POLYAMINES IN CELL CULTURE

A thesis submitted to the

University of London

for the degree of Ph.D.

Kevin Michael Fallon

Department of Biology
University College London

1990

Described is an investigation into the role of polyamines in plant cells. The study was performed throughout with the use of a liquid suspension culture of carrot (Daucus carota cv Chantenay) cells as the model system.

Polyamine synthesis and accumulation were studied during the culture growth period, with biosynthetic and catabolic enzyme assays performed to determine rates of polyamine synthesis and free-polyamine analysis to determine cellular polyamine levels.

Methods of manipulating polyamine levels within the cell were studied. These included varying the constituents of the growth medium of the suspension culture, such as the plant hormone levels and the balance and level of the available nitrogen sources.

The possibility that polyamine supplements might replace plant hormones in the growth medium was investigated and polyamine precursors were added to the medium in an attempt to boost cellular polyamine levels.

A substantial comparative study was also made into the effects of inhibitors of polyamine biosynthesis on polyamine biosynthesis and accumulation. Included were determinations of the effects of a range of inhibitors on polyamine biosynthetic and catabolic enzyme activities, on the accumulation of polyamines within the cell and on growth over the culture period.

Finally, the relationship between polyamines and other solutes, and cellular responses to short and long-term stress conditions was studied - both in cells previously adapted to stress conditions, and unadapted cell lines.

Principal findings were:

a) Difluoromethylarginine strongly inhibits arginine decarboxylase and radically reduces putrescine and total amine content, while having no effect on cell number and stimulating fresh weight through increased cell expansion.

b) Difluoromethylornithine strongly inhibits ornithine decarboxylase activity but has little effect on either polyamine levels or culture growth.

c) Ethylmethylglyoxalbisguanylhydrazone strongly inhibits S-adenosylmethionine decarboxylase activity with little effect on fresh weight.

d) Proline and other organic solutes increase in concentration in response to polyethylene glycol induced water stress in adapted and unadapted cell lines while polyamines and glycinebetaine do not increase significantly.

TABLE OF CONTENTS

Abstract	i
Table of Contents - text	iii
List of figures and tables	v
Acknowledgements	viii
Chapter One - Introduction	1
Chapter Two - Materials and Methods	13
2.1 Carrot cell culture	14
2.2 Growth determinations	16
2.3 Solute determinations	19
2.4 Enzyme assays	23
2.5 Solute potentials	25
2.6 Inhibitor studies	26
2.7 Amines as auxin replacements	27
2.8 Short and long term stress	27
2.9 Anoxia	28
Chapter Three - Polyamine Metabolism	29
3.1 Polyamines and growth rates during culture	30
3.2 Amine biosynthesis during culture	38
Chapter Four - Manipulation of Amine Levels	49
4.1 Inhibitors of amine biosynthesis	50
4.2 Plant hormones and amines in relation to culture growth	58
4.3 Amines as auxin replacements	61

4.4 Amine precursors 67

4.5 Available nitrogen and growth 69

4.6 Potassium levels in relation to amine content and growth 71

Chapter Five - Polyamines and Stress 75

5.1 Water stress 76

5.2 Short and long term stress 87

5.3 Anoxia 96

5.4 Heat stress 98

Chapter Six - Discussion 100

Appendix One - Bibliography 115

Appendix Two - Publications 136

List of Abbreviations

EMGBG	Ethylmethylglyoxalbisguanylhydrazone	FNBT	4-fluoro-3-nitrobenzotrifluoride
MGBG	Methylglyoxalbisguanylhydrazone	OPA	O-phthalaldehyde
DFMA	Difluoromethylarginine	DFMO	Difluoromethylornithine
ADC	Arginine decarboxylase	DAO	Diamino oxidase
ODC	Ornithine decarboxylase	SAMDC	S-adenosylmethionine decarboxylase
AIH	Agmatine iminohydrolase	SAM	S-adenosylmethionine
CDC	Citrulline decarboxylase	CPH	N-carbamoylputrescine hydrolase
MTA	Methylthioadenosine		

LIST OF FIGURES AND TABLES

Figure i. Di-and polyamines

Figure ii. Synthesis of putrescine

Figure iii. Synthesis of spermidine and spermine

Figure iv. A typical HPLC spectrum - polyamine standard mixture

Figure v. A typical HPLC spectrum - amino acid standard mixture

Figure vi. A typical GC spectrum - sugar standard mixture

Figure 1a. Changes in fresh weight over a 14 day culture period

Figure 1b. Changes in amine content over initial 48 hours of culture

Figure 1c. Changes in amine content over a 14 day culture period

Figure 2. Changes in ADC activity over initial 24 hours of culture

Figure 3. Changes in ODC activity over initial 24 hours of culture

Figure 4. Changes in SAMDC activity over initial 24 hours of culture

Figure 5. Changes in DAO activity over initial 24 hours of culture

Figure 6. Effect of DFMA on amine levels after 10 days in culture

Table 1. Cell number, fresh weight, and dry weight per culture of carrot cells grown for 10 days in the presence of 1 mM DFMA, 1 mM DFMO, 30 μM EMGBG or no inhibitor.

Figure 7. Effect of DFMO on amine levels after 10 days in culture

Figure 8. Effects of DFMO, DFMA and EMGBG on ADC activity after 10 days in culture

Figure 9a. Effects of DFMO, DFMA and EMGBG on ODC activity after 10 days in culture

Figure 9b. Effects of DFMO, DFMA, and EMGBG on SAMDC activity after 10 days in culture

Figure 10a. Growth of auxin-depleted cells in auxin-free, kinetin-free and hormone-free medium at 10 days growth

Figure 10b. Cell number of cell cultures transferred between hormone-supplemented and unsupplemented medium at 10 days growth

Figure 11a. Growth of auxin-depleted cells in auxin-free polyamine-supplemented medium at 10 days growth - fresh weight

Figure 11b. Growth of auxin-depleted cells in auxin-free polyamine-supplemented medium at 10 days growth - cell number

Figure 12. Growth of undepleted cells in inhibitor and amine-supplemented medium at 10 days growth

Figure 13a. Growth of undepleted cells in arginine and ornithine supplemented media at 10 days growth

Figure 13b. Amine content of cells in arginine and ornithine supplemented media at 10 days growth

Figure 14a. Effect of variation in NH_4^+/NO_3^- balance on growth at 10 days growth

Figure 14b. Effect of variation in NH_4^+/NO_3^- balance on amine content at 10 days growth

Figure 15. Effect of variation in nitrogen content on growth

Figure 16a. Effect of variation in media potassium level on growth at 10 days growth

Figure 16b. Effect of media potassium level on amine content at 10 days growth

Table 2. Stress and amine content in carrot cells

Table 2a. Stress and amine content in carrot cells continued

Figure 17a. Growth of adapted cells in media supplemented with 0-25% PEG

Figure 17b. Growth of unadapted cells in media supplemented with 0-25% PEG

Figure 18. Relative growth of adapted and unadapted cells in PEG-supplemented medium at 20 days growth

Table 3. Free amine concentrations in unadapted (U) and adapted (A) cell lines at 10 hours growth

Figure 19. Water potentials of adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

Figure 20a. Total amino acids in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

Figure 20b. Proline concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

Figure 21a. Sucrose concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

Figure 21b. Fructose concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

Figure 21c. Glucose concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

Table 4. Free amino acid and glycinebetaine concentrations in unadapted (U) and adapted (A) cell lines at 10 hours growth

Table 5. Medium and cell osmolarity and percentage contribution of each solute in unadapted (U) and adapted (A) cell lines at 10 hours growth

Figure 21. Sucrose a), fructose b) and glucose c) concentrations in (A) adapted and (U) unadapted cell lines in PEG-supplemented media at 10 h growth

Note regarding Figures: Except where otherwise indicated, error bars indicate standard deviation of three replicates

Acknowledgements

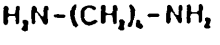
To Richard Phillips for his patience, George Stewart for his valuable time, Mike Kerr for his guidance, and not least Beattie for herself.

CHAPTER ONE

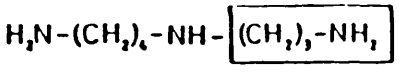
INTRODUCTION

This thesis reports an investigation into a class of amino acid derivatives known as the di- and polyamines. These are long-chain aliphatic polycationic compounds formed by the removal of a carboxyl group from an amino acid and the subsequent addition of aminopropyl groups (see Fig. i). The relative lack of information regarding the function of these compounds compared to other amino acid metabolites has led to their being labelled the 'cinderellas of cell biology' by one author (Walters, 1987). The history of their study provides some insight into why they should presently have earned such a title. They were first described as crystals in seminal fluid by the microscopist Leouwenhoek in 1678 - but the discovery was not pursued to identify these crystals until comparatively modern times. Cell biological research was initiated in work involving microorganisms, with putrescine first described as a growth factor of Haemophilus parainfluenzae (Herbst and Snell, 1948). This discovery was the first of many which indicated that amines were essential in the growth of some microorganisms and mammalian cells (Cohen, 1971; Bachrach, 1973; Tabor and Tabor, 1976). Though substantial work involving prokaryotes and animal cells has now taken place, it has taken longer for botanists to recognise the important role which these amines may play in plant growth and development, and which is indicated by their ubiquity throughout the plant and animal kingdoms. Recently however, this situation has been remedied to some extent by a series of studies which have sought to demonstrate an important role for amines as plant growth regulating substances (Bagni, 1966; 1970; Bertossi et al, 1965; Bagni and Fracassini, 1973). These studies however, depend to a large extent on knowledge of

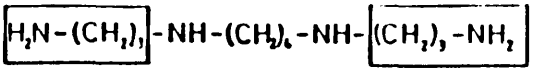
Figure i Di- and polyamines



PUTRESCINE



SPERMIDINE



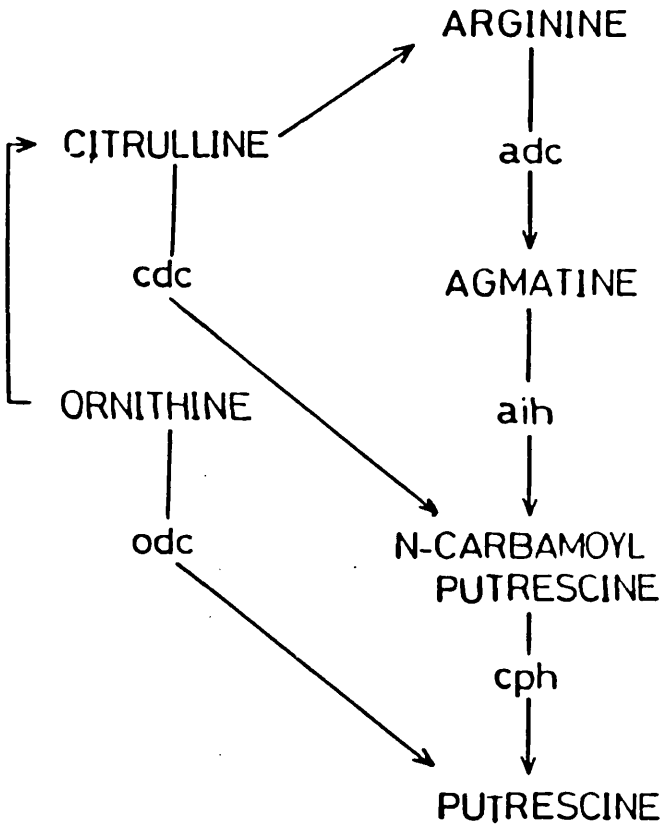
SPERMINE

amine metabolism gained from animal studies. In particular the use of specially developed amine biosynthetic inhibitors has proved invaluable. These serve to block individual stages in the biosynthetic pathway and together with the application of synthetically produced amines may be used to alter cellular levels of specific amines. To fully appreciate the use of such techniques it may be helpful to briefly consider the present knowledge of amine metabolism.

The metabolic routes to amine synthesis in plants have not received the level of attention which amine biosynthesis in non-plant systems has enjoyed, though some of the differences and similarities between the pathways in plants and in other organisms have been established.

In mammalian cells amine biosynthesis commences with the ornithine decarboxylase (ODC)-mediated decarboxylation of ornithine to form putrescine (Pegg and McCann, 1982) and this is also true of amine biosynthesis in fungi (Tabor et al, 1983). In plant cells, putrescine may be synthesized from ornithine directly or in a less direct manner from arginine after decarboxylation via arginine decarboxylase (ADC) and following a number of other steps (see Fig. ii). Though there has been shown to be a degree of variation in the production of putrescine in this way between different plant species, in all plants the general situation involves the conversion of arginine to agmatine through an ADC-mediated decarboxylation step (Smith, 1979). This is then followed by its hydrolysis via agmatine iminohydrolase to yield N-carbamoylputrescine (NCP) (Sindhu and Desai, 1979) and this intermediate compound is then converted to putrescine by the enzyme NCP-amidohydrolase (Yanagisawa and Suzuki, 1982). It is from the diamine putrescine that the polyamines

Figure ii Synthesis of Putrescine

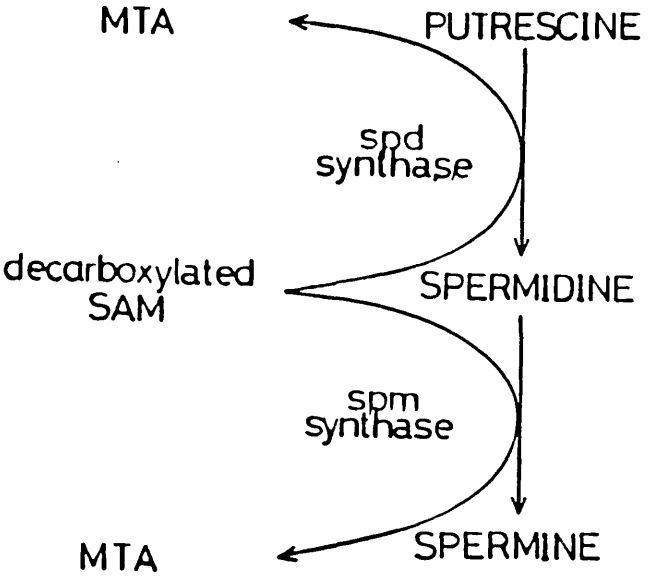


spermidine and spermine are formed. This synthesis of polyamines is now known to be accomplished by the spermidine and spermine synthase catalyzed addition of aminopropyl groups to the putrescine molecule (see Fig. iii). These groups are donated by S-adenosylmethionine (Baxter and Coscia, 1973; Hirasawa and Suzuki, 1983; Sindhu and Cohen, 1984) through S-adenosylmethionine decarboxylase (SAMDC) (Suresh and Adiga, 1977; Suzuki and Hirasawa, 1980). The catabolism of these amines may now be considered as an introduction to the catabolic investigations carried out in this study.

The breakdown of these amines is achieved through oxidation via diamine and polyamine oxidases (DAO, PAO). The present investigation has concentrated on the enzyme DAO which catalyzes the oxidation of putrescine to pyrroline (Yanagisawa et al, 1981; Floris et al, 1983; Chaudhuri and Ghosh, 1984) in those plant species which have so far been examined. With the outline of amine metabolism and catabolism which this summary has provided, a number of those reports whose results served to trigger the initiation of the present study may now be briefly considered:

Considerable interest accompanied the publication of the results of studies which suggested that amines might have significant growth stimulating and regulating properties. Such stimulation has been reported in Helianthus tuberosus explants where exogenous amines were reported to cause marked growth increments (Bagni et al, 1982) and in Alnus glutinosa and Alnus incana mesophyll protoplasts (Huhtinen et al, 1982) where stimulation of cell division was reported with putrescine application. Similarly, in Nicotiana sylvestris growth was increased with application of putrescine (Oshmarina et al, 1982).

Figure iii Synthesis of Spermidine and Spermine



Cell division was promoted when fruit trees were treated with amines (Costa and Bagni, 1983; Costa et al, 1984). Germination of mung bean seeds was increased on treatment with spermine (Altman et al, 1982) and growth inhibition in lettuce was relieved by application of amines (Cho, 1983). Correlations have also been found between levels of amines and growth activity: Growth of Scorzonera hispanica, Sesamum and Nicotiana glauca habituated tissue was accompanied by elevated putrescine and spermidine content (Audisio et al, 1976; Bagni and Serafini-Fracassini, 1974; Kung et al, 1973; Speranza and Bagni, 1977). In carrot suspension cultures ODC activity was found to increase with rapid cell division activity (Heimer et al, 1979) and increased amine biosynthesis and accumulation in carrot has also been correlated with differentation activity (Montague et al, 1978; 1979). Seed germination has been correlated with fluctuations in amine content and biosynthesis (Anguillesi et al, 1978; Bagni, 1970; Ramakrishna and Adiga, 1975; Smith and Wiltshire, 1975; Villanueva et al, 1978).

In addition to such studies which suggest the fundamental importance of amines in cellular functions, a further body of evidence indicating their significance has been gathered through the use of amine biosynthetic inhibitors such as alpha-difluoromethylarginine (DFMA) inhibiting ADC, alpha-difluoromethylornithine (DFMO) inhibiting ODC and methylglyoxybisguanylhydrazone (MGBG) which inhibits SAMDC.
Spermidine concentration was reported to increase during development of crown gall tumours and exogenously applied spermidine enhanced tumour growth. The growth was inhibited by exposure to MGBG and this inhibition was counteracted through application of spermidine (Galsky and Kulpa, 1983). In a similar way the growth of Nicotiana, Vigna and Petunia

cultures was inhibited by amine biosynthetic inhibitors and in some examples this inhibition was relieved on application of exogenous amines (Kaur-Sawhney et al, 1984). Application of DFMO halted tomato fruit development and the application of putrescine reversed this inhibition. In addition, when tomato fruits were exposed to putrescine without the inhibitor then ODC activity decreased indicating that levels of putrescine are regulated through feedback control of putrescine synthesis (Cohen et al, 1982).

Carrot culture embryogenesis was reduced by more than 50% with DFMA application and this effect counteracted on application of exogenous putrescine, spermidine or spermine. Also when tobacco cell lines resistant to MGBG were isolated and regenerated into whole plants these were green but infertile with aberrant reproductive structures (Malmberg and McIndoo, 1983). In mung beans MGBG prevented root formation and the amine increase which accompanies root initiation. In tobacco cell cultures DFMO treatment prevents cell division activity and leads to cell enlargement (Berlin and Forche, 1981). However, though excellent as investigative tools, data generated through the use of such inhibitors does require careful interpretation as it is important to show that any effects attributed to a decline in polyamine levels may be counteracted by an application of polyamines. Without such controls a causal relationship is difficult to justify. In addition these inhibitors may be altered or metabolized regardless of their specificity in in vitro enzyme kinetic studies (Evans and Malmberg, 1989).

There is some evidence that amines are involved in the regulation of nucleic structure and function: In mutants of E. coli a reduction in the levels of available putrescine was found to reduce the rate of synthesis

of RNA, DNA and protein through a reduction in the speed at which components were added to the molecules (Morris and Hansen, 1973; Geiger and Morris, 1978). The effects of putrescine starvation on synthesis of these molecules has been postulated to be a result of effects of amines on DNA gyrase (Geiger and Morris, 1978). These studies have also indicated that DNA polymerase too may be affected by reductions in amine contents. Studies with lymphocytes have also shown reduced DNA synthesis rates with inhibition of amine accumulation (Otani et al, 1974; Fillingame et al, 1975; Morris et al, 1977). In these systems a minimum cellular level of amines is necessary for optimal growth and DNA replication. Why this should be so is still not fully understood though some reports have suggested that amines may stimulate DNA polymerase activity (Chiu and Sung, 1970; Stalker et al, 1976). Spermine can bind to DNA and protect it from denaturation at high temperature in vitro (Liquori et al, 1967) and lowered cellular amine levels result in increasing susceptibility to DNA alkylation (Hung et al, 1983). In addition spermidine and spermine can promote changes in polynucleotide conformation (Behe and Felsenfeld, 1981) and take part in the regulation of DNA conformation (Russell et al, 1983). Spermidine has been identified as an important part of the RNA in the core of one plant virus (Cohen and Greenberg, 1981) and other data suggests that amines may regulate tRNA conformation (Sakai and Cohen, 1976) while aminoacylation rates are increased when tRNAs are treated with spermine (Loftfield et al, 1981).

In a number of studies in higher plants amines have been shown to interact with nucleic acids. They have been shown to influence protein synthesis activation in isolated ribosomes and to bind to RNA in Jerusalem artichoke, spinach and pea (Bagni et al, 1973; Bagni and Serafini-Fracassini,

1974). In soybean interaction between spermidine and the RNA polymerase of hypocotyls has been demonstrated (Nakamura et al, 1972) as has the replacement of magnesium ions by amines during ribosome incorporation in polyphenylalanine synthesis (Cohen and Zalik, 1978). Also amines may inhibit increasing ribonuclease activity while blocking senescence in protoplasts of oat (Galston et al, 1978). Such enzyme activity regulation has been noted in a number of studies and may be an indirect result of the mediation of protein phosphorylation by amines (Slocum et al, 1984). One of the first studies implicating amines in such phosphorylation was that of Kuehn et al (1979) which demonstrated an amine modulated phosphorylation of a nucleolar protein governing the rate of Physarum rRNA synthesis, and later found that the nucleolar protein was ODC (Kuehn and Atmar, 1982). Amines may also influence enzyme activities through ionic interactions. This appears to be the case in spinach where amines increase NADPH oxidation rates mediated by a flavoprotein iron-sulphur protein complex (Nakamura et al, 1972). Amines are also thought to be involved in photosynthetic regulation with reports that spermidine and spermine can suppress the activation of fructose 1,6 bisphosphate - a key carbon fixation enzyme (Cohen et al, 1981). Amines may also accomplish protein regulation through covalent binding (Folk, 1980). Some reports suggest that the activity of ODC may be modulated through the binding of the enzyme protein with putrescine (Russell, 1981).

In addition to reports that amines may be implicated in the determination of growth activity, other recent reports have indicated that amines may be of some importance in plant metabolism during exposure to stress conditions. In response to such conditions putrescine levels within the plant often rise with other amines showing little change in

concentration. Some authors have suggested that such responses may be fundamental to plant survival (Slocum et al, 1984) and it is evident that any mechanism involved in plant stress responses is worthy of some attention in view of the fact that the majority of the world's plant life completes the life cycle under far from ideal conditions, where a number of different forms of stress may be present. Stress conditions under which amines have been shown to accumulate include osmotic stress, acid stress and potassium deficiency. The accumulation of putrescine in response to potassium deficiency was first reported by Richards and Coleman (1952). This initial work was supplemented by Murty et al (1971) who found very large increases in putrescine accompanying potassium deficiency. In fact the extent of the accumulation led the authors to suggest that putrescine may serve to replace potassium under such circumstances.

Acid stress may occur under a variety of conditions where excess protons are generated such as when there is an excess of acid in the environment, when the nitrogen source consists predominantly of ammonium or there is pollution by sulphur dioxide. This has been shown to be accompanied by an accumulation of putrescine in several studies (Priebe et al, 1978; Smith and Sinclair, 1967; Young and Galston, 1983). It is not clear exactly what, if any, importance this putrescine accumulation may have. It may be merely a perturbation of amine metabolism which is of no general importance but if the phenomenon is of value then it is possibly due to a buffering effect which increased accumulation of amines may achieve. Due to their polycationic nature, amines bind to anionic compounds and structures within the cell such as nucleic acids and membranes, thereby conferring increased stability (Srivastava and Smith, 1982). Such properties have also been implicated in the increased amine

levels reported in plants subjected to osmotic stress. Again putrescine levels are dramatically increased on exposure to stress via immersion of roots of whole plants in osmotica or floating detached leaves on solutions of low water potential (Flores and Galston, 1982b). This may also be due to amines contributing to cellular molarity. In the study above ADC activity rose in response to the stress conditions although ODC activity remained unchanged. DFMA treatment prevented ADC activity from increasing and also prevented putrescine from accumulating while ODC was unaffected by DFMO *in vivo* or *in vitro*. DFMO treatment did, however, increase the sensitivity of the putrescine osmotic response. Treatment with cycloheximide prior to incubation in sorbitol prevented any rise in putrescine levels from occuring which suggests that *de novo* protein synthesis is required to produce ADC for this response. Though putrescine accumulates in response to osmotic stress in the protoplasts of non-regeneratable cereal leaves, in other regeneratable protoplasts there is no measurable accumulation of putrescine under such conditions (Dumortier and Galston, 1984). It has further been suggested that putrescine increases under stress conditions may be due to its use as a storage product under adverse environments or as a preservative for sensitive proteins (Smith, 1984).

Amines may also play a role in the response of the plant to wounding or senescence. Exogenously applied spermine, spermidine and cadaverine halted senescence in oat leaf protoplasts and increased osmotic activity (Altman *et al*, 1977; Galston *et al*, 1978; Galston *et al*, 1979; Kaur-Sawhney *et al*, 1979; Kaur-Sawhney *et al*, 1980). Amines can also retard chlorophyll breakdown in detached leaves and prevent an increase in RNAase and proteases (Cohen *et al*, 1979; Kaur-Sawhney and Galston, 1979; Altman,

1980). The biosynthesis of senescence-promoting ethylene was inhibited in apple fruit by spermidine and spermine (Apelbaum et al, 1981) and some studies have suggested that amines may facilitate seed germination at elevated temperatures (Ashour and Thalouth, 1971).

From the studies discussed above, it is clear that a role for amines has been implicated in many areas of plant growth and development. Such investigations have employed a wide range of whole plant species and plant cell and tissue cultures, but it is apparent that as yet a unifying treatment has not been attempted with a number of these investigative methods applied to a single easily manipulated system. Perhaps this explains why the authors of a recent review stated that it was impossible to say exactly how polyamines function in higher plant physiology (Evans and Malmberg, 1989). A number of workers such as Montague, Bagni and Feirer have identified such a system in the carrot suspension culture.

Unfortunately however, it is not always possible to combine the results from these investigations in order to provide a more complete picture of the role of amine metabolism in general cell function - as can be seen when two similar studies such as those of Montague et al (1979) and Fienberg et al (1984) are compared. While the conflicting results of these investigations may be rationalised as the result of a slight difference in methodology, it does indicate that a coherent pattern of investigation incorporating a single experimental system would be of great value - thus providing comparative data for further metabolic analysis. Indeed, the confusion concerning the role of polyamines in plants is due in part to the profusion of experimental systems and experimental treatments employed. Many studies cannot be compared because

different systems have been used and different parameters have been chosen to study the polyamine pathways. The decision by several authors to choose the carrot suspension culture system however, has allowed their results to be compared and these studies have been thorough enough to be convincing (Evans and Malmberg, 1989).

The initial application of the carrot suspension culture system to the search for the answer to the amine question arose out of previous studies involving whole plants which attempted to replicate osmotic stress studies in which putrescine was reported to show massive increases (Flores and Galston, 1984). Two separate investigations failed to provide similar dramatic data and served to suggest that these increases might have been phenomena of methodological origin rather than a response to stress conditions.

Clearly a concise, repeatable and controllable methodology was required to avoid the generation of inconclusive and conflicting data and in order to approach a solution to the 'polyamine question' in plants. And therefore, in a single, controllable and reproducible cell culture system the relationship between amines and cell growth and stress was studied, with the system's flexibility allowing each part of the study to incorporate similar techniques so that each part could be meaningfully compared with the other parts. Growth investigations would attempt to provide a thorough evaluation of amine metabolism during different phases of growth while stress studies tried to place amine changes in response to stress in the context of other metabolic changes occuring simultaneously as a result of the stress stimulus.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Carrot cell culture

A culture of Daucus carota (cv Chantenay) carrot tissue was established and maintained after a series of transfers to fresh media resulted in vigorous growth. The culture was grown on agarized Murashige and Skoog (1962) medium (Flow Labs, Irvine, Scotland) with the addition of 0.2 mg/L 2,4-dichlorophenoxyacetic acid, 0.1 mg/L Kinetin and 2% sucrose, henceforth referred to as 'MDK'. At each subculturing of the tissue, the tissue which appeared most vigorous and healthy in appearance was selected.

In 1985 a suspension culture was initiated by the transfer of a small quantity of tissue to a 20 mL vial containing 5ml MDK medium without agar under sterile conditions. The vial was incubated in a rotary shaker at 25°C for one week, at the end of which time the culture was sufficiently thick to be transferred to a 250 mL Ehrlenmeymer flask containing identical medium, again under sterile conditions.

The inoculum density was kept high initially to minimise the degree of acclimatisation which was required before vigorous growth could resume. At the end of a 14 d period the culture was sufficiently thickened to allow for its subculture. From this point onwards a number of 250 mL flasks were maintained by subculturing into fresh medium at the end of each 14 d culture period. On all occasions the flasks were inoculated at a ratio of 15 mL inoculum to 75 mL fresh MDK medium. The cultures were incubated at 25°C on an orbital shaker at 80 rev/min and an amplitude of

5cm in the dark except for intermittent exposure to light while being inoculated and sampled.

2.2 Growth determinations

Fresh and dry weights

The growth pattern of the carrot suspension culture was established by growth determinations performed every 2 days during the culture period. 250 mL flasks of carrot suspension culture were removed from the orbital shaker and placed within the sterile laminar air flow cabinet which had been previously disinfected with a dilute (10%) solution of 'Domestos'. The apparatus for filtering off the carrot cells consisted of four (10ml) syringe barrels fitted with sintered polypropylene filter discs (40 µm pore size). These four were first filled with distilled water which was driven out through the filter disc using a Buchner flask fitted to a vacuum pump. The tubes were now spun within syringe cases in a bench top centrifuge at 2000 rpm for 5 min to drive off remaining water. After centrifugation the excess water remaining within the narrow tips of the syringes was removed with a piece of absorbent tissue paper and the syringe barrels were weighed and the weights noted. They were then brought within the flow cabinet and placed in a 100 mL glass beaker. The culture flask to be sampled was now taken and the foil cap covering the neck of the flask was passed through the flame of a bunsen burner to sterilise it. The foil was now carefully removed with dry, washed and scrubbed hands without contaminating the sterile interior surface of the foil cap or flask neck. The suspension was swirled rapidly to distribute the cells evenly within the culture medium and rapidly poured into each syringe barrel in 5 mL aliquots. As soon as the samples had been taken the flasks were carefully resealed using the original foil caps and the flasks of suspension culture replaced onto the orbital shaker once again.

The syringe barrels filled with suspension culture were now taken and the culture medium was drawn through the filter discs under vacuum as before. The syringe barrels were then placed in the centrifuge and spun as before. After removing excess fluid once again the 'spin-dried cells' were weighed within the barrels, the weights noted, and the cell fresh weights calculated by subtracting the weights of the syringe barrels as previously determined.

The dry weights of the suspension cultures was determined as described above except that the spin-dried cells were removed from the syringe barrels by air pressure applied to the tip of the syringe and the tissue wrapped in weighed foil. The foil wrapped package of cells was then labelled and weighed and both weights noted. The package was now oven dried at 60°C overnight and weighed once more. Dry weights were calculated and noted.

Cell counts

Cell counts were made on weighed tissue by a modification of the method of Brown and Rickless (1949). 100 mg portions of tissue were macerated in 5% chromium trioxide in 5% HCl within glass scintillation vials, separated by drawing the solution repeatedly through a hypodermic needle, and counted under a phase-contrast microscope with a haemocytometer after appropriate dilution with deionised water.

Protein content

The protein content of the cells was determined by a modification of the Lowry et al (1951) method. A known weight of spin-dried cells was extracted in 0.1 M NaOH for 30 min. To 1 mL of this extract was added an equal volume of alkaline copper reagent, and the mixture allowed to stand for 10 min. 4 mL of diluted Folin- ciocalteau solution was now added, and after mixing the tube was placed in a water bath at 55°C for 5 min. The tube was cooled rapidly in water for 1 min, and its absorbance at 650 nm measured. The protein concentration was then read from a calibration curve. (bovine albumen)

2.3 Solute determinations

Free-amines

The free-amine content of the sample was determined according to the method of Spragg and Hutchings (1983). Spin-dried cells were extracted for 30 mins in 5% perchloric acid, at 0°C with continuous stirring. The resulting extract was filtered into a plastic container and stored at 20°C. To the thawed extract was added 60 µL of 10 mM diaminooctane as an internal standard. At the same time a standard sample was prepared by adding 250 µL of a standard polyamine mixture and 60 µL of diaminooctane to a volume of perchloric acid equal to that used to extract the cell sample. To each sample a few drops of methyl orange were now added. The samples were neutralised with 1.0M KOH, and after the precipitate had been allowed to settle the supernatant was withdrawn with a pasteur pipette, and transferred to a plastic beaker. The samples were freeze-dried overnight and redissolved in 500 µL 1.0M sodium carbonate. 150 µL of the resulting solution was transferred to a 1mL 'Reacti-vial' and 450 µL of FNBT derivatizing reagent added. The vial was sealed and mixed thoroughly. It was then incubated at 60°C for 20 mins, mixing again after the first 5 mins. After being allowed to cool 60 µL of 1.0M histidine in 1.0M sodium carbonate was added and when the two phases had separated the upper organic phase was drawn off with a pasteur pipette and transferred to a sample vial. The lower phase was re-extracted with a further 2 mL of 2-methyl butane. The organic phase was reduced to dryness under a stream of nitrogen and the residue redissolved in 500 µL AnalaR methanol. This was then filtered through a 0.5 micrometre size millipore filter.

The bright yellow polyamine derivatives were quantified by HPLC against authentic standards (Sigma).

The separation was performed isocratically on a Varian 5040 liquid chromatograph with a UV50 variable wavelength detector set at 254nm and equipped with a 25cm reverse phase C18 column (Spherisorb ODS2 5µm particle size) and a 20µl injection loop. The mobile phase was 85% acetonitrile (HPLC grade, Fisons)/15% double-distilled water at a flow rate of 1.5ml/min (Fig. iv).

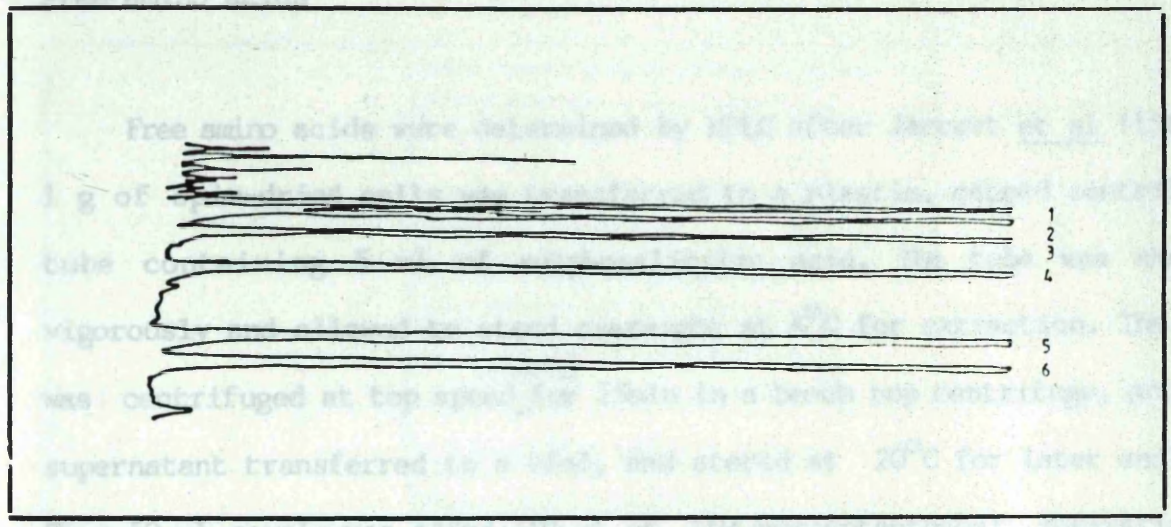
Proline

Proline was determined according to the method of Troll and Lindsley (1955). 1 g spin-dried samples were extracted in 5 mL AnalaR methanol within glass boiling tubes sealed with 'Nescofilm' (Nescofilm, Osaka, Japan) after initial vigorous shaking, on ice for 30 min. 1 mL of the extract was mixed with 1 mL acid ninhydrin and 0.5 mL 6 M orthophosphoric acid. The mixture was boiled for 45 min and extracted with 5 mL toluene. After centrifugation and phase separation, the absorbance of the sample (upper phase) was measured at 515 nm. Standards of 0 - 100 nmol proline were similarly treated, and a calibration curve constructed.

Glycinebetaine

Glycinebetaine was determined colorimetrically by the method of Stumpf (1984). Cells were harvested and spin-dried as above before extraction in AnalaR methanol at 24°C overnight with initial vigorous shaking. 1 mL of extract was transferred to a 1.5 mL Eppendorff along with 100 mL modified Dragendorff reagent. After centrifuging for 2 min at top

Figure iv A typical HPLC spectrum for a Polyamine standard mixture



Key: 1. diaminopropane 2. putrescine 3. cadaverine 4. spermidine
5. diaminooctane (internal standard) 6. spermine

speed (10000g) in a microcentrifuge the supernatant was removed and the pellet redissolved in 1 mL 2.45 M potassium iodide solution. A 10 µL aliquot was then added to 1 mL 0.49 M KI and the absorbance (OD467) read against a blank of 0.49 M KI. Betaine content was read from a standard curve.

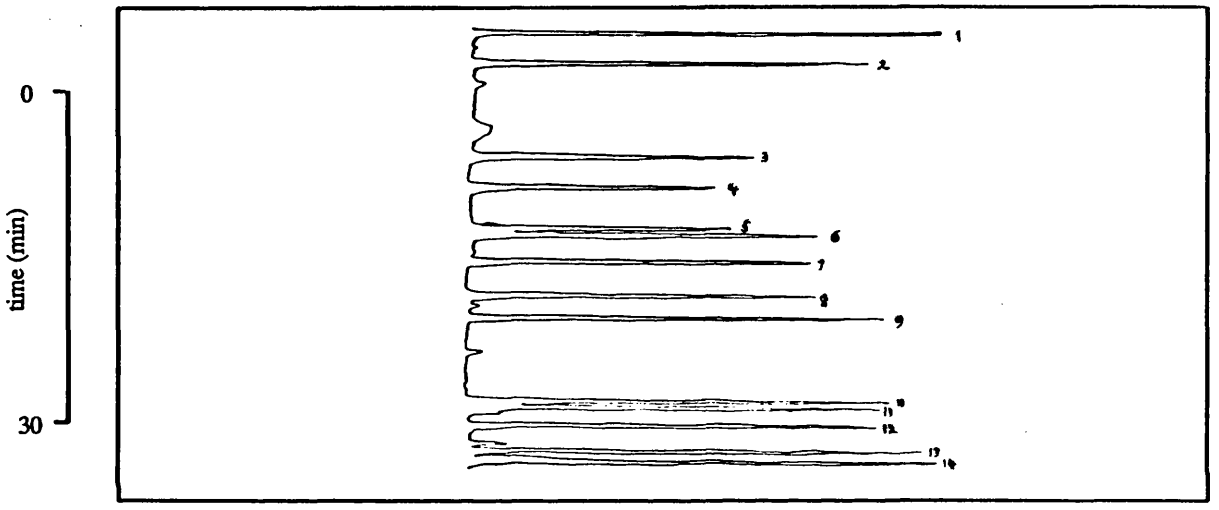
Free amino acids

Free amino acids were determined by HPLC after Jarrett et al (1986). 1 g of spin-dried cells was transferred to a plastic, capped centrifuge tube containing 5 mL of sulphosalicylic acid. The tube was shaken vigorously and allowed to stand overnight at 4°C for extraction. The tube was centrifuged at top speed (10000g) for 15min in a bench top centrifuge, and the supernatant transferred to a vial, and stored at 20°C for later analysis. To a 50 µL sample was added 150 µL of OPA/mercaptoethanol derivatizing reagent, and the mixture left to stand for 2 mins. A 20 µL sample was then analysed on a Varian HPLC with a 4.6 x 120 mm Varian octyl silica C-18 column and a mixture of tetrahydrofuran:sodium acetate(45 mM), pH 5.7, (4:96) as aqueous solvent and a linear gradient of 10 to 65% methanol over 15 min by reversed-phase chromatography (see Fig. v).

Sugars

Sugars were determined by the method of Sweeley (1963) with the modifications recommended by Holligan (1971): 1 g of spin-dried cells was transferred to a glass, capped centrifuge tube with 5 mL of extraction medium: methanol, methylene chloride, and water (12:5:3 by volume), along with 500 µL of 5 mM xylitol as internal standard. After mixing well the tube was transferred to a fridge and left overnight at 4°C. To the extract was added 5 mL methylene chloride followed by 5 mL distilled water to induce phase separation between the methanol and water and the methylene

Figure v A typical HPLC spectrum for an amino acid standard mixture

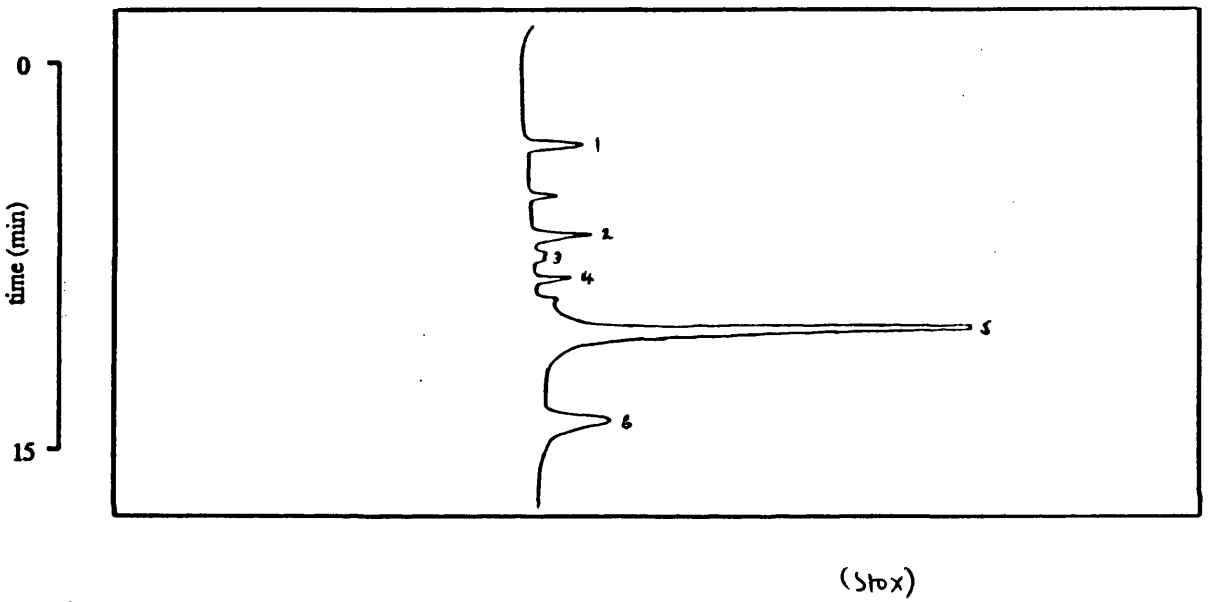


Key: 1. aspartate 2. glutamate 3. serine 4. histidine 5. glycine
6. threonine 7. arginine 8. alanine 9. tyrosine 10. methionine
11.valine 12. phenylalinine 13. isoleucine 14. leucine

chloride. The upper phase containing sugars, sugar alcohols, amino acids and related compounds was drawn off using a pasteur pipette and dried down in a rotary evaporator at 40°C. The dry material was then resuspended in 1 mL of distilled water. Separation was performed on a Dowex $50H^+$ strongly acidic ion exchange column (5cm deep) composed of a pasteur pipette plugged with cotton wool. 0.5 mL of the extract was applied was applied to the top of the column and sugars and organic acids eluted with 7.5 mL distilled water. This mixture was dried down in a freeze-drier and redissolved in 0.5 mL water. This was then applied to the top of a Dowex-1 Formate strongly basic ion exchange column (5ml Gilson tip, 4cm deep). Sugar alcohols and sugars were eluted with 7.5 mL distilled water and the sample dried down in a freeze drier, redissolved in 0.5 mL water and stored in a freezer prior to derivatization. For derivatization a 100 µL sample was dried down under nitrogen gas, 200 µL methylene chloride added, the samples dried again and redissolved in 100 µL "Stox" solution. The mixture was heated at 70°C for 30min and cooled to room temperature, and left at room temperature for 1 h after addition of 100 µL trimethylsilylimidazole. 2 µL of the resulting derivatised mixture was injected onto an SE30 packed column at 150°C. After 10 mins the temperature was raised to 280°C at 13°C/min. Sugar derivatives were detected using a flame ionization detector, with helium as carrier gas (see Fig. vi).

* STOX Pyridine + 25mg/ml hydroxyammonium chloride + 6mg/ml phenyl-B-D-glucopyranoside

Figure vi A typical GC spectrum for a sugar standard mixture



Key: 1. xylitol 2. fructose 3. glucose 4. myo-inositol 5. internal standard 6. sucrose

2.4 Enzyme assays

Arginine decarboxylase

0.5g spin-dried, weighed tissue was homogenised with 1.5 mL 0.1M HEPES pH7 containing 5 mM dithiothrietol with 30 strokes of a glass homogenizer at 0°C. After centrifugation at 12000 g for 15 min, the supernatant was frozen and stored at -20°C for ADC assay, while the pellet was resuspended in ODC buffer(50 mM Tris pH 8.5, 0.3 mM EDTA, 5 mM dithiothrietol, 50 mM pyridoxal-5-phosphate) and frozen as above, for later ODC assay. It has been reported that 75% of ODC activity is associated with the 4000 g pellet in barley seedlings (Kyriakidis et al, 1983), and preliminary assays confirmed that over 90% of ODC activity was present in the pellet.

Enzyme reactions were carried out in glass tubes within sealed scintillation vials containing 0.5 mL Packard Carbosorb, to absorb released CO_2.

The ADC assay was initiated by injection of 2 µL of DL-[U-^{14}C] arginine monohydrate(Amersham 0.1 µCi, 342 mCi/mmol) through a serum cap on the vial into 150 µL of extract mixed with 10 µL pyridoxal-5-phosphate, and the vial incubated at 30°C for 60min on a shaker at 100 rpm. The reaction was then terminated by addition of 50 µL 0.2N perchloric acid. After shaking for a further 60 min, the reaction tubes were removed and the radioactivity of the absorbed CO_2 determined in a scintillation counter after addition of 10 mL scintillation fluid (Optiphase,Fisons).

Ornithine decarboxylase

For ODC assay the above procedure was used, except that 150 µL of pellet suspension was mixed with 50 µL of ODC buffer and the reaction initiated by addition of 2 µL L-[1-^{14}C] ornithine hydrochloride (Amersham 0.1 µCi, 61 mCi/mmol) as substrate.

S-Adenosylmethionine decarboxylase

SAMDC activity was determined in a similar fashion to that of ADC. Cells were extracted as above with ADC buffer at pH 7.5. The enzymic reaction was started by adding 0.075 mL of crude extract to 0.01 mL of S-Adenosyl-L-[carboxyl-^{14}C]-^{14}C] methionine (Amersham, 0.25 µCi, 59.3 mCi/mmol) and 0.01 mL of 5 mM pyridoxal-5'-phosphate.

Diamino oxidase

DAO activity was determined by measuring the change in oxygen levels due to the oxidation of putrescine by DAO via an oxygen electrode. To 20 µL of crude extract (pellet and supernatant) was added 1 mL of buffer (0.1 M KPO4, 2 mM Na Azide pH 7). The reaction was initiated by the addition of 10 µL of 0.1M putrescine at 30°C. (McGowan and Muir, 1971; Yanagisawa et al, 1981)

In every case enzyme activities were quantified against protein content which was determined as above, but with additional precipitation with perchloric acid and dissolving in NaOH, to avoid the effects of HEPES, as described by Montague et al (1979).

2.5 Solute potentials

Media solute potential

The osmotic potential of the cell contents and media were determined by the use of a vapour pressure osmometer (Wescor 5100C). Samples of fresh and spent media were sampled directly, and the following procedure observed: The osmometer was calibrated using a precise quantity of a reference solution of known osmolarity. Once calibrated a similar quantity of medium was pipetted into the sample chamber and after allowing time for equilibration the osmolarity noted.

Cell solute potential

For cellular solute potential, a similar procedure was followed. Collected, filtered and spin-dried tissue was stored frozen at -20°C, and allowed to reach room temperature. A quantity of the cell contents, liberated by the freeze-thaw process, was transferred by pipette to the sample chamber, and the osmolarity determined as above.

2.6 Inhibitor studies

The treatment of the cultures with the specific inhibitors was carried out through incorporation of the inhibitors in the standard culture medium. The MDK medium was prepared as previously described and autoclaved. Meanwhile, the inhibitor was dissolved in distilled water in a glass vial to produce a standard solution. The standard solution was then filter-sterilized by pipetting it through a 10 µm millipore filter into a sterile vial. A known quantity of the sterile solution was then pipetted into the sterile culture medium under aseptic conditions using a Gilson pipette, the barrel and pipette tips of which had been previously sterilized by autoclave. In this way, flasks of media containing a range of inhibitor concentrations were produced. Aliquots of stationary-phase cultures were inoculated into supplemented and control (unsupplemented) media as before and the inoculated flasks were incubated as previously described. 10 d after inoculation cells were extracted, and a range of growth parameters, amine contents and amine metabolic enzyme activities determined.

In the case of the diamine synthesis inhibitors DFMA and DFMO, the medium was supplemented with 0.1, 0.3, 1 and 3 mM concentrations of the inhibitor, and in the case of the polyamine synthesis inhibitor EMGBG, with 3, 10 and 30 µM concentrations of the inhibitor. Cells were incubated for 10 d in the supplemented medium and then cell numbers (DFMA, DFMO only), fresh weights and dry weights determined along with amine levels and ADC, ODC and SAMDC activities. Samples were stored on ice prior to enzyme determinations, which were carried out on the day of harvesting. Amine determinations were carried out on samples which had been extracted and stored at -20°C prior to analysis.

2.7 Amines as auxin replacements

Inocula of auxin-depleted* stationary-phase cells were transferred to hormone-free medium supplemented with a range of concentrations of filter-sterilized amines and incubated as described previously.

1, 10, 100 and 1000 μM concentrations of putrescine, spermidine, spermine and cadaverine were used in the study. In two separate experiments, both depleted and normal cells at stationary phase were inoculated into the media, and after 10 d growth in the experimental medium, the cells were harvested and fresh weights and cell numbers determined on triplicate samples from identical flasks.

* cells grown for one passage in auxin-free medium

2.8 Short and long-term stress

Five cell-lines were used; unadapted cells were grown in standard culture medium without PEG, while four cell-lines were adapted to growth in medium containing (w/v) 10, 15, 20, and 25% PEG (BDH-4000 MW, purified by ion-exchange with BDH-Duolite MB5113 mixed resin) by repeated subculture until consistent final fresh weights were obtained in consecutive passages; the adaptation period was for a minimum of 8 subcultures. For the experimental treatments, adapted cells were transferred to fresh medium of corresponding PEG concentration. Unadapted cells were transferred both to standard medium without PEG (control treatment) and to media containing each PEG concentration. For each cell-line, cells from stationary phase cultures were collected by nylon mesh filtration, allowed to drain, and resuspended in the same volume of fresh medium. Water potential and solute determinations were made 10 h after transfer.

2.9 Anoxia

In order to produce anoxic conditions in the carrot culture medium without producing other physical changes which would be the case if the flasks were not shaken an apparatus was set up to feed sterile nitrogen gas through the medium whilst other conditions remained as under standard conditions. The apparatus consisted of a rubber bung in which two vertical holes had previously been bored fitted with two glass tubes, one 3 cm longer than the other. The longer of the tubes which extended below the surface of the medium was used to feed nitrogen gas into the medium while the shorter tube served as exhaust for excess gas. Each tube was fitted with a cotton wool bung to prevent contamination of the medium and prior to use the entire apparatus was sterilized by autoclaving.

To initiate the experiment stationary phase cells were inoculated into standard medium and all flasks fitted with the gas delivery apparatus and transferred to the orbital shaker. Three experimental flasks were then connected to a nitrogen supply via a three-way adaptor and the gas supply adjusted to approximately 300 bubbles/min. The flasks were then incubated for 8 h under standard conditions and cells harvested, fresh weighted and analyzed for amine content as previously described.

CHAPTER THREE

POLYAMINE METABOLISM

3.1 Polyamine levels and growth rates during culture

Introduction

Substantial evidence for an amine requirement in cellular growth has been produced in the last decade, first in bacteria (Herbst and Snell, 1948) and later in fungi (Sneath, 1955) and mammalian cells (Pohjanpelto et al, 1981); with some evidence of a link between amines and growth in plants (Smith, 1985; Slocum et al, 1984). In plants active growth and cell division are accompanied by increased polyamine synthesis rates in Scorzonera hispanica (Speranza and Bagni, 1977) and Nicotiana glauca tumour tissues (Audisio et al, 1976) and Helianthus tissue (Bagni et al, 1980). A similar increase in amine synthesis was also found in tobacco cells during rapid division (Heimer et al, 1979), in developing tomato ovaries (Cohen et al, 1982) and in growing buds and leaves of Phaseolus (Palavan and Galston, 1982). However, in a recent study Felix and Harr (1987) analysed polyamine levels in various organs of a number of plant species and found that increases in polyamine levels were not always seen in those organs showing rapid cell division. In fact, the situation in plants remains less clear than in prokaryotes where polyamine auxotrophic mutants have been available.

Much of the data reported from previous studies comes from measurements of free polyamine content and synthesis during periods of varying growth activity and in tissues with differing growth rates. Since

amines have been shown to be elevated in rapidly dividing tissues in some studies, it would appear possible that changes in amine levels are associated with growth regulation.

To test this hypothesis an initial experiment was designed to determine whether exogenous amine levels correlated to growth rates throughout a single culture passage. Stationary phase cells were inoculated into fresh medium and both fresh weight and free amine levels determined at regular intervals.

Consistent fresh weight values over successive days indicated that stationary phase and the end of the culture period had been reached and successive experiments indicated a consistent growth pattern, which involved an increase in cell mass of reproducible size.

Results and Discussion

3.1.1 Fresh weights

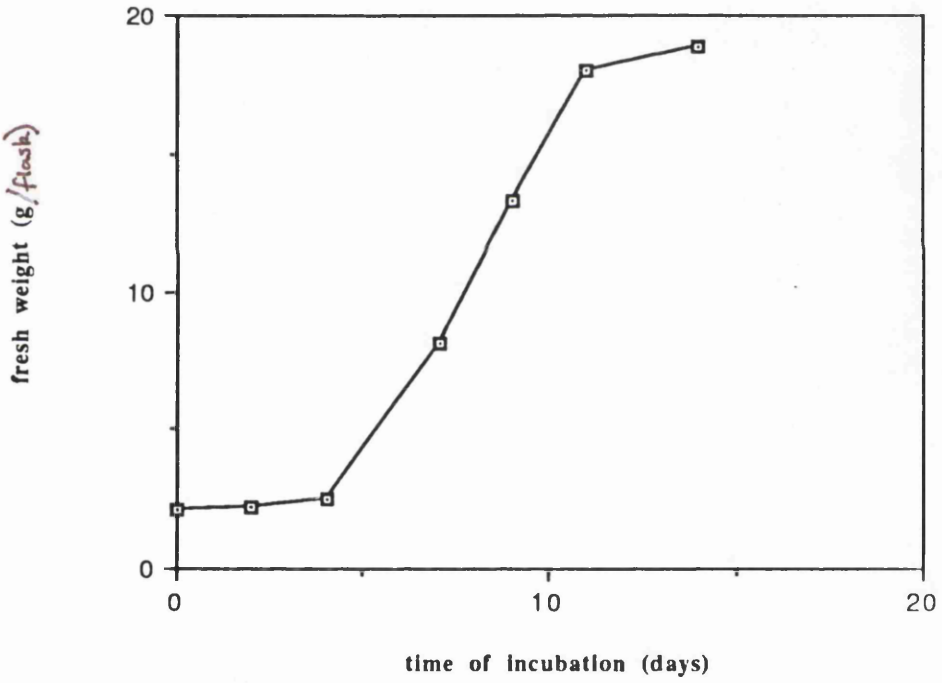
During the first four days of culture, little change in fresh weight was observed, with only a slight increase apparent - a characteristic lag phase (Fig. 1a). A marked change in growth rate occured after this four day period however, with fresh weight increasing rapidly and at a consistent rate up until 11 d after subculture, which signalled the end of the exponential phase. Between 11 and 14 d after subculture, growth was reduced markedly, with fresh weight increasing very little compared to earlier increases.

Having established the growth pattern under standard conditions, it was possible to investigate its relationship to the pattern of change in amine level over a similar period of growth:

3.1.2 Free polyamine levels

A similar experimental framework to that above was employed once more with samples weighed and then extracted for amine determination as previously described. Samples were extracted immediately in every case, and concentrations presented in terms of the means of triplicate samples. Fig. 1b shows changes in amine levels during the initial 24 h of culture. Putrescine content increases rapidly over the first 2 h of culture, while spermidine and spermine concentrations decline. Putrescine doubles in concentration, the spermidine level declines to half its initial value,

Figure 1a Changes in fresh weight over a 14 day culture period



and spermine content falls by one third. Between 2 and 4 h after subculture the increase in putrescine content continues but is not so rapid, increasing by approximately 20% over the 2 h period. Spermidine levels begin to increase at 2 h and show a 25% increase by 4 h while spermine levels show little change. The initial peak in putrescine levels is followed by a sharp fall after 6 h in culture. All polyamines do however show a general increase from this period up until the 24 th hour of culture, when levels appear to stabilize and are little changed at the 48 th hour of culture. Having considered the initial hours of culture, the general pattern of amine change over the 14 d culture period may be studied (Fig. 1c). The sharp increase in the main amines up to 48 h after subculture is followed by a decline, which is sharper in the case of putrescine than spermidine. Putrescine concentration falls by more than 70% between 2 d and 7d after subculture with spermidine level falling by 50% during the same period and only spermine content increasing. Putrescine exhibits a second peak after 9 d which is less than half the initial peak level. Spermidine meanwhile continues to decline reaching a level of only 114 nmol/gfwt after 11 d, compared to a 2 d peak of 788 nmol/gfwt. Towards the end of the culture period spermidine levels once again increase as putrescine levels fall, and the consistent increase in spermine levels is followed by a final decline. At the end of the culture period (14 d), the stationary phase cells have similar levels of all 3 major amines to those which were present in the initial inoculum.

If the initial period of culture is first considered, in terms of growth a four day lag period - then clear differences in the general trends of growth rates and polyamine levels may be seen. The initial period of very little growth in terms of fresh weight gain coincides with

Figure 1b Changes in amine content over initial 48 hours of culture

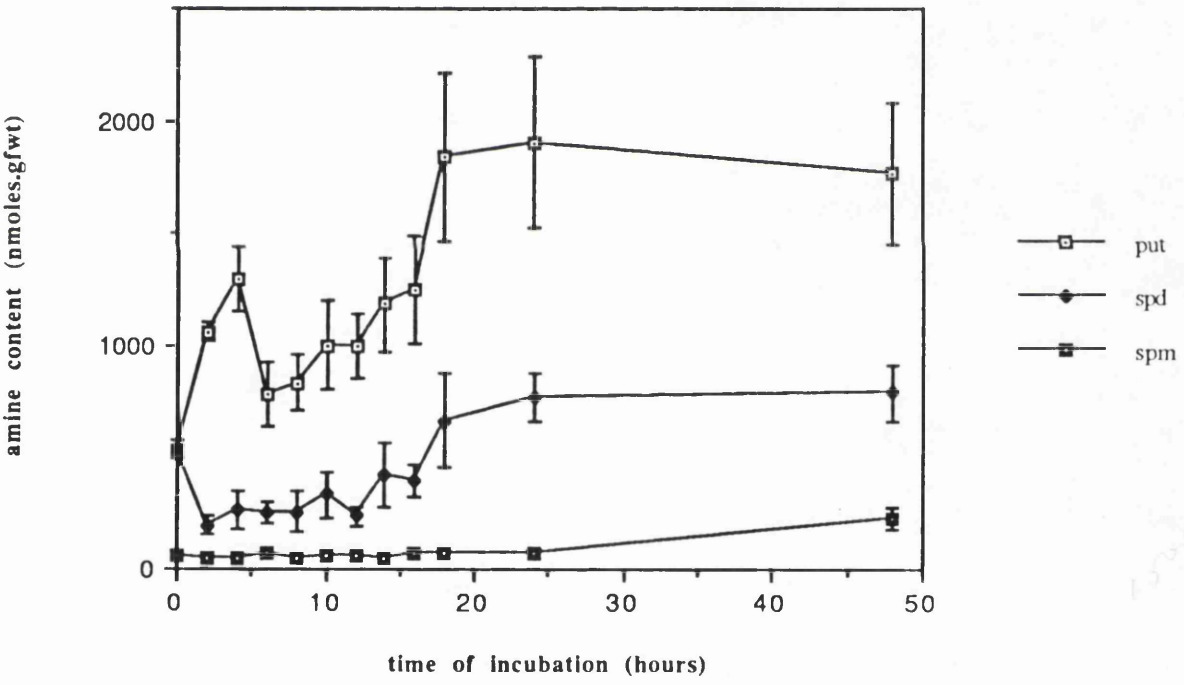
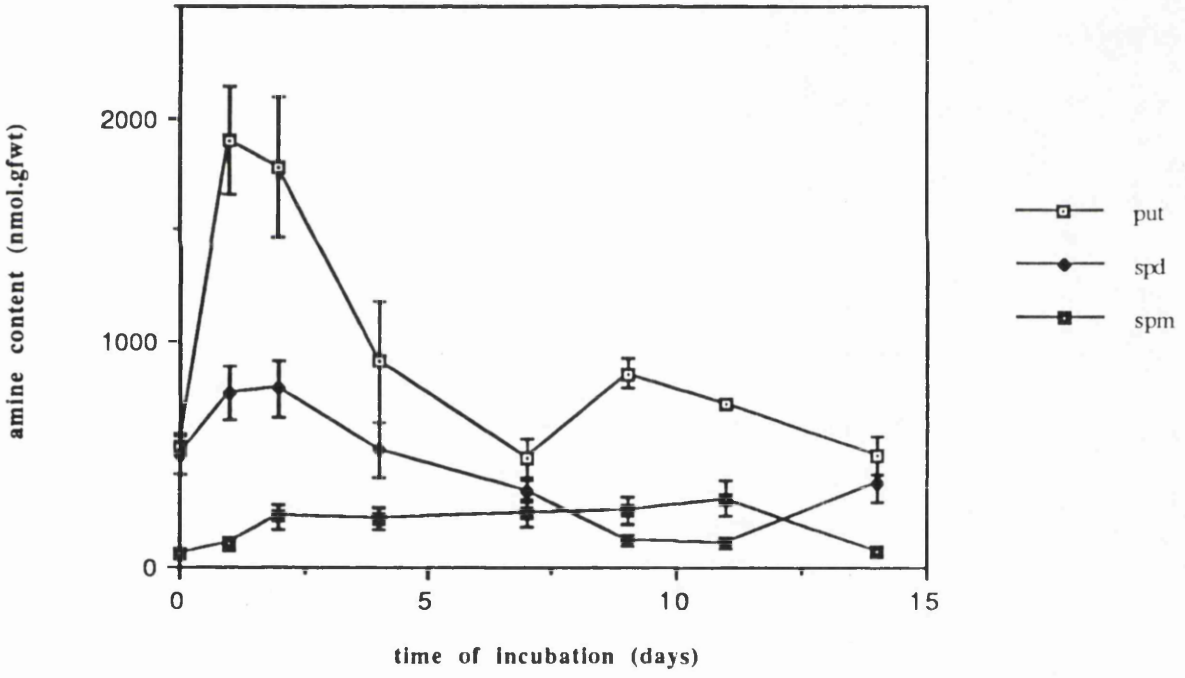


Figure 1c Changes in amine content over a 14 day culture period



large fluctuations in concentrations of the major amines. This initial period of sharply increasing amine concentrations was observed repeatedly, and was found to be a characteristic of newly subcultured suspensions.

If the period of rapid increase in fresh weight is next considered, (in the case of this culture the period 4 - 11 d after subculture), a lack of correlation is once again observed between changes in fresh weight and changes in the content of the major amines.

Levels of both putrescine and spermidine fell during the initial period of rapid growth which occured 4 d after subculture, and only the minor component spermine displayed any increase during this period - with its level showing a close correlation with the pattern of fresh weight change. Over the period of rapid fresh weight gain as a whole, levels of the major amines putrescine and spermidine declined, and while putrescine levels did increase between 7 d and 9 d after subculture, spermidine content declined during this period and increased as cells entered the stationary phase. Levels of the minor component spermine showed a very different pattern of change however. Levels of this component rose steadily over this entire period of rapid growth, showing a significant increase over initial post-subculture levels. After 11 d in culture, and before the end of the culture period at 14 d, the rate of fresh weight gain declined as spermine concentration returned to a level similar to that immediately following subculture.

It is in the pattern of change in spermine content that a parallel is found to changes in rates of fresh weight gain, with increasing spermine concentration during the exponential phase of rapid growth and a gradual decline in spermine level during the progression of the lag phase, coinciding with the decline in rate of fresh weight gain.

To summarise therefore, in terms of the major amines putrescine and spermidine - there is little correlation between changes in amine concentration and changes in rates of fresh weight accumulation. There is in fact a negative correlation , with levels of putrescine and spermidine elevated prior to the onset of rapid cell growth, and levels depressed during the succeeding rapid increase in fresh weight.

There is however, a clear positive correlation between levels of the minor component spermine and rates of fresh weight gain. Though levels of this amine are an order of magnitude less than those of the major amine components. Spermine may have a different role from that of the other polyamines detected and could be required for the progression of those processes initiated by a possible 'triggering' function for the free-amines. The accumulation of spermine during growth is not widely reported however, with Smith et al (1978) reporting a decline in spermine level during growth and an accumulation of the major amines putrescine and spermidine during rapid growth.

The pattern of change of putrescine and spermidine levels found above is similar to that found in other studies: Smith et al (1978) found a near doubling in putrescine 6 h after subculture of rose suspension culture cells into fresh medium, and a 1.4-fold increase in spermidine 24 h after subculture. As in the present study, levels rose and fell rapidly showing a large degree of fluctuation over the initial 24 h period. Changes in 24 h spermine levels in both studies showed a great similarity, with levels showing very little change except for a very gradual increase over the period. Montague et al (1978) also found an increase in levels of the major amines during the initial 24 h of culture of carrot suspensions. Putrescine levels rose and then fell during the initial 24 h while

spermidine levels rose slightly and spermine levels remained quite constant for the first 6 h and then rose considerably. Audisio et al, (1976) found increases in both putrescine and spermidine after 4 d culture in Nicotiana glauca habituated tissue with a second peak after 11 d similar to the 9 d peak in the present study and the 6 d peak of Smith et al (1978). In yeast cells Castelli and Rossoni (1968) detected peaks in spermidine and spermine during the lag phase 2 h after inoculation. There is little consistency in the pattern of change in polyamine levels between different systems however: Rolle et al (1971) found a decline in spermidine level in Scenedesmus algae over a 7 d growth period in contrast to the present study and Fienberg et al (1984) reported a 6-fold drop in putrescine level after 1 d culture of carrot cells. Smith et al (1978) has noted the considerable variation in the pattern of polyamine change between different species. Indeed spermine which alone among the polyamines studied increased during rapid carrot fresh weight gain actually declined in cultured rose cells. While no correlation between active growth and raised amine levels was found in the present study, the elevated levels of putrescine and spermidine observed prior to rapid growth may indicate a link between these amines and growth initiation. Kaur-Sawhney et al (1980) induced DNA synthesis and mitosis in oat leaf protoplasts and Huhtinen et al (1982) induced mitosis in alder protoplasts with exogenously applied amines, while other studies have reported increased growth in dormant Jerusalem artichoke tissue with similar treatments (Bertossi et al, 1965; Bagni, 1966). The present study cannot alone exclude such a growth initiatory effect.

A clearer indication of the role of amine accumulation in growth may be gained by measurement of amine synthesis and breakdown rates during

culture growth, with simultaneous monitoring of culture growth, for it is unclear whether such accumulation is due to enhanced synthesis or reduced rates of conversion to other forms.

The rise in spermine levels as putrescine and spermidine levels decline might thus be accounted for in terms of conversion of these amines to spermine through enhanced relative rates of spermidine synthesis compared to synthesis of putrescine. Declining levels of putrescine and spermidine in terms of fresh weight during active growth might also be explained in terms of dilution through fresh weight gain, unaccompanied by increased synthesis of these amines. It should also be noted that spermine levels decline at a period when declining growth rates would tend to raise levels in terms of fresh weight were active synthesis continuing, suggesting a sharper fall in spermine synthesis (or sharper increase in breakdown rates) than would be immediately apparent.

3.2 Amine biosynthesis during culture

Introduction

It has already been noted that changing amine levels may result from a number of factors, and that measurements of amine concentration alone provide only an indication of the metabolic framework within which these accumulations or declines are occuring.

A clearer picture of the causes of fluctuations in the levels of these chemicals may be gained by the study of the regulation mechanisms underlying these trends. While a unified representation of such mechanisms is very difficult to achieve, information concerning some key points of metabolic regulation may be more easily obtained. One of the key areas of regulation is in the control of biosynthesis, in the case of these amines through variation in the activity of ornithine decarboxylase (ODC) which catalyzes the decarboxylation of ornithine to form putrescine (Tabor and Tabor, 1984) and especially (as indicated later) arginine decarboxylase (ADC) which catalyzes the production of agmatine through decarboxylation of arginine (Smith, 1985b). ADC has been identified as a regulator of amine synthesis in several plant and bacterial systems (Le Rudelier and Goas, 1975; Smith, 1963, 1970; Speranza and Bagni, 1977; Suresh et al, 1978; Wu and Morris, 1963; Montague et al, 1979) while ODC activity is affected by growth, development and environment (Mc Anulty and Williams, 1977; O'Brien et al, 1975; Prouty, 1976; Yamasaki and Ichihara, 1976, 1977)

The biosynthesis of spermidine and spermine is also regulated by the activity of the enzyme SAMDC, which catalyzes the decarboxylation of S-adenosylmethionine to yield S-5-deoxyadenosyl-(5')-3-

methylthiopropylamine which serves as an aminopropyl donor in the conversion of putrescine to spermidine and spermidine to spermine (Tabor and Tabor, 1984). In addition, the level of accumulation of the amines will be determined by the rate of oxidative breakdown via the enzyme DAO which catalyzes reactions such as the conversion of putrescine to pyrroline and spermidine to aminopropylpyrroline (McGowan and Muir, 1971).

A study of the activities of these biosynthetic and oxidative enzymes during culture growth may provide a more complete picture of the background to fluctuation in amine levels.

Since the greatest fluctuation in amine concentrations takes place within the first 24 h of culture, it was this period which was investigated in detail. As mentioned above, some of the most important amine-related enzymes from the point of view of regulation of amine content are the biosynthetic enzymes ADC, ODC and SAMDC along with the catabolic enzyme DAO. Levels of all of these enzymes were determined in cells harvested at intervals during the initial 24 h.

Stationary phase cells from 14 d cultures were inoculated into 100 mL Ehrlenmeyer flasks at an inoculum ratio of 2 mL suspension : 28 mL fresh MDK medium. Flasks were incubated under standard conditions and sampled at 3 h intervals up to 12 h, and after 24 h with the above technique. Triplicate samples were taken from 3 identical flasks in each case, and cells extracted immediately for assay. Assays were all perfcrmed using the standard procedures described above.

Results and Discussion

All of the enzymes studied showed an increase in activity immediately after subculture into fresh medium. The activity of ADC increased almost 5-fold after 6 h in culture, though by 12 h after subculture the activity was only 50% of its peak value (Fig. 2). Similarly, ODC activity increased during the first hours of culture, with more than a 3-fold increase after 6 h (Fig. 3). Like ADC there was then a fall in ODC activity with levels less than half those at peak by 24 h. ODC activity was generally about half that of ADC.

To summarise the behaviour of these two enzymes therefore, which together largely control the input of the diamine putrescine into cellular metabolism: A large increase in activity immediately after subculture was followed by a fall to levels between peak and initial values after 12 h in culture. Levels then fluctuated up until the end of the 24 h culture period.

It is interesting to compare this pattern with that reported in other studies where these synthetic enzymes were determined under similar circumstances. The rise in ADC activity to a peak after 6 h is very similar to that reported by Montague et al (1979) in similarly cultured carrot cells and both studies also detected a subsequent fall in activity, though the study of Montague reports a slight increase between 12 h and 24 h whereas in the present study a slight fall was observed (Fig. 2). Fienberg et al (1984) report a peak in ADC activity after 24 h growth in carrot cell cultures, while Torrigiani et al (1987a) report a peak after 15 h in activated Helianthus tuber slices and Speranza and Bagni (1977) report a peak in ADC levels during the lag phase in Agrobacterium. As in the present study, all these periods of enhanced activity were brief and

Figure 2 Changes in ADC activity over initial 24 hours of culture

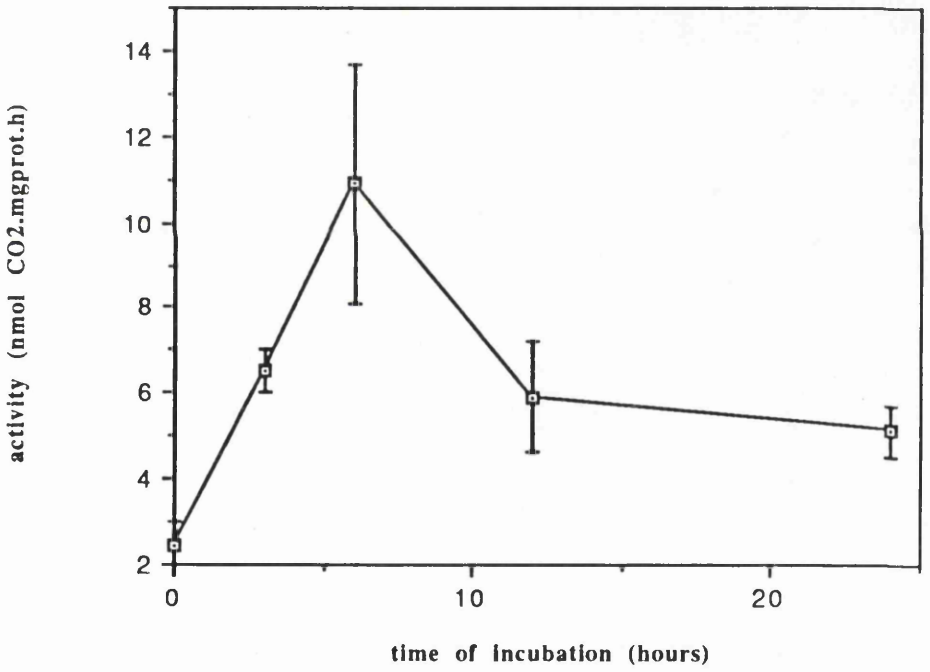
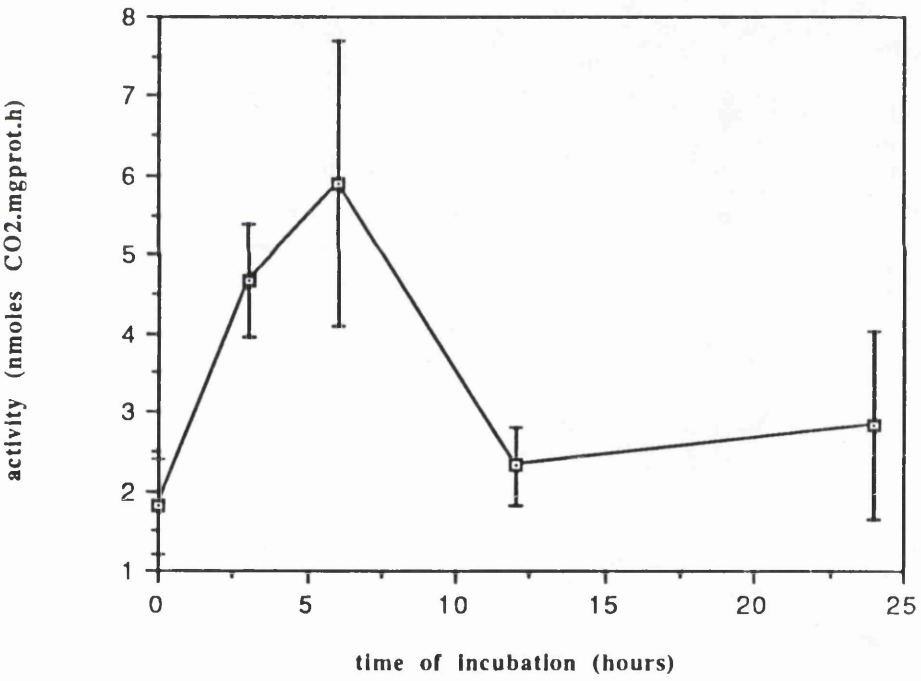


Figure 3 Changes in ODC activity over initial 24 hours of culture



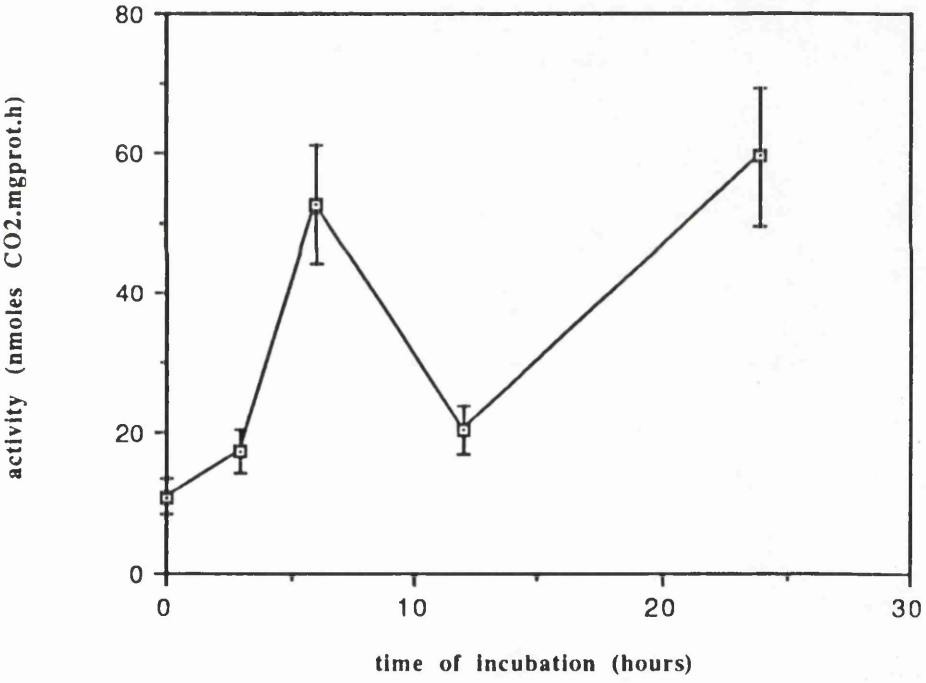
were followed by sharp falls in activity. Absolute ADC activities reported varied significantly between species, though levels reported by Fienberg et al, (1984) and Montague et al, (1979) in carrot culture were similar to those reported here. In the latter study a further investigation was conducted to determine whether the initial sharp rise in ADC activity was due to fresh medium, lowered cell density or both; and concluded that fresh medium was the primary cause of the rapid increase in activity; an effect also noted by Sung and Okimoto (1981) and Sung and Dudits (1981).

The post inoculation increase in ODC activity was also reported in the study of Montague et al (1979) where ODC changes were similar to those of ADC. A similar peak during lag phase was once again reported by Speranza and Bagni (1977) in Agrobacterium and Torrigiani et al (1987a) in Helianthus, while Heimer et al (1979) reported an ODC activity peak during log phase in tobacco suspensions between the 4th and 6th days in culture. It would appear likely that the ODC increase is also due primarily to the fresh medium effect.

Further down the amine biosynthetic pathway, diamines are converted to polyamines such as spermidine and spermine via SAMDC, (Fig. 4). In common with activities of ADC and ODC, SAMDC activity sharply increased on subculture, though this increase was not as pronounced as were changes in ADC and ODC activities. The change in activity was more gradual, with levels almost doubling after 3 h and then increasing 3-fold between 3 and 6 h. Activity was halved between 6 and 12 h after subculture. Its activity did not continue to fall however, and unlike ODC and ADC, SAMDC activity exhibited a second peak after 24 h in culture.

In the present study, the increase detected in the level of SAMDC after

Figure 4 Changes in SAMDC activity over initial 24 hours of culture



subculture would be expected, in the light of the increases in spermidine and spermine levels already found, due to increased rates of biosynthesis. Spermidine levels increased 3-fold after 2 h in culture, with this rise possibly facilitated by the relatively high levels of SAMDC present in the stationary phase cells.

The second peak in spermidine levels which occured after 24 h was accompanied by a second peak in SAMDC levels of similar magnitude to the first, suggesting the possibility that spermidine levels are regulated by SAMDC activity. Putrescine levels also rose, even though ADC and ODC levels alone would not account for this, and even though putrescine was being actively converted into polyamines. It would appear feasible that an increased rate of conversion of bound to free-amines may perhaps account for this anomaly.

Averaged over the whole 24 h cycle, SAMDC activity was about 10-fold greater than ADC activity. This suggests that a high turnover of putrescine is taking place, since any putrescine produced via ODC and ADC will be rapidly converted via SAMDC to spermidine and other polyamines.

If the changes in SAMDC activity over the 24 h period are compared to the changes observed by Montague et al (1979) in a similar study with carrot suspensions, then a clear similarity is seen in the general trends of both. A sharp peak in activity was observed in both immediately after subculture with levels rising 9-fold in the study mentioned and 5-fold in the present study, and absolute activity values similar in both. This first peak was followed by a sharp fall in both cases, though the decrease was twice as great in the present study. The following increase to give a second peak after 24 h was noted in both studies, though the increase was sharper in the present study with 12 h levels far lower than in the study

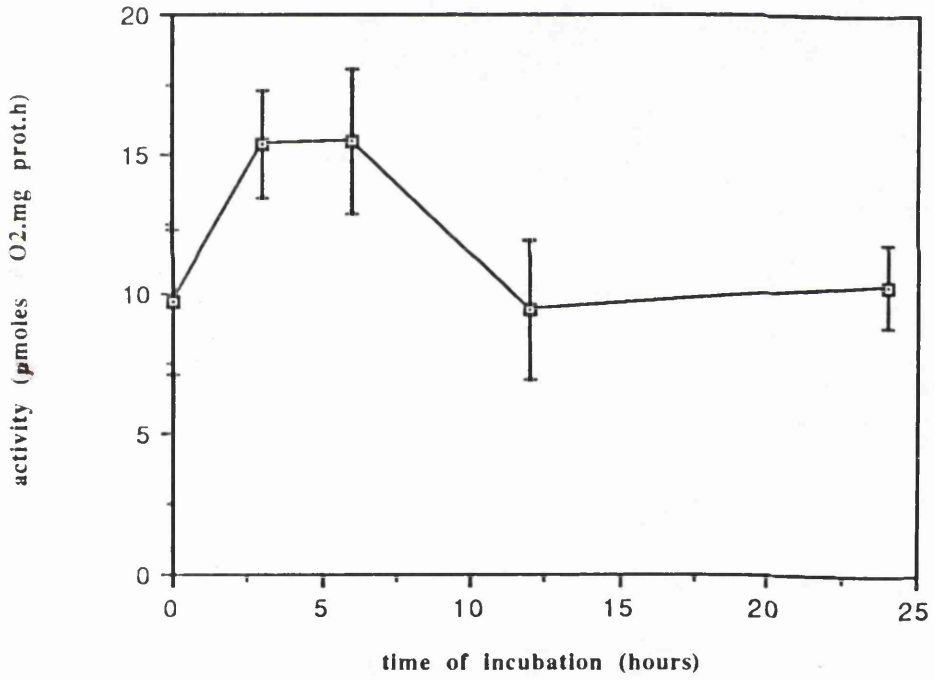
of Montague et al (1979). The close similarity between these two studies also extends to that of Fienberg et al (1984) where carrot cultures also exhibited a peak of SAMDC activity 24 h after subculture though levels were only 50% greater than time zero levels in this case and absolute SAMDC activities were 10-fold lower than in the other studies. Both Fienberg et al (1984) and Montague et al (1979) report a subsequent fall in activity after 48 h by 70% in the former and 20% in the latter. The close agreement of these three reports suggests that post subculture SAMDC activity in carrot cell culture follows a clearly definable pattern. In addition, the pattern of SAMDC activity in activated Helianthus explants as reported by Torrigiani et al (1987a) shows some similarity to the situation in carrot culture investigations. Once again a biphasic pattern of activity was noted which occured at 6 h and 21 h after subculture. The 6 h peak involved a 3-fold increase followed by a similar decrease with a gradual increase representing an approximate doubling in activity up to 24 h which closely resembled the change in activity in the present study. The main SAMDC peak in this follows the main accumulation of putrescine, a phenomenon also noted by Torrigiani et al (1987a) in Helianthus who suggest that SAMDC may be putrescine stimulated and further theorize that if spermidine and spermine are required for DNA synthesis (Heby, 1981; Smith, 1985b) then the peaks in SAMDC activity may be related to DNA synthesis. This theory is supported by the results of the present study where increasing levels of spermidine and spermine occur along with increasing SAMDC activity.

The turnover of putrescine is not only brought about via conversion to spermidine, the diamine is also broken down by the enzyme DAO. Diamine oxidising enzymes are sporadically present within the plant kingdom and

are particularly active in the Leguminosae (Slocum et al, 1984). Extracts of lentil seeds, leaves of Vicia faba, pea epicotyls and also the non-leguminous Euphorbia characius contain copper amine oxidases which oxidise mostly diamines (putrescine, cadaverine and 1,6-diaminohexane) and some mono and polyamines (Smith, 1985a). Diamine oxidases seem to be mainly located in the cell wall though a limited amount has been detected in the cytoplasm (Federico and Angelini, 1986). Despite such studies however the physiological importance of these substances in plants remains unclear - this investigation should help to clarify matters. The activity of this enzyme was determined at intervals in the same fashion as the amine biosynthetic enzymes and as described above, and its pattern of activity over the 24 h period showed interesting parallels with the pattern of activity of the other enzymes.

As in the case of ADC, ODC and SAMDC, there was a peak in DAO activity in the initial hours of culture (Fig. 5). The level of DAO activity present was also high relative to the other enzymes - three orders of magnitude greater. If the enzyme were induced by increased levels of its substrate, then such an increase would be expected, since putrescine levels rise on subculture. The maintenance of these high levels of activity of the oxidative enzyme suggests a rapid cycling of amines, with both synthesis and oxidation taking place at a rapid rate. The subsequent fall in its level of activity after the initial peak, when synthesis of amines is taking place at an increased rate suggests that its level of activity is linked to amine synthesis and accumulation. The maintenance of high levels of DAO also suggests that amine synthesis is followed by rapid breakdown. This would suggest that amines would only accumulate due to continuous amine synthesis. This may be due to a requirement for the input

Figure 5 Changes in DAO activity over initial 24 hours of culture



of newly-synthesized amines, or perhaps because amine accumulation does not benefit the cell and amines accumulating within the cell must be removed by oxidation.

In a recent study in Helianthus tuberosus Torrigiani et al (1987b) investigated the pattern of DAO activity throughout two different physiological changes, the period of tuber formation in vivo and the period comprising the first cell cycle of hormone activated tuber slices in vitro. If the period of tuber formation is considered first then we see that DAO activity was higher during cell division than before flowering in vivo and peaked along with ADC (Bagni et al, 1983) before dormancy. Furthermore, in tuber slices released from dormancy in vitro, DAO activity peaked during the first cell cycle 15 h after activation (S phase) along with ODC and ADC (Torrigiani et al, 1987a).
It was concluded that a correlation existed between DAO activity and cell division. Such a correlation had been demonstrated previously only in animal cells (Sessa et al, 1982). A similar conclusion was drawn by Torrigiani et al (1987b) to that arrived at in the present study: that DAO has a probable role in the regulation of amine content, through putrescine degradation.

To briefly summarise the picture of amine synthesis and breakdown which the present studies provide: Amine biosynthesis accumulation and oxidation are elevated immediately after subculture, but synthesis soon slows along with oxidation activity. Polyamine biosynthesis increases again 24 h after subculture. As was reported in the case of amine accumulation, there appears to be little relationship between rates of amine synthesis and growth rates. It is possible however that polyamines

may serve as a hormone-like trigger which enables growth to be initiated. They have certainly been linked to DNA synthesis (Torrigiani et al, 1987a; Heby, 1981; Smith, 1985) and are thought to function in the regulation of nucleic acid structure at several levels of organisation (Slocum et al, 1984). Though other reports have correlated active growth and cell division with increased amine synthetic activity (Speranza and Bagni, 1977; Audisio et al, 1976; Bagni et al, 1980) such a direct temporal correlation is not evident in the present study. It would therefore appear that in carrot culture at least the link between amine synthesis and growth is not a simple one - but is more likely to be indirect. Other reports in fact, have raised questions about the functions of amines in the Helianthus system (Phillips et al, 1987, 1988) which has been the basis of many studies of plant polyamine physiology and which in turn have supported some of the theories concerning the role of amines in plant growth and development (Bertossi et al, 1965; Bagni, 1966; Bagni et al, 1980; Torrigiani et al, 1987a). It is apparent from the degree of disparity between the patterns of polyamine accumulation during growth in some of the systems investigated: Rose, (Smith et al, 1978); carrot, (Montague et al, 1978); Jerusalem artichoke, (Phillips et al, 1987) and the different patterns found by different authors even within the same systems of Jerusalem artichoke, (Bagni et al, 1980; Phillips et al, 1987) and carrot, (Montague et al, 1978; Fienberg et al, 1984), that the relationship between polyamine synthesis and accumulation and plant growth may not be simply defined.

These methods of enzyme study involve the use of crude plant extracts containing the decarboxylating enzymes. These enzymes decarboxylated a substrate, and levels of the decarboxylated substrate were then measured.

It has been noted by Birecka et al (1985a:1985b) and Smith and Marshall (1988) that such a crude extract can contain other decarboxylating molecules in addition to the enzymes in question. Consequently, excess substrate decarboxylation may occur and artefactually high enzyme activities may be recorded. This difficulty emphasises the importance of a broadly based approach, employing a range of reliable techniques, and yielding comparable data.

The accumulation of amines in the 24 h post subculture period is accounted for by a similar increase in activities of amine synthetic enzymes in the hours immediately following transfer to fresh medium. A rise in the activity of the amine oxidative enzyme DAO at the same time may account for the fall in amine levels following this peak. Also, with amine levels detected at a low level during rapid fresh weight gain, the lack of a requirement for high amine levels during active growth is evident from this study. However, the sharp rise in amine synthesis and accumulation prior to the onset of rapid growth activity is interesting. It indicates that rapid amine synthesis during the lag phase may be an important factor in pre-cell division processes taking place at this time. Finally it should be noted that spermine accumulation proce eds throughout the rapid growth period with SAMDC activity peaking at 24 h. Indeed, another study of SAMDC activity in carrot culture has found steady increases throughout the growth period (Fienberg et al, 1984). Together, these two studies suggest that spermine may have a role in the active growth of carrot cell cultures, despite its comparatively low cellular levels throughout the growth period. A very similar change in spermine levels was observed in embryogenic carrot cultures over a 14 d culture period by Fienberg et al (1984). This suggests that increased spermine

levels are not of themselves a feature of embryogenesis but may have a more fundamental growth function, although Sung and Jacques (1980) have concluded that non-differentiating and embryogenic carrot cultures differ markedly in arginine metabolism.

CHAPTER FOUR

MANIPULATION OF AMINE LEVELS

4.1 Inhibitors of amine biosynthesis

Introduction

In previous sections , two methods of investigating amine biosynthesis have been described: Measurement of amine biosythetic enzyme activities and determination of cellular amine levels. Both of these methods help to clarify the relationship between growth and amine content, but though it may be shown that there is little connection between the pattern of either amine synthesis or general amine accumulation and rates of growth over the culture period, this alone does not preclude a cellular requirement for certain levels of amines in order for culture growth to proceed.

A clearer indication of the importance of physiological levels of amines during culture growth may be obtained by the manipulation of amine synthesis in order to reduce cellular amine levels, while carrying out growth determinations to find whether growth is adversely affected by a lowering of amine levels. In studies involving animal systems inhibitors of amine biosynthesis were capable of halting the progression of the cell cycle and have indicated that DNA replication is the phase of the cycle which is most strongly affected by reductions in cellular amine levels. In animal cells such depletion is simply effected through blocking of the single pathway via which amines are synthesized from ornithine through the action of the enzyme ODC. In mammalian cells for example synthesis of

amines requires the sequential action of two decarboxylases and two transferases. The amine synthesis inhibitors so far produced have tended to be directed at the two decarboxylases and this may be due to the fact that the decarboxylation step is thought to be rate limiting (Mamont et al, 1980).

In plants however, the situation is rather more complicated since at least two pathways to putrescine are in operation: One pathway is from ornithine to putrescine via ODC and one pathway is from arginine to putrescine via ADC. Recently however a number of irreversible competitive inhibitors of plant amine metabolism have become available (Stevens and Stevens, 1980; Smith, 1985b) which has made possible the testing of relationships between variations in amine biosynthesis and the accumulation of amines, and between particular aspects of growth and development. Two such inhibitors are: alpha-difluoromethylarginine (DFMA) (Kallio et al, 1981) and alpha-difluoromethylornithine (DFMO) (Metcalf et al, 1978), both of which are enzyme activated irreversible inhibitors belonging to the novel class known as suicide enzyme inactivators (Mamont et al, 1980) and which have been reported to inhibit the activity of these enzymes specifically in a number of plant species (Smith, 1985b). Also, the incorporation of putrescine into polyamines may be blocked by the inhibitor ethylmethylglyoxalbisguanylhydrazone (EMGBG) (Elo et al, 1986) which is a far more potent derivative of the widely used polyamine biosynthesis inhibitor methylglyoxalbisguanylhydrazone (MGBG) (Pegg, 1983), (which has limitations of use since it is a reversible inhibitor, is not absolutely specific, inhibits enzymes such as DAO and is toxic (Pegg and McGill, 1978)). EMGBG is far more suitable as an inhibitor of polyamine production than MGBG since smaller quantities are required and

toxicity effects are not so apparent. Indeed, with the inhibition of SAMDC activity via EMGBG and the inhibition of ADC and ODC with DFMA and DFMO respectively, both initial diamine and subsequent polyamine synthesis could be inhibited as required, and thus both the quantity and balance of amines modulated.

Results and Discussion

DFMA treatment (Fig. 6) had no effect on the growth of the culture (as fresh weight) at 0.1 and 0.3 mM concentrations, and markedly stimulated growth at 1 mM and 3 mM, while dry weight remained unaffected (Table 1), with a maximum value of 0.177 g/flask and a minimum value of 0.164 g/flask. A dramatic fall in putrescine content with increasing DFMA concentration was observed such that at 1 mM DFMA the putrescine concentraton was reduced by 95% compared to the controls, and spermidine levels also declined markedly. The spermine content on the other hand increased steadily with increasing DFMA concentration. These findings point to a lack of correlation between fresh weight changes and endogenous levels of the major amines putrescine and spermidine, but to a possible link between the minor component spermine and growth.

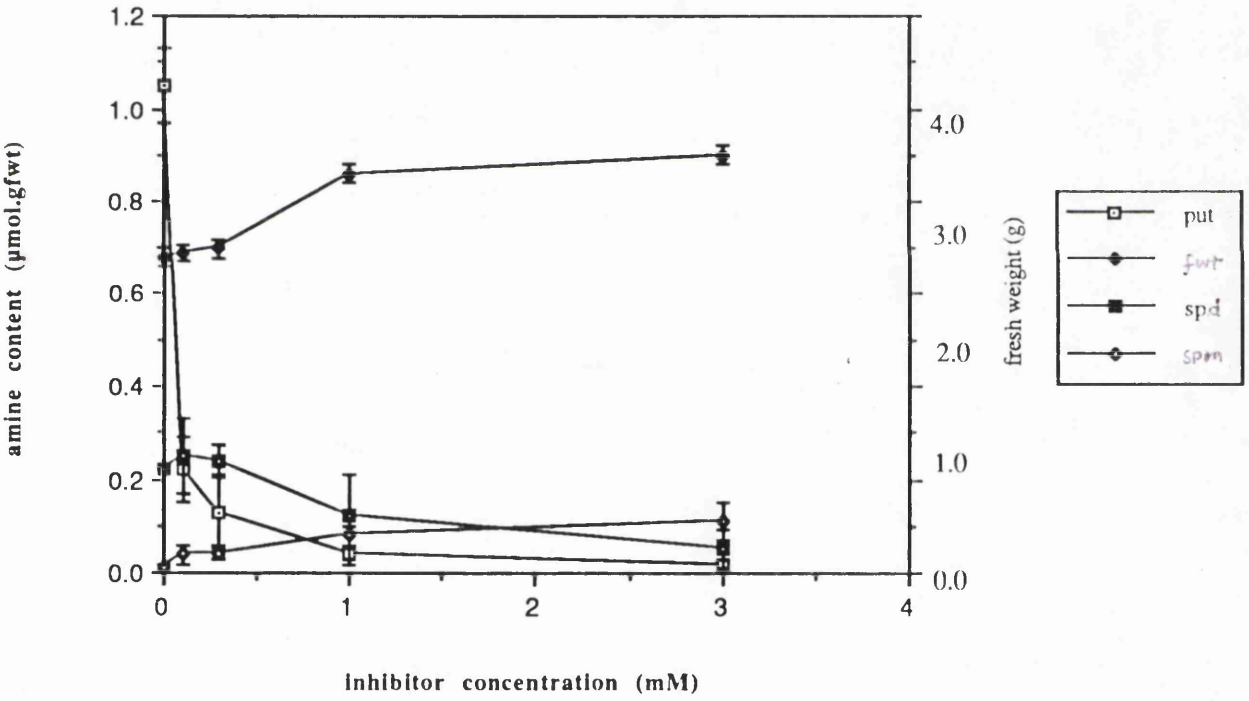
A similar experiment was also carried out but this time using varying concentrations of DFMO. Once again, stationary phase cells were incubated in inhibitor-supplemented medium and after 10 d incubation, no significant effects on fresh weight, dry weight (max 0.180 g, min 0.173 g), or content of spermidine and spermine was detectable. Furthermore, a much smaller reduction in putrescine level was observed with DFMO compared to that observed with DFMA treatment (Fig. 7).

Table 1 Cell number, fresh weight, and dry weight per culture of carrot cells grown for 10 days in the presence of 1 mM DFMA, 1 mM DFMO, 30 μM EMGBG, or no inhibitor

Treatment	Cell Number x 10^7	Fresh Weight	Dry Weight
		g	g
Control	11.9 ± 0.5*	2.75 ± 0.31	0.180 ± 0.006
DFMA (1 mM)	12.5 ± 0.6	3.63 ± 0.29	0.173 ± 0.004
DFMO (1 mM)	12.1 ± 0.9	2.80 ± 0.22	0.170 ± 0.009
EMGBG (30 μM)	-	2.58 ± 0.25	-

* Means ± standard errors

Figure 6 Effect of DFMA on amine levels after 10 days in culture



In addition to amine determinations cell counts were also made on cultures incubated for 10 days with 1.0mM DFMA, 1.0mM DFMO, or no inhibitor, as above. Cell numbers were not significantly different from control values with either inhibitor, in two separate experiments (Table 1). The increase in fresh weight resulting from DFMA treatment is thus due solely to an increase in cell expansion of the order of 25%; there is no effect on cell division. DFMO treatment had no significant effect on either cell division or cell expansion.

Following investigations of the influence of amine synthesis inhibitors on cell growth, further work was carried out to confirm the specificity and site of action of DFMO and DFMA. This involved measurement of the activities of ADC in the supernatant and ODC in the pellet in cells incubated for 10days. Measurements were carried out in the presence of either DFMA, DFMO or EMGBG at varying concentrations as before. It was found that in the presence of DFMA, ADC activity was dramatically reduced at concentrations as low as 0.1mM, and virtually no activity was detectable in cells incubated at 3mM DFMA (Fig. 8). When ODC activity was determined under similar conditions however, its activity proved to be unaffected by any concentration tested (Fig. 9a). Together with these studies, a complementary experiment was carried out to determine SAMDC activity in cells incubated in media supplemented with DFMO, DFMA and EMGBG. This showed that SAMDC activity was reduced by approximately 30% at 3 mM. DFMO treatment reduced the activity of ODC by 75% at a concentration of 0.3mM in the medium, and more than 98% at 3 mM. Again ADC activity was unaffected by any concentration of DFMO (Fig. 8), while SAMDC activity doubled at 3 mM (Fig. 9b). Both ODC and ADC activities found in this study are comparable with those in which the supernatant alone was used for

Figure 7 Effect of DFMO on amine levels after 10 days in culture

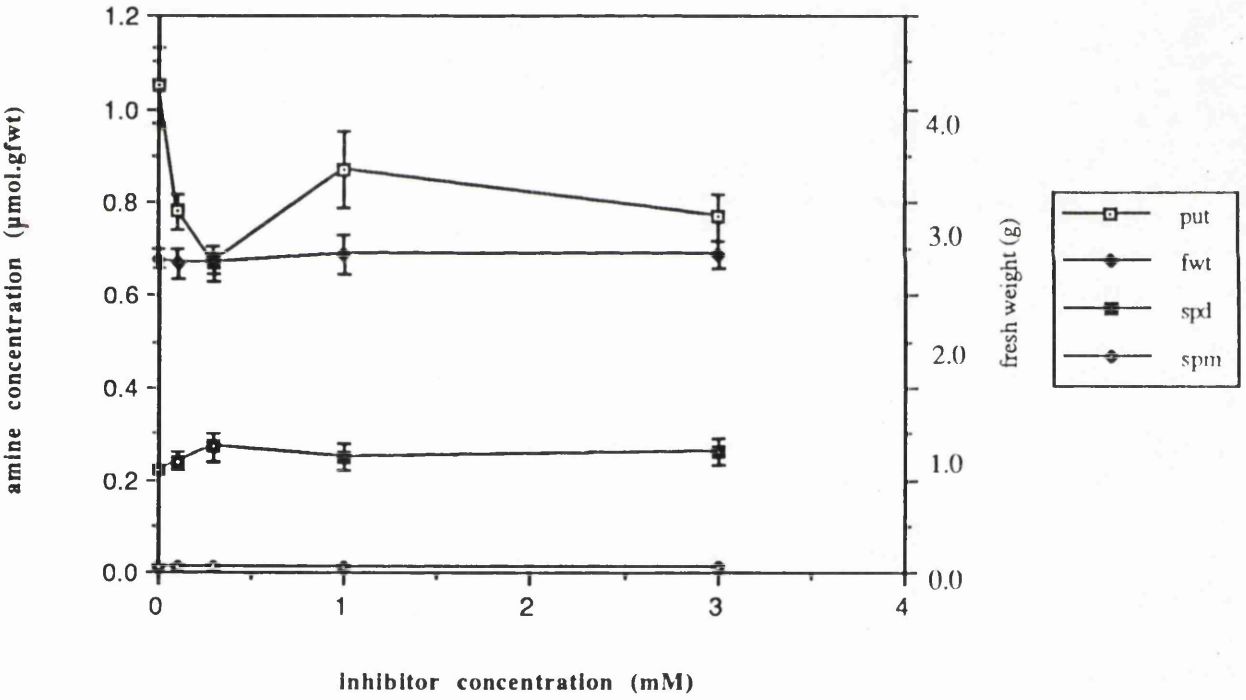


Figure 8 Effects of DFMO, DFMA, and EMGBG on ADC activity after 10 days in culture

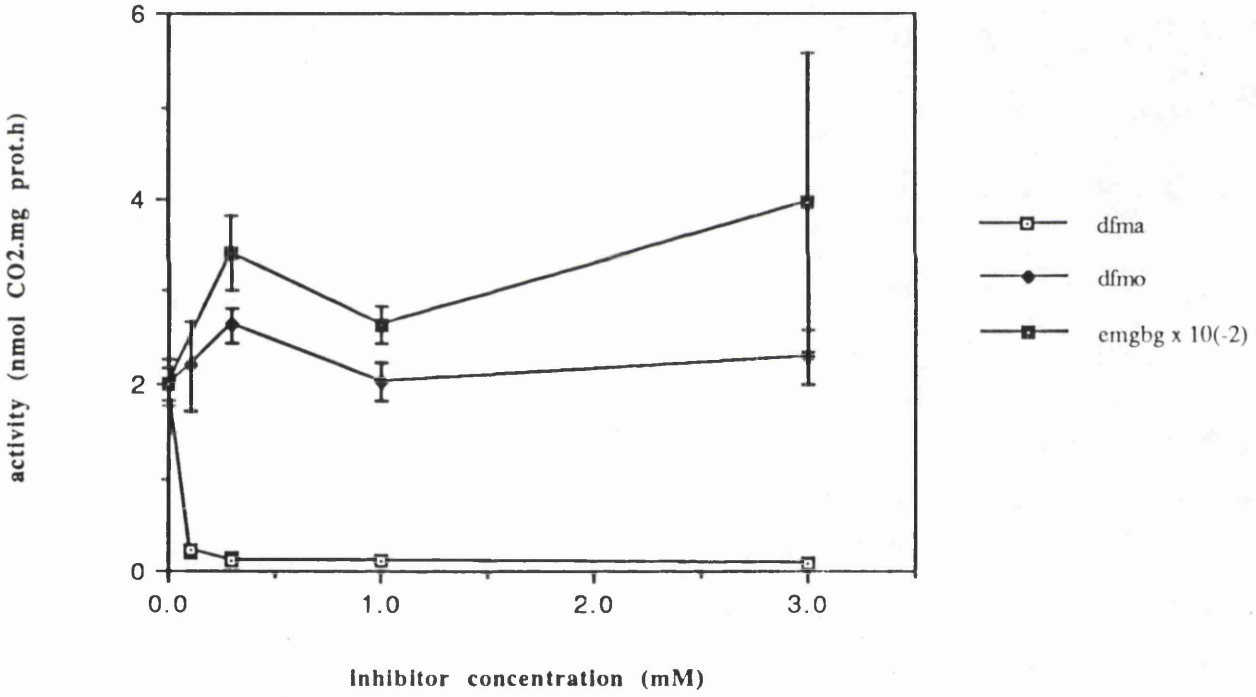
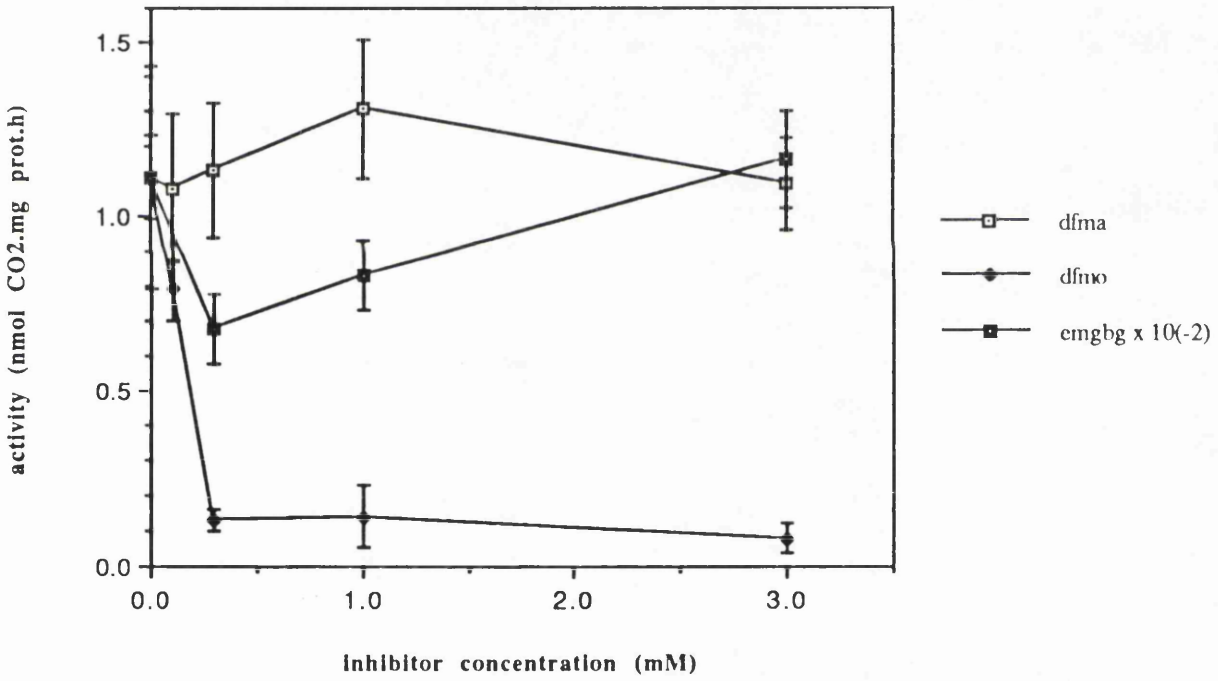


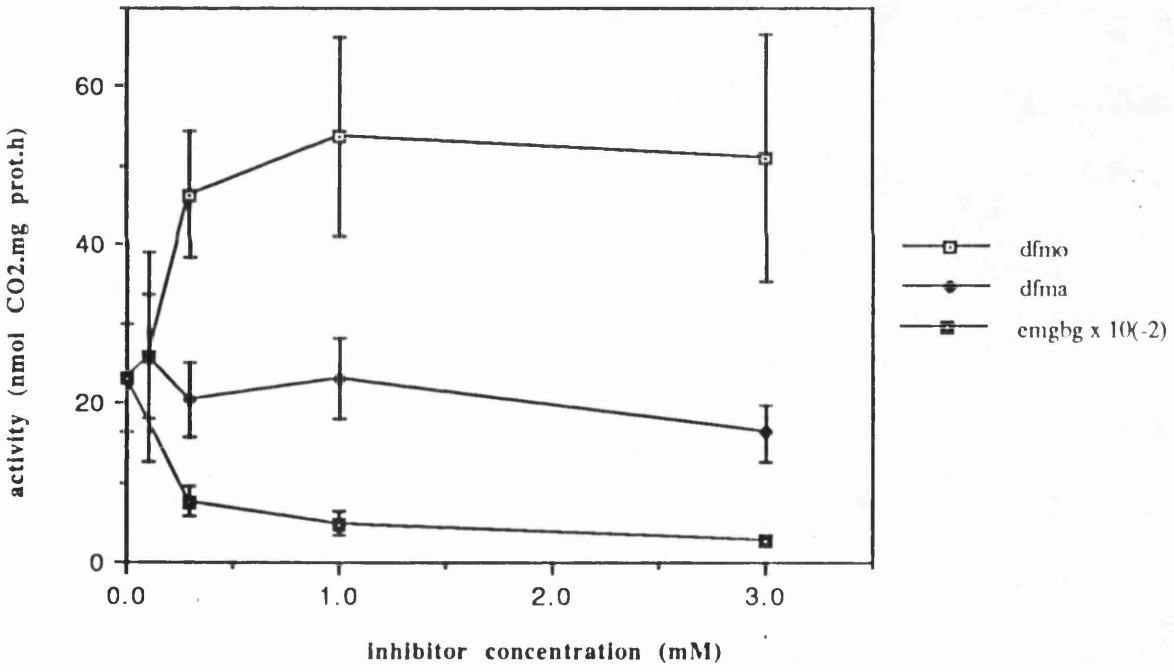
Figure 9a Effects of DFMO, DFMA & EMGBG on ODC activity after 10 days in culture



enzyme assay of ADC (Montague et al, 1979), and studies in which activities of both pellet and supernatant fractions were determined for ODC and ADC assay (Cohen et al, 1984).

In the case of EMGBG treatment, there was little effect on ADC activity in the supernatant (Fig. 8) except at the strongest inhibitor concentration employed (30 μM). When cells were incubated for 10 d in medium supplemented with 30 μM EMGBG there was a doubling in ADC activity over control values. ODC activity (Fig. 9a) however, was not significantly affected at any concentration tested. The specificity of the enzyme was powerfully demonstrated by the change in SAMDC activity relative to controls (Fig. 9b). SAMDC activity was reduced at every concentration tested, with the greatest reduction occuring at the maximum EMGBG concentration of 30 μM, when SAMDC activity was reduced by more than 90%. Together with the lack of inhibitory effects on ADC and ODC activities, these results show the fine degree of specificity associated with the inhibitory effects of EMGBG, together with its great potency. This potency has been reported previously (Elo et al, 1986) and is thought to be due to its enhanced structure compared to MGBG. In terms of growth (as fresh weight), none of the concentrations of EMGBG tested was effective (Table 1), as was also the case with DFMO treatment. It may thus be concluded that DFMO, DFMA and EMGBG are effective in inhibiting the activity of their specific enzymes while having no apparent inhibitory effects either on the other enzymes studied or on growth. The increased activity of ADC found in cells incubated in EMGBG supplemented medium suggests that this inhibitor may have an unspecific activity enhancing property, which may perhaps be related to an inhibitory quality of SAMDC activity on the activity of these biosynthetic enzymes.

Figure 9b Effects of DFMO, DFMA & EMGBG on SAMDC activity after 10 days in culture



To summarise, the major finding of this study of amine biosynthesis inhibition has been that cell division can be unaffected under conditions causing significant alterations in polyamine concentrations and relative proportions. In the presence of 1 mM DFMA, spermine increased 2-3-fold; the combined free amine titre fell by more than 80%. In contrast with the lack of effect on cell division, cell expansion of the order of 25% was reproducibly induced by DFMA. Interestingly, spermine levels increased roughly in parallel with final fresh weights. This, together with the pattern of change occuring during the growth passage suggests a possible correlation between spermine content and cell expansion.

Studies of the response of other cultured tissues to inhibitors of polyamine biosynthesis have been inconclusive and in only a few cases have precise measurements of cell division activity been made. In a recent study, where cultured artichoke explants were incubated on agarized medium supplemented with various inhibitors (Phillips et al, 1988) neither DFMA nor DFMO at concentrations up to 10 mM affected mitotic rates in the short-term Jerusalem artichoke explant tissue. Mengoli et al (1987) found DFMO at 5 mM incorporated in the growth medium to be without effect on cell numbers in carrot suspension cultures similar to those made use of in the present study although cell expansion was stimulated in the post-exponential phase. In contrast however, cell division in tobacco cultures incubated in inhibitor-supplemented medium was completely inhibited by DFMO while cell expansion was stimulated to a remarkable extent (Berlin and Forche, 1981). It was not clear whether this was due to changes in polyamine metabolism since putrescine levels were not reduced although ODC activity was completely inhibited.

In some contrast to such reports of cell expansion, reductions in fresh weight have been reported in response to exposure to DFMA in both embryogenic carrot cultures (Fienberg et al, 1984) and in Jerusalem artichoke tissue (Bagni et al, 1980) although in both cases neither cell counts nor amine determinations were carried out. Also, in another study when tobacco calli were treated with mM DFMO fresh weights and putrescine levels were reduced by almost 50% (Tiburcio et al, 1987). At 3 mM DFMO reductions in final fresh weight were even more dramatic (71%) though polyamine determinations were not made in this case. In Chlorella cell numbers were reduced by up to 50% and putrescine content by 40% at 25 mM DFMO (Cohen et al, 1984). In addition, fresh weights of Helianthus tubers were reduced by more than 50% on growth in 5 mM DFMO and by over 30% with exposure to 5 mM DFMA. Incorporation of MGBG in the medium at 1 mg/ml inhibited the growth of crown gall tumours but the inhibition could be reversed specifically on adding spermidine (Galsky and Kulpa, 1983). Also the growth of petunia, Nicotiana and Vigna tissue cultures was inhibited by polyamine biosynthesis inhibitors but inhibition was countered by addition of putrescine or spermidine in some cases (Kaur-Sawhney et al, 1984). Tomato fruit development too was inhibited by DFMO, and alpha-methylornithine, if applied during the period of active cell division and the inhibition was relieved by application of putrescine. Also, a mutant tobacco cell line with low ODC/SAMDC activities when regenerated into whole plants grew poorly and never flowered.

When considering the results of the inhibitor studies mentioned here, it is useful to bear in mind points raised by Evans and Malmberg (1989) in a recent review. They point out that not all studies have demonstrated that the biological effects of inhibitors are reversible by

adding the inhibitor plus the appropriate polyamine. In addition, some have not demonstrated that the inhibitors reduce the internal concentration of the polyamines. In the present study however, enzyme assays and amine determinations in conjunction with the use of inhibitors provide a powerful indication of inhibitor specificity.

It is evident that a number of studies have found that application of polyamine biosynthesis inhibitors to whole plants and tissue and cell cultures has a measurable effect on subsequent levels of growth. It is also the case however, that a number of studies have been unable to conclude that inhibition of amine biosynthesis has any such effect. In the absence of any clear consensus on the role and importance of polyamines in plant cell function, there remains further scope for the application of inhibitor studies in providing a solution to the polyamine question. In any case, these studies together suggest that neither diamines nor polyamines are required at physiological concentrations in order for growth to proceed in carrot cells under these standard suspension culture conditions.

4.2 Plant hormones and amines in relation to culture growth

Introduction

The importance of hormone supplements

High levels of auxin have generally been found to be beneficial for establishment and growth of suspension cultures, especially in the form of 2,4-dichlorophenoxyacetic acid (2,4-D) (Street, 1977; King, 1984). The results of studies with tissue cultures of Helianthus tuberosus carried out by Arnott (1982) suggested that 2,4-D exogenously applied in the medium was a requirement for cell cycle events including cell division. In addition, previous studies have found that growth is markedly reduced when auxin is not available to growing cells (Bagni et al, 1982). In order to complement these Jerusalem artichoke studies and to confirm unpublished observations of the effects of auxin withdrawl on growth in carrot suspension cultures a set of studies were performed to precisely determine the changes in fresh weight accumulation occuring as a result of reductions in 2,4-D levels in the carrot suspension culture medium. Along with 2,4-D the carrot culture medium is routinely supplemented with the synthetic cytokinin 'kinetin', a phytohormone which in whole plants is associated with cell division activity. The relative importance of this supplement in media preparation has received little investigation and its inclusion in carrot suspension medium has been mainly due to its presence in the MDK formula in which long term carrot culture had been shown to be feasible. In order to more clearly determine the significance of this compound in the MDK medium, the effect on growth of kinetin withdrawl was studied in conjunction with the auxin studies already mentioned.

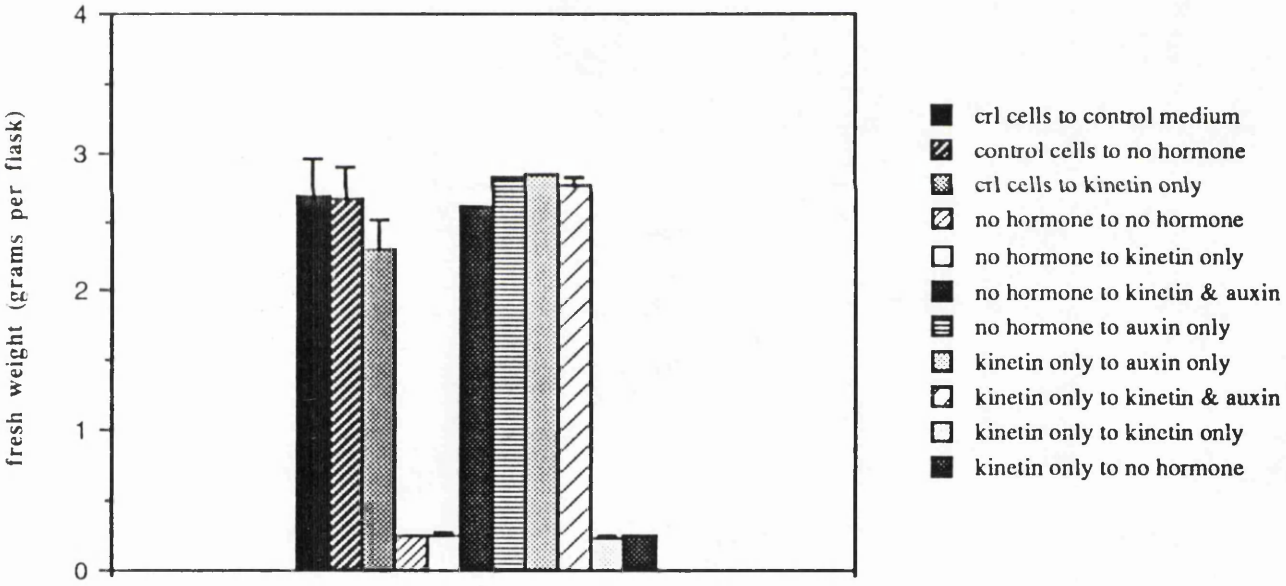
The initial stages of the study involved the transfer of carrot suspension inocula of known fresh weight to flasks of hormone-free medium under standard conditions. The flasks were then incubated as before for a two week period, at the end of which the contents of the flasks were fresh weighted. Fig. 10a shows the fresh weights of cultures grown in auxin and kinetin-free media for 14 d along with fresh weights of carrot cultures grown in supplemented medium.

The second part of this study consisted of the transfer of the cultures grown for one passage in hormone-free media to a variety of experimental media with and without kinetin and auxin to determine whether prolonged growth in auxin-free and kinetin-free media resulted in a reduction in culture growth over control levels. Two growth parameters were measured: fresh weight and cell number.

Results and Discussion

It may be seen that there is no significant difference in the fresh weight accumulated by cultures deprived of hormone supplements compared to those in control media. It may be concluded therefore that cells grown previously in auxin and kinetin supplemented media may grow for one passage in hormone-free, kinetin-free and auxin-free media with no detectable reduction in final fresh weight gain. Fig. 10a shows the effects of an auxin-free, kinetin-free and hormone-free single passage on subsequent growth. Cells incubated for a single passage in hormone-free medium ('depleted-cells') inoculated into medium without auxin showed no signs of fresh weight gain over the initial inoculum value after 10 d incubation (Fig. 10a).

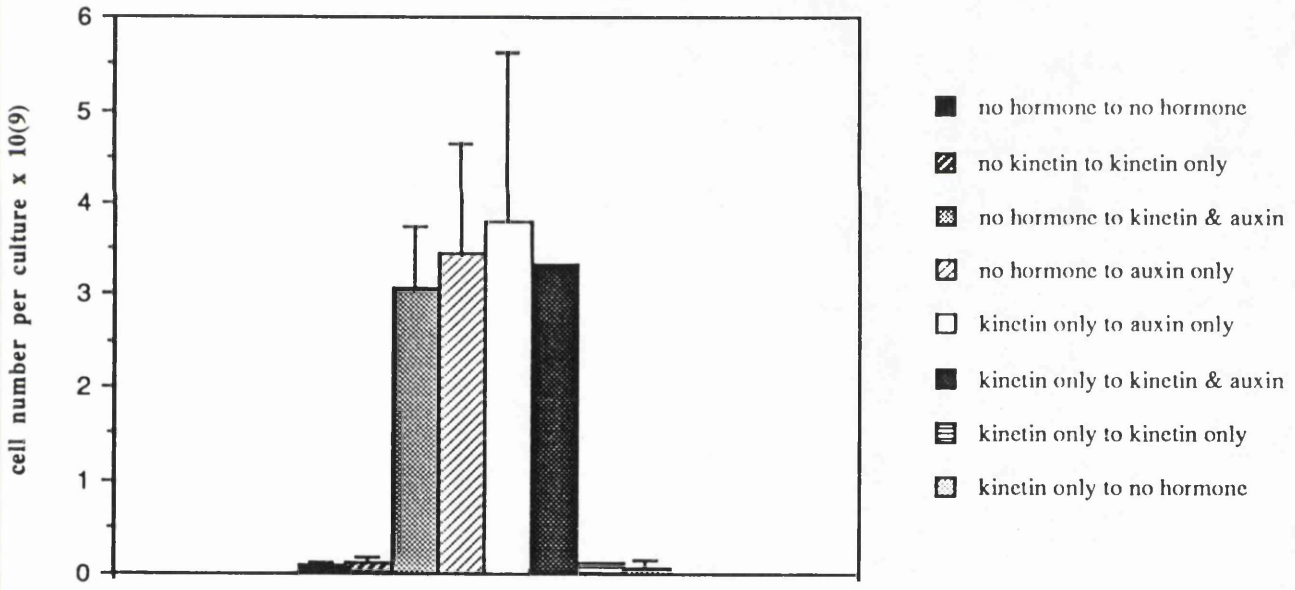
Figure 10a Growth of auxin-depleted cells in auxin-free, kinetin-free and hormone-free medium at 10 days growth



In contrast, auxin-depleted cells transferred to media containing auxin achieved full growth both in the presence of and without kinetin. A similar picture was evident after analysis of cell number data (Fig. 10b). It would appear that although growth for a single passage in hormone-free medium may take place without any obvious change in growth, the following passage presents a very different picture: in the absence of auxin, final fresh weights of treated cells were reduced by more than 90% compared to auxin-supplemented controls. Furthermore, in all cases growth was reduced to the same degree in kinetin-supplemented and hormone-free cultures. All cultures grown in media supplemented with auxin, with or without the addition of kinetin showed near control (auxin-supplemented) final fresh weights. Indeed, those cultures which were incubated in media to which kinetin had been added in addition to auxin showed a slightly lower final fresh weight than cultures incubated in media supplemented with auxin alone. Results of cell number determinations (Fig. 10b) present a similar picture with all cultures transferred to auxin-free media showing negligible growth and only cultures incubated in auxin-supplemented media reaching final fresh weights similar to control (auxin-supplemented) cultures.

It is evident from both of these parameters that auxin alone is the hormone supplement upon which growth depends in these carrot cultures, as in the case of Jerusalem artichoke cultures (Bagni et al, 1982). It is also clear that during repeated episodes of growth and subculture the carrot cells build up an endogenous level of auxin which, together with any auxin transferred during subculture is sufficient to permit growth to proceed for a full growth passage without an exogenous supply of auxin being available in the medium. This endogenous supply would seem to be

Figure 10b Cell number of cell cultures transferred between hormone-supplemented and unsupplemented medium at 10 days growth



consumed to some extent during the growth passage in amine free medium leaving insufficient levels to facilitate normal growth. This does indeed provide powerful evidence for the involvement of auxin in the regulation of plant growth at the cellular level. In addition these results also indicate a questionable role for kinetin as a requirement for culture growth at least in the short time period to which this study applies. However, though the results indicate that kinetin is not required for culture growth over at least two passages and is indeed slightly inhibitory when compared with the effects of auxin alone on growth, as has been reported in cotton callus by Osmama and Peter (1984). It remains unclear whether kinetin is necessary for long term culture growth and whether it is possibly involved in processes which if impaired might produce symptoms at some later stage.

With the importance of auxin as a requirement for carrot suspension culture growth now established, a further investigation was carried out to determine whether amines, as postulated growth regulators (Galston and Kaur-Sawhney, 1982), would to some degree replace auxin in the culture medium and allow growth to proceed in its absence.

4.3 Amines as auxin replacements

Introduction

As we have seen, amines have been the subject of a number of investigations in relation to their possible role in cellular growth processes. The results of such studies have led to their identification as plant growth regulators by some authors such as Galston and Kaur-Sawhney

(1982) who have noted reports linking polyamines to growth as evidence for this conclusion. These include reports of high polyamine levels in meristematic tissues as opposed to senescent tissue (Kaur-Sawhney et al, 1982a), and stimulation of cellular growth associated with exposure to polyamines (Bagni et al, 1982). Also, some authors have reported that polyamines may stimulate growth in dormant artichoke tissue in the place of auxin which is normally applied to achieve such stimulation (Bertossi et al, 1965). In order to test the basis of such hypotheses regarding the role of plant amines in cellular growth, the well-tried carrot suspension culture system was used along with altered culture media. It was envisaged that these procedures would indicate the ability of amines to replace auxin as a growth regulating compound in the cell culture medium. Cells which had been grown for a single passage in auxin-free media as described previously and which were hence depleted of cellular auxin (depleted cells) were used in the study.

Results and Discussion

Hormone-depleted cells showed no increase in fresh weight over initial inoculum fresh weight levels after 10 d growth in hormone-free medium supplemented with any of the amines used at any of the concentrations tested (Fig. 11a). Indeed, the growth (as fresh weight) of depleted cells appeared to be slightly inhibited at the higher concentrations of putrescine employed, when compared to depleted cells incubated in auxin and amine-free medium.

The pattern of growth when depleted cells were transferred to auxin-supplemented medium provided a striking contrast to that observed on

incubation in hormone-free medium. The final fresh weight was similar to that in undepleted controls incubated under standard conditions. It would seem therefore that amine supplementation of the medium does not allow growth to proceed in the absence of 2,4-D,

The lack of a growth stimulatory effect with exogenously applied amines was also indicated in the results of the cell number determinations carried out together with fresh weightings. As in the case of fresh weight, cell numbers per culture were similar to those of the original inoculum after 10 d incubation, in all cases in which depleted cells were incubated in auxin-free media (Fig. 11b). In the auxin-supplemented controls, cell numbers were similar to those obtained when undepleted cells were incubated under standard conditions. To complement the above experiments, a similar investigation was performed with cultures grown in auxin-supplemented and amine-supplemented MDK media. In these experiments cells were not depleted prior to the experimental incubation period, and therefore the study was solely concerned with studying the effects on cellular growth (as fresh weight) of increased media amine levels. Similar amine concentrations were employed to those in previous experiments, and stationary-phase cells were once again incubated for 10 d in experimental and standard (control) media, with fresh weights determined on harvested cells at the end of the culture period. Fig. 12 shows how final fresh weights were affected by these amine treatments. Growth was not stimulated at any of the amine concentrations tested - and was indeed reduced at some of the greatest amine concentrations used.

Figure 11a Growth of auxin-depleted cells in auxin-free polyamine-supplemented medium at 10 days growth - fresh weight

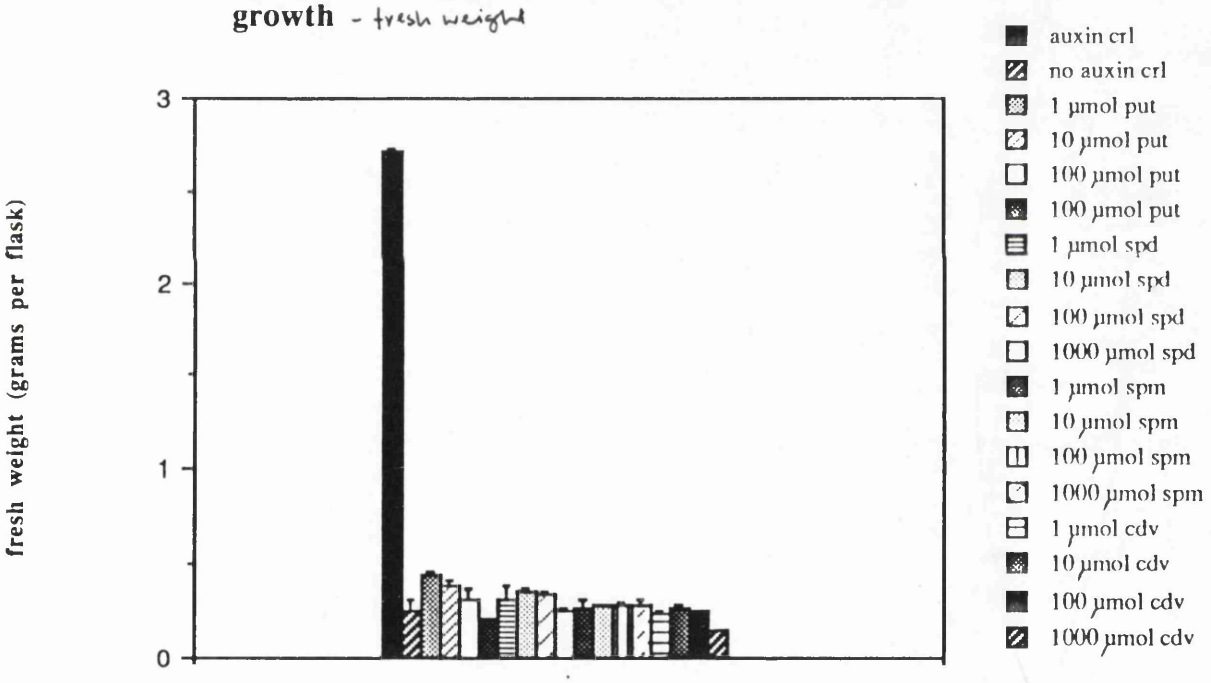


Figure 11b Growth of auxin-depleted cells in auxin-free polyamine-supplemented medium at 10 days growth - cell number

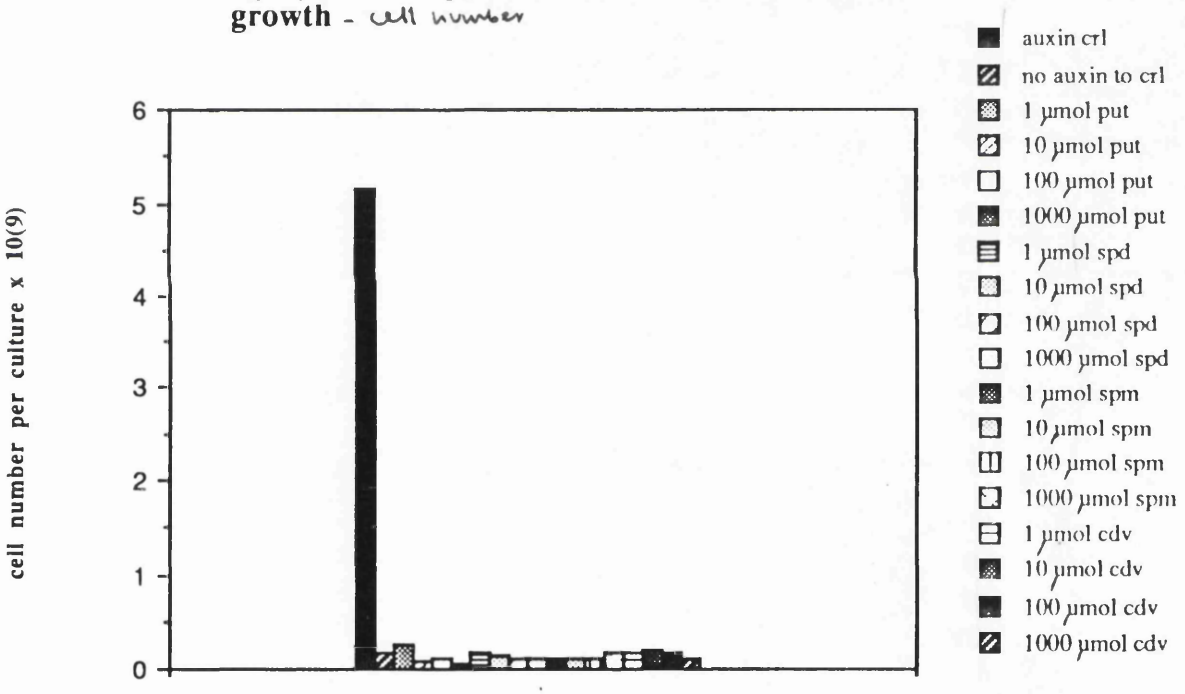
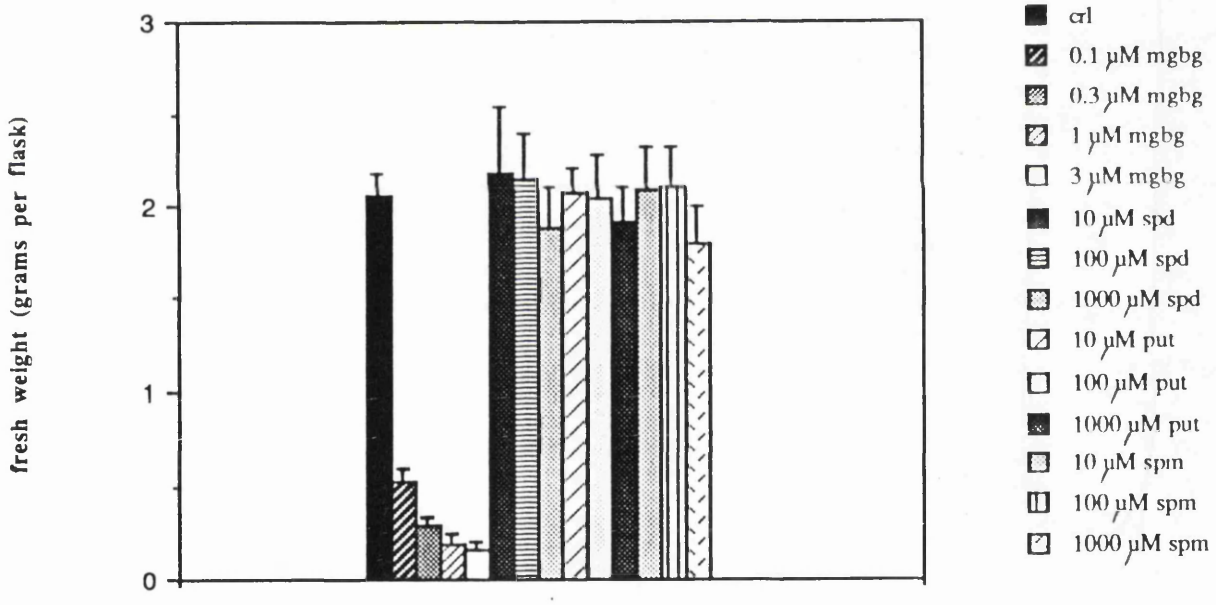


Figure 12 Growth of undepleted cells in inhibitor and amine-supplemented medium at 10 days growth



The results of this study provide a contrast to those of previous studies which found that when exogenous polyamines were supplied at 10-100 µM concentrations to explants of Helianthus tuberosus dormant tuber cultivated in vitro the tuber cells were stimulated to divide and grow without exposure to any other growth substance. Growth stimulation and differentiation (Serafini-Fracassini and Alessandri, 1983) in the explants was similar to that observed with IAA treatment (Bertossi et al, 1965; Bagni, 1966). Amines were also reported to generally increase active cell proliferation during seed formation (Bagni et al, 1967), germination (Bagni, 1970; Smith and Best, 1977; Ramakrishna and Adiga, 1975) and tuber sprouting (Serafini et al, 1980; Kaur-Sawhney et al, 1982b). Exogenously applied polyamines also exerted weak auxin-like effects on leaf explants of Datura inoxia (Chriqui et al, 1986) while the polyamine biosynthesis inhibitor DCHA inhibited growth in Helianthus tuber explants (Barbieri et al, 1983). In Hiproly barley callus putrescine, spermidine and spermine levels were elevated after withdrawl of 2,4-D and were associated with root formation (Katoh et al, 1985). Evidence for a link between auxin and polyamines also comes from the study of Tiburcio et al (1985) on polyamines in tobacco callus who conclude that NAA regulates polyamine metabolism in tobacco callus since levels of polyamines were altered by changing NAA levels. Clearly a significant body of work has been based on studying the role of polyamines and auxin in plant growth and yet despite such studies the evidence for a polyamine role remains unproven. After the present investigation the results may be summarised as follows:

Exogenous amines are not capable of replacing auxin as a growth factor in carrot culture in standard suspension culture medium at any of the concentrations tested, under these standard conditions. Moreover,

amines may inhibit growth when present at high concentrations in the media both with and without the presence of auxin. Auxin it would appear, is vital for long term culture growth, at least under these conditions - while kinetin is certainly inessential in the short term. In interpreting these results however, it is important to consider how much of the exogenously applied amine is entering the cell and whether the exogenous concentrations chosen were effective: With respect to exogenous amine concentrations, previous determinations have indicated amine levels within the cell (Fig.1b) and exogenous concentrations were of this order of magnitude. Diffusion and active transport would be expected to result in movement of these amines into the cell and the raising of cellular amine levels. Furthermore, Pistocchi et al (1987) found that putrescine and spermidine were rapidