS	10 µM		
Total volume	200 µl	pH 8.0	

The particulate suspension was transferred into the prepared reaction mixture in 1 cm x 5 cm bacteriological test tubes, taking care to avoid pipetting extract on to the sides of the tube. The

tubes were incubated in a stationary Grant water bath at 37°C for 15 minutes.

The reaction was terminated by adding 1 ml of water and immersing in a boiling water bath for 5 minutes. A micro-spatula tip (3 mg) of powdered cellulose was added to each tube and boiled for a further 5 minutes. The bulked cellulose was transferred with 3 complete tube washings to a 15 ml centrifuge tube and centrifuged at 25×10^3 g for 15 minutes. The pellet was further washed twice by hot 4% NaOH and finally once by water, by heating in a boiling water bath for 5 minutes and pelleting by centrifuging at 25×10^3 g for 10 minutes (MSE 18).

The bulked cellulose pellet was transferred with several water washings on to a glass fibre filter (2.1 cm diameter Whatman GFA) under gentle suction. The suction was turned off and the filter washed by 10 ml of chloroform methanol (1:1V) in 1 ml aliquots. The filter was given a final water wash under suction and completely dried under a heat lamp.

The filter was immersed in 15 ml of scintillation fluid (Butyl PBD). The radioactive label incorporated was counted in the scintillation counter (Intertechnique) for 10 minutes or up to 10,000 cpm.

CHAPTER THREE

CHAPTER 3

EXPERIMENTAL RESULTS

Development of Techniques

In the following chapter, an attempt has been made to standardise the techniques used for segment culture and assay of enzymes. A considerable effort has been made to bring uniformity to the system.

Section 1 - Growth of Peas and Root Segments

From preliminary experiments it was obvious that problems encountered in developing such a culture system were not only concerned with obtaining optimal growth responses but also with minimising the variability in growth. Extensive work was done to analyse and reduce the sources of variability in the system. The variability introduced by working with genetically variable seeds and not clonal tissue, was probably not significant.

a. Germination Conditions

The growth of the seedling determines to a large extent the growth response of the root fragments in culture, therefore the germination conditions are important. The medium must be sufficiently aerated, contain the correct amount of moisture and be otherwise inert. A popular medium which fulfils these requirements is vermiculite, a flaked mica. Accordingly, experiments were performed to determine the best ratio of vermiculite to water for seed germination.

The peas, P. sativum L. var Feltham First were imbibed overnight and sown in vermiculite mixed with water in known ratios, by volume. The germination response and the response of the penultimate 2 mm root segment to culture in 2% sucrose were noted. The results are summarised in Table 1.

Table 1

Seedling growth and root segment culture response from various vermiculite mixes.

Vermiculite: Water by volume	Description of Seedling Root at Harvest	Response of 2 mm Root Segment to culture - length after 24 hours - mm
4 : 1	long, thin	4.74 ± 1.05
3 : 1	straight, strong	4.39 ± 0.35
2 : 1	short, thick	3.54 ± 0.72

S.D. calculated with n = 80 segments.

It is apparent that the germination medium has a large influence on the growth of the seedlings and the cultured root fragments. The roots which had been grown in the 4 : 1 vermiculite provided segments which grew the best in culture, nearly 2½ fold in 24 hours. However, the response was highly variable, SD of ± 1.05 mm. The roots grown in the 2 : 1 vermiculite did not respond as well in culture, some of the segments tended to thicken instead of lengthen. The 3 : 1 vermiculite provided the most satisfactory roots. An original mixture of 2 : 1 (v/v) of vermiculite to water provided seedlings at harvest similar to those from the 3 : 1 vermiculite which was not autoclaved and in which imbibed peas were germinated. Therefore, in all future experiments a 3 : 1 mixture was used for germinating imbibed peas and a 2 : 1 (v/v) mixture of vermiculite to water mixture when dry peas were planted.

b. Shaking Speed and Volume of Culture Medium

Segments may be cultured satisfactorily on a porous surface in a petri dish of medium, but in this static system nutrient gradients may be established and the segments tend to curve, making measurements more difficult. Therefore, the provision of a more uniform

environment with some degree of agitation would seem to be desirable. The segment growth is depressed by violent agitation but in contrast, the growth may be depressed by poor aeration due to gentle shaking. Initially, enough culture medium (2% sucrose) was added to the 25 ml extraction flask to cover the 20 segments and the flasks shaken on a rotary shaker to provide gentle agitation of the segments.

Table 2

Segment length after 24 hours under various culture conditions

Volume of Medium ml.	Shaking Speed 80 rpm	110 rpm	160 rpm
1.5	4.00 ± 0.63	4.42 ± 0.75	2.57 ± 0.23
2.5	3.40 ± 0.45	3.35 ± 0.65	2.77 ± 0.34
3.5	3.33 ± 0.33	2.95 ± 0.60	3.42 ± 0.46

Table 3

Segment length after 6 hours under various culture conditions

	110 rpm x 1.5 ml	100 rpm x 1.5 ml	100 rpm x 1.0 ml
Shaking speed rpm	2.52 ± 0.23	3.15 ± 0.29	2.60 ± 0.19
x	2.52 ± 0.18	2.96 ± 0.18	2.82 ± 0.28
Volume of Medium ml.	2.28 ± 0.12	2.95 ± 0.21	3.10 ± 0.18

S.D. calculated with n = 80 segments

The results indicate that the optimal conditions for segment elongation are a shaking speed of 100 rpm with 1.5 ml of medium. 20 segments were in each flask. Therefore these conditions were employed in all future experiments.

c. Size and Type of Root Segment

The root represents a time sequence in the development of the plant root cell. Above the root cap, the majority of the cells are dividing, beyond this zone the cells are in progressively advanced

stages of development. The cellular changes associated with the extension growth are efficiently studied by taking a fragment from the zone of the root in the earlier stages of expansion, but without a large occurrence of divisions. The shorter root fragments will provide cells in similar stages of development but the damaged cells at the cut ends will contribute to a larger percentage of the total cells than in the larger root fragments. Therefore the type of segment is important. The percentage increase in segment length after 24 hours in culture in sucrose from various types of root fragments is shown in Table 4.

Table 4

Percentage increase in segment length after 24 hours culture in 2% sucrose

Segment - mm from apex	2-4	1-3	0-1	1-2	2-3	3-4	4-5	5-6
% increase in length	128	119	50	20	62	47	27	32

Vaughan (1965) found that in the Meteor variety of pea, the penultimate 2 mm segment gave the optimal growth responses. The provisional results for the Feltham First variety, as given in Table 4, verify the optimal response of the 2-4 mm segment. An increase of about 2¼ fold was observed after 24 hours in culture. These results were obtained at an early stage when the culture conditions were 180 rpm with 3 ml of 2% sucrose.

Subsequently in all future experiments, a 2 mm fragment taken from 2-4 mm behind the root apex was used.

d. Variety of Pea

In earlier related work e.g. Vaughan (1965), the Meteor variety of pea was used but the supply of this old variety could not be readily obtained. The Feltham First variety was used initially as it was a popular variety for other areas of research in this department, was a

dwarf variety and the testa was not too wrinkled. It was compared with Meteor and a selection of other varieties which were chosen mainly as they were dwarf varieties and readily available. The germination response was examined and the acceptable varieties were compared by their increase in length under culture. The results are summarised in Tables 5 and 6.

Table 5

Germination response of different varieties of peas

Variety of Pea P. Sativum	Days to Harvest	Percentage Recovery of Roots or Comment on Roots
Early Onward	3	Variable
Feltham First	3	40
Gradus	4	Not straight
Little Marvel	4	Not straight
Meteor	3	40
Onward	4	10
Pioneer	3	30

Table 6

Length and Weights of segments (2-4 mm apical) after 48 hours culturing in 2% sucrose

Variety	Length mm	Fr. wt. mg.	Dry wt. mg.	Residual dry wt. mg.
Feltham First	4.69	5.28	0.40	0.17
Meteor	3.72	4.52	0.37	0.15
Pioneer	3.93	4.58	0.39	0.15

On the basis of yield of acceptable roots and the response of the 2-4 mm segment culture, Feltham First was selected as the most suitable variety for use in all future experiments.

e. Selection of Roots

The variability of the response of segments to culture may be

overcome by careful selection of the seedling roots. Roots 2.5 - 3.5 cm long were selected (Vaughan 1965). Noticeably thin and thick roots were avoided and the roots selected were 0.99 - 1.03 mm thick. When the segments were removed from longer roots, up to 4 cm, a 4-fold increase in segment length in 24 hours was not uncommon but the variability within a flask was very high. Shorter roots provided segments which tended to thicken instead of elongate in culture. A sample of results are compared in Table 7.

Table 7

Length of Seedling root cm	Segment length after 24 hours in culture mm	± Standard Deviation n = 40
1.5 - 2.5	2.90	± 0.27
2.5 - 3.5	4.31	± 0.60
2.5 - 4.0	5.59	± 1.44

It may be concluded from the results that seedling roots of length between 2.5 cm and 3.5 cm in the Feltham First variety, provide the most suitable type of subapical 2 mm segment for culture experiments.

Sections 2 and 3 - Enzyme Assays and Kinetics

In an investigation in which the activity of an enzyme is to be used as a parameter of development it is essential to extract and assay the enzyme under optimum conditions. Great care has been taken in this study to ensure that the activity recorded is a reflection of the activity present in the extract and not limited by the conditions of extraction, composition of the assay medium or physical conditions under which the activity of the enzyme is measured. In this section an attempt has been made to establish these conditions for glucose-6-phosphate dehydrogenase and cellulose synthetase.

Section 2 - Assay of Glucose-6-Phosphate Dehydrogenase

G6PDH catalyses the first step of the pentose phosphate pathway

and is an important regulatory enzyme. It is stable in crude homogenates and much is known about its catalytic properties, however before proceeding with the investigation it was necessary to determine with precision the assay kinetics of the enzyme. The results of this study are presented in the following section.

The reaction medium at optimum conditions comprised of

Extract - 0.15 ml - from total 1.5ml/20 segments - 1.7×10^{-4} g protein

NADP	0.50 μ M
G6P	0.20 μ M
50 mM TRIS	100 μ M
50 mM Mercaptoethanol	7.5 μ M
BSA	60 μ g pH 7.6

In total volume of 2.05 ml buffer

In the following kinetics experiments all other conditions were at optimum except the one being varied.

a. Preparation of Extracts

Extracts prepared from plant tissues frequently contain compounds which denature proteins and can lead to the underestimation of enzyme activity (Anderson 1968; Aitchison and Yeoman 1973). The addition of various reducing agents to the extraction and incubation media prevents this interaction and should result in active enzyme preparations. In this experiment a number of sulphydryl protecting compounds have been added to the extraction media and their effects measured.

It can be seen from the results in Table 8 that the most active preparations were obtained in the absence of any reducing agent, although the addition of 50 mM Mercaptoethanol or 2 mM Dithiothreitol

did not decrease the activity of the enzyme in the extracts. Subsequently the enzyme extract was prepared in the cold in 50 mM TRIS buffer at pH 7.6 in the presence of 40 mg/100 ml of BSA and 50 mM Mercaptoethanol.

Although, the presence of 50 mM Mercaptoethanol did not increase the activity recorded with T_o tissue, it was retained in the extraction medium in case the inhibitors such as polyphenol oxidases developed during culture. BSA serves to saturate any systems which destroy or adhere to protein molecules.

Table 8

Effect of the addition of sulphydryl protecting compounds to the G6PDH activity recorded

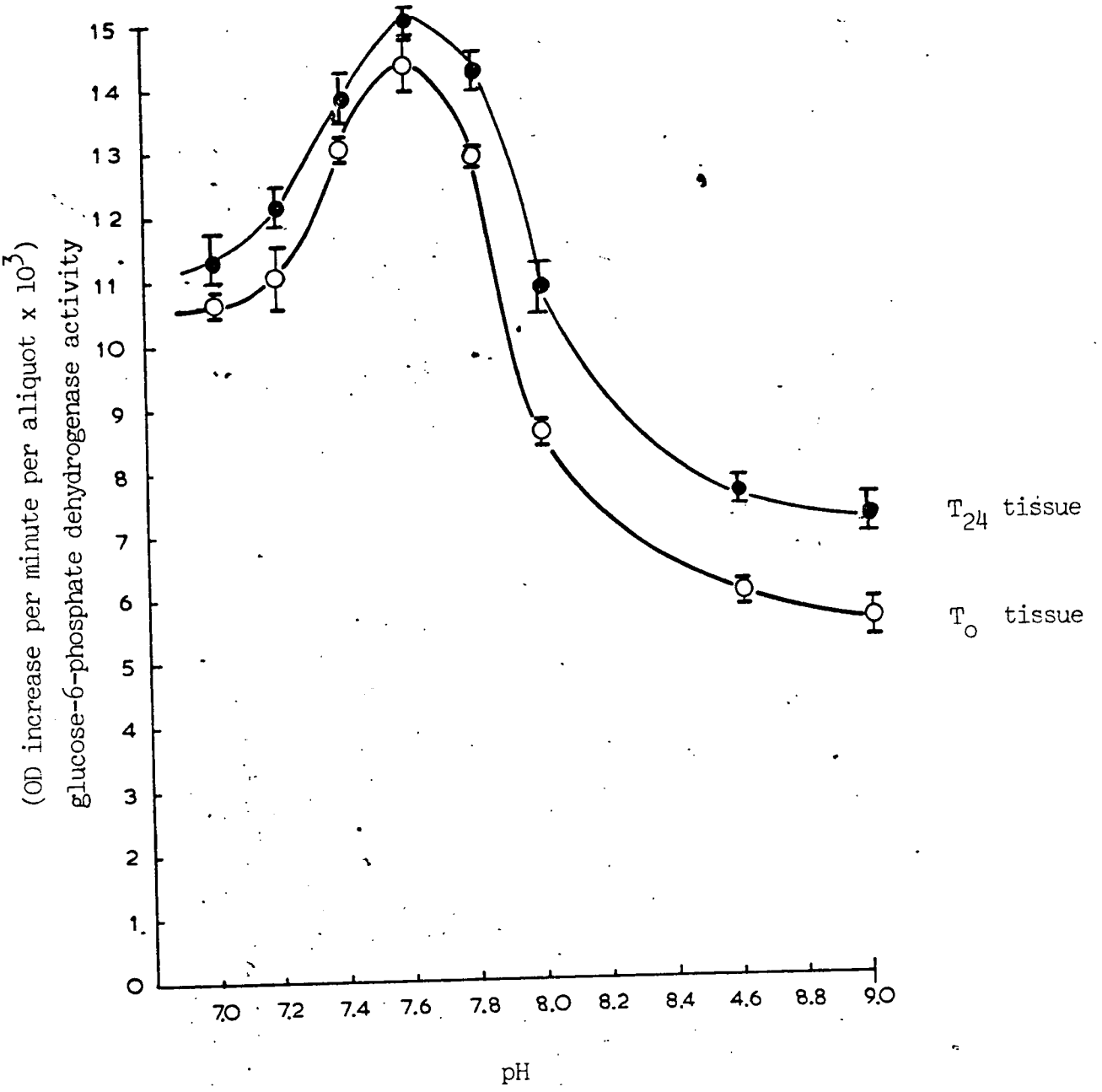
Extraction Medium (50 mM TRIS + 40 mg/100 ml BSA) +	G6PDH Activity $OD_{340\ mm}$ increase per minute per segment x 10^3
0	11.5 ± 0.2
50 mM Mercaptoethanol	11.3 ± 0.3
2 mM Dithiothreitol	11.1 ± 0.2
10 mM Dithiothreitol	10.6 ± 1.3
2 mM Potassium Metabisulphite	8.6 ± 1.3
10 mM Potassium Metabisulphite	8.5 ± 1.0

b. pH of Assay Medium

It has been established with a variety of tissues (Methods in Enzymology 1953) that G6PDH has an optimum pH of 7.6. In this experiment the pH optimum was determined for both freshly excised pea root tissue (T_o) and segments cultured for 24 hours (T_{24}) in case the pH optimum should change in culture and subsequently lead to an underestimation of the enzyme activity. Results presented in Figure 3-3 show that the pH optimum for both T_o and T_{24} tissue is pH 7.6. This

FIGURE 3-3

THE EFFECT OF ASSAY pH ON THE RECORDED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE



pH was used in all subsequent assays.

c. Cofactor Requirement

(i) Concentration of NADP

NADP is an obligatory coenzyme of the dehydrogenase and the activity is measured by the rate of production of the reduced form $NADPH_2$. Therefore an experiment was designed to ensure that the concentration of NADP used did not limit the activity measured. The concentration of NADP in the reaction cuvette was altered by varying the volume of solution added, the final total volume being kept constant by adjusting the volume of buffer. The results of activity against μ Moles of NADP added are shown in Figure 3-4. All other conditions of the assay were kept constant. 0.50 μM NADP was the lowest concentration which provided the highest activity. Therefore this concentration was used in all future assays.

(ii) Presence of Magnesium Chloride

Aitchison and Yeoman (1973) found that the inclusion of $MgCl_2$ (2 μM) in the assay mixture when assaying G6PDH from Jerusalem artichoke tissue resulted in a higher recorded activity. G6PDH from the pea root tissue was assayed with and without $MgCl_2$ in the reaction mixture. All other conditions were kept the same. The results are shown in Table 9 and show that $MgCl_2$ does not appreciably affect the recorded activities. Therefore, it was not routinely included in the assay mixtures.

The average results from two separate experiments are given.

FIGURE 3-4

THE EFFECT OF NADP CONCENTRATION ON THE RECORDED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

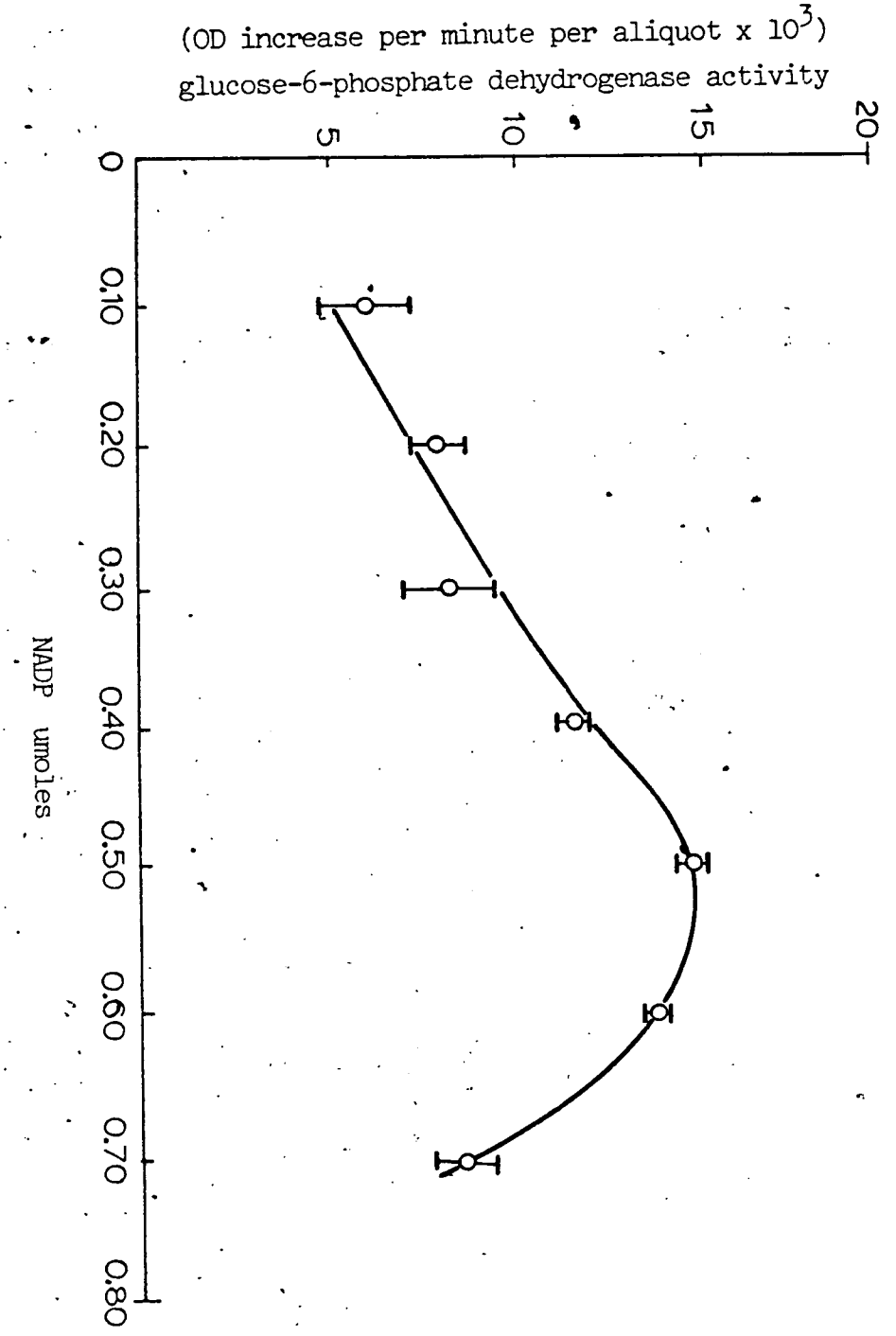


Table 9

Assay Medium 50 mM TRIS + 50 mM Mercaptoethanol	G6PDH Activity $OD_{340\ mm}$ increase per minute per segment
+ $MgCl_2$ 2 µM	9.60 ± 1.0
- $MgCl_2$	9.46 ± 0.5

d. Concentration of Glucose-6-phosphate

G-6-P is the substrate of the dehydrogenase activity being measured. It must be established that the concentration of substrate being used is not limiting or indeed wastefully high. Subsequently, the activity was measured with the same extract using a range of concentrations of G-6-P. Figure 3-5 shows the activity recorded against the amount of G-6-P. The activity increased with increasing amount of G-6-P to 1 mM but not beyond. Therefore, in all future experiments a final concentration of 1 mM was used, providing 0.2 µM per reaction cuvette.

Figure 3-6, of OD against time, obtained using fresh root material, shows that no reduction of NADP is detected before the addition of the G-6-P substrate. Therefore, no correction need be made in the calculation of activity.

e. Temperature of Assay

The term 'temperature optimum' cannot be applied as simply as the pH optimum term. Although with any one time of exposure to a given temperature, the activity might appear to increase at first, go through a maximum and eventually decline, the situation is more complex. The temperature optimum is decided by a combination of two factors, the thermal stability of the enzyme and the kinetic effect of temperature on reaction rates. The latter accounts for the increase in activity with increase in temperature until the denaturation from

FIGURE 3-5

THE EFFECT OF GLUCOSE-6-PHOSPHATE CONCENTRATION ON THE RECORDED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

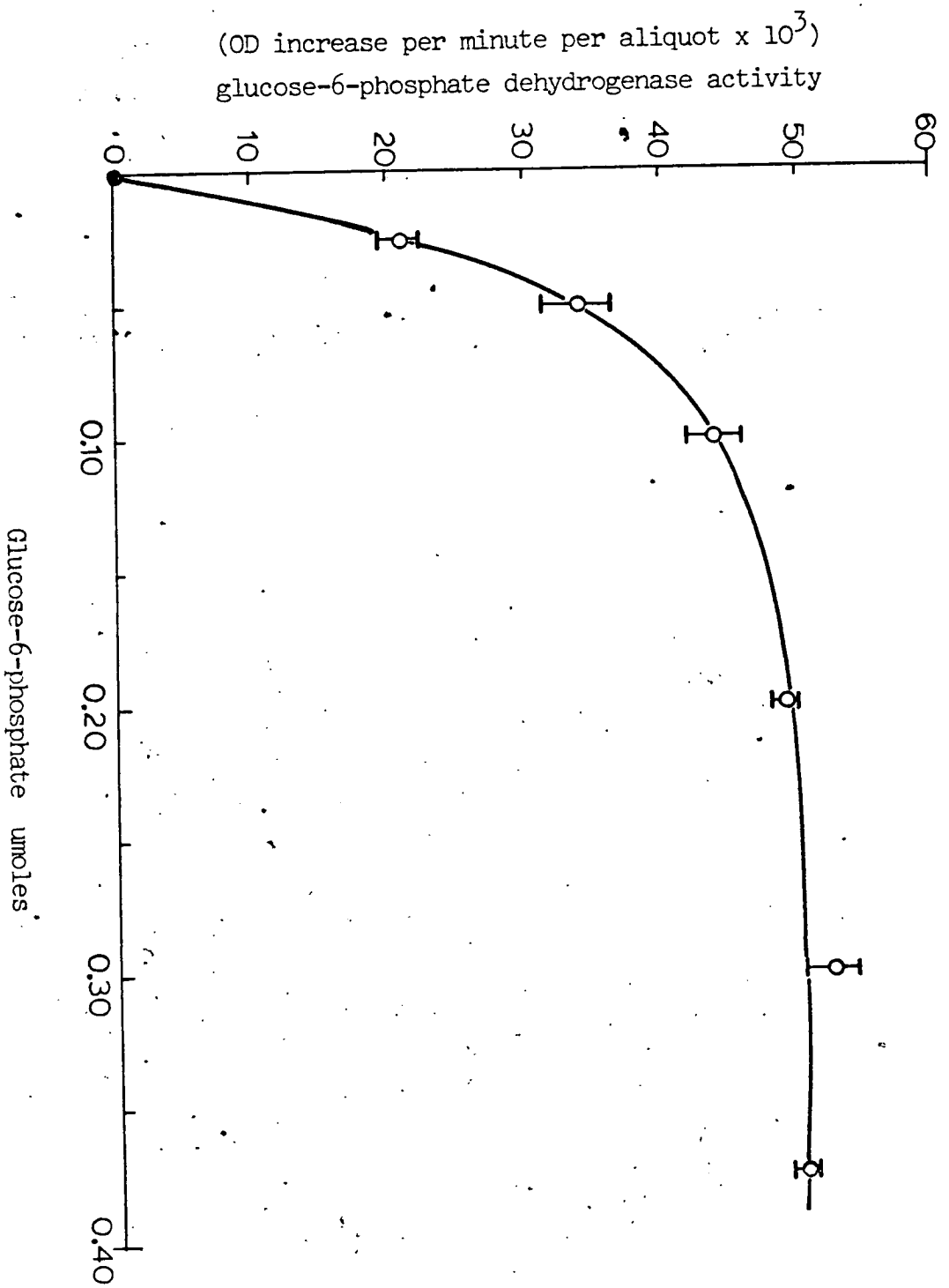
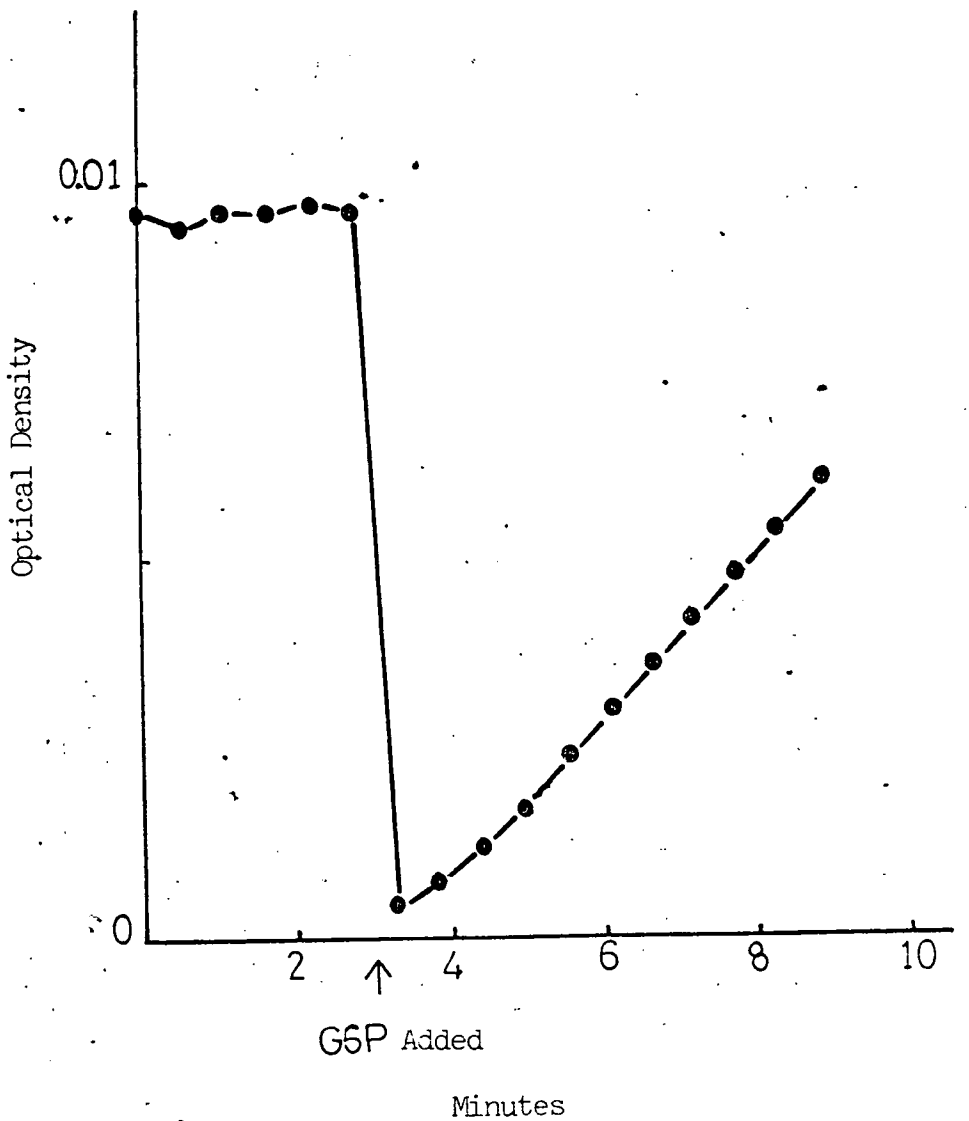


FIGURE 3-6

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY

CHANGE IN OPTICAL DENSITY BEFORE AND

AFTER ADDITION OF GLUCOSE-6-PHOSPHATE



the former brings about a reduction in activity at higher temperatures.

The temperature of the spectrophotometer (Unican SP800) block which holds the reaction cuvettes was controlled by pumping through water from a temperature controlled water bath. Time was allowed between each temperature stage to allow the block and reaction cuvettes to equilibrate at the desired temperature. Figure 3-7 shows the temperature sensitivity of the G6PDH from pea root tissue.

It is apparent from the results that the enzyme is very temperature sensitive. The graph peaks at around 50°C but with consideration to the temperature sensitivity of the enzyme and the high temperature, a temperature on the lower side of the peak was chosen. The temperature was routinely kept at 37°C during assays.

f. Volume of Extract

Preliminary experiments revealed that 20 segments extracted in a total volume of 1.5 ml would provide an extract with a significant activity. The extract volume used has to be checked to ensure that it bears a linear relationship to the activity measured. Volume of extract against activity is not linear overall ratios. The activity measured was compared against the aliquot volume of extract taken from a total of 1.5 ml/20 segments from fresh root material. These tests were repeated using an extract of 20 segments in a total of 1.5 ml, using material from segments cultured for 24 hours in 2% sucrose. The results are given in Figure 3-8. 0.15 ml aliquots were taken in all future assays as this point lies about mid-way on the linear portion of the graph.

g. Time Course

The velocity of a reaction is measured by following the dis-

FIGURE 3-7

THE EFFECT OF ASSAY TEMPERATURE ON THE RECORDED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

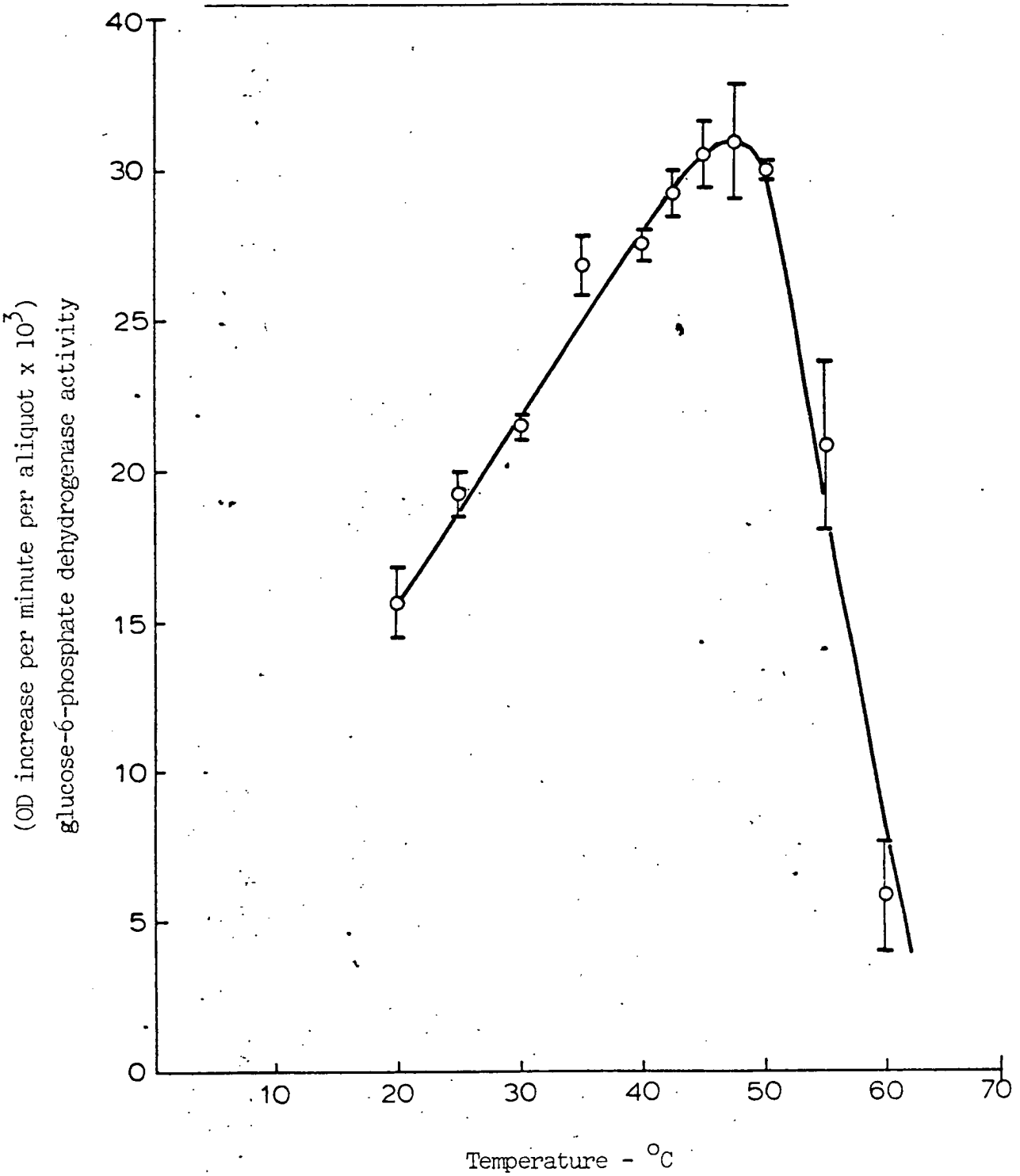
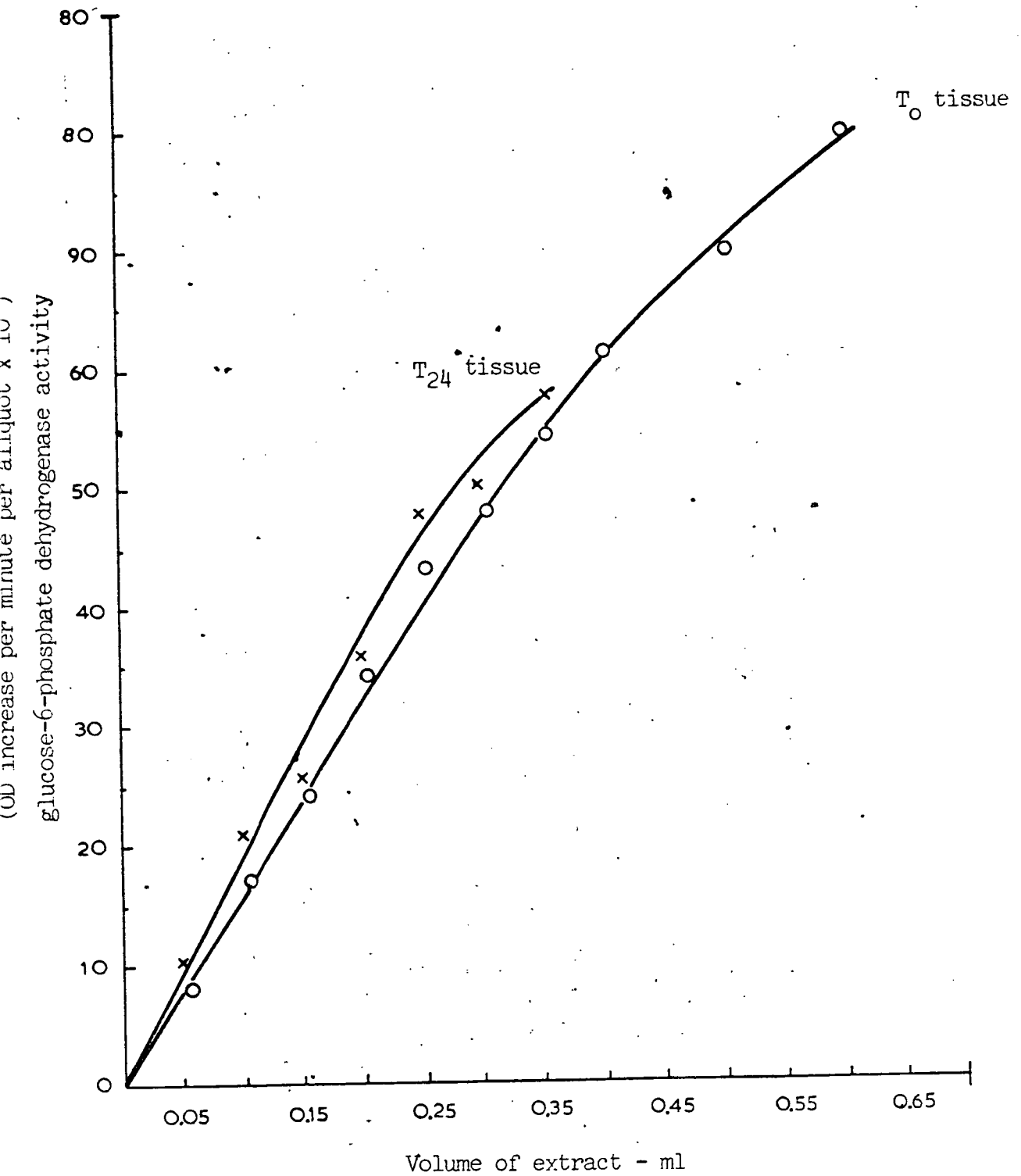


FIGURE 3-8

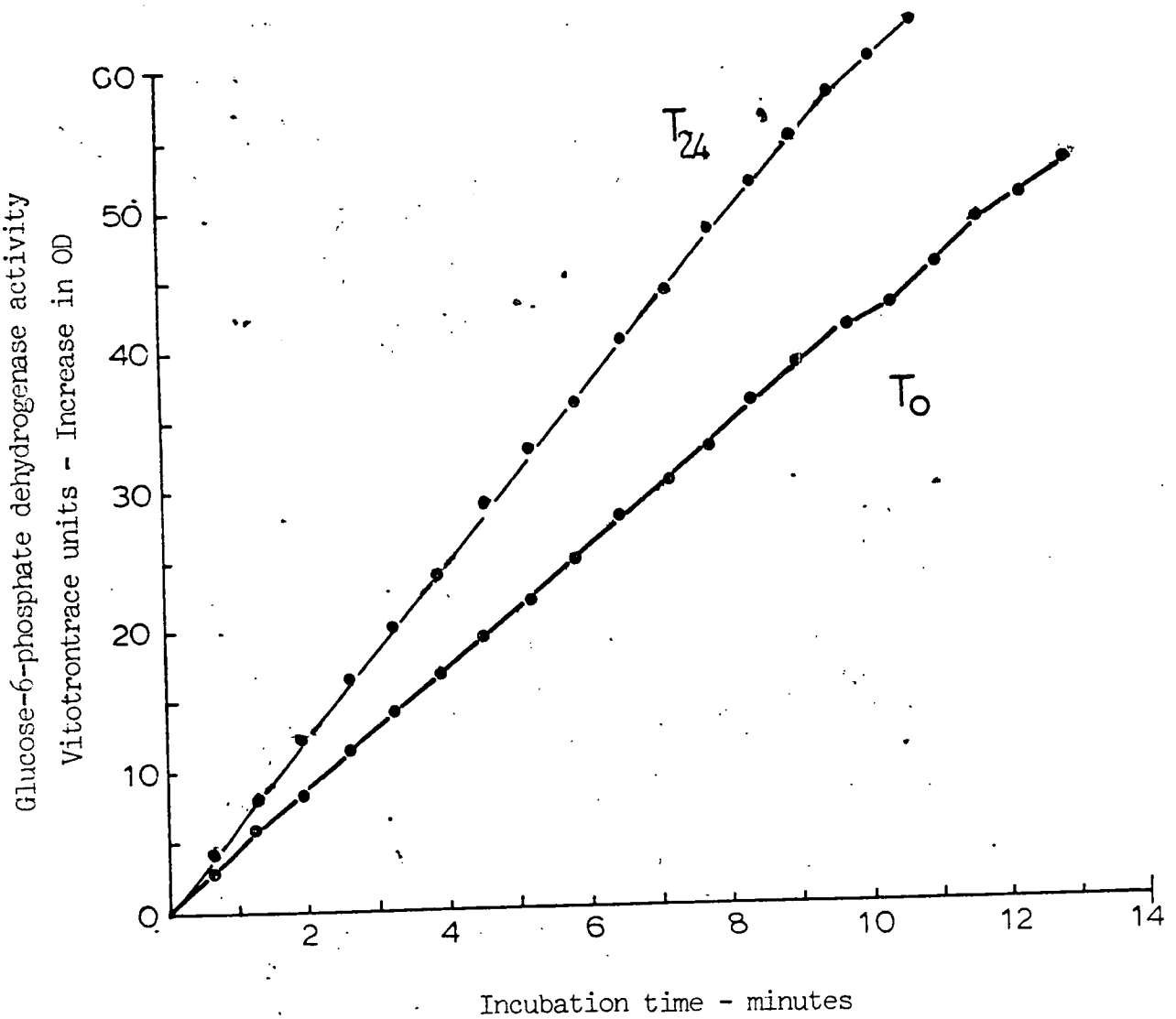
THE EFFECT OF VOLUME OF EXTRACT ON THE RECORDED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE



Protein Content 1.2 mg/ml of Extract

FIGURE 3-9

TIME COURSE OF OPTICAL DENSITY INCREASE
IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY



appearance of substrate or formation of product with time. For a single substrate-enzyme system such as G6PDH the rate of change of substrate with time will be linear at first, and then decrease as the substrate is used until equilibrium is established between substrate and product. The rate at which equilibrium is reached is determined by the amount of enzyme present.

The initial average increase in $OD_{340\ nm}$, on addition of the G6P after the equilibration period, was used for calculation of the activity. Under the conditions of assay, the OD increased linearly for 10 minutes before gradually levelling off, using T_o and T_{24} tissue. Routinely the activity was followed over a 5 minute period. The result is exemplified in Figure 3-9.

Conclusion

The accumulated results from this section enables one to assay G6PDH in pea root tissue under optimum conditions. The final assay conditions were in fact the same as those adopted in the early assay attempts using the method detailed for artichoke tissue (class schedule). Further, by comparison of the results taken from pH, time course and extract volume experiments, from assays using extracts from fresh roots and segments cultured for 24 hours, it appears that the G6PDH does not change grossly in character during the culture period. Therefore, the same assay technique may be confidently used for all extracts.

Section 3 - Assay of Cellulose Synthetase

The basis of the cellulose synthetase assay involves isotope labelling of fractions of cell homogenates from the precursor UDPG.

Hassid et al (1959) showed that elementary fibrils of cellulose

were formed by the bacterium Acetobactor Xylinium with UDPG as precursor, but G-I-P itself was ineffective. Barber, Elbein and Hassid (1964) demonstrated using etiolated mung bean seedlings that only GDPG could act as a glucose donor in cellulose synthesis. However, Ordin and Hall (1966) found that the particulatecell wall polysaccharide synthetase from oat coleptiles could use either GDPG or UDPG, with the latter being a much more effective glucose donor.

The products derived from the UDPG precursor, after cellulose digestion were cellobiose and an unidentified trisaccharide. Whereas from the GDPG precursor, cellobiose alone was identified. However, Franz (1967) did not detect GDPG in higher plants and preference was given to UDPG, as it occurs in growing tissues.

More recently, Delmer, Beasley and Ordin (1974) have studied the capacity to synthesise cellulose, in isolated cotton fibres at various stages of development. Incorporation of ^{14}C-glucose was highest from GDP-G(^{14}C) during the period of rapid elongation and declined in older fibres undergoing active deposition of secondary walls. Incorporation from UDPG was low in young fibres but increased to high levels in older fibres. The product was soluble in chloroform-methanol solvent. This product is thought to be the gluco-lipids as found by Forsee and Elbein (1972) in 15 day post anthesis cotton fibres.

The method used for assaying cellulose synthetase was adopted from the methods used by Barber, Elbein and Hassid (1964), Ordin and Hall (1967) and Delmer, Beasley and Ordin (1974).

The assay reaction mixture composed -

UDPG - 5.24×10^{-3} µM (20×10^{4} cpm) UL^{14}_{3} C - G - In early expts.
UL^{3} H - G - In later expts.

$MgCl_2$ - 4 µM

Extract - 100 µl (12 segments) 1.1×10^{-3} g protein

In total volume of 200 µl 50 mM TRIS (10 µM TRIS) at pH 8.0.

The method of assay of cellulose synthetase in the pea root tissue was developed and the results from the preliminary experiments are summarised in the following section. In these experiments, the 1 cm tip of seedling roots were used. The tissue was approximately quantitatively related to the 2 mm segments by fresh weight. All other assay conditions were at optimum except the one being varied.

a. Preparation of Extracts

Extracts prepared from plant tissues frequently contain compounds which denature proteins and can lead to the underestimation of enzyme activity but reducing agents can be added to the extraction and incubation media to prevent this deactivation. Spencer, Ziola and MacLachlan (1971) working with peas, found that the inclusion of dithiothreitol had no effect on the activity yield but other workers routinely include sulphydryl protecting compounds in the extraction and assay media, e.g. Ordin and Hall (1968) and Delmer, Beasley and Ordin (1974) included 4 mM EDTA and 50mM maleate, respectively.

The tissue was extracted in pH 8.0, 50 mM TRIS buffer supplemented with B.S.A. (40 mg/100 ml), and with three different sulphydryl protecting compounds. The assays were carried out in TRIS buffer alone. The results are given in Table 10 and show that the highest activities were obtained when the 50 mM TRIS was supplemented by 50 mM Mercaptoethanol, the other supplements reduced the activity.

Therefore, 50 mM Mercaptoethanol was routinely included in the extraction medium in all future assays.

Table 10

Extraction Medium - 50 mM TRIS +	Average cellulose synthetase activity - cpm incorporated per segment
0	21.3
50 mM Mercaptoethanol	27.6
20 mM Dithiothreitol	21.2
10 mM Dithiothreitol	18.2
2 mM Metabisulphite	10.3
10 mM Metabisulphite	9.8

b. pH of Assay Medium

Previous workers have used a range of pH's in different tissues to assay cellulose synthetase - Ordin and Hall (1968) pH 8 in oat coleptiles, Spencer, Ziola and MacLachan (1971) pH 8 in peas, Barber, Elbein and Hassid (1964) pH 7.5 in mung beans and Delmer, Beasley and Ordin (1974) pH 6.5 in Gossypium. In this experiment the pH optimum was determined for both freshly excised pea root tissue (T_o) and segments cultured for 24 hours (T_{24}) in case the pH optimum should change in culture and subsequently lead to an underestimation of the enzyme activity.

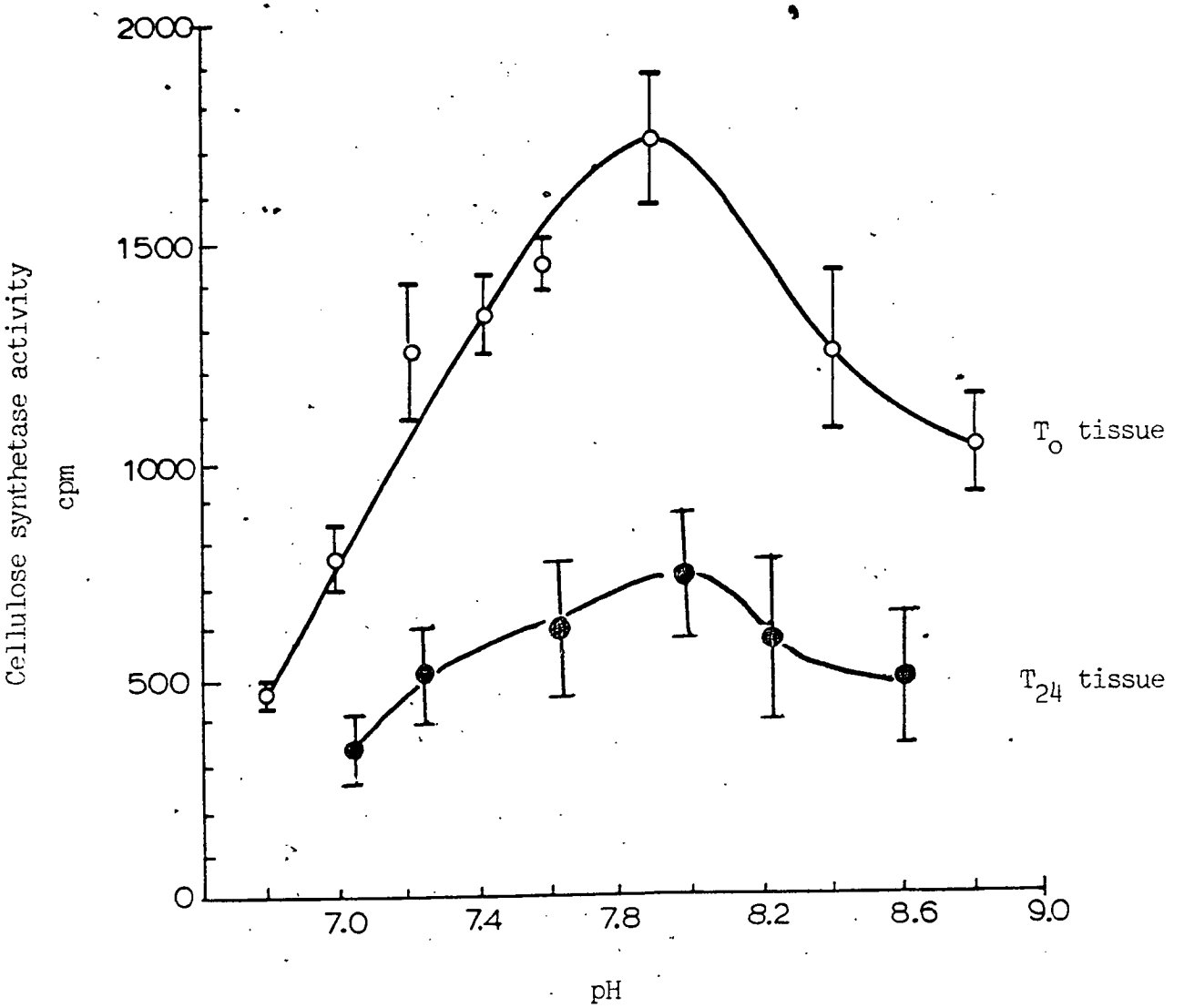
The root tissues were extracted in pH 8.0 medium and assayed under various pH regimes. The results of the activity against pH are given in Figure 3-10 and show a pH 8.0 optimum for the enzyme system in both fresh root and cultured for 24 hours pea root tissue. Therefore, this pH was used in all future assays.

c. UDPG - Substrate Concentration

It is essential to ensure that the concentration of UDPG in the assay mixture is not limiting the activity recorded . In the experiment to check this, the radioactively labelled UDPG concentration was kept constant, providing about 20×10^4 cpm and the total UDPG

FIGURE 3-10

THE EFFECT OF ASSAY pH ON THE
RECORDED ACTIVITY OF CELLULOSE SYNTHETASE



concentration was varied by increasing the amount of cold carrier. The activity recorded was evaluated by considering the cpm incorporated and the label to carrier ratio. The graph obtained is shown in Figure 3-11. The system was not saturated probably as there are many other reactions using up the substrate. The cpm incorporated against time of incubation graph may be used to ensure that the amount of substrate is not limiting. 5.24×10^{-3} µmoles (20×10^{4} cpm) UDPG was used routinely in assays.

d. Temperature of Assay

As discussed under G6PDH, the temperature optimum is decided by a combination of the thermal stability of the enzyme and the kinetic effect of temperature on the reaction rate.

The reaction tubes were incubated at various temperatures from $0^{o}C$ to $45^{o}C$. All other conditions were kept constant. The results for incubation temperature between 0 and $45^{o}C$ plotted against cpm (^{14}C) present in the insoluble residue are shown in Figure 3.12. The activity peaked at $37^{o}C$.

Therefore, with consideration to these experimental results and the G6PDH assay temperature, an incubation temperature of $37^{o}C$ was used in all future experiments. No cpm above background were counted in the insoluble product where the extract which had been boiled for 5 minutes was used.

e. Volume of Extract

The activity measured is not linearly related to the volume of extract over all ratios. Therefore, an experiment was performed to ensure that the volume of extract used lies within the linear portion of the relationship. The plot of activity, recorded as cpm incorporated, against fresh weight of tissue, shown in Figure 3-13 demonstrates

FIGURE 3-11

THE EFFECT OF UDPG CONCENTRATION ON THE RECORDED ACTIVITY OF CELLULOSE SYNTHETASE

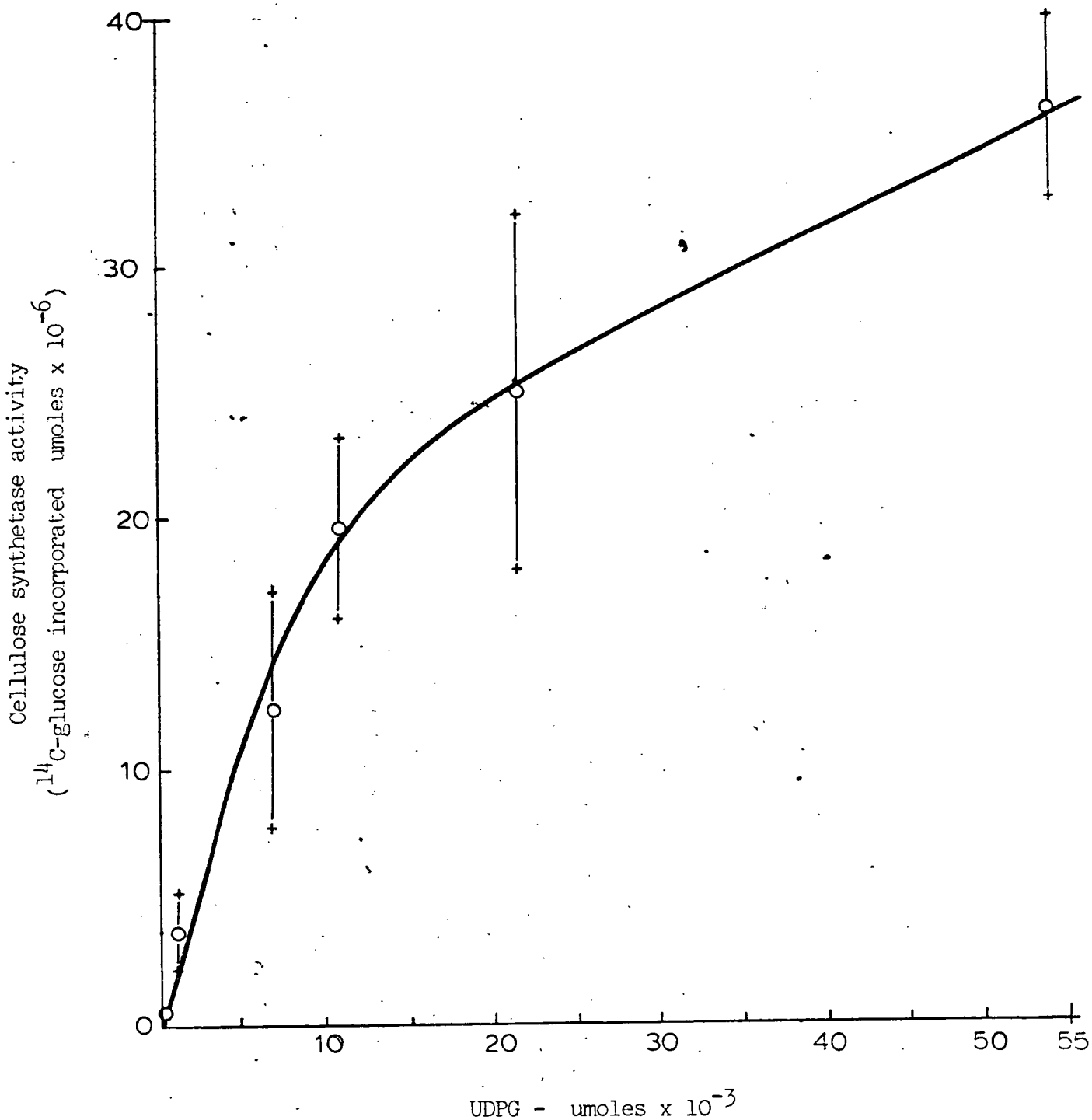


FIGURE 3-12

THE EFFECT OF ASSAY TEMPERATURE ON THE RECORDED ACTIVITY OF CELLULOSE SYNTHETASE

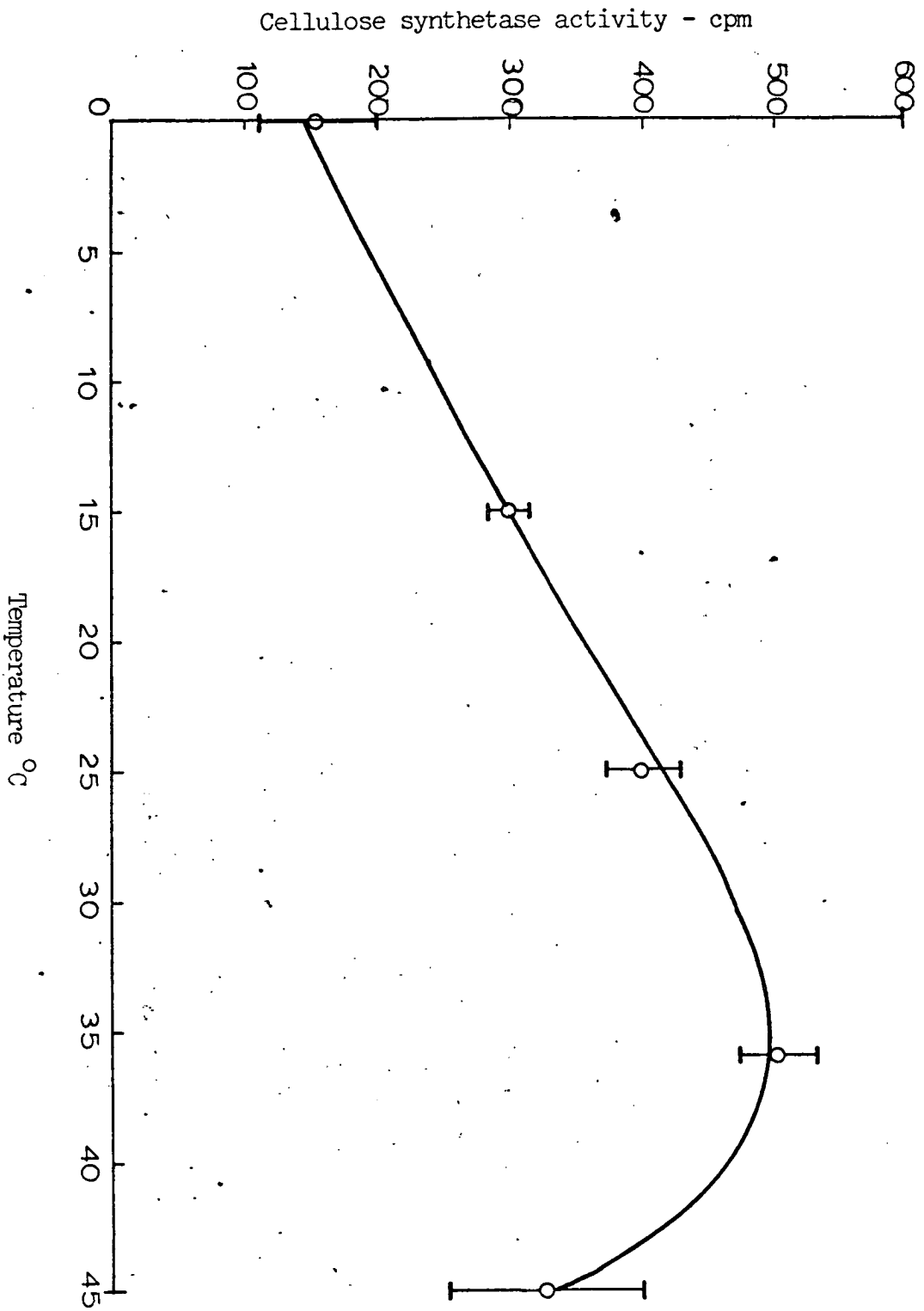


FIGURE 3-13

THE EFFECT OF SIZE OF EXTRACT ON
THE RECORDED ACTIVITY OF CELLULOSE SYNTHETASE

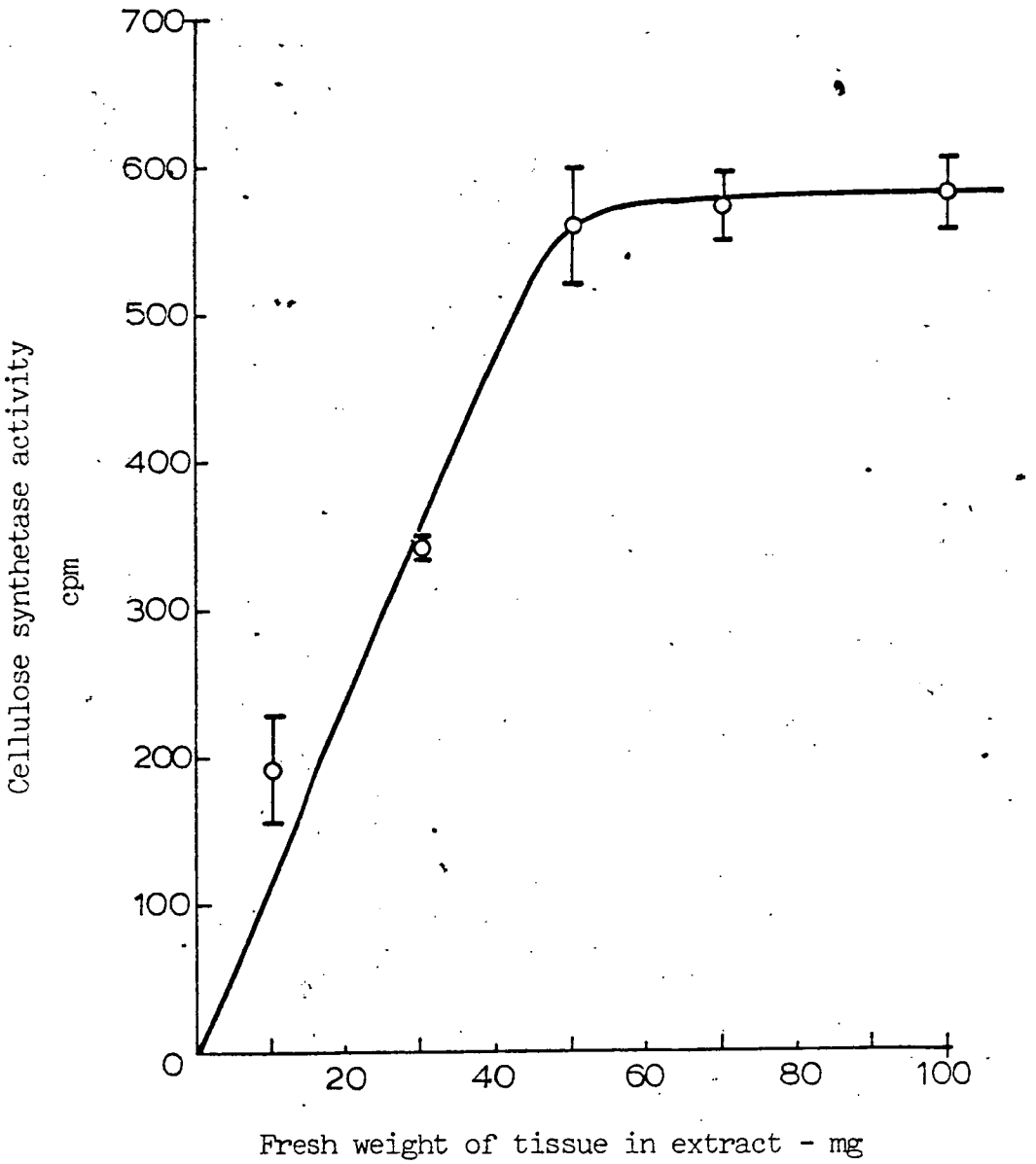
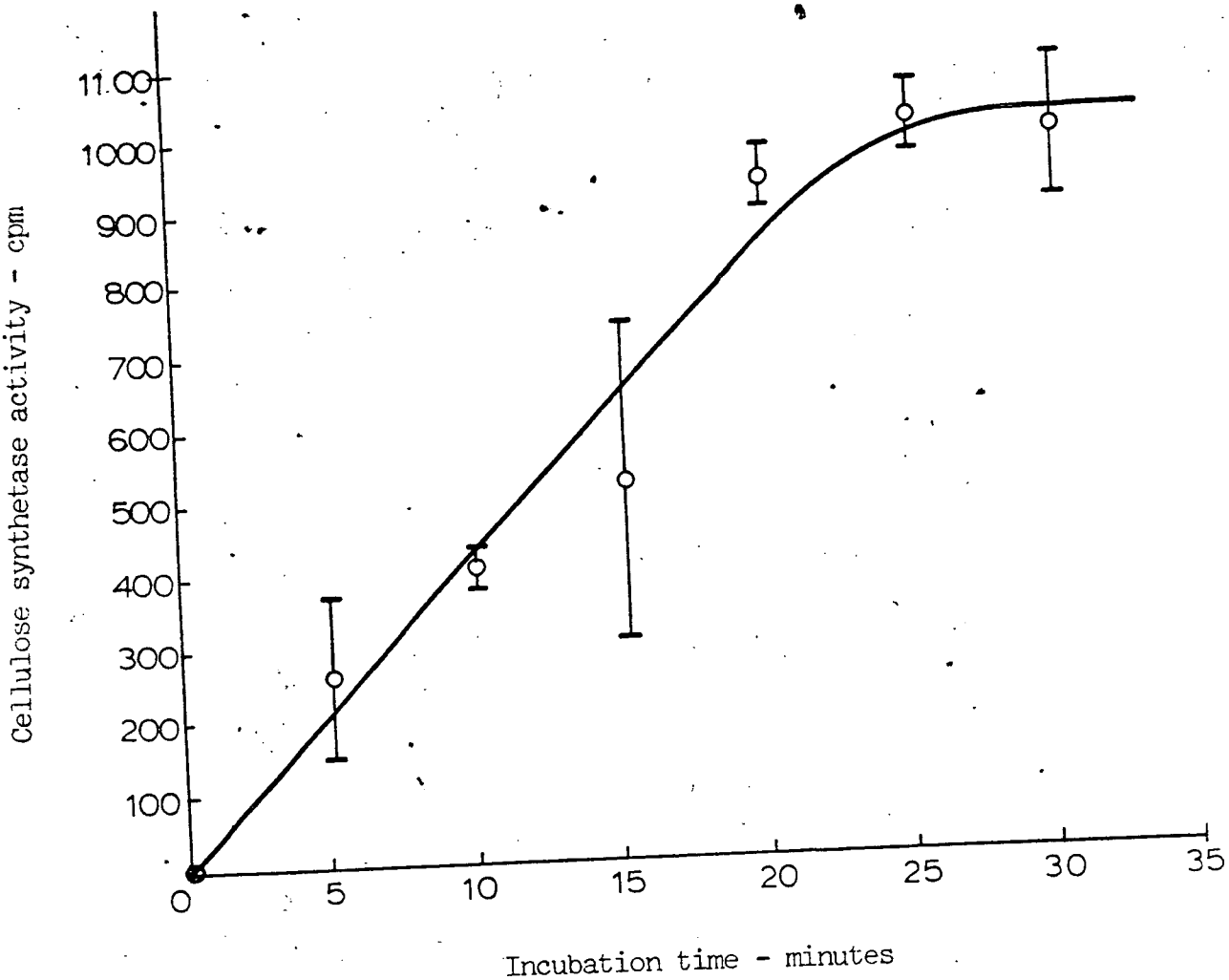


FIGURE 3-14

THE EFFECT OF LENGTH OF INCUBATION TIME ON
THE RECORDED ACTIVITY OF CELLULOSE SYNTHETASE



that the activity increases linearly to 50 mg fresh weight then levels off sharply. The fresh weight of 20 segments is about 40 mg at time zero and 90 mg after 24 hours culturing. Therefore, with due consideration to the increased water content of the 24 hour material, 12 segments provide an acceptable amount of tissue for the assay under the conditions stated.

f. Incubation Time

Due to the nature of the assay technique the reaction rate cannot be followed within a single sample as with the spectrophotometrically monitored assay of G6PDH. The rate of change of substrate with time will be linear and then decrease as the substrate is used until equilibrium is established between substrate and product.

The extract and substrate were allowed to react for various lengths of time before the reaction was terminated. The cpm incorporated increased linearly for 30 minutes and then quickly levelled off, as shown in Figure 3-14 . An incubation time of 20 minutes was used routinely in assays.

g. Distribution of Counts in Residue Washings

The fate of the radioactively labelled precursor molecules was followed through the various washing procedures.

The majority of the counts originally added were washed out in the initial water wash. These represent the unreacted precursor. A further significant proportion was removed from the residual bulk cellulose by the hot alkali wash. These represent the glucose molecules which were synthesised into water-in soluble, non-cellulose molecules. By the traditional definition the remaining fraction is cellulose but a further fraction is removed by the final chloroform wash. This represents a gluco-lipid fraction. Results obtained are

summarised in Table 11.

Table 11

Distribution of Counts in Cellulose Washes and Filter

cpm	Washes Water	Hot Alkali	Chloroform	Residual Cellulose Filter
	84258	4450	647	337
	81354	5370	2764	216
	83838	6743	2790	237
	79686	3785	1129	257
	89633	6013	2070	168
	79814	5056	1688	321
	79574	4592	1318	318
	65806	5072	1342	257
	74519	5177	1067	301
	74512	4981	1055	174

All counts added are not accounted for as the efficiency of counting varies under the different methods used and some counts would have been lost by adhering to the apparatus.

Only one chloroform wash was given as the small extra number of counts removed by a second wash did not justify the extra labour involved. Results obtained are summarised in Table 12.

Table 12

Counts Removed by First and Second Chloroform Washes

cpm in first chloroform wash	cpm in second chloroform wash	cpm in residual cellulose filter
2681	109	237
2030	99	257
1900	70	268
2414	174	321

The proportion of counts remaining on the filter after the chloroform wash appeared to decrease slightly through the 24 hour culture period. The proportionate production of the gluco-lipid fraction therefore increased through the culture period. Results obtained are given in Table 13

Table 13

Proportion of Counts remaining after Chloroform Wash through Culture Period

	Hours in Culture				
	0	3	6	10	24
	72	71	73	65	63
%	80	78	78	80	44
Average	89	82	78	80	73
	80	77	76	75	60

$$\frac{\text{Filter cpm, with chloroform wash}}{\text{Filter cpm, with no chloroform wash}} \times 100\ \%$$

The decrease in proportion of counts through the culture period may be significant and would be a topic worthy of future attention.

h. Guanosine Diphosphoglucose (GDPG) Substrate in Cellulose Assay

Ordin and Hall (1967) found that a particulate fraction from oat coleptile tissue could use either GDPG or UDPG. Franz and Meier (1969) and Delmer, et al (1974) also suggested that GDPG and UDPG are both precursors on cellulose synthesis in cotton fibres.

Therefore, the cellulose synthetase assay was carried out on the same fresh root segment extract, using the two precursor substrate molecules, uridine diphosphoglucose (UDPG) and guanosine diphosphoglucose (GDPG).

The procedure as described for the cellulose synthetase assay in 'Materials and Method' was followed. The conditions as found optimal for UDPG were initially used.

Assay Mixture

Extract - 20 segments (2-4 mm) - 17×10^{-4} g protein

UDPG - 5.2×10^{-3} (3.0×10^{4} cpm) or

GDPG - 5.2×10^{-3} (5.5×10^{4} cpm)

TRIS - 10 μ moles

$MgCl_2$ - 4 μmoles

Mercaptoethanol - 10 μmoles

Total Volume - 200 μl

pH 8.0 or 6.5

Incubation - 20 minutes 37°C

The results obtained from the initial assay, comparing the activities with UDPG and GDPG, are shown in Table 14.

Table 14

Assay pH	Chloroform Wash	Substrate	cpm incorporated into insoluble residue: UDPG	GDPG
8.0	+		4120 ± 120	215 ± 20
8.0	-		709 ± 51	230 ± 9
6.5	+		1434 ± 310	78 ± 25
6.5	-		311 ± 119	14 ± 10

Standard Deviations calculated from 4 Replica Assays

The counts incorporated represent about 0.4% conversion for glucose from UDPG and GDPG, in the pH 8.0, chloroform washed samples.

The results with GDPG could not be repeated. When the conditions of assay were kept the same with fresh root and 24 hour cultured segments, the assay pH ranged from 6.0 to 9.0, the concentration of GDPG was varied 100 fold from 0.19×10^{-3} μmoles and the incubation time increased from 5 to 60 minutes, only a maximum

of double the background number of counts was recorded in the cellulose fraction. Therefore, GDPG was not used routinely as the substrate in cellulose synthetase assay.

Conclusion

In this section, a technique for the assay of cellulose synthetase in pea root tissue has been presented. The technique is not grossly different from any of the existing techniques. In other sections of this work, the cellulose synthetase assay were carried out with the UDPG precursor and not GDPG. Although, the use of GDPG in this assay does deserve attention in future research.

Section 4

Combination of Glucose-6-Phosphate Dehydrogenase and Cellulose Synthetase Assays

It was desirable to be able to assay the G6PDH and CS activities within the same group of segments, partly due to the amount of tissue involved and to be able to assay both enzymes in same sample of segments. The optimal pH's of the two enzymes were found not to differ too widely and so the possibility of using the same initial homogenate to provide the extracts was explored.

The activities of the two enzymes were measured completely separately in similar samples of segments and using the same initial homogenate from one sample of segments. The results presented in Table 15 show that no loss of activity is encountered when the assays

are combined.

Table 15

Comparison of G6PDH and CS Activities measured in entirely Separate Extracts and in Extracts from a Common Homogenate

Enzyme System	Activity Measured in Separate Extracts	Activity Measured in Extracts from Common Homogenate	
G6PDH	9.5 ± 0.9	9.5 ± 0.1	OD increase per min. per segment x 10^3
Cellulose Synthetase	18.0 ± 1.3	20.5 ± 0.9	cpm incorporated per segment

Standard deviations calculated from three replica experiments.

Therefore, in all future experiments, both enzymes were assayed in extracts taken from the same tissue homogenised at pH 7.8. Further, from the results from experiments concerned with the preparation of extracts for each of the enzymes, it is evident that a 50 mM supplement of Mercaptoethanol to the 50 mM TRIS buffer will be suitable for both enzymes. It will therefore be possible to combine the extraction and assay of Glucose-6-phosphate dehydrogenase and cellulose synthetase.

Section 5 - Conclusions

In this chapter, results have been presented which have permitted the development of a successful root segment culture method. The source of variability in growth response was reduced to a tolerable level. The standardized methods of segment culture were described in Methods, Chapter 2, Section 1.

The kinetics studies of the enzymes glucose-6-phosphate dehydrogenase and cellulose synthetase have shown that the assay techniques are suitable for the pea root tissue and that the assays were carried out under optimal conditions. The assay methods are detailed in Methods, Chapter 2, Sections 2 and 3.

CHAPTER FOUR

CHAPTER 4

EXPERIMENTAL RESULTS

Changes in Root Segments During Expansion Growth in Culture

It has been shown in the previous chapter that the maximal extension growth in culture was obtained with the 2-4 mm sub-apical root segment with the Feltham First variety of pea. This tissue is suited to the study of expansion growth as the greatest majority of the cells in the sub-apical 2 mm fragment have completed the division phase of growth and are in various phases of expansion growth; the further the cells are situated from the tip, the older the cells and the later the stage of expansion growth. Also, with the culture system developed, it was possible to reduce the variability in length increase among the segments within the same treatment.

The examination of a system working under what are considered 'normal' conditions is a starting point for the study of expansion growth. In this work the 'normal' conditions are considered to be the culture of the isolated sub-apical 2 mm root fragment in 2% sucrose. Isolation in culture results in a smaller expansion of the cells but it ensures that the root fragments being studied are not under the influence of unknown variables from the cells in the rest of the plant, in other stages of development. It is unlikely that the growth pattern observed in culture will be entirely different from that occurring in the intact plant.

A greater understanding of a working system is obtained by noting the changes in behaviour when the system is perturbed and comparing it with the 'normal' behaviour. The growth response and the selected cellular changes were noted when the segments were cultured in the presence of either 6-methyl purine (6MP) or indole

acetic acid (IAA). 6MP is an analogue which inhibits normal RNA synthesis. In the concept of cell development presented by Brown (1963), an inhibition of RNA synthesis would arrest cell development or maturation. The concept postulates that cell development is divided into a number of merging stages each with its own characteristic proteins, enzymes, metabolic status and growth type. The change from one stage to the next requires the synthesis of RNA. Therefore, in this work, segments in a rapid phase of expansion growth were exposed to 6MP to discover if the high rate of growth was maintained over an extended period, and how the activities of selected enzymes change during this period. Segments were also cultured in the presence of a growth factor, IAA. It was chosen because of its general effect on expansion growth and to obtain comparative results for root tissue as have been obtained by numerous workers using shoot material.

The development of the assays of the two enzymes, G6PDH and cellulose synthetase, in the pea root tissue was presented in Chapter 3. The changes in the enzyme activities relative to each other and to the growth of the segments were considered. Also the effect of the inhibitor and the growth factor on the pattern of enzyme activity during culture was examined. The majority of experiments extended over 24 hour periods as the greatest changes occurred during this period and infection increased after this time.

Results have been presented from single experiments, also in the case of segment length, G6PDH activity and cellulose synthetase activity, the results from numerous experiments have been accumulated and plotted together. It was considered that the greater number of points provided by this method would enable a clearer idea of what was happening during culture to be formulated. Also, the enzyme

activities have been expressed as percentage changes from the T_o activity. This method of data presentation was adopted as although the activity patterns through the culture experiments were very similar within the same treatment, and varied similarly relative to the T_o activity, the T_o activity varied slightly among the experiments. Therefore, as it was the relative changes in the enzyme activities which were of interest, the data was more usefully expressed as percentage changes from the T_o activity.

a. Extension Growth in Sucrose

Sucrose has been shown e.g. Brown and Sutcliffe (1950) and Vaughan (1965) to be the most effective sugar in expansion growth, with Zea and pea root segments respectively. Vaughan (1965) showed that 2% was the optimal concentration for extension growth in sub-apical 2 mm pea root segments. The results accumulated from 10 separate experiments, performed over an 18 month period, on the culture of 2-4 mm pea root segments in 2% sucrose are plotted in Figure 4-15. The curve shows that the length increased linearly over the first 9 hours, at a rate of 0.23 mm per hour, doubling the segment length in 9 hours. Over the following 9 hours, the rate decreased smoothly until at 18 hours, and a segment length of 4.8 mm, the rate was zero and no further increase was observed within a 48 hour culture period. Although great efforts were made to reduce the variability in the segment growth response, it could not be entirely eliminated. The results from a single experiment on culture in 2% sucrose are given in Table 16. It is evident that variability in growth response as expressed by Standard Deviations, was present, being a maximum of $\pm$ 18% after 24 hours in culture. The three replica flasks at one sample time were not significantly different but there

FIGURE 4-15

Growth of 2-4 mm Root Segment in 2% Sucrose

Legend

The open circles show the average segment lengths calculated from 1-3 flasks each of 20 segments in 10 separate experiments. The crosses show the overall average lengths calculated for each sample time from the separate experiment values. The curve is drawn with reference to the crosses.

The T_o values for the separate experiments are not shown.

The data are tabulated in Appendix 1.

FIGURE 4-15

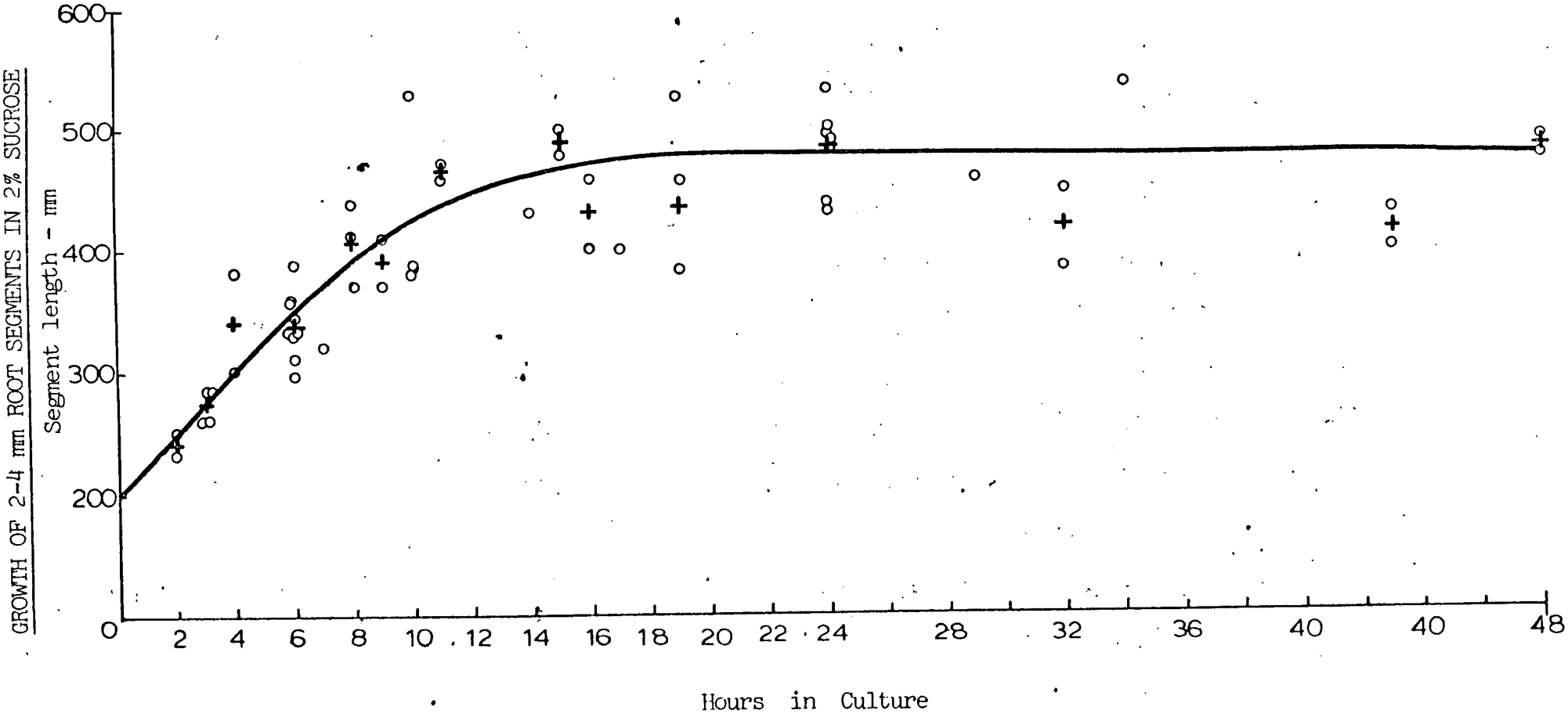

GROWTH OF 2-4 mm ROOT SEGMENTS IN 2% SUCROSE
Segment length - mm
600
500
400
300
200
0
2
4
6
8
10
12
14
16
18
20
22
24
28
32
36
40
40
48
Hours in Culture

TABLE 16

GROWTH OF 2-4 mm ROOT SEGMENTS IN 2% SUCROSE,

SHOWING THE VARIATION IN SEGMENT LENGTH

EXPERIMENT PERFORMED 12-13/4/73

CULTURE TIME HOURS	SEGMENT LENGTH ± SD (mm)			
	REPLICA FLASKS			AVERAGE
0	1.99 ± 0.08	1.97 ± 0.13	2.11 ± 0.17	2.02
3	2.44 ± 0.22	2.53 ± 0.09	-	2.49
6	3.38 ± 0.40	3.41 ± 0.27	2.83 ± 0.52	3.21
9	4.28 ± 0.83	4.10 ± 0.43	3.30 ± 0.37	3.89
15	4.46 ± 0.48	4.57 ± 0.85	3.93 ± 0.43	4.32
24	4.62 ± 0.60	5.10 ± 0.81	5.59 ± 0.98	5.10

Standard Deviations calculated with n = 20

was a significant increase in segment length during the culture period. The variability was greater than that recorded by Vaughan (1965) but his micrometer method of measuring the segments is probably less sensitive than the 'shadow' technique used in this work and this may account for the differences in the magnitude of the variation in segment length.

b. The Effect of 6-Methyl Purine on Extension Growth

Having established the pattern of growth in 2% sucrose, the effect of selected agents on this pattern was studied. Woodstock and Brown (1963) found that the pyrimidine analogue, 2 thio-uracil, produced a maximum stimulation in root segment extension growth at a concentration of 2×10^{-3}M. Vaughan (1965) also produced a maximal stimulation of the 2-4 mm pea root segment at the same concentration. The latter work showed that only the growth rate was affected but in the former the duration of growth was extended. In the present work, the effect of the nucleotide analogue, 6-methyl purine was examined. Key (1966) found 6MP, at concentration 2.5×10^{-3}M inhibited RNA synthesis, protein synthesis and growth in excised soybean hypocotyls.

The accumulated results from experiments on culturing root segments in 2% sucrose supplemented with 3 concentrations of 6MP throughout the 24 hour culture period are plotted in Figure 4-16. The curves are drawn through the average length for each sample time from all experiments. A 6MP supplement at a concentration of 2×10^{-3}M brought about a complete inhibition of extension growth and bleaching of the segments occurred in this culture medium. At 2×10^{-5}M, the length increases linearly over the initial 11 hour period, at a rate of 0.23 mm per hour. The rate then slowly decreased and was zero at 19 hours and through the remaining hours of the 24 hour culture

FIGURE 4-16

Growth of 2-4 mm Root Segments in 2% Sucrose
in the Presence of 6-Methyl Purine

Legend

The small symbols show the lengths calculated from 40 segments, cultured in 2 sets of 20, from up to 3 separate experiments.

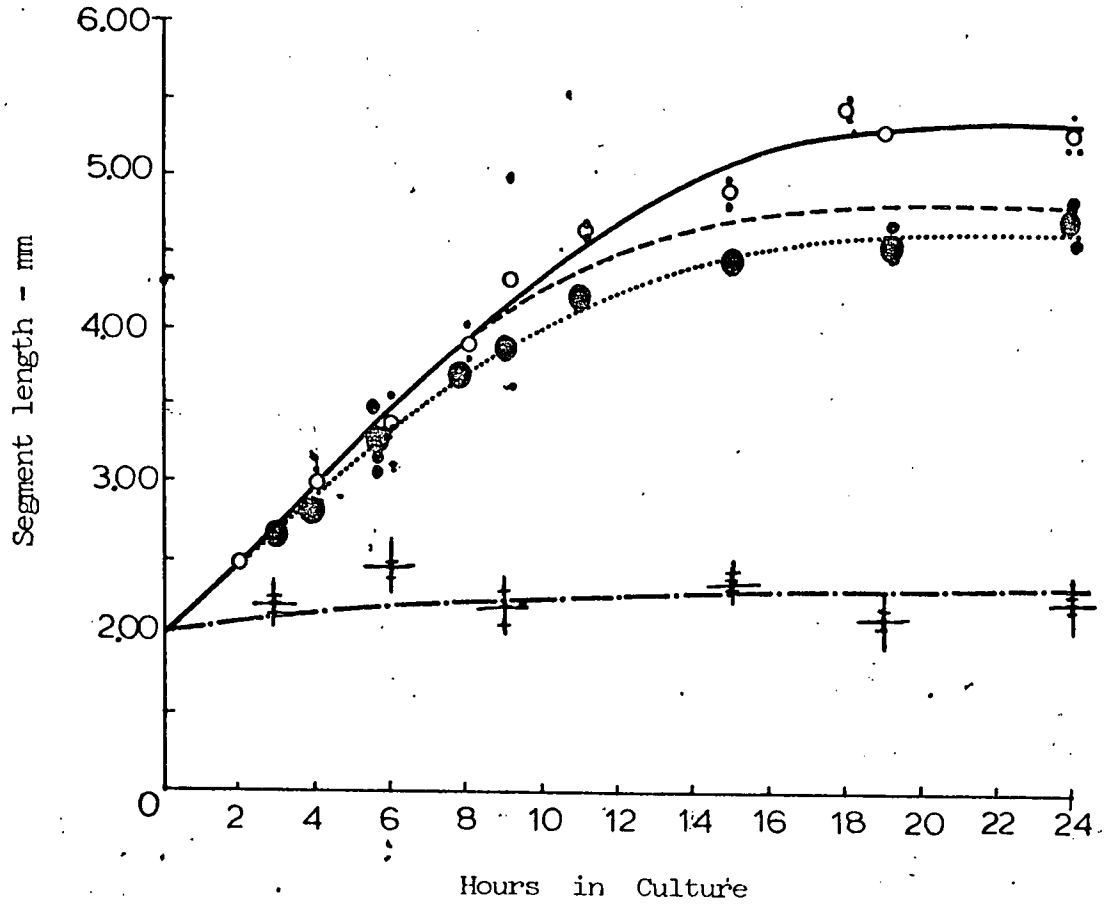
The large symbols show the average segment lengths calculated from the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The data are tabulated in Appendix 2.

The 2% sucrose curve shown was taken from Figure 4-15, the accumulated data for segments cultured in 2% sucrose.

FIGURE 4-16

GROWTH OF 2-4 mm ROOT SEGMENTS IN 2% SUCROSE IN PRESENCE OF 6-METHYL PURINE



2% sucrose + 2 x 10^{-5}M 6MP

2% sucrose

2% sucrose + 2 x 10^{-7}M 6MP

2% sucrose + 2 x 10^{-3}M 6MP

period. The segments attained a maximum length of 5.2 mm. When the sucrose was supplemented with 2 x 10^{-7}M 6MP, the length increased linearly for 8 hours, the rate then decreased from 0.21 mm per hour to zero by 18 hours and remained at zero, with a segment length of 4.5 mm for the remaining hours of the culture period. Therefore, by comparison with the 2% sucrose curve, 6MP at the concentration 2 x 10^{-5}M appears to stimulate extension growth by extending the period of growth, 2 x 10^{-7}M was ineffective. When the results from a single experiment are examined, with consideration to the variation in the segment growth response, as given in Table 17 ., the stimulation by 2 x 10^{-5}M 6MP, apparent from the accumulated results, is not statistically significant. The inhibitory effect of 2 x 10^{-3}M 6MP is significant.

c. The Effect of Indole-3-acetic Acid on Extension Growth

Auxin has a great diversity of effects on plant growth. Its stimulatory effect on shoot growth is well known but it also stimulates extension growth in root tissue but at lower concentrations. The 2% sucrose medium was supplemented with a range of indole acetic acid concentrations and the segment lengths obtained after 6 hours and 24 hours in culture were measured. The results are presented in Figure 4-17. 10^{-8}M and 10^{-10}M IAA supplements produced a slightly stimulatory effect compared to the sucrose control, 14% and 21% at 24 hours, respectively. But, when the variation is taken into account the stimulation is not statistically significant. Of the other concentrations used, 10^{-4}M was the only one which produced a significant inhibition of length increase; the inhibitory effect of 10^{-6}M was not statistically significant. 10^{-12}M was ineffective compared with the sucrose control.

TABLE 17

GROWTH OF 2-4 mm ROOT SEGMENTS IN 2% SUCROSE

IN THE PRESENCE OF 6-METHYL PURINE,

SHOWING THE VARIATION IN SEGMENT LENGTH

EXPERIMENT PERFORMED 8-9/8/74

CULTURE TIME HOURS	CULTURE MEDIUM		
	2% SUCROSE	2% SUCROSE + 2×10^{-3}M 6MP	2% SUCROSE + 2×10^{-5}M 6MP
0	(2.01 ± 0.15)	-	-
2	2.28 ± 0.15	2.23 ± 0.11	2.40 ± 0.16
4	2.97 ± 0.13	2.49 ± 0.21	2.75 ± 0.16
6	3.28 ± 0.36	2.37 ± 0.17	3.12 ± 0.31
8	3.72 ± 0.54	2.29 ± 0.14	4.00 ± 0.34
16	4.51 ± 0.50	2.25 ± 0.12	4.93 ± 0.41
24	4.95 ± 0.68	2.17 ± 0.10	5.24 ± 0.39

Segment Length ± SD (mm)

Standard Deviations calculated with n = 40

Consequently, the pattern of segment extension was examined through a 24 hour culture period when 10^{-10}M and 10^{-4}M IAA were added to the sucrose medium throughout the experiment. The accumulated results of increase in segment length with time from 3 separate experiments are plotted in Figure 4-18. In the presence of the inhibitory concentration (10^{-4}M) of IAA, the length increased linearly at a rate of 0.09 mm per hour for 8 hours. Then the rate declined to zero by about 18 hours, giving an average segment length of 3.14 mm or a 64% increase by the end of the 24 hour period.

When the sucrose is supplemented with 10^{-10}M IAA throughout the experiment, the length increased linearly at a rate of 0.23 mm per hour in the initial 12 hours. Over the remaining 12 hours the growth rate gradually declined to half the initial rate by the end of the experimental period, the segments attaining an average length of 6.29 mm, an increase of 214%. Therefore, by comparison with the 2% sucrose curve, the 10^{-10}M IAA appears to stimulate extension growth by extending the period of growth. It did not appear to affect the initial rate of extension growth. The significance of these results on the effect of IAA on extension growth is lost when a single experiment is considered, the results of which are given in Table 18 . The inhibitory effect of 10^{-4}M is no doubt a true one but due to the variation inherent in the experimental system, the results are marginally outside the range for being statistically significantly different from the sucrose control. Similarly the stimulatory effect of the 10^{-10}M IAA supplement is not statistically significant.

d. Extension Growth in the Absence of Sucrose

Sucrose is required in the culture medium probably as an energy source and it also accumulates in the vacuole and so maintaining the

FIGURE 4-17

Legend

Effect of Range of Concentrations of Indole Acetic Acid on Growth of Root Segments

The bars show the average segment length attained after 6 and 24 hours in culture. The average length was calculated from 40 segments grown in 2 flasks of 20 segments. The ranges shown are Standard Deviations calculated with n = 40.

The data are tabulated in Appendix 3.

FIGURE 4-17

EFFECT OF RANGE OF CONCENTRATIONS OF INDOLE ACETIC ACID ON GROWTH OF ROOT SEGMENTS

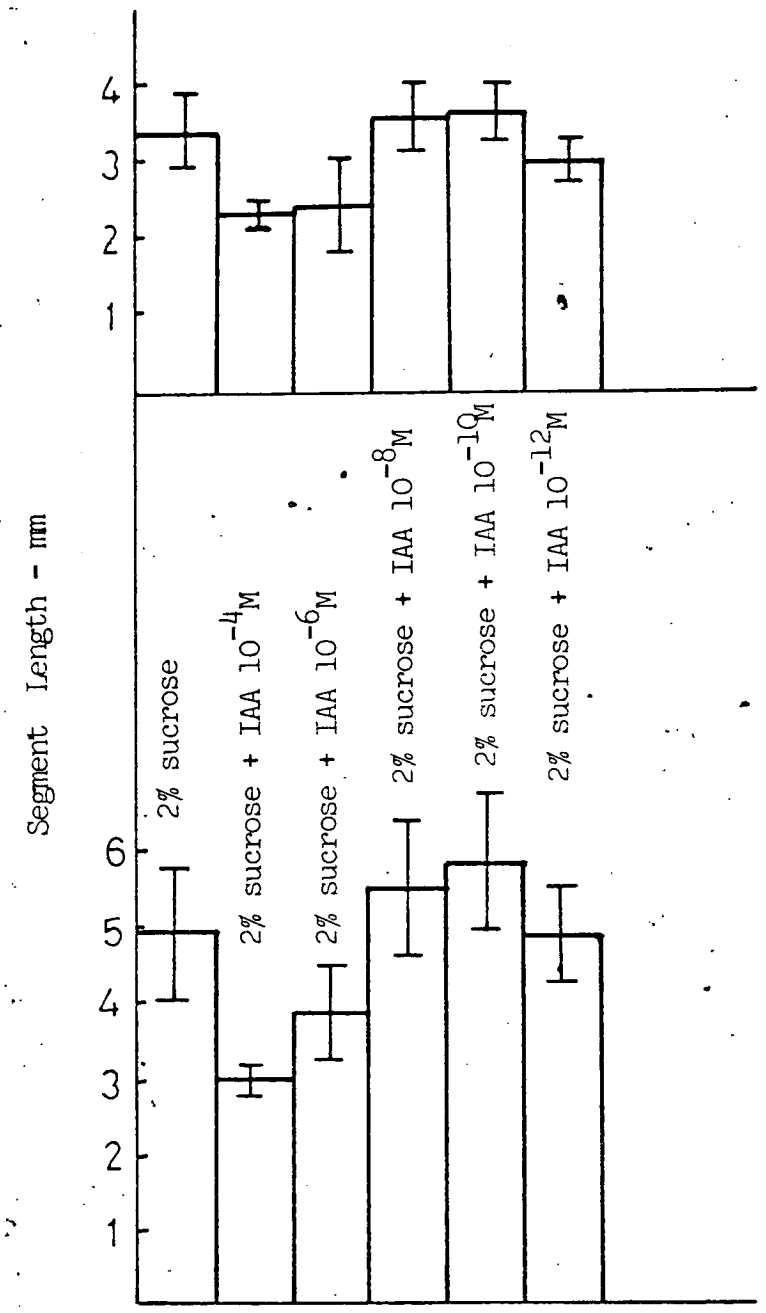


FIGURE 4-18

Growth of 2-4 mm Segments in 2% Sucrose in the Presence of Indole Acetic Acid

Legend

The small symbols show the lengths calculated from 40 segments cultured as 2 flasks of 20, from at least 3 experiments. The large symbols show the segment lengths calculated from the average of the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The data are tabulated in Appendix 4.

The curve for segment growth in 2% sucrose was that shown in Figure 4-15, obtained from the accumulated data.

FIGURE 4-18

GROWTH OF 2-4 mm ROOT SEGMENTS IN
2% SUCROSE IN PRESENCE OF INDOLE ACETIC ACID

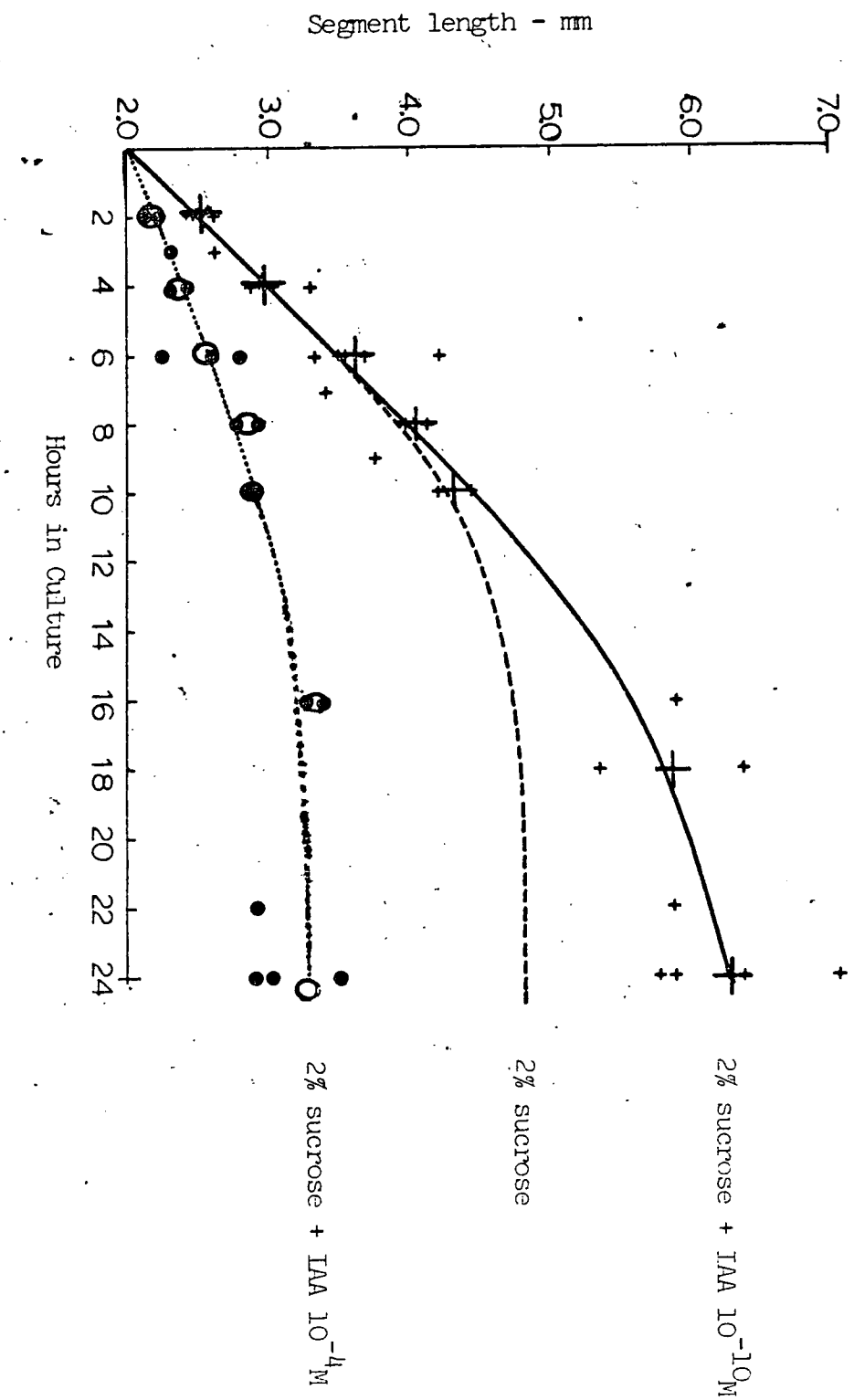


TABLE 18

GROWTH OF 2-4 mm ROOT SEGMENTS IN 2% SUCROSE IN THE PRESENCE OF IAA, SHOWING THE VARIATION IN SEGMENT LENGTH

EXPERIMENT PERFORMED 23-24/7/74

CULTURE TIME HOURS	CULTURE MEDIUM		
	2% SUCROSE	2% SUCROSE + 10^{-4}M IAA	2% SUCROSE + 10^{-10}M IAA
0	(1.97 ± 0.07)	-	-
3	2.59 ± 0.17	2.30 ± 0.17	2.60 ± 0.23
6	3.56 ± 0.40	2.63 ± 0.83	3.49 ± 0.27
9	3.70 ± 0.55	2.66 ± 0.24	4.14 ± 0.33
16	4.60 ± 0.72	3.41 ± 0.60	4.78 ± 0.59
24	4.84 ± 0.71	3.48 ± 0.58	5.94 ± 1.21

Segment Length ± SD (mm)

Standard Deviations calculated with n = 40

osmotic pressure of the expanding cells (Vaughan 1965). In the absence of sucrose, Brown and Sutcliffe (1950) found limited extension growth and that the cellulose content of the Zea root segments decreased; with sucrose present cellulose synthesis occurred. Vaughan (1965) also showed that the stimulatory effect of 2 thio-uracil was lost in the absence of sucrose. In this work, when sucrose was omitted from the culture medium, either in the presence or absence of either 2×10^{-5}M 6MP or 10^{-10}M IAA, the segments did not elongate more than 50% in the 24 hour period. Results are presented in Table 19 . The variation in length, as measured by the range from the mean, was never greater than 19%. The changes occurring in the absence of sucrose were not examined further as interest was centred on stimulatory culture conditions compared with the 'normal' or 2% sucrose cultures.

e. Conclusions - Changes in Segment Length during Culture in Various Media

Both 6MP (2×10^{-5}M) and IAA (10^{-10}M) appear to stimulate extension growth in cultured pea root segments by extending the period of growth, by 2 and 3 hours respectively. Neither agent brought about an increase in the initial rate of growth compared with the growth in 2% sucrose alone. However, due to the variability in the segment growth response, the stimulation was not statistically significant but the stimulatory effects may be considered with reasonable confidence due to the large numbers of points through which the curves were drawn. Further, sucrose must be present for the stimulations to occur.

Parameters of growth, in addition to the segment length were examined and compared when the segments were cultured in 2% sucrose and sucrose supplemented with either 6MP or IAA.

BLE 19

THE GROWTH OF 2-4 mm ROOT SEGMENTS IN WATER

SEGMENT LENGTH - mm.

CULTURE MEDIUM	HOURS IN CULTURE							
	0	3	6	9	12	16	21	24
Water	2.03	2.09	2.36	2.44	2.53	2.74	2.89	2.91
2×10^{-5}M 6MP	2.08	2.13	2.20	2.49	2.55	2.56	2.69	2.79
10^{-10}M IAA	2.00	2.10	2.19	2.35	2.48	2.53	2.90	2.93

6MP - 6-methyl purine

IAA - Indole acetic acid

Legend

Each figure represents the average length calculated from 40 segments, cultured as 2 flasks of 20. The variation was never greater than a 20% range from the mean.

f. Segment Width During Culture

Expansion is an increase in cell volume but in the present work, length was used as a measure of the expansion increase. It is a reliable indicator as the segment breadth or width did not increase significantly during the 24 hour culture experiments. The results given in Table 20 show that the segment breadth never increased more than 20% during culture in 2% sucrose, and sucrose supplemented with either 2 x 10^{-5}M 6MP, 10^{-4}M IAA or 10^{-10}M IAA. Therefore the segment length will give an acceptable measurement of expansion growth.

g. Cell Number During Culture

When the fragments removed from the extending zone of the root are supplied with suitable media, Brown and Sutcliffe (1950) and Vaughan (1965) showed that the growth was due only to cell extension. Therefore the growth being measured as increase in segment length may be attributed to cell extension. The absence of cell divisions was checked by counting and comparing the number of cells per segment through the culture period. The values obtained at times 0, 9 and 24 hours during culture in 2% sucrose and in the presence of 2 x 10^{-5}M 6MP or 10^{-10}M IAA are presented in Table 21 . The results confirm that no significant change in cell number per segment occurs during culture, the number remaining about 70,500 and therefore any changes occurring during culture may be confidently associated with expansion growth.

h. Changes in Weight During Culture

The segment length has been used as the basic parameter of growth. A greater increase in segment length may be brought about by a greater increase in the length of the constituent cells, a greater number of cells undergoing expansion growth or a combination of both.

TABLE 20

WIDTH OF 2-4 mm ROOT SEGMENTS CULTURED IN 3 MEDIA

Culture Medium	HOURS IN CULTURE 0	2	4	8	17	24
2% sucrose	1.01 ± 0.02	1.09 ± 0.06	1.07 ± 0.07	1.10 ± 0.12	1.07 ± 0.08	1.06 ± 0.06
2% sucrose + 6MP 2×10^{-5} M	-	1.13 ± 0.01	1.06 ± 0.08	1.15 ± 0.06	1.11 ± 0.07	1.00 ± 0.13
2% sucrose + IAA 10^{-4} M	-	1.14 ± 0.08	1.09 ± 0.06	1.13 ± 0.06	1.20 ± 0.07	1.14 ± 0.08
2% sucrose + IAA 10^{-10} M	-	1.17 ± 0.09	1.14 ± 0.07	1.15 ± 0.07	1.12 ± 0.06	1.22 ± 0.07

6MP - 6-Methyl Purine

IAA - Indole Acetic Acid

Standard Deviation calculated with n = 20

TABLE 21

NUMBER OF CELLS IN 2-4 mm SEGMENTS AFTER CULTURING IN SUCROSE IN PRESENCE AND ABSENCE OF 6-METHYL PURINE AND INDOLE ACETIC ACID

HOURS IN CULTURE	MEDIUM	NUMBER OF CELLS PER SEGMENT
0	Fresh Tissue	69,700 ± 3,900
9	S	70,700 ± 3,600
	S + 6MP	71,300 ± 4,700
	S + IAA	72,100 ± 3,900
24	S	68,900 ± 4,600
	S + 6MP	71,000 ± 3,200
	S + IAA	69,500 ± 2,800

Standard deviations calculated from the results of three sets of 5 segments

S - 2% Sucrose

6MP - 6-methyl purine 2×10^{-5}M

IAA - Indole acetic acid 10^{-10}M

Detailed work on cell measurements in segment longitudinal sections would be required to distinguish which of these was predominant. Such an investigation would be tedious and probably fruitless. The volume increase, reflected by the length increase, is mainly due to the uptake of water, but earlier workers have shown that synthesis of cell components occurs. The fresh, dry and residual dry weights were measured in segments cultured in sucrose and sucrose supplemented with 6MP (2×10^{-5}M) and IAA (10^{-10}M). The fresh weight will show the greatest increase due to the water uptake which is excluded in the dry weight. The residual dry weight, excludes the weight due to the sucrose and represents mainly the cell wall polysaccharide, protein and nucleic acid components of the segment cells. The results obtained are given in Table 22 and plotted in Figures 4-19, 4-20 and 4-21. The addition of 6MP or IAA to the sucrose medium slightly stimulated the increases in fresh, dry and residual dry weights. The fresh weight steadily increased over the 24 hour culture period from 1.93 g $\times 10^{-3}$ to 4.65, 5.39 and 5.77 g $\times 10^{-3}$, increases of 240%, 280% and 300% in sucrose, 6MP and IAA media, respectively; the dry weight increased by 100%, 120% and 120%, respectively, from 1.73 g $\times 10^{-4}$ per segment. The residual dry weight increased from 0.99 g $\times 10^{-4}$ per segment to 1.51, 1.76 and 2.03 g $\times 10^{-4}$, increases of 50%, 80% and 100% in sucrose, 6MP and IAA respectively in the 24 hour period but most of the increase occurred in the initial 14 hours.

i. Protein Content During Culture

The residual dry weight, comprising the cell wall polysaccharides, protein and nucleic acid cell components, increased under the three culture conditions. Abbott (1972) found cultured, excised pea root

TABLE 22

SEGMENT WEIGHTS OF 2-4 mm ROOT SEGMENTS

DURING CULTURE IN THREE MEDIA

HOURS IN CULTURE	MEDIUM	g x 10^{-3} SEGMENT FRESH WT.	g x 10^{-4} SEGMENT DRY WT.	g x 10^{-4} SEGMENT RESIDUAL DRY WT.
0	(fresh tissue)	1.93	1.73	0.99
2	S	2.13	2.06	1.20
	S + 6MP	2.30	2.12	1.24
	S + IAA	2.32	2.23	1.37
4	S	2.56	2.17	1.26
	S + 6MP	2.73	2.36	1.34
	S + IAA	2.76	2.44	1.49
6	S	3.01	2.23	1.24
	S + 6MP	3.09	2.66	1.39
	S + IAA	3.34	2.95	1.55
14	S	4.11	2.79	1.41
15	S + 6MP	4.49	2.81	1.64
16	S	4.19	3.03	1.67
	S + 6MP	4.74	3.14	1.79
	S + IAA	5.41	3.54	1.98
18	S + 6MP	4.97	3.25	1.81
	S + IAA	5.38	3.52	1.96
24	S	4.65	3.48	1.51
	S + 6MP	5.39	3.79	1.76
	S + IAA	5.77	3.77	2.03

S = 2% Sucrose

6MP = 6-methyl purine 2 x 10^{-5}M

IAA = Indole acetic acid 10^{-10}M

Data accumulated from two successive experiments, with sample times as shown:

Expt. A = T_0 T_4 T_{14} T_{15} T_{18} T_{24}

Expt. B = T_0 T_2 T_6 T_{10} T_{24}

At each sample time, 20 segments were weighed for fresh and dry weights and 40 segments for residual dry weight, for each treatment.

FIGURES 4-19 - 4-21

CHANGE IN SEGMENT WEIGHTS DURING CULTURE IN DIFFERENT MEDIA

FIGURE 4-19

FRESH WEIGHT

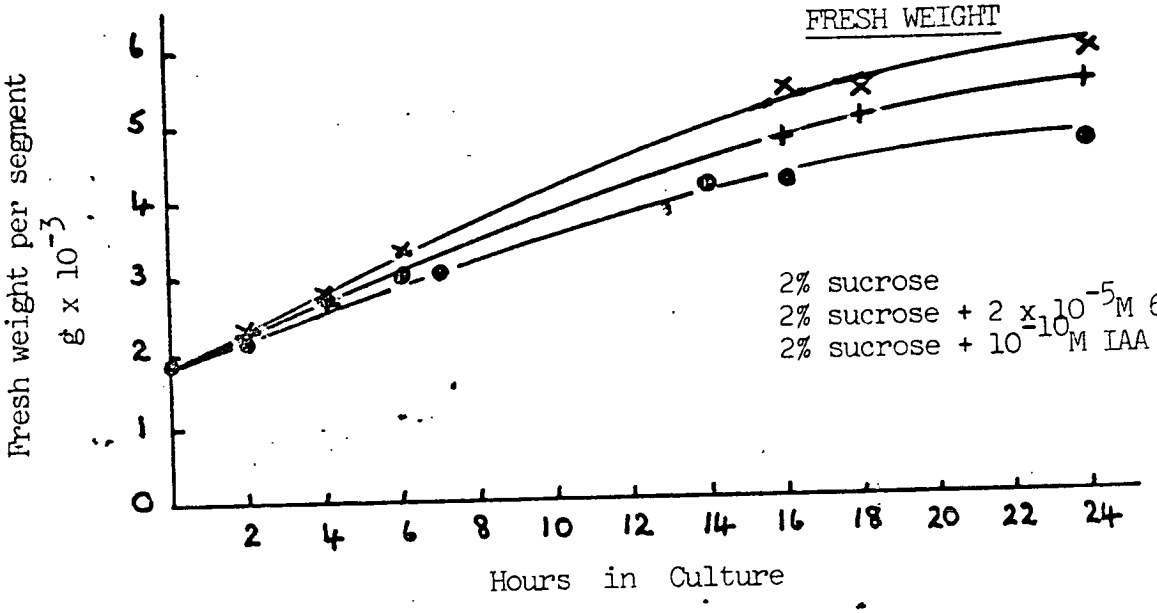


FIGURE 4-20

DRY WEIGHT

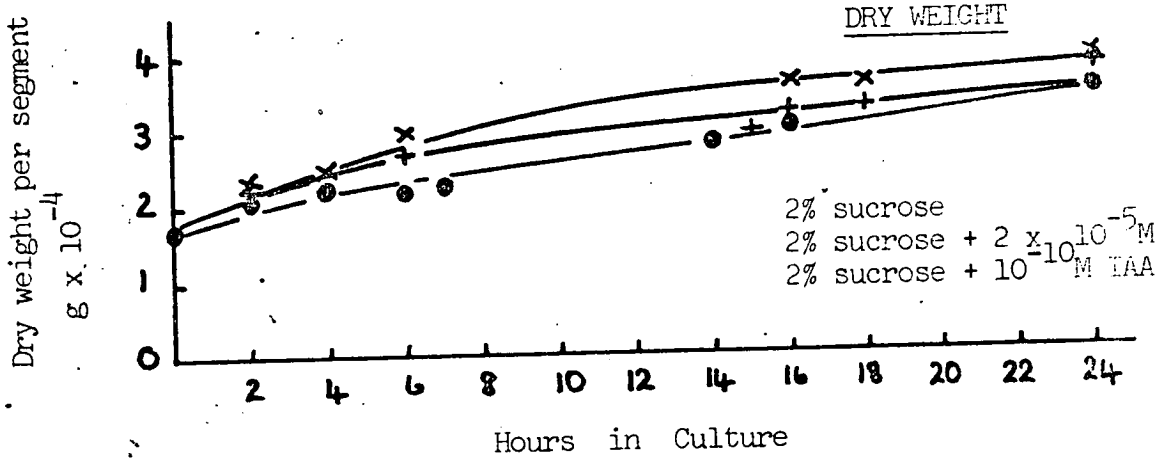


FIGURE 4-21

RESIDUAL DRY WEIGHT

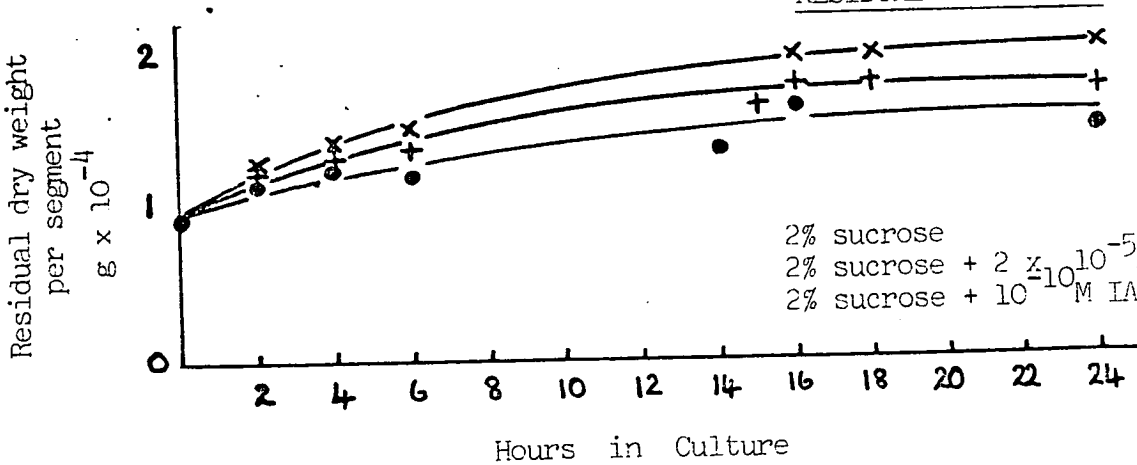


TABLE 23

PROTEIN CONTENT OF 2-4 mm SEGMENTS AFTER CULTURING IN SUCROSE IN PRESENCE AND ABSENCE OF 6-METHYL PURINE AND INDOLE ACETIC ACID

HOURS IN CULTURE	MEDIUM	PROTEIN PER SEGMENT g x 10^{-5}
0	Fresh Tissue	8.7
3	S	8.9
	S + 6MP	8.9
	S + IAA	8.8
6	S	8.7
	S + 6MP	8.8
	S + IAA	9.0
9	S	9.0
	S + 6MP	8.8
	S + IAA	8.9
15	S	8.7
	S + 6MP	8.8
	S + IAA	8.7
24	S	8.8
	S + 6MP	8.9
	S + IAA	8.9

S - 2% Sucrose
6MP - 6 methyl purine 2 x 10^{-5}M
IAA - Indole Acetic acid 10^{-10}M

Each value calculated from duplicate measurements from each of two flasks for each treatment. The variation from the mean was never greater than 1.0 g x 10^{-5} per segment.

tips, showed a reduction in nitrogenous and nucleic acid content. The results presented in Table 23 demonstrate that the protein content of segments cultured in 2% sucrose and sucrose supplemented with 6MP (2×10^{-5}M) or IAA (10^{-10}M) did not change over the 24 hour culture period. The protein content remained at about 8.8 g x 10^{-5} per segment. Therefore, the increase in residual dry weight was probably brought about by the increase in cell wall polysaccharides, of which the main constituent is cellulose.

j. Enzyme Changes During Culture - Introduction

Although, the total protein content of the cultured segments does not change, there is evidence from previous workers that shows that the pattern of the protein complement changes during growth and differentiation (e.g. Heyes and Brown 1965). A variety of enzymes have been assayed in the root culture system e.g. Robinson and Brown (1954) found the invertase activity decreased and the phosphatase activity increased during culture; Vaughan (1965) found invertase, phosphatase and ribonuclease activities peaked at 10, 12 and 6 hours, respectively, in a 24 hour culture period in 2% sucrose. In the present work, glucose-6-phosphate dehydrogenase and cellulose synthetase were assayed. The former was chosen as a general metabolic indicator and also because of its involvement in respiration and the production of pentose sugars which are used in nucleic acid synthesis and indirectly for cell wall synthesis. Cellulose synthetase was chosen as the increase in cell wall polysaccharide is the most notable increase in weight, second to the uptake of water during extension growth in the root segments.

k. Changes in Glucose-6-Phosphate Dehydrogenase Activity in Excised Segments during Culture in Various Media

The G6PDH activity in pea root segments growing in different

culture media was monitored through 24 hour experiments. The activities are expressed as a percentage of the activity in fresh root, or T_o, segments. In the accumulated activity data presented, Figures 4-22, 4-23 and 4-24, the T_o values in each experiment are always expressed as 100%, the actual values were 8.7 ± 0.5 $OD^{(340\ nm)}$ increase per minute per segment, with the Standard Deviation calculated with $n = 8$.

1. 2% Sucrose

During culture in 2% sucrose, Figure 4-22, the G6PDH activity increased almost linearly to a maximum of 145% at 12 hours, increasing at a rate of 3.75% per hour. The recorded activity then decreased to 10% above the initial T_o rate by the end of the 24 hour period. In some early experiments, the activity took a sharp upward trend at 24 hours but it is believed that this high activity was due to infection. When the average of all the 24 hour points was taken, the activity fell in the latter 12 hours of the culture period to 27% above the initial value.

The G6PDH activity was measured in separate extracts from freshly cut segments and from segments cultured for 24 or 48 hours. The extracts were then mixed in known proportions and the recorded activity compared with that expected from the separate assays. The results given in Table 24 show that the activities are additive, no inhibitive or stimulatory effects were noted.

FIGURE 4-22

The Change in Glucose-6-Phosphate Dehydrogenase Activity During Culture in 2% Sucrose

Legend

The small symbols show the activities calculated from 2 flasks each of 20 segments from at least 3 experiments. The large symbols show the average activities calculated from the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o activity within each separate experiment.

FIGURE 4-22

THE CHANGE IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY DURING CULTURE IN 2% SUCROSE

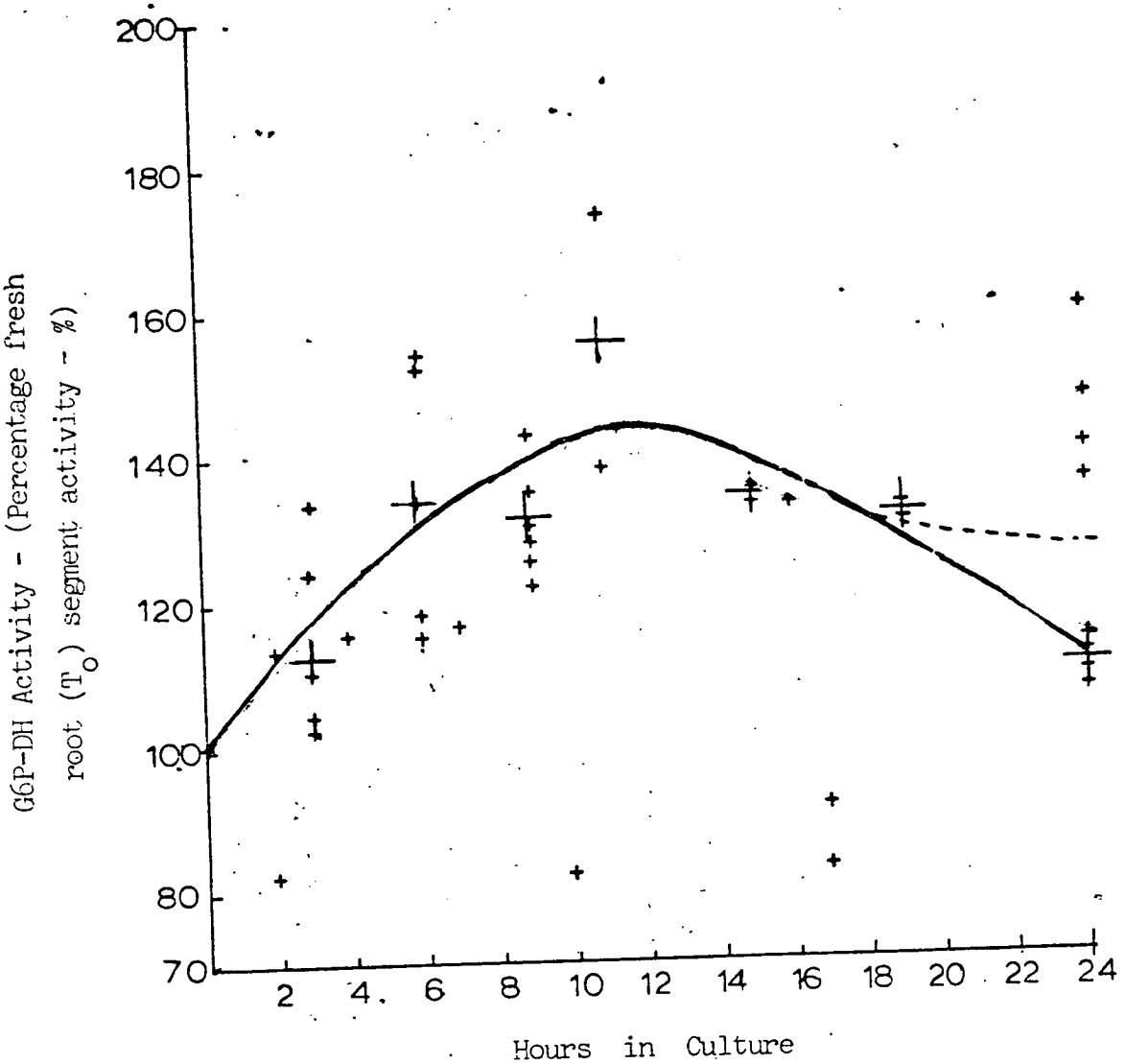


Table 24

G6PDH Activities in Mixed Extracts

Expected Activity	T_o ml	T_{24} ml		OD Increase per minute per segment x 3 Observed Activity
	0.10 = 10.6	0.10 = 15.1	Total = 25.7	25.1
	0.15 = 15.9	0.15 = 22.6	" = 38.5	34.5
	0.05 = 5.3	0.15 = 22.6	" = 27.9	26.3
	T_o ml	T_{48} ml		Observed Activity
	0.10 = 7.8	0.10 = 14.4	Total = 22.2	22.7
	0.15 = 13.1	0.15 = 22.2	" = 35.3	34.2

Therefore, as the T_{24} and T_{48} extracts did not reduce the activity present in the T_o extract, it may be considered that the loss of activity during culture is not due to the production of inhibitors of enzyme activity.

2. In the Presence of 6-Methyl Purine

6-Methyl purine, as an inhibitor of RNA synthesis would be expected to stop any rise in enzyme activity if the rise was dependent on 'de novo' protein synthesis. Aitchison and Yeoman (1973) found that 6MP prevented the increase in G6PDH activity which preceded the early period of synchronous division in Jerusalem artichoke. In the presence of a concentration (2×10^{-3}M) of 6MP which completely inhibited segment extension, the G6PDH activity decreased throughout the experimental period

to 15% at 24 hours. See Figure 4-23. The G6PDH activity in the 2 x 10^{-5}M 6MP supplement medium, increased to 120% in the first 4 hours, decreased steeply to 80% by 16 hours and then decreased a further 10% in the remaining 8 hours to 70% of the T_o level. The 20% increase observed with 2 x 10^{-5}M 6MP in the initial 4 hours may reflect the period required for the inhibitor to enter the cells and become effective.

When the segments were cultured for 12 hours in 2% sucrose and the G6PDH activity assayed in the presence of 6MP (2 x 10^{-5}M) the activity was 13.7 $\pm$ 0.3 OD increase per minute per segment compared with 12.7 $\pm$ 0.18 in the absence of 6MP. Therefore 6MP appears not to be reducing the recorded G6PDH activity by an allosteric effect on the G6PDH molecules.

3. In the Presence of IAA

When 10^{-4}M IAA was present in the culture medium, segment elongation was inhibited compared to sucrose alone and the G6PDH activity decreased throughout the culture period, Figure 4-24 . A small peak may be present between 4 and 10 hours but it may be a product of the variability in the results. The 20% rise to 55% between 16 and 24 hours may be real and possibly due to the reduction in the IAA concentration and release from inhibition. During culture in sucrose with 10^{-10}M IAA, the G6PDH activity increased by just over 30% in the initial 5 hours, then decreased by 20% in the following 5 hours and remained at 110% to the end of the 24 hour culture period. Figure 4-24.

4. Conclusions

In 2% sucrose, the G6PDH activity increased by about 40% in

FIGURE 4-23

The Change in Glucose-6-Phosphate Dehydrogenase Activity During Culture in 2% Sucrose in Presence of 6-Methyl Purine

Legend

The small symbols show the activities calculated from 2 flasks each of 20 segments from at least 3 experiments. The large symbols show the average activities calculated from the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o activity within each separate experiment.

FIGURE 4-23

THE CHANGE IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY DURING CULTURE IN 2% SUCROSE IN PRESENCE OF 6-METHYL PURINE

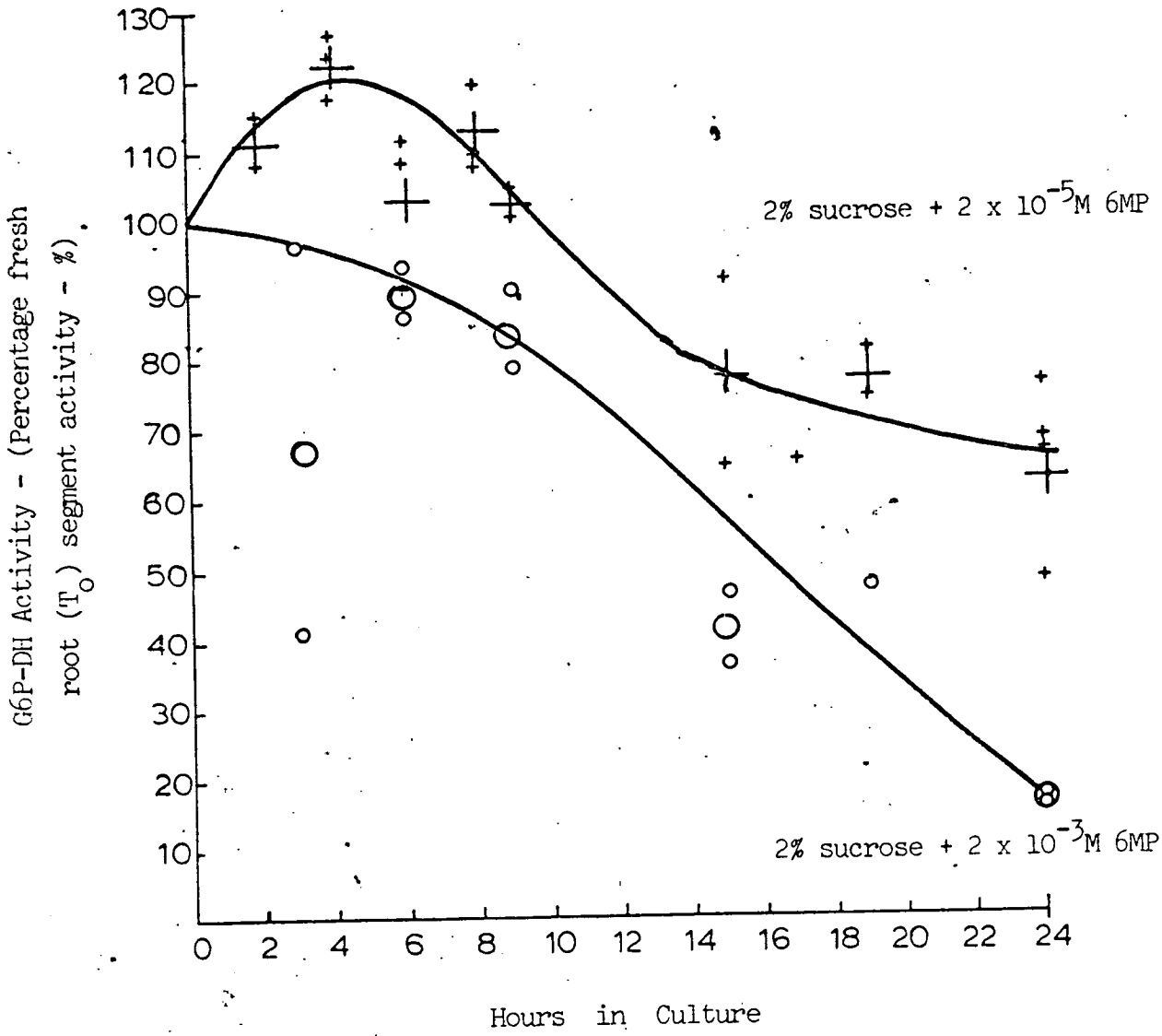


FIGURE 4-24

The Change in Glucose-6-Phosphate Dehydrogenase Activity During Culture in 2% Sucrose in Presence of Indole Acetic Acid

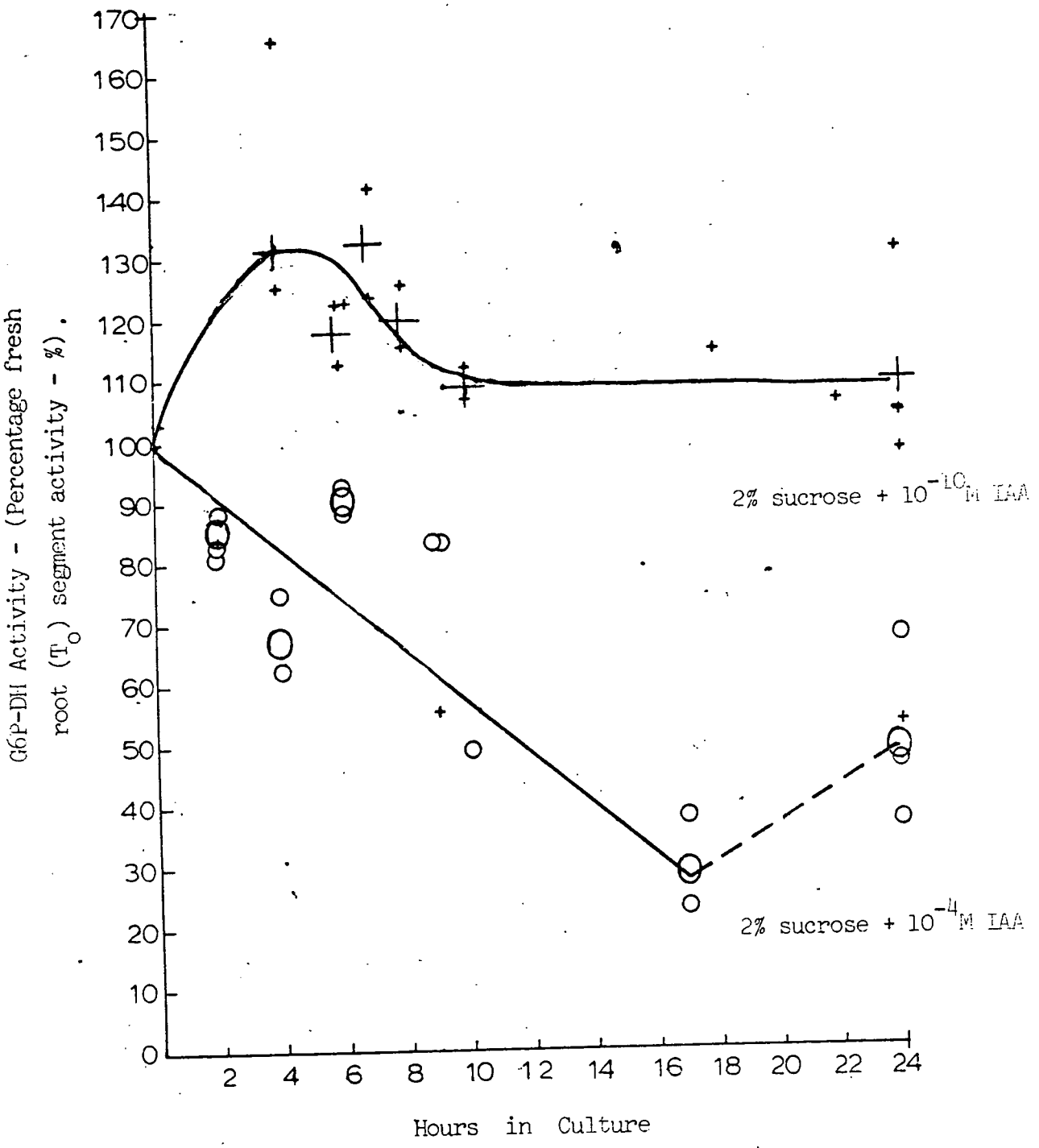
Legend

The small symbols show the activities calculated from 2 flasks each of 20 segments from at least 3 experiments. The large symbols show the average activities calculated from the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o activity within each separate experiment.

FIGURE 4-24

THE CHANGE IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY DURING CULTURE IN 2% SUCROSE IN PRESENCE OF INDOLE ACETIC ACID



12 hours then decreased to the original level by 24 hours. The addition of 6MP prematurely terminated the increase in activity after a 20% increase at 4 hours, the activity then declined at a greater rate than in 2% sucrose. The 10^{-10}M IAA supplement brought about a similar initial peak as 6MP (2×10^{-5}M) but the activity did not decrease to such an extent over the remaining hours of the culture period.

1. Changes in Cellulose Synthetase Activity in Excised Segments during Culture in Various Media

Cellulose synthetase was assayed to compare the pattern of activity during culture with the G6PDH pattern. Also, this enzyme was considered to be of interest as the cell wall polysaccharide increase appeared to be the only synthesising pathway in an otherwise decaying system. The CS activity has also been expressed as the percentage change from the fresh root or T_o segment activity. In all experiments, the T_o values have been expressed as 100%; the actual activity was 21 $\pm$ 4 cpm per segment, the Standard Deviations calculated with n = 8.

1. 2% Sucrose

When the root segments were cultured in 2% sucrose, the CS activity decreased to 36% in the first 2 hours before rising in a sharp peak to 305% at 9 hours then decreased again to 54% by 16 hours and 13% at 24 hours; see Figure 4-25.

2. In the Presence of 6-Methyl Purine

In the presence of 2×10^{-5}M 6MP the peak of CS activity was lower but more widely based, as shown in Figure 4-26, the activity increased to 181% at 8 hours then decreased in a slightly convex curve to 55% at 18 hours and levelled to 41% at 24 hours.

FIGURE 4-25

The Change in Cellulose Synthetase Activity
During Culture in 2% Sucrose

Legend

The small symbols show the activities calculated from 2 flasks each of 20 segments from at least 3 experiments. The large symbols show the average activities calculated from the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o activity within each separate experiment.

FIGURE 4-25

THE CHANGE IN CELLULOSE SYNTHETASE ACTIVITY DURING CULTURE IN 2% SUCROSE

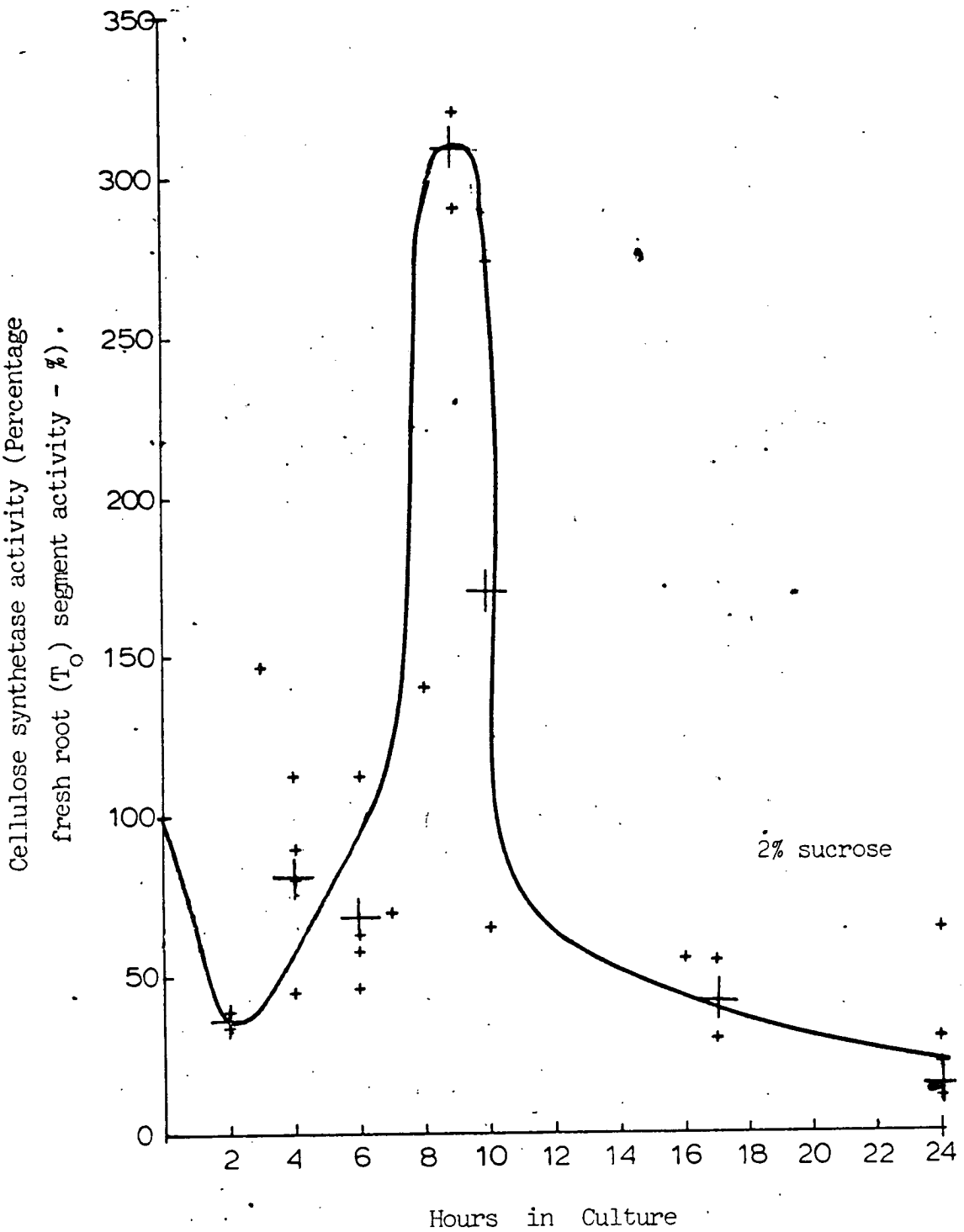


FIGURE 4-26

The Change in Cellulose Synthetase Activity During Culture in 2% Sucrose in Presence of 6-Methyl Purine

Legend

The small symbols show the activities calculated from 2 flasks each of 20 segments from at least 3 experiments. The large symbols show the average activities calculated from the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o activity within each separate experiment.

FIGURE 4-26

THE CHANGE IN CELLULOSE SYNTHETASE ACTIVITY DURING CULTURE IN 2% SUCROSE IN PRESENCE OF 6-METHYL PURINE

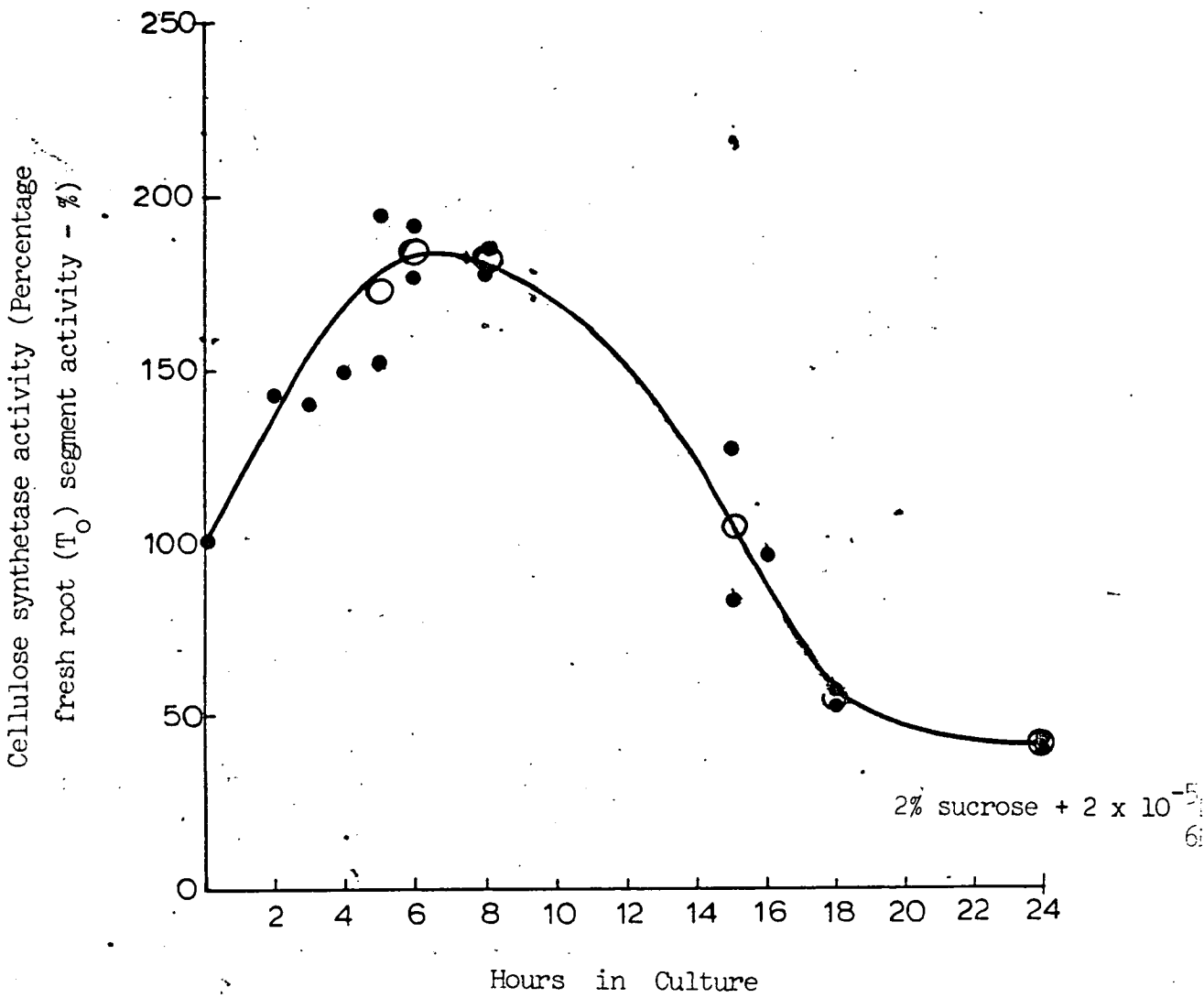


FIGURE 4-27

The Change in Cellulose Synthetase Activity During Culture in 2% Sucrose in Presence of Indole Acetic Acid

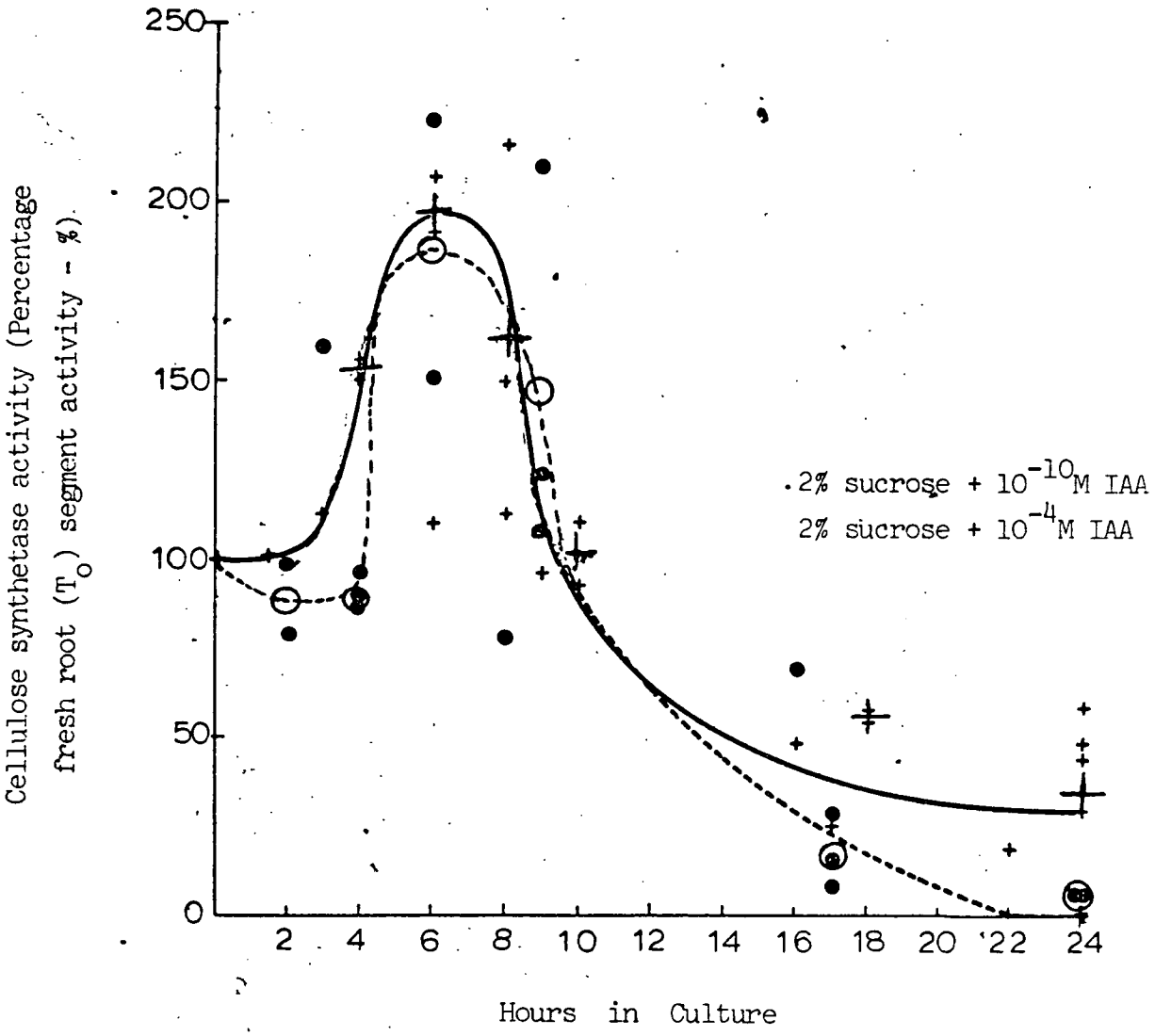
Legend

The small symbols show the activities calculated from 2 flasks each of 20 segments from at least 3 experiments. The large symbols show the average activities calculated from the separate experiments for each sample time. The curves are drawn with reference to the large symbols.

The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o activity within each separate experiment.

FIGURE 4-27

THE CHANGE IN CELLULOSE SYNTHETASE ACTIVITY DURING CULTURE IN 2% SUCROSE IN PRESENCE OF INDOLE ACETIC ACID



3. In the Presence of IAA

The CS activities during culture in 2% sucrose with 10^{-4}M IAA were similar even although the latter stimulated and the former inhibited extension growth, compared to growth in sucrose alone. In both culture media the activity peaked at 6 hours. In 10^{-4}M IAA, the CS activity decreased by 10% over the initial 4 hours before rising to a 187% peak at 6 hours. The activity then fell throughout the culture period to 0% at 24 hours. In 10^{-10}M, IAA the activity increased to a 198% peak at 6 hours after an initial 2 hour lag, the activity then dropped to 35% at 18 hours and levelled to 30% at 24 hours. With the overlap of points, the two curves may be considered to be the same. Figure 4-27.

4. Conclusions

The addition of 6MP (2×10^{-5}M) or IAA (10^{-10}M) to the 2% sucrose medium, generally decreased the height of the peak of activity and broadened the base of the peak, compared to the pattern of activity in sucrose alone. Both these effects were greatest in the 6MP cultures.

The two enzymes G6PDH and CS showed different patterns of activity during the culture of root segments in the same culture conditions, and the patterns vary with different culture conditions. The activities are not directly related to the segment size or the amount of cell protein.

CHAPTER FIVE

CHAPTER 5

DISCUSSION

Section 1 - Segment Culture System

Fragments taken from the expanding zone of roots undergo considerable extension growth when cultured in contact with suitable media, and provide a good system for the study of cell expansion. (Brown and Broadbent 1951, Vaughan 1965). In this investigation, 2 mm fragments taken 2 mm behind the apex of pea seedling primary roots were cultured in a liquid medium in flasks on a rotary shaker. Preliminary experiments were performed to show the best variety, size of root and type of segment, and the optimum conditions for segment growth i.e. germination conditions, shaking speed and volume of culture medium.

Agitated cultures were used in preference to stationary cultures to ensure the segments grew straight. Further, in stationary cultures actively growing segments use up the medium in their vicinity and concentration gradients of nutrients and gases will be formed within the culture.

The greatest problem encountered with the culture system was the variability in growth response of the excised root fragments. This variability may originate from the fact that the constituent cells of the fragments are at slightly different stages of development and respond differently to culture. Segments taken from long roots (3.5 - 5.0 cm) where the differences in development of the constituent cells within the fragment are fairly large show a more variable growth response in culture when compared with segments taken from shorter roots (2.5 - 3.5 cm) (Table 7). The variation could also arise from differences between growth capacities of the segments. Some of these differences probably originate from the mixed genetical nature of the seeds, but this is not

considered to be important. However, the variability is appreciably reduced when care is taken in selecting the seedling roots, paying particular attention to the root width. Roots about 1 mm wide and 2.5 - 3.5 cm long were selected. The widths of the excised segments from the selected roots were determined by the shadow technique (see Chapter 2) and were selected to lie within the range 0.99 - 1.03 mm.

Another problem, general to culture systems, is that of maintaining sterility. In this study, the materials and apparatus were carefully sterilised but it proved impossible to preserve complete asepsis because of the bulk of tissue used in each experiment. However, the culture period was not usually extended beyond 24 hours so that any contaminants did not grow sufficiently to distort the growth response. Fortunately, a 24 hour culture period was long enough to observe the main changes which occur in the segments during extension growth in culture.

It must be emphasised that the careful selection of plant material and standardisation of culture methods is extremely important in studies of this type. However, a degree of variability in the growth response of segments was present despite the steps taken and had to be taken into consideration when the results were analysed.

Section 2 - Enzyme Kinetics

There is evidence from previous work (Heyes and Brown 1965, Tulett 1967) that the pattern of the cell protein complement changes during growth and differentiation. In this investigation, the activities of the enzymes glucose-6-phosphate dehydrogenase (G6PDH) and cellulose synthetase (CS) were examined in excised root segments during extension growth under a range of culture conditions.

Whenever a new enzyme is introduced to a study preliminary investigations must be performed to establish that the activity measured

is directly related to the catalytic protein present in the tissue. This is especially important when working with crude homogenates. The physical conditions and the concentration of reactants which give the optimal recorded enzyme activity must be found. In a crude homogenate other reactions may interfere with the one being examined. Interference can be due to complexing with the substrate, formation of a similar product or by inhibition of the enzyme under examination. The preliminary investigations are vital to give confidence to the validity of the pattern of enzyme activities found during culture of the segments. The activities have been calculated on a per segment basis but expressed in some cases as percentage change from the fresh root activity (T_o). The values presented are directly proportional to the activity expressed on a per cell basis, as the cell number is constant. Also, the values are directly proportional to the specific activity i.e. with respect to the total protein content of the segment, as during the 24 hour culture period the total amount of protein stays constant. Enzyme activity and not the amount of catalytic protein present was measured in the segment extracts. Mixed extract assays, with extracts taken from segments cultured for different times T_o, T_{24}, T_{48}, verified that inhibitor or promoter factors of enzyme activity were not present and so the enzyme activities gave an approximate value for the catalytic protein present in the segments.

a. Glucose-6-phosphate Dehydrogenase

The spectrophotometric assay for G6PDH is straightforward and no major difficulties were encountered during attempts to find the optimal assay conditions. These are detailed in Chapters 2 and 3. The enzyme proved to be very temperature sensitive and so it was important to control the temperature of the reaction cuvettes during

the assays. The character of the G6PDH enzyme is not markedly different in T_o and T_{24} extracts, as shown by the results of experiments on pH, volume of extract i.e. amount of tissue, and the time course of change in optical density (Chapter 3). Therefore the same assay technique was applied to all the segment extracts.

b. Cellulose Synthetase

The pathway of cellulose synthesis in higher plants still evades complete understanding. Cellulose synthetase may be compared to the closely examined and well documented enzyme DNA polymerase. Both enzymes are components in complex systems although the constituent units of DNA polymerase are larger and varied. The difference in knowledge of the two synthesising systems reflects the attention by research workers but cellulose synthetase is now becoming more favoured as a research topic.

Cellulose is composed of units of cellobiose (C_{12} H_{22} O_{11}) a disaccharide isomer of maltose, formed from two molecules of glucose (Robertis, et al, 1954). Hassid, et al (1959) found that glucose-1-phosphate was ineffective as a donor molecule in cellulose synthesis and for many years it has also been debated whether UDPG or GDPG is the glucose donor molecule. The literature was reviewed in Chapter 1. In this work, both UDPG and GDPG were used as substrates in the assay of cellulose synthetase with T_o and T_{24} root segment extracts. Incorporation of radioactively labelled glucose was observed from UDPG but with the exception of one isolated assay, no incorporation was noted from GDPG. This result would not be entirely unexpected considering the work of Franz (1967) who showed the presence of UDPG but failed to show the presence of GDPG in a range of plant tissues. However, GDPG can be an effective donor in cellulose synthesis. Brummond and Gibbons (1965)

found cellulose could be synthesised from either UDPG or GDPG by Lupinus albus extracts. Ordin and Hall (1967) showed that a particulate fraction from oat coleoptiles could use either UDPG or GDPG. Delmer, Beasley and Ordin (1974) showed that the incorporation into cellulose in intact young cotton fibre hairs from GDPG is high during rapid elongation and primary wall synthesis, but UDPG is more important in older fibres. Delmer et al (1974) performed their assays with intact tissues at pH 6.5 which differs from the assay conditions adopted in this investigation where a particulate extract was used, although no incorporation of glucose was noted from GDPG at pH 6.5 in the pea root tissue extracts. The degree of integrity of the tissue and the plant species may affect the type of donor molecule and the optimal pH conditions for the reactions. Much work still remains to be done before it can be said that the synthesising processes of cellulose are widely understood; only a few of the problems have been mentioned here.

The cellulose synthetase activity was measured in this investigation by following the incorporation of radioactively labelled glucose into a cellulose fraction, from UDPG by a particulate tissue extract. The cellulose fraction was initially, by convention, recognised by its solubility characteristics - it was the fraction insoluble in hot and cold water and hot alkali. But more recent work (Delmer et al, 1974) showed that the insoluble product remaining may be further characterised. They showed that 90% of the hot alkali insoluble product from UDPG, in cotton fibre hairs, was soluble in chloroform - methanol. This soluble fraction was tentatively recognised as glucolipid and when chromatographed in lipid solvents, separated into 3 components. Further, two of the components increased with the increasing age of the cotton hairs. Therefore as UDPG is used as the glucose donor for cellulose synthesis

in this investigation the alkali insoluble product was examined. It was found that about 20% of the radioactive counts are washed by chloroform-methanol from the water and alkali insoluble 'cellulose' product obtained from the reaction of T_o segment particulate extracts of segments cultured for 24 hours in 2% sucrose. The proportion is much smaller than the 90% recognised by Delmer et al (1974) in cotton hairs, but the trend of increasing proportion of 'glucolipid' fraction with aging of the tissue is similar. Only the counts incorporated into the fraction insoluble in hot water, hot alkali and chloroform-methanol, are used as a measure of the cellulose synthesising capacity of the segments.

Although it is not within the scope of this work, the role of lipids in cellulose synthesis and the complex nature of the product of the cellulose synthetase reaction deserve some attention. The role of lipids in cellulose synthesis has been mentioned by various workers but apparently always where UDPG is used as the precursor to cellulose synthesis, and not GDPG. A lipid carrier is involved in the secretion of UDPG (Hassid et al 1959). Cellulose is formed by an alcohol soluble fraction from Acetobacter (Colvin 1959). Later, Pinsky and Ordin (1959) working with oat seedling particulate fractions, which synthesised cellulose from UDPG, concluded that a lipid intermediate was used for transporting glucose across membranes. Villemez and Clark (1969) concluded from rate, isotope dilution and direct incorporation studies that a lipid soluble material is a direct precursor of higher plant polysaccharides and the intermediate is a glucolipid tightly bound to the particulate enzyme system. The intermediate was physically recognised as a branched lipid by Manley, Jonker, Cooper and Pound (1971).

Characteristics of the cellulose product have been shown to be

dependent on the assay conditions. In oat coleoptiles with a low concentration of UDPG, 1-3 and 1-4 glycosyl linkages are produced in the hot alkali - insoluble product; with higher concentrations of UDPG, the 1-4 linkages predominate. The addition of $MgCl_2$ under both conditions decreases the fraction of 1-3 linkages (Tsai and Hassid 1973). Similar results were found with oat coleoptiles by Ordin and Hall (1968).

Although the detailed nature of the cellulose synthetase assay product was not analysed in this investigation, the levels of incorporation of radioactively labelled glucose may be confidently used as a physiological indicator, monitoring at least part of the cellulose synthesising capacity of the segments, provided the conditions of assay are not varied. The results of enzyme kinetic experiments on pH and volume of extract against activity showed that the cellulose synthetase does not markedly change during culture. Isozymes, which are multiple forms of an enzyme and catalyze the same general reaction but have distinctive chemical and physical properties, were not obviously detectable. The same assay technique was applied to all cultured segment extracts.

The basis of an assay technique for cellulose synthetase in pea root tissue has been developed. It is suggested that in any further investigations the nature and behaviour of the organic soluble and insoluble fractions of the cellulose synthetase reaction should be examined with particular attention given to the change during culture.

Section 3 - Segment Growth During Culture - The Effect of Medium Activities

In the following discussion on growth and in the subsequent section on the enzyme pattern in root segments during culture, the figures and data which will be considered have been obtained from the combined results of a number of experiments. In some cases, the differences between

treatments within a single experiment are not statistically significant, as shown in the single experiment results presented in Chapter 4. It is believed that a more reliable impression of the patterns of change in the segments during culture in the various media is obtained from the figures where the curves are drawn through many points. The larger number of points is particularly desirable due to the inherent variability present in this type of culture system. The differences which may exist between separate experiments due to slightly varying conditions are overcome by expressing the results as a percentage change from the T_o segments.

The basic parameter used to measure growth is change in segment length. The segment width increased by up to 20% in the 24 hour culture period. This increase, in terms of effect on volume is equivalent to a 44% increase in length. However, it was felt that the extra work involved in measurement of width and calculation of segment volume was not justifiable. Further, the cell number is constant throughout culture in all media used (Table 21) and so the interpretation of the changes in the segments during expansion growth are not complicated by cell divisions. Previously, Vaughan (1965) also showed that there was no change in the cell number of pea root segments during culture in a range of media, including one which contained 2-Thiouracil (2-TU) and stimulated extension growth.

a. Growth in the Presence of 6-Methyl Purine

In this investigation, the addition of 6-methyl purine (6MP), at a concentration of 2×10^{-5}M, to the 2% sucrose medium stimulates extension growth of the root segments by extending the duration of the initial rate of growth. (Figure 4-16). The length increases measured in 3 media are shown in Figure 5-28, and Table 25 gives a summary of the rates and

FIGURE 5-28

COMPARISON OF INCREASE IN SEGMENT LENGTH

DURING CULTURE IN VARIOUS MEDIA

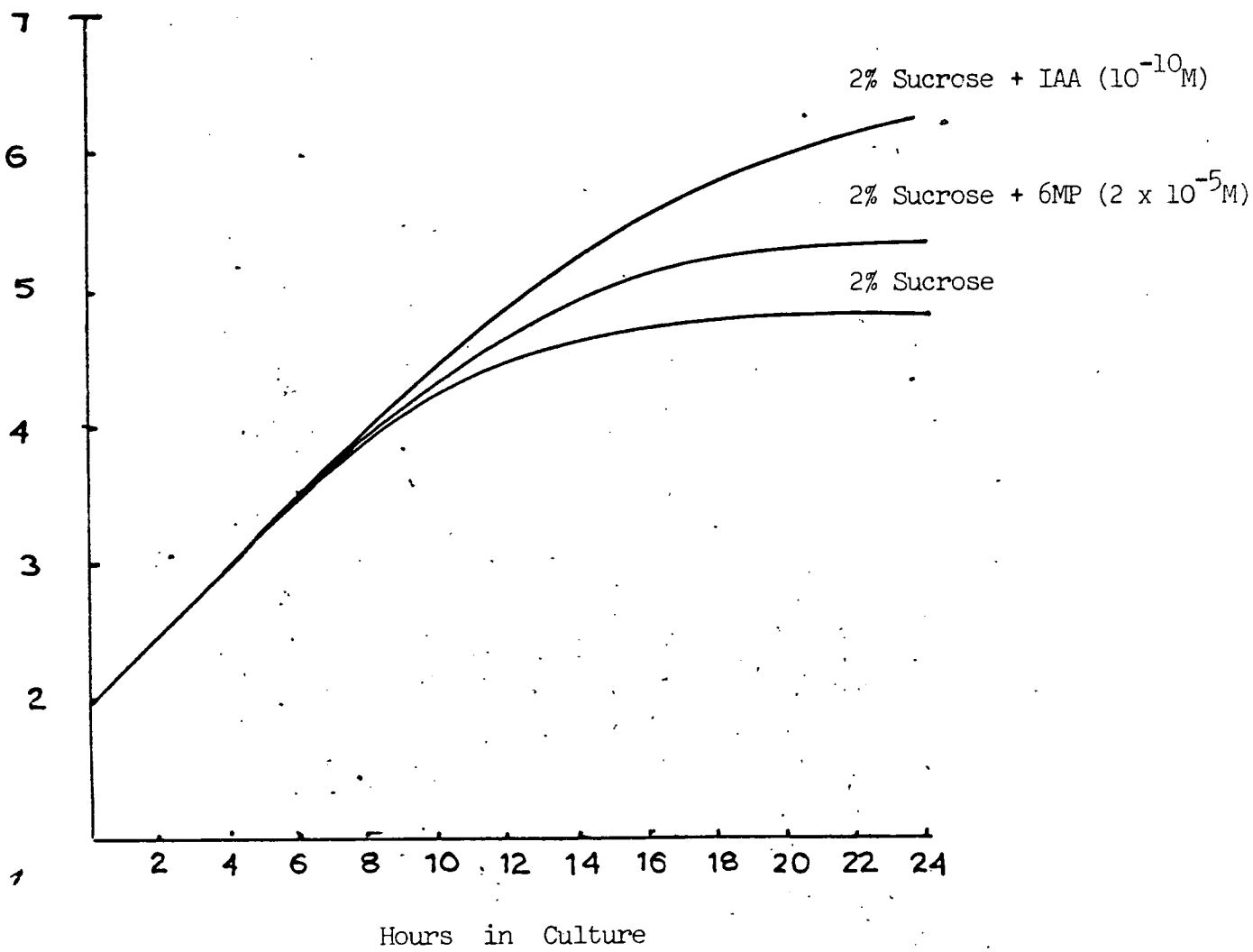


TABLE 25

COMPARISON OF SEGMENT GROWTH

RESPONSE IN VARIOUS CULTURE MEDIA

Medium	Initial Growth Rate mm/hr	Duration of Initial Growth Rate - Hours	Time Zero Rate Attained - Hours	Average Segment Length at T_{24} Hours - mm
Sucrose	0.23	9	18	4.8
Sucrose + 6MP	0.23	11	18	5.4
Sucrose + IAA 10^{-4} M	0.09	8	18	3.1
Sucrose + IAA 10^{-10} M	0.23	12	(0.12 mm/hr at T_{24} hours)	6.3

Legend

Values obtained from curves drawn through points from accumulated data.

duration of extension growth, calculated from the accumulated data. The initial growth rates are the same, 0.23 mm/hour, both in sucrose alone and in sucrose supplemented throughout the culture period with 2×10^{-5}M 6MP. Woodstock and Brown (1963) reported that 2-thiouracil (2-TU) stimulates elongation in pea root cells in the expanding phase of growth by extending the period of active growth, whereas Vaughan (1965) found that 2-TU (2×10^{-3}) stimulates only the growth rate. However, he found 8-Azaguanine (10^{-4}M) increases the duration of extension growth in pea root segments in culture and that other analogues, not including 6MP, have no effect on extension growth of the segments.

The stimulatory effect of 6MP on the duration of extension growth can be interpreted as supporting the concept of cell development presented initially by Brown (1963) from work on the analysis of serial sections of roots and root fragments grown in culture. He stated that as each cell expands, the activities of the enzymes change relative to each other and so the metabolic status of the cell is considered to be changing. Brown (1963) and Heyes and Brown (1965) characterised expansion by a series of enzyme complexes, E_1, E_2 and E_3. E_1 expressed the complex of the meristematic state, E_2 the expanding state and E_3 the state in which expansion ceases. The enzymatic proteins of the states differ quantitatively and not qualitatively. However, the notation E_1, E_2 and E_3 gives the impression that cell development is occurring in discrete steps, however this is not how Brown (1963) visualises it. The notation is used only to make the explanation easier and to denote the grossly definable stages. Development is a continuous process with each more or less definable phase such as rapid expansion, merging gradually into the next phase, the expansion rate slowing down until cell expansion ceases. When cells in the rapid expansion growth stage are examined, the physiological

characteristics such as enzyme activities, and protein and carbohydrate content may be noted. Brown (1963) suggests that these physiological characteristics define the cellular conditions necessary for the particular growth phase. However, it may be alternatively suggested that the cells undergoing, for example, rapid expansion growth, may have a physiology characteristic of the next growth phase, i.e. the cells are in preparation for the next stage of development. For example dividing cells synthesise DNA during the S period of the cell cycle, in preparation for the following cell division. It will be difficult to distinguish experimentally between the two possibilities but the former seems at present more acceptable if only for its simplicity.

The question of how the cells pass through cell development, from the newly formed cell in the meristem to the full differentiated form, is intriguing. The gradual change from one enzyme complex to the next is not an autonomous process and requires changes in the RNA synthesised (Heyes and Brown 1965). Greater overall growth may be obtained by either, or both, a greater growth rate when the cell is in the E_2 rapid expansion state or to remain in the E_2 state for a longer period of time before transition to the mature or E_3 non-expansion state.

The greater growth rate in the E_2 state may be attained by a general increase in metabolic rate, brought about by conditions such as a change in the level of IAA. The increased metabolic rate would also result in a quicker transition from the E_2 to E_3 state, assuming that development continued along the same pathway in the presence of IAA. The quicker transition is assumed to take place by increasing the metabolic rates, increasing the rates of RNA synthesis and transition through the phases of development. However, an increase in metabolic rate need not necessarily result in a quicker transition from E_2 to E_3 if the metabolic

state depends on the pools or stores of compounds in the cell. The quantity of materials in a pool may remain constant, in equilibrium, even though the rates of production are increased, provided the rates of removal of compounds from the pool are increased proportionally.

By the concept presented initially by Brown (1963) inhibitors of RNA synthesis should stop the development from the E_2 to the E_3 state and so maintain the cell in the expanding growth phase for a longer period of time. The phase will not be maintained indefinitely as turnover of proteins and RNA species will occur and there will be little or no replacement synthesis of RNA species occurring in the presence of the inhibitor. The time the rapid elongation phase is extended will depend on a number of factors but mainly the 'life' of the RNA and protein species of the E_2 state and the quantities present. When 2-thiouracil, an RNA synthesis inhibitor, and ribonuclease are applied in the early stages of expansion growth, they have a marked inhibitory effect on extension, but when applied in the later stages, they have a marked stimulatory effect (Brown 1963). Yeoman (1962) from his work on extension growth in pea root segments concluded that ribonuclease affects only the rate and not the time of expansion. In the present investigation, 6-methyl purine is presumed to inhibit the synthesis of RNA such that the RNA species necessary for the continuation of cell development are not produced and so the cells remain in the rapidly expanding state, as removed from the fresh seedling root, for a longer period of time.

The effects of 6MP on uridine and leucine incorporation were not measured in this investigation but previous work shows that 6MP does inhibit RNA synthesis in other tissues. Key (1961) found in soyabean hypocotyls after 3-4 hours exposure to 6MP that $^{14}(C)$ - leucine incorporation is inhibited by 87%. White (1970) found 6MP effective in

blocking 'de novo' amylase synthesis in response to barbiturates in barley endosperm. Aitchison and Yeoman (1973) found 6MP inhibits both uridine and amino acid incorporation in growing Jerusalem artichoke cultures but could not distinguish whether 6MP affects protein synthesis directly by preventing the production of m-RNA, or indirectly by restricting the supply of other RNA required for continued protein synthesis. Therefore, it is not unreasonable to assume that 6MP is inhibiting RNA synthesis in the pea segments growing in culture and thus stimulating extension growth as postulated by Brown (1963). However, the effects of 6MP on uridine and leucine incorporation in cultured pea root segments must be examined to verify this suggestion.

b. Growth in the Presence of Indole Acetic Acid

Indole acetic acid (IAA), at the concentration of 10^{-10}M also stimulates extension growth of excised pea root segments in culture by increasing the duration of the initial rate of growth (Figure 5-28). The effect is more marked than the increase in duration of rapid growth observed when 6MP (2×10^{-5}M) is added to the basic sucrose culture medium. The data are compared in Table 25. The duration of the initial rate of growth, of 0.23 mm/hour, is lengthened by 12 hours in the presence of IAA (10^{-10}M), compared with 11 hours in 6MP. Also, the growth rate in the presence of IAA only gradually declines until it is halved by the end of the 24 hour culture period, whereas in 6MP the rate is zero by 18 hours. It cannot be assumed that IAA is acting in a similar manner to 6MP, even though it brings about a similar effect.

Auxins have a range of effects on plant tissues and organs, and many theories exist for their mode of action. Some of the literature was reviewed in Chapter 1. Briefly, the initial action of auxin is probably (Barkley and Evans, 1970 and Andreae, 1967) in the cell wall,

possibly associated with the proteins of the wall for which an effect on RNA and protein synthesis is required for continued stimulation. In most of the works on the mode of action of auxin, growth is measured over a period of minutes and a stimulation of growth rate is noted. In the present work, this early stimulation of growth rate may have occurred but was not detected. Over the 24 hour culture period IAA has the effect of extending the period of rapid elongation growth. In this case, IAA may be acting by maintaining the conditions necessary for the rapid growth rate, either by its effect on the cell wall or on RNA and protein synthesis. The segments cultured in sucrose alone may have exhausted their endogenous supply of auxin at an early stage of culture.

c. Growth in the Absence of Sucrose

Sucrose must be present for the stimulation of extension growth observed when 6MP or IAA are added. Also no significant increase in segment length can be detected in the absence of sucrose (Table 19). It is probably required to maintain the osmotic pressure in the expanding cells and to provide an energy supply to the rapidly growing tissue, although no data were gathered on the exact role of sucrose in the growth of the segments in this study. A future topic of research might be to examine the precise role of sucrose in the growth of cultured segments.

Section 4 - Changes in Enzyme Activities During Growth in Culture

The patterns of activity of G6PDH and CS which occur during segment extension growth in culture will be compared and considered along with the growth of these segments in the various media.

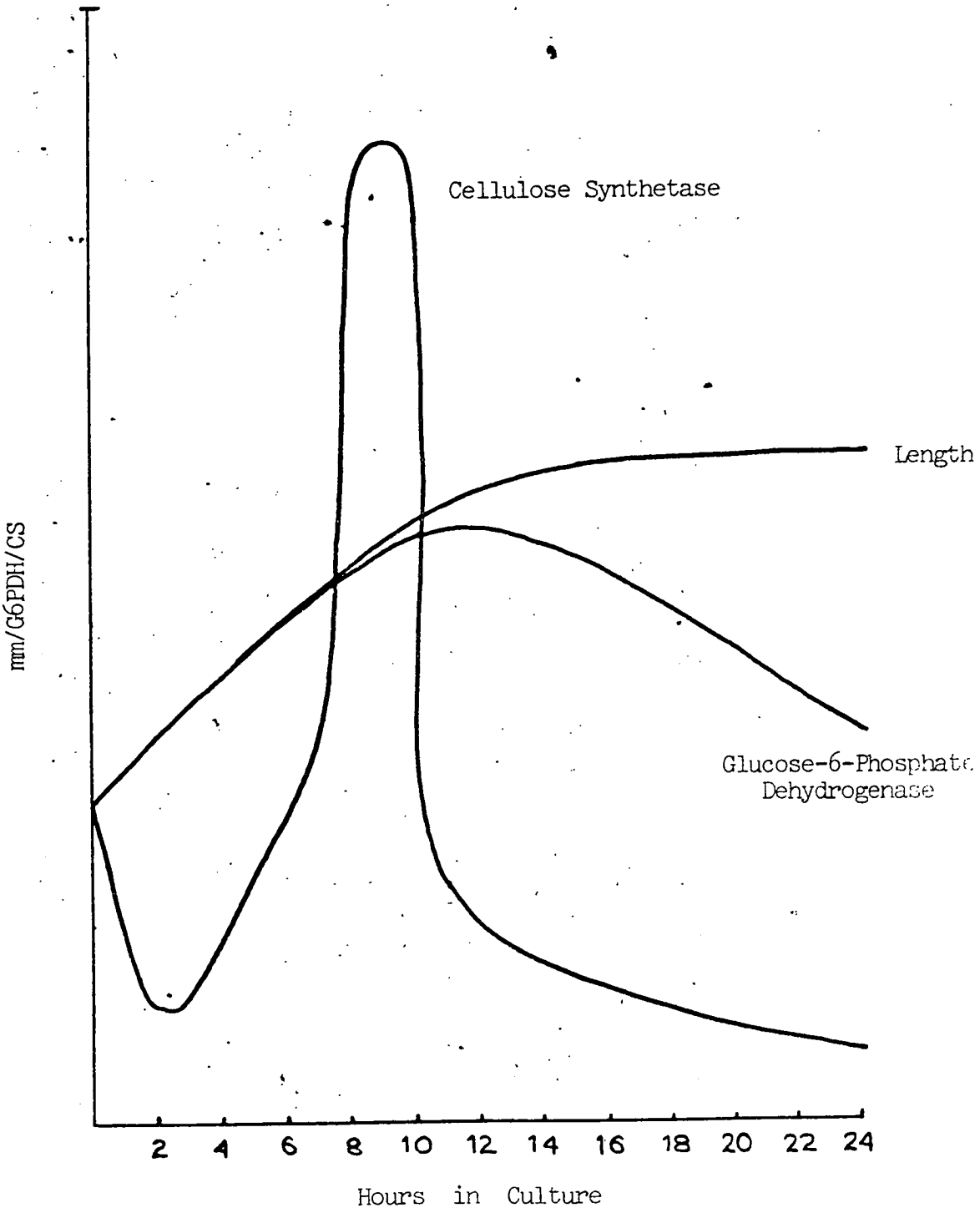
a. In 2% Sucrose

1. Glucose-6-phosphate Dehydrogenase

In Figure 5-29 a comparison is made of three parameters which

FIGURE 5-29

COMPARISON OF SEGMENT LENGTH, GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY AND CELLULOSE SYNTHETASE ACTIVITY DURING CULTURE IN 2% SUCROSE



were measured in root segments cultured in 2% sucrose for up to 24 hours. It is evident that the decline in G6PDH activity coincides temporally with the point where the rate of extension growth becomes zero. Over the first 12 hours of culture, the plot of change in G6PDH activity with time is almost coincident with the plot of segment length increase with time. Thereafter, growth ceases and a loss in enzyme activity ensues. The decline in activity is almost certainly not due to the production of an enzyme inhibitor because when extracts taken from T_o and T_{24} segments are mixed, the G6PDH activities are additive.

An examination of Figure 5-34, where growth rate is plotted against enzyme activity for each sample time, shows that there is no simple relationship between these two parameters over the 24 hour culture period. Initially, the growth rate is constant and the activity increases, then in the latter half of the culture period, the growth rate quickly approaches zero and the activity gradually falls. This would suggest that G6PDH is not directly involved in the control of growth of the segments, probably the increase is associated with the increase in cell size. However, the data do not permit a distinction to be made between cause and effect. Similarly, whether the decline in activity is causal or as a result of the cessation of growth cannot be confirmed. Presumably, the fall in activity reflects the general decay of the system after 12 hours of culture.

2. Cellulose Synthetase

When cellulose synthetase (CS) activity is plotted against time, in segments cultured in sucrose the peak coincides with the broad G6PDH activity peak and precedes the termination in growth by

approximately 4 hours (Figure 5-29). The CS peak is extremely sharp and narrowly based. The initial loss of activity is unlikely to be due to a leaching of enzyme from the cut ends. However, the cutting process will promote a wound response which may account for the initial loss of CS activity. This loss is not observed with G6PDH activity. The segments may take some time to become established in the culture medium and recommence cellulose synthesis. The greatest increase in residual dry weight, comprised mainly of cell wall polysaccharides, occurs within the first 12 hours of culture, although some increase was detected in the second half of the culture period (Figure 4-21) when the recorded CS activity is low and the expansion growth rate is zero. This latter increase in residual dry weight may be due to deposition into the wall of cellulose already synthesised but not yet deposited from Golgi vesicles (Shore and Maclachlan 1974). The increase may also be due to the deposition of wall polysaccharides other than cellulose, such as hemicellulose which is also a component of the residual dry weight. Some thickening of the walls has probably began with the cessation of cell expansion growth and it is at this stage that the wall components, other than cellulose are deposited. Brown and Sutcliffe (1950) found with Zea root fragments cultured in the presence of sucrose, that a rapid synthesis of cellulose occurs which is linear with time but was noted over a longer period than 12 hours.

Figure 5-34 shows that the CS activity, as with G6PDH activity, is not simply related to the rate of length increase. No simple relation could be shown, due to the distinct peak of CS activity around the 9th hour of culture. The coincidence of the cessation

of increase in length and loss of appreciable CS activity is possibly significant but which is cause and which is effect cannot be distinguished from the available data. It would be interesting in future work to examine the change in CS activity under culture conditions in which expansion growth was osmotically inhibited with mannitol.

3. Conclusions

The two enzyme patterns observed in segments cultured in 2% sucrose alone, may at first glance appear very different but there are some features in common. (Figure 5-29). Both activities form a peak at approximately the 10th hour of the culture period and then subsequently fall to more or less zero. The temporal coincidence of the cessation of growth with the loss of activities is interesting but whether it is causal or resultant cannot be decided from the available date. CS is likely to be more important in the control of growth than G6PDH as there are alternative pathways bypassing the latter enzyme.

b. In the Presence of 6-Methyl Purine

1. Glucose-6-phosphate Dehydrogenase

When 6MP is present in the sucrose medium at the concentration which stimulates extension growth (2×10^{-5}M), the G6PDH activity increases slightly over the first 4 hours and then gradually declines to 20% of the original activity at 24 hours (Figure 5-30). From the pattern of G6PDH activity observed in segments cultured in 2% sucrose alone, it was expected that the activity would be maintained when the period of extension growth was increased, in the presence of 6MP.

However, this expectation was not realised. From Figure 5-32, it

FIGURE 5-30

COMPARISON OF SEGMENT LENGTH, GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY AND CELLULOSE SYNTHETASE ACTIVITY DURING CULTURE IN 2% SUCROSE IN THE PRESENCE OF 6-METHYL PURINE (2×10^{-5}M)

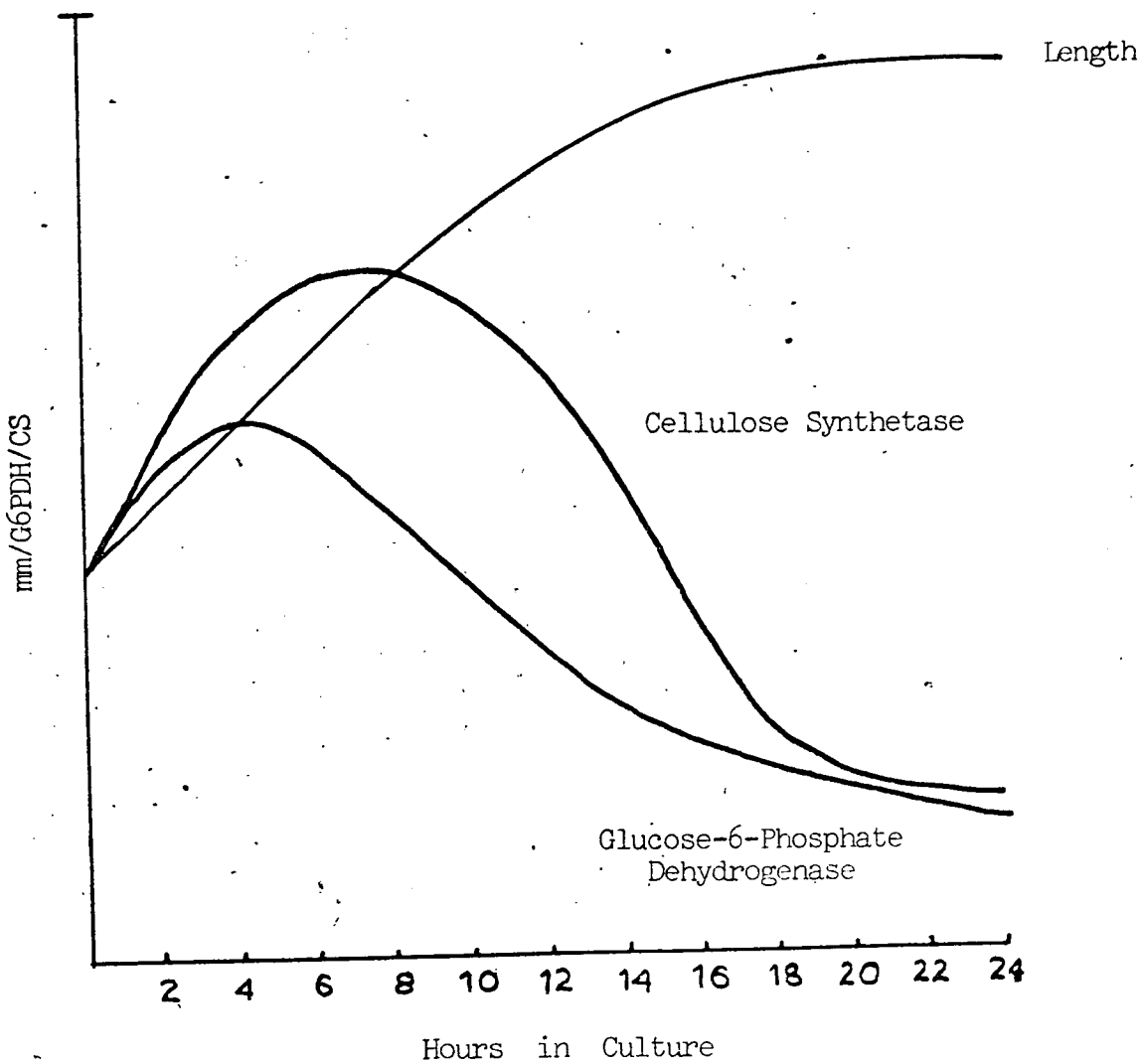
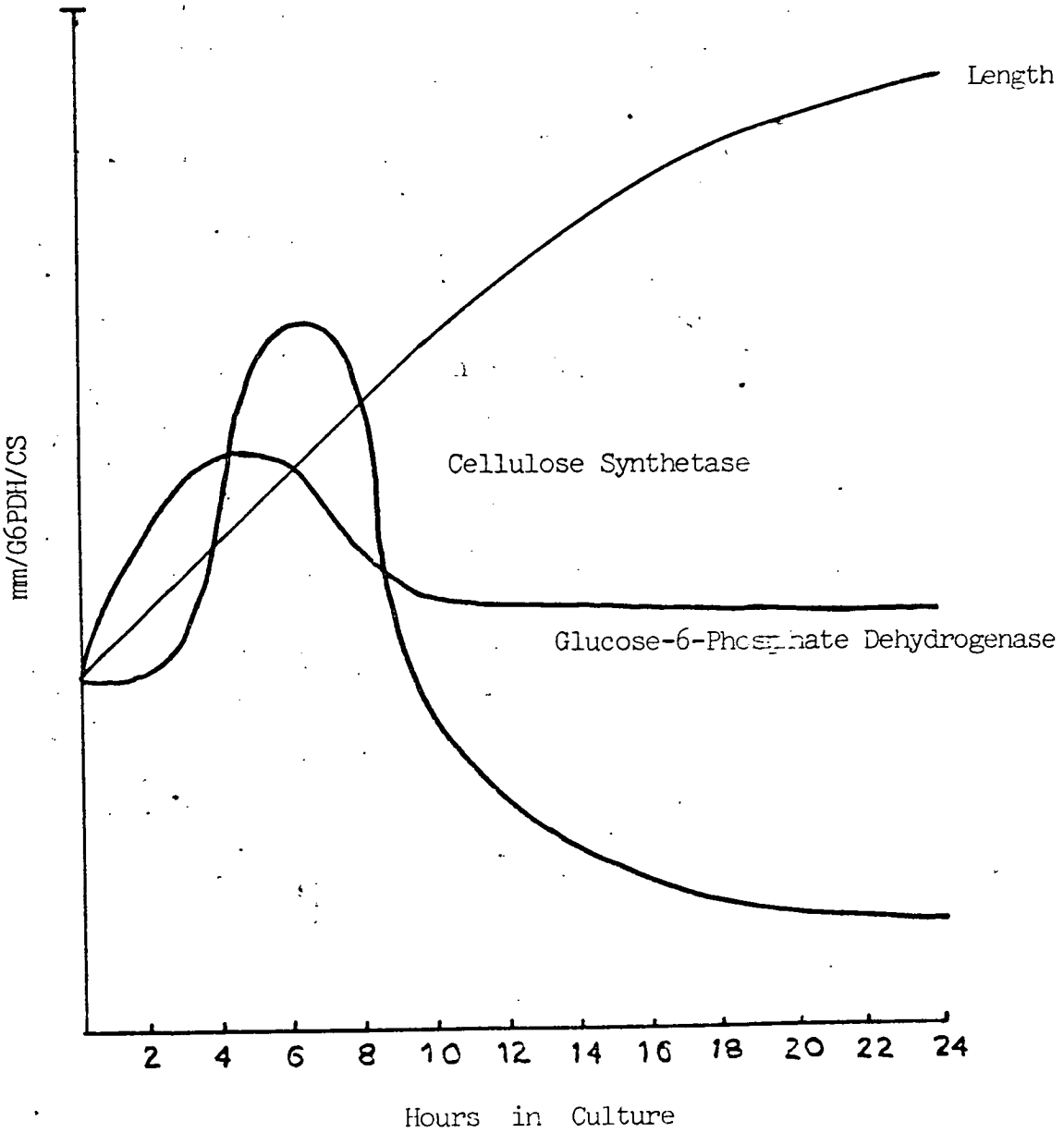


FIGURE 5-31

COMPARISON OF SEGMENT LENGTH, GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY AND CELLULOSE SYNTHETASE ACTIVITY DURING CULTURE IN 2% SUCROSE IN THE PRESENCE OF INDOLE ACETIC ACID (10^{-10}M)



can be seen that the rate of increase in G6PDH in the presence of 6MP (2×10^{-5}M) coincides with the increase in sucrose alone, over the initial 3 hours of culture. These 3 hours probably do not represent the time the 6MP takes to be effective because Aitchison (1975) found that 6MP inhibits RNA synthesis within minutes in Jerusalem artichoke tissue. The 3 hours could be the time interval during which the RNA species involved in G6PDH synthesis are viable, but this suggestion assumes that G6PDH is an unstable molecule, with a high rate of turnover, and new G6PDH protein has to be continually synthesised. This is probably not true. Aitchison (1975) also found that the half life of G6PDH in Jerusalem artichoke tissue to be in excess of 48 hours. Yagil and Feldman (1969) using rat fibroblasts and KB cells could not detect any decay in G6PDH when protein synthesis and RNA synthesis were blocked. However, the properties of G6PDH protein in pea tissue could differ but such a difference is unlikely.

The rate of decline of G6PDH activity is greater in the presence of 6MP (Figure 5-32), even when the segment growth response is greater than in sucrose alone. (Figure 5-28). 6MP could be having a direct inhibitory effect on the G6PDH activity. However, the addition of 2×10^{-3}M 6MP to the assay cuvette does not reduce the G6PDH activity recorded. (Chapter 4 Section k-2). The effect of 6MP 'in vivo' on G6PDH could however differ from the 'in vitro' effect and in the intact cell the 6MP could combine with the dehydrogenase protein, thus inactivating the enzyme.

A positive growth rate is maintained by the segments in the presence of a declining G6PDH activity (Figure 5-30). Therefore, the enzyme is either not required for growth of the segments in culture or the

activities measured are in excess of that required for growth. Alternative pathways of metabolism e.g. the Glycolytic pathway and the TCA cycle, are available to provide energy from carbohydrates for growth.

2. Cellulose Synthetase

The cellulose synthetase activity in the presence of 6MP (2×10^{-5}M) forms a widely based peak approximately 6 hours after the start of the experiment. As can be seen from Figure 5-34, there is no direct correlation between CS activity and rate of growth. Over the first 7 hours, activity increases proportionally to the length, after which time the growth rate and CS activity decline, until the cessation of the change in activity coincides with the establishment of a zero growth rate at 18 hours (Figure 5-30).

In segments cultured in the presence of 6MP, the peak in the plot of CS activity against time, although slightly lower than observed in segments cultured in sucrose alone, is more broadly based and the activity does not decline so sharply with time. (Figure 5-33). This is the pattern which would be expected considering the concept of Brown (1963) on cell development. (See Chapter 5 Section 3 a). The cells when treated with 6MP remain in the actively expanding state, as they were when removed from the fresh root, for a longer period, accompanied by a high CS activity, also for an extended time. This feature can be seen in Figure 5-33. The increase in residual dry weight is also slightly stimulated in the presence of 6MP (Figure 4-21). The final decline in CS activity would be determined by the stability of the cellulose synthetase system and any direct effects which 6MP may have on this molecule. Unfortunately, no data exist on the half life of the cellulose synthesising

system, to compare with G6PDH.

3. Conclusions

Considering not only the pattern of CS activity but also that of G6PDH, in the presence of 6MP, an alternative but not unrelated explanation to that of Brown (1963) may be considered for the stimulatory effect of 6MP on segment growth.

6MP is known to inhibit RNA synthesis. RNA comprises, in part, 5-carbon sugars (ribose) which are one of the major products of the pentose phosphate pathway, a part of which is catalysed by G6PDH. It is suggested that when the use of 5-carbon sugars for RNA synthesis is inhibited by 6MP, there is a build up of these sugars which could bring about end product depression of at least some of the pentose phosphate pathway. Under these conditions G6PDH may be unstable for the activity has been shown experimentally to fall sharply in tissue extracts. Cellulose synthetase is linked into this concept as it requires sugars, 5-carbon sugars for the precursor donor molecule (UDPG) and 6-carbon sugars (glucose) for cellulose. With the cessation of RNA synthesis, more energy will also be available to the cell for cellulose synthesis and other facets of cell expansion growth.

The lengthening of the period of active expansion growth of the cultured segments in the presence of 6MP is visualised due to the extra materials and energy available to the expansion growth of the cell, following on the inhibition of RNA synthesis.

This concept is similar to that of Brown (1963) as it relies on the inhibition of RNA synthesis but differs in that the important factor is not that the RNA present is retained but that no new RNA is synthesised. The system would eventually decay due to the

failure of the active RNA and protein species to be replaced. The experimental approach to test this concept would involve testing whether the Pentose Phosphate pathway enzymes of pea root tissue can be inhibited by the accumulation of end products. Also, the pool of 5-carbon sugars in the cell should be examined to follow their fate in the presence and absence of 6MP.

c. In the Presence of Indole Acetic Acid

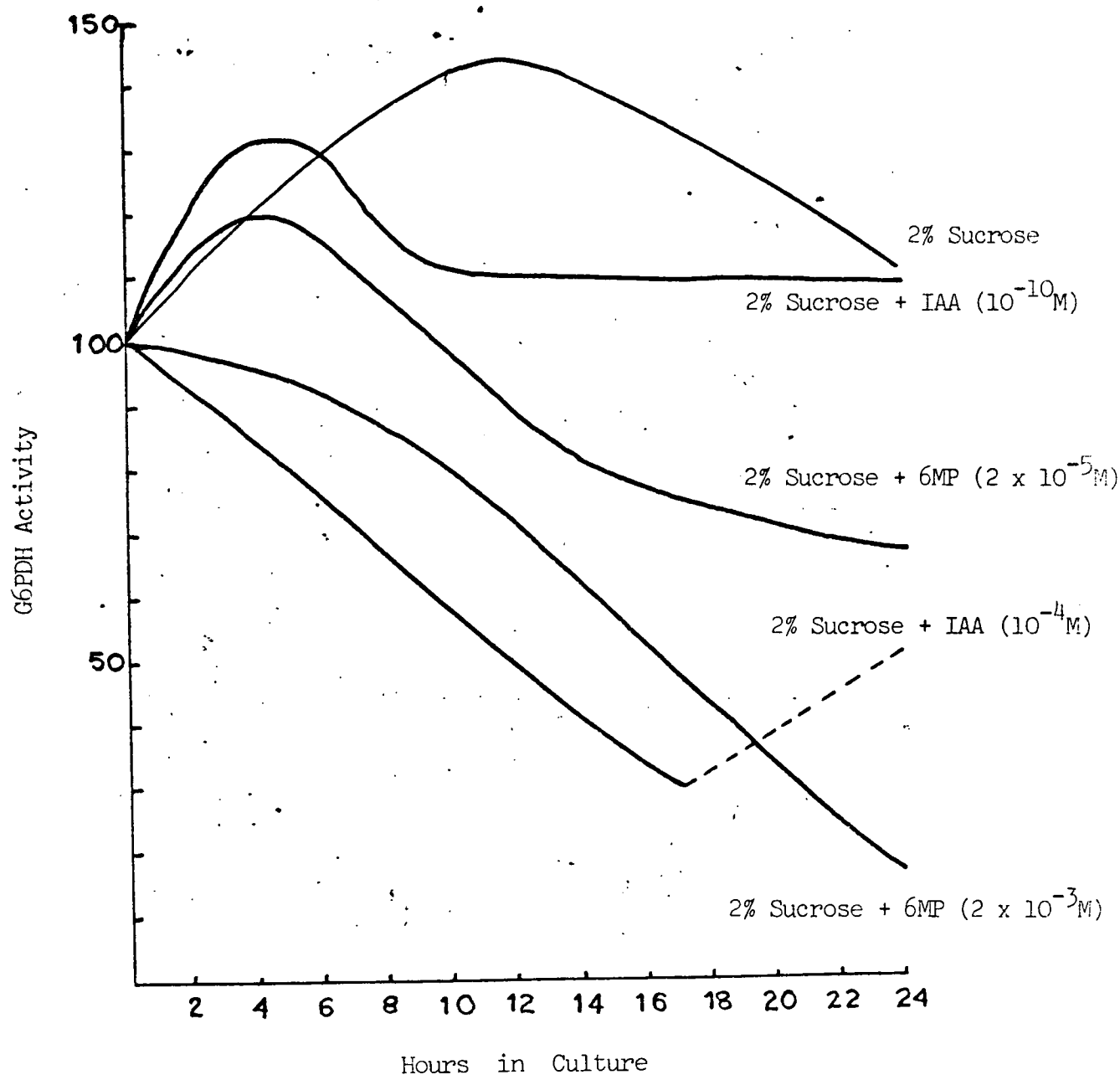
1. Glucose-6-phosphate Dehydrogenase

In the presence of 10^{-10}M IAA, which stimulates extension growth, G6PDH activity increases initially at a greater rate than in either sucrose alone or in the presence of 6MP (Figure 5-32). After 5 hours the activity reaches a maximum value and then decreases over the following 3 hours to 1½ times the initial level at which it stays until the end of the 24 hour culture period. The activity is appreciably lower than that measured in segments cultured in 2% sucrose but the activity is maintained above the T_o value and does not decline to zero as in the segments cultured in the medium supplemented with 6MP. Therefore, the stimulatory effects of IAA (10^{-10}) on G6PDH activity appear to be, the greater initial rate of increase and the maintenance of the level of activity in the last 16 hours of culture.

Figure 5-34 shows there is no simple coorelation between growth rate and G6PDH activity in the presence of IAA. It was suggested from the results of growth and G6PDH activity in the presence of 6MP that G6PDH is probably not limiting the growth of the segments. In the segments cultured in the presence of IAA, it is observed that the maintenance of a high growth rate is concurrent with the maintenance of the level of G6PDH activity. Whether the high

FIGURE 5-32

COMPARISON OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY DURING CULTURE IN VARIOUS MEDIA



activity is the cause of the maintained growth or as a result of it, cannot be distinguished from the available data. In segments cultured in sucrose alone, the G6PDH activity declined when the growth rate approached zero. IAA appears to maintain the conditions required for continued growth but again whether the maintained G6PDH is the direct result of the addition of IAA or brought about by the growth response, or only incidental, cannot be decided from the available data. The possibilities might be distinguished by examining G6PDH in the presence of IAA when the segments are cultured, in conditions e.g. mannitol, which osmotically inhibit extension growth. This might show whether IAA was the direct cause of the G6PDH activity or whether the activity was closely linked to growth; but it could not distinguish whether it was the cause or as a result of the growth.

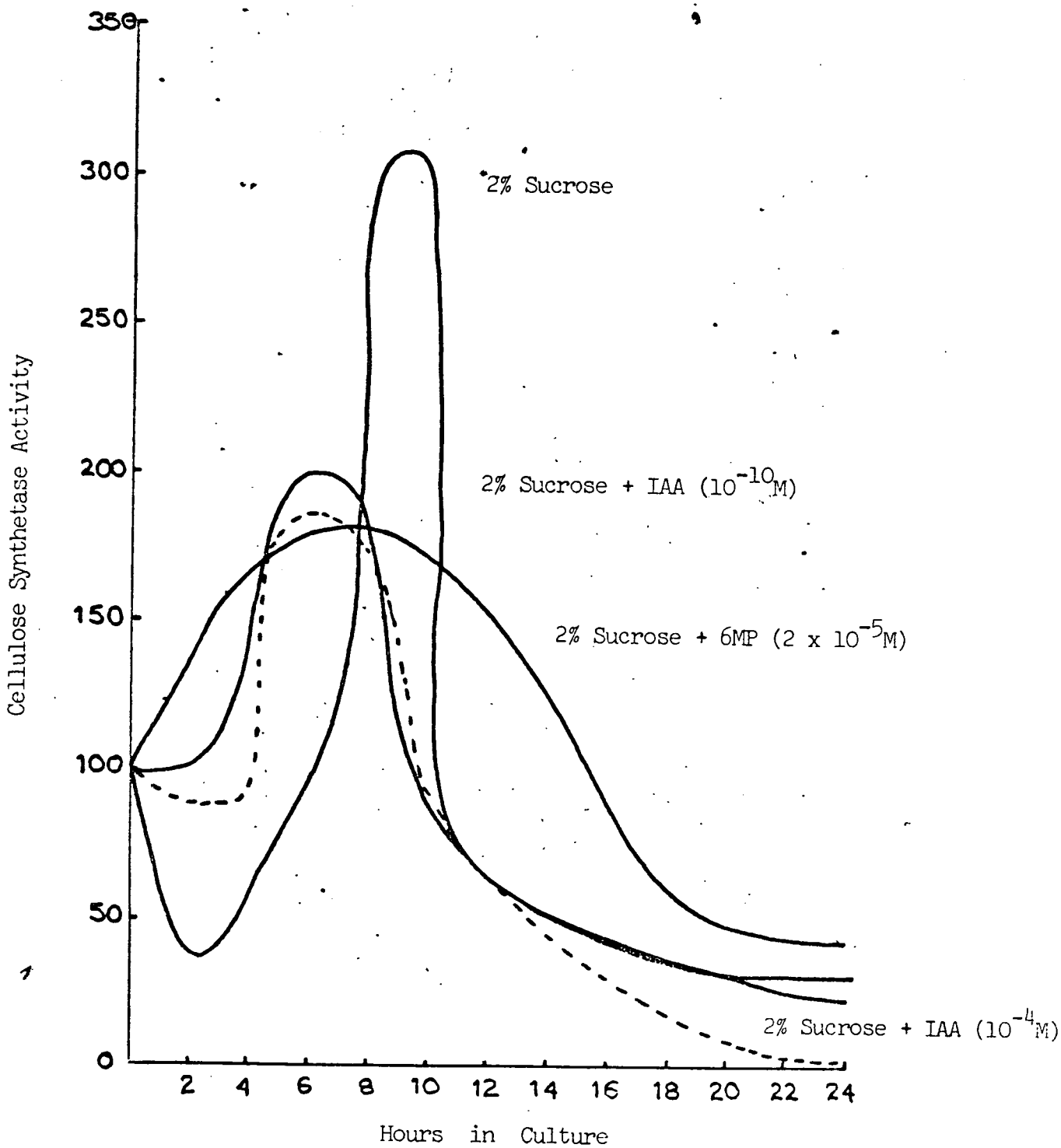
2. Cellulose Synthetase

The CS activity in the presence of IAA (10^{-10}M) shows a variation of the pattern already described in sucrose alone and in cultures supplemented with 6MP, with the peak of activity preceding the peaks in sucrose alone and 6MP cultures by about 2 hours (Figure 5-33). The CS activity is not maintained over a longer time as was observed in the presence of 6MP, but the stimulatory effect of IAA on segment length (Figure 4-18) and residual dry weight (Figure 4-21) is even greater than the stimulatory effect on segments cultured in the presence of 6MP. A similar pattern of CS activity is observed in segment cultures where extension growth is inhibited by the addition of 10^{-4}M IAA to the sucrose medium. Therefore, the CS activity probably does not increase in response to the extension growth of the segments. Also there is no simple

FIGURE 5-33

COMPARISON OF CELLULOSE SYNTHETASE ACTIVITY

DURING CULTURE IN VARIOUS MEDIA



Legend

FIGURE 5-34

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY

AND CELLULOSE SYNTHETASE ACTIVITIES AGAINST

RATE OF EXTENSION GROWTH IN 3 MEDIA

Glucose-6-phosphate dehydrogenase

- - 2 % sucrose
- - 2 % sucrose + 6MP (2×10^{-5} M)
- - 2% sucrose + IAA (10^{-10})

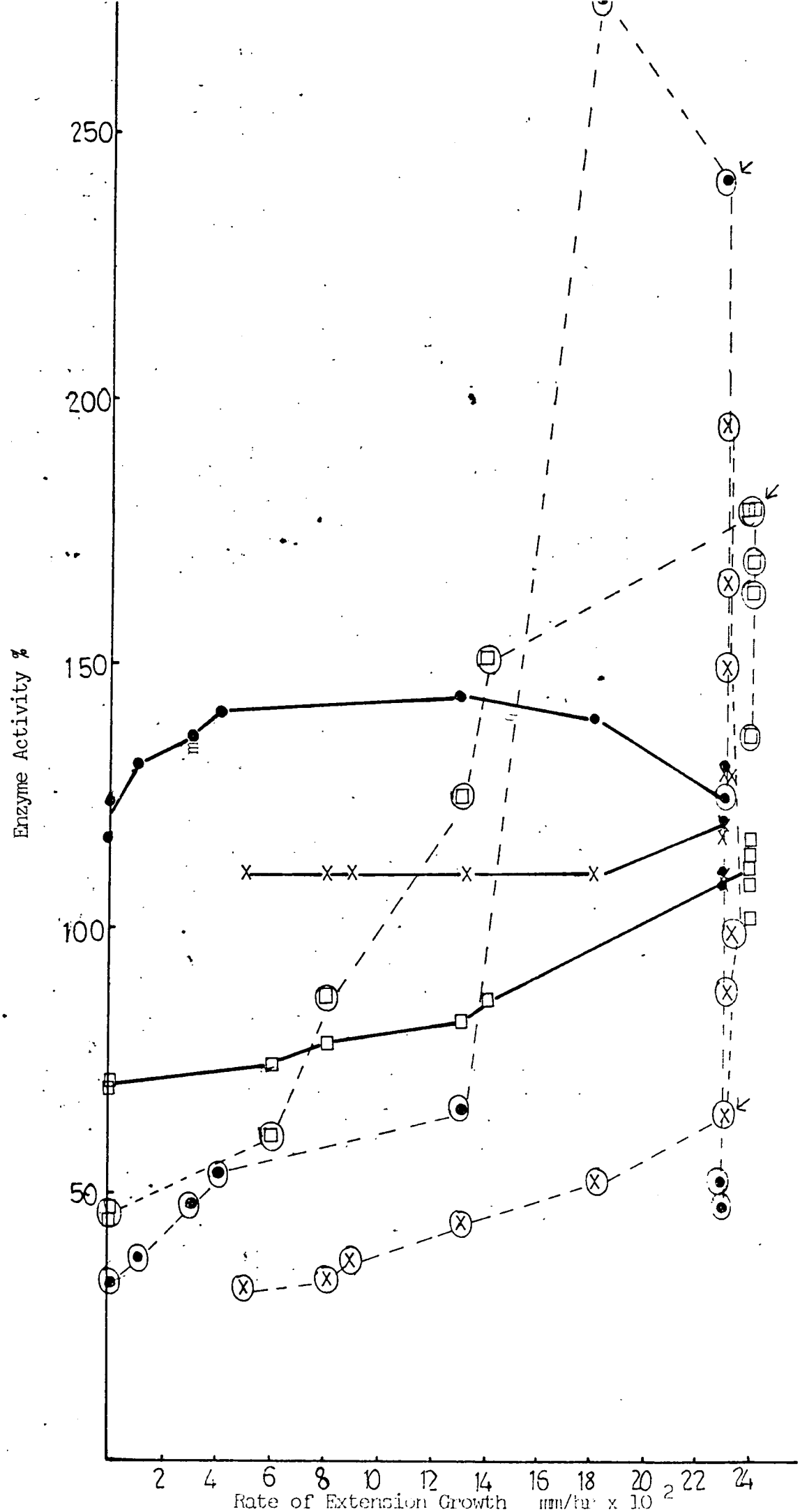
Cellulose synthetase

--- --- 2% sucrose
--- --- 2% sucrose + 6MP (2×10^{-5} M)
--- --- 2% sucrose + IAA (10^{-10} M)

Arrows indicate enzyme activity at end of linear growth period.

Data taken from Figures 15, 16, 18 and 22-27, accumulated data graphs.

FIGURE 5-34


250
200
150
100
50
Enzyme Activity %
2
4
6
8
10
12
14
16
18
20
22
24
Rate of Extension Growth mm/hr x 10 2

relation between CS activity and rate of growth. (Figure 5-34). Abdul-Baki and Ray (1971) found that IAA promoted cell wall synthesis, even in the presence of calcium chloride and mannitol, both of which inhibit elongation, in pea stem segments. They suggested that the stimulation of polysaccharide synthesis by IAA is partly due to changes in the reaction rate of hexokinase resulting from an increase in the metabolic glucose pool, caused by increased glucose uptake. Also, they suggested that an increase in the β-glucan synthetase activity could be attributed to the stimulated cell wall synthesis. Considering their work, the similar levels of CS activity in segments cultured in the presence of the stimulatory (10^{-10}M) and inhibitory (10^{-4}M) IAA, in this study, would not be expected as cell wall synthesis does not necessarily occur in response to increasing cell size.

In the expanding regions of decapitated pea epicotyls, cellulose deposition ceases and the particulate, GDPG - dependent synthetase activity declines to a barely detectable level within two days (Shore, Spencer, Horowitz and Maclachlan 1972). The addition of cycloheximide to the cut surface reduces the rate of decline and the addition of auxin at the time the material is cut maintains the initial level of synthetase activity for several days. A rough comparison may be drawn with the present investigation, where 6MP reduces the rate of decline and although IAA (10^{-10}M) did not appear to maintain the CS activity level, it did stimulate the increase in residual dry weight. Ray (1973) treated pea shoot segments with IAA and found a 2-4 fold increase in the activity of the particulate, UDPG - dependant glucan synthetase. Although, this IAA induced increase is only seen if the level of activity

is first allowed to fall below the intact plant level, and, only if sucrose is supplied. In the present investigation sucrose was supplied to the segments throughout the culture period and exposure to IAA was started immediately after excision from the intact plant. Therefore, stimulation of the CS activity was not observed possibly because the endogenous IAA level of the segments was not allowed to fall below that of the intact plant before exposure to IAA.

The activity of the membrane-bound synthetase which synthesis 1-3 and 1-4 glucans from UDPG is stimulated in the presence of growth promoting concentrations of 2,4-D in the 'in vitro' assay (Van der Wande, Morre and Williamson 1972). The 2,4-D is most effective in stimulating the synthesis of polysaccharides soluble in hot water and increasing the proportion of 1-4 linkages in polysaccharides insoluble in hot alkali. Ray (1973) found that IAA did not change the composition of the mainly 1-4 linked product from UDPG. In the pea root segments cultured in the presence of the concentration (10^{-10}M) of IAA which stimulated extension growth and residual dry weight, the cellulose synthetase activity was not maintained. However, the activity is measured as the incorporation of radioactively labelled glucose into a cellulose fraction which has been washed by hot water and hot alkali. Therefore, from the work of Van der Wande, et al (1972), had any stimulation which occurred would not have been detected in the cellulose product.

3. Conclusions

It appears from the results presented here that IAA is stimulating extension growth through a general increase in cell metabolism. Work by Trewavas et al (1967) suggested that the auxin effect on cell wall synthesis may be an indirect one related to a general

'upstep' in metabolism. They found IAA induces a drop in the amount of radioactive label in the hexose phosphate pool relative to the control, in Avena sativa coleoptile sections, both in the absence and presence of sucrose. This result suggested to them that the rate of some reaction, or reactions, leading from the pool, had been increased. Systems which showed an immediate and consistent increase when IAA was present, directly involved UDPG and RNA. UDPG is related to the hexose phosphate pool by UDPG pyrophosphorylase and RNA synthesis utilises ribose-5-phosphate which comes from the pentose phosphate pathway. Therefore, in this present investigation, the stimulatory effect of IAA on the G6PDH activity may be a reflection of these reactions described by Trewavas et al (1967). This work would also tend to suggest that the G6PDH activity is increasing in response to the accelerated system and is not itself bringing about the stimulation. Ray (1973) also noted that IAA induced a rise in glucan synthetase activity in pea shoot segments. The addition of concentrations of Actinomycin-D or Chloramphenicol that inhibit growth did not prevent the IAA induced rise. However, it was prevented by inhibitors of energy metabolism and by high osmotic pressure, but not by calcium ions at the concentration that inhibits auxin-induced elongation.

Section 5 - Summary

During expansion growth in excised pea root fragments the G6PDH and IE activities have been found to change, even though the total protein content of the segments remains constant (Table 23). This confirms the results of Brown (1954) who also found that the total protein content in excised root fragments grown in culture stays constant but the invertase

activity decreases and the phosphatase activity increases, the greatest changes being in the first 12 hours when growth is vigorous. The patterns of G6PDH and CS activity changes are affected by the addition of 6MP or IAA to the sucrose medium. Some features of the patterns of enzyme activities during culture are compared in Table 26.

Table 26

Comparison of Peaks of Enzyme Activities in Pea Root Segments Cultured in 3 Media

Medium	Hours/Relative Peak Size	
	G6PDH	CS
2% Sucrose	10	9
2% Sucrose + 6MP	5/-	7/+/-
2% Sucrose + IAA	5/-	6+

Notes

G6PDH	Glucose-6-phosphate dehydrogenase
CS	Cellulose synthetase
6MP	2×10^{-5}M 6-Methyl purine
IAA	10^{-10}M Indole acetic
Hours	Hours into the culture period of the peak of enzyme activity. Taken from graphs 4-22 to 4-27
Relative Peak Size	Areas under peaks of enzyme activity against time. Relating activities from segments cultured in the presence of 6MP or IAA against those cultured in sucrose alone

The G6PDH activity and the CS activity peak at 10 and 9 hours respectively, and the addition of 6MP or IAA causes both activities to peak earlier by 5 hours in the case of G6PDH, and by 2 to 3 hours in the case of CS for 6MP and IAA respectively. A comparison may be drawn with other enzyme activities examined by Vaughan (1965). He assayed invertase, phosphatase

and ribonuclease at intervals during the culture of the 2-4 mm pea root segment (Meteor variety) in various media. In sucrose the activities increase and peak at 10, 12 and 6 hours, respectively. The invertase and phosphatase activities then gradually decline but the ribonuclease drops sharply. In the absence of sucrose, where segment extension growth is limited, Vaughan (1965) found the invertase activity steadily decreases with time and phosphatase and ribonuclease show a slight increase.

The stimulation of segment growth by the addition of 6-methyl purine to the sucrose medium was brought about by an extension of the duration of the rapid phase of growth. This mode of stimulation and the widening of the peak of cellulose synthetase activity by 6MP (2×10^{-5}M) are interpreted as supporting the concept of Brown (1963) on cell development. (See Section 3 a). Although the G6PDH activity behaved differently, gradually declining over the culture period after a short initial rise and so an alternative theory is presented based on a re-distribution of available sugars in the cell, to explain the stimulatory effects of 6MP.

A stimulation of segment length, also by extending the duration of the rapid phase of growth, is observed when IAA (10^{-10}M) is added to the sucrose medium. CS activity follows a similar pattern to that in sucrose alone. The same pattern is also observed in segments when extension growth is inhibited by 10^{-4}M IAA. The G6PDH activity is maintained at a level $1\frac{1}{2}$ times the T_o activity. The results are interpreted as suggesting that IAA is bringing about a general increase in metabolic rate, thus maintaining the growth phase in a system which would otherwise be in decay due to a deficiency of IAA in the latter half of the culture period.

A catalogue of the enzyme changes, as well as other cellular changes, during extention growth of root segments in culture is gradually

being formed and it is hoped that with the aid of further studies the process of cell expansion in higher plants will eventually be understood. This work, as well as augmenting the data available on the changes accompanying extension growth in root segments in culture, also provides the basis of the assay technique for cellulose synthetase and glucose-6-phosphate dehydrogenase in pea root tissue. This former assay is perhaps the most interesting aspect of this work and the technique used will be a helpful aid to others who wish to commence a study of cellulose synthesising systems in pea root and other tissue. Many more questions remain to be answered concerning both the cellulose synthetase assay and aspects of extension growth. Those which became apparent during the course of this investigation and for which time was not available, were introduced in the various sections throughout this chapter.

APPENDICES

APPENDIX 1

GROWTH OF 2-4 MM ROOT SEGMENTS IN 2% SUCROSE

SEGMENT LENGTH (mm)

HOURS IN CULTURE											
48				4.86	4.84						4.85
43				4.01	4.33						4.17
34					5.41						(5.41)
32				4.52	3.85						4.19
29					4.59						(4.59)
24	4.42		5.33	5.02	4.93	4.84	4.90	4.30	5.00	4.90	4.85
23											
22											
21											
20											
19			5.28	4.58	3.78		3.68				4.33
18											
17										4.00	(4.00)
16								4.60	4.00		4.30
15				5.02	4.75						4.89
14			4.29								4.29
13											
12											
11				4.68	4.58						4.63
10						3.86	3.74			5.30	4.30
9					4.10			3.70			3.90
8									3.70	4.40	4.05
7		3.21									(3.21)
6	3.30		3.38	3.12	3.50	2.92	3.57	3.60	3.30	3.90	3.40
5											
4									3.00	3.80	3.40
3				2.75	2.60	2.88	2.88	2.60			2.74
2			2.46						2.30	2.50	2.42
0											
	2.04	1.95	2.08	1.97	2.06	1.96	2.01	1.97	2.06	1.99	2.01

AVERAGE

Legend: Growth of 2-4 mm segments in 2% sucrose - segment length (mm) The column of figures show the segment lengths measured within single experiments. The rows show the average segment lengths at the same culture times. The end column is the average lengths calculated from the 10 separate experiments. Each length figure was calculated from 40-60 segments, grown in 2-3 flasks of 20.

APPENDIX 2

GROWTH OF 2-4 mm ROOT SEGMENTS IN 2% SUCROSE

IN PRESENCE OF 6-METHYL PURINE

SEGMENT LENGTH (mm)

CULTURE MEDIUM	HOURS IN CULTURE												
	0	2	3	4	6	8	9	11	15	18	19	24	
2% Sucrose +			2.20		2.48		2.29		2.30		2.13	2.20	
2×10^{-3}M 6MP			2.30		2.42		2.11		2.40		2.17	2.30	
Average	2.00		2.25		2.45		2.20		2.35		2.15	2.25	
2% Sucrose +		2.40	2.60	2.80	3.30	4.00	5.00	4.70	4.80	5.42	5.30	5.20	
2×10^{-5}M 6MP		2.43	2.70	3.05	3.40	3.79	3.70	4.60	5.00	5.43		5.33	
		2.71		3.08	3.10							5.20	
					3.41								
					3.63								
Average	2.00	2.51	2.65	2.98	3.37	3.90	4.35	4.65	4.90	5.43	5.30	5.24	
2% Sucrose +		2.47	2.92		3.54		3.90		5.01			4.91	
2×10^{-7}M 6MP		2.43	2.61		3.41		3.89		4.83			5.00	
			2.73		3.36							4.79	
Average	2.00	2.45	2.75		3.44		3.90		4.92			4.90	

SEGMENT LENGTH - mm

6MP - 6 Methyl purine

Legend Each figure is the average segment length calculated from 40 segments cultured as 2 sets of 20. The average length for each sample time is calculated from the values from the separate experiments

2×10^{-3}M - 2 experiments

2×10^{-5}M, 2×10^{-7}M - 3 experiments

APPENDIX 3

COMPARISON OF SEGMENT LENGTHS AFTER 6 HOURS AND 24 HOURS IN CULTURE IN 2% SUCROSE AND 2% SUCROSE SUPPLEMENTED WITH VARIOUS CONCENTRATIONS OF IAA

CULTURE MEDIUM	HOURS IN CULTURE 6	24
2% Sucrose	3.40 ± 0.48	4.85 ± 0.85
2% Sucrose + IAA 1 x 10^{-4}M	2.29 ± 0.15	2.93 ± 0.19
+ IAA 1 x 10^{-6}M	2.42 ± 0.62	3.78 ± 0.59
+ IAA 1 x 10^{-8}M	3.59 ± 0.44	5.38 ± 0.91
+ IAA 1 x 10^{-10}M	3.65 ± 0.35	5.75 ± 0.88
+ IAA 1 x 10^{-12}M	2.98 ± 0.26	4.76 ± 0.63

Standard Deviations calculated with n = 40

APPENDIX 4

GROWTH OF 2-4 mm ROOT SEGMENTS IN 2% SUCROSE IN PRESENCE OF INDOLE ACETIC ACID - IAA

SEGMENT LENGTH - mm

2% Sucrose + IAA	HOURS IN CULTURE 2	3	4	6	7	8	9	10	16	18	22	24
x 10^{-10}M	2.45	2.60	3.01	3.53	3.28	3.87	3.70	4.23	5.90	5.35	5.90	5.78
	2.60		2.72	3.59		4.10		4.40		6.39		6.24
			3.30	3.30								5.90
				3.65								7.10
				4.20								6.41
Average	2.53	2.60	3.01	3.54 / 3.64	3.28	3.99	3.70	4.32	5.90	5.87	5.90	6.29
x 10^{-4}M	2.20	2.30	2.30	2.80		2.90	2.70	2.80	3.40		2.90	2.93
	2.73		2.41	2.60		2.74		2.81	3.27			3.50
				2.29								3.00
Average	2.47	2.30	2.36	2.56		2.82	2.70	2.81	3.34		2.90	3.14

Legend Each figure is the average segment length calculated from 40 segments, cultured as 2 sets of 20. The average length for each sample time was calculated from the values of the separate experiments.

APPENDIX 5

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN ROOT SEGMENTS DURING CULTURE IN 2% SUCROSE

Activity Expressed as a Percentage of the Activity recorded in Fresh Root (T_o) Segments

	HOURS IN CULTURE														
G6P-DH	0	2	3	4	6	7	8	9	10	11	15	16	17	19	24
Activity	100	113	102	115	115	116	137	126	82	173	134	133	91	134	147
Percentage		113	102		152			135		138	133		82	133	111
of			110		153			143							140
T_o%			104		118			128							107
			124		133			121							135
			134					130							159
															111
															108
Average	100	113	113	115	134	116	137	131	82	156	134	133	87	134	127

Legend: The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o value within each separate experiment. Each value is the average of assays on 2 flasks of 20 segments. The overall average is calculated within each sample time from the values of all experiments.

APPENDIX 6

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN ROOT SEGMENTS DURING CULTURE IN 2% SUCROSE IN PRESENCE OF 6-METHYL PURINE

Activity Expressed as a Percentage of the Activity in Fresh Root (T_o) Segments

	HOURS IN CULTURE											
MEDIUM	0	2	3	4	6	8	9	15	17	18	19	24
2% Sucrose +	100		96		93		90	36			48	16
2×10^{-3}M 6MP			46		86		79	46				15
Average	100		71		90		85	41			48	16
2% Sucrose +	100						100	91			81	68
2×10^{-5}M 6MP							104	64			74	48
		108		123	108	109			65			
				117	90	119				58		66
				126	111	107				48		66
Average	100	108		122	103	112	102	78	65	53	78	62

Legend: The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o value within each separate experiment. Each value is the average of assays on 2 flasks of 20 segments. The overall average is calculated within each sample time from the values of all experiments.

APPENDIX 7

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN ROOT SEGMENTS DURING CULTURE IN 2% SUCROSE IN PRESENCE OF INDOLE ACETIC ACID

Activity Expressed as a Percentage of the Activity Measured in Fresh Root (T_o) Segments

CULTURE MEDIUM	HOURS IN CULTURE - T												
	0	2	4	6	7	8	9	10	16	17	18	22	24
2% Sucrose +	100	88						49					68
1×10^{-4} M IAA	100	82	75	88			83			37			36
	100	81	62	92			83			23			46
Average	100	84	69	90			83	49		30			50
2% Sucrose +	100		136		123			106					103
1×10^{-10} M IAA	100		165		141			111					130
	100	81	104	112			55					105	
	100		125	122		125					114		97
				122		115							
Average	100	81	133	119	132	120	55	109			114	105	110

Legend: The T_o activity is always expressed as 100% and all other activities calculated relative to the T_o value within each separate experiment. Each value is the average of assays on 2 flasks of 20 segments. The overall average is calculated within each sample time from the values of all experiments.

APPENDIX 8

RESULTS FROM SINGLE EXPERIMENT GIVING SEGMENT LENGTH, GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND CELLULOSE SYNTHETASE ACTIVITIES

Legend

Results from a single complete experiment showing actual values of enzyme activities.

Length and both activities measured in the same flask for each treatment.

Standard Deviation calculated with n = 20.

Experiment performed 6-7/9/74

APPENDIX 8

RESULTS FROM SINGLE EXPERIMENT GIVING SEGMENT LENGTH, GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND CELLULOSE SYNTHETASE ACTIVITIES

CULTURE TIME HOURS		CULTURE MEDIUM	SEGMENT LENGTH	GLUCOSE-6-PHOSPHATE DEHYDROGENASE OD INCREASE PER MINUTE PER SEGMENT	CELLULOSE SYNTHETASE CPM PER SEGMENT
0		-	1.99 ± 0.01	8.7	23
2	a	2% Sucrose	2.50 ± 0.15	9.8	--
	b	" + 6MP 2×10^{-5} M	2.40 ± 0.17	9.5	6.2
	c	" + IAA 10^{-4}	2.23 ± 0.54	7.6	5.1
	d	" + IAA 10^{-10}	2.62 ± 0.23	8.4	14.4
4	a		3.84 ± 0.36	11.5	28.9
	b		3.07 ± 0.10	10.7	
	c		2.34 ± 0.12	8.1	22.3
	d		3.26 ± 0.25	10.1	39.9
6	a		3.88 ± 0.40	11.3	21.6
	b		3.52 ± 0.11	9.8	31.3
	c		2.75 ± 0.14	13.3	7.6
	d		4.15 ± 0.34	6.5	35.8
8	a		4.41 ± 0.22	-	-
	b		3.48 ± 0.28	8.7	31.6
	c		2.94 ± 0.12	-	20.1
	d		4.12 ± 0.25	10.5	28.8
10	a		5.32 ± 0.60	12.8	26.5
	b		-	-	-
	c		2.84 ± 0.30	4.7	4.8
	d		4.36 ± 0.51	5.2	13.3
18	a		5.29 ± 0.59	9.9	5.2
	b		5.49 ± 0.30	4.7	9.5
	c		2.93 ± 0.19	3.1	1.4
	d		5.86 ± 0.75	11.1	11.7
24	a		4.90 ± 0.61	3.5	0.3
	b		6.92 ± 0.87	6.7	7.3
	c		2.98 ± 0.22	6.5	0.7
	d		6.41 ± 0.79	8.9	0.7

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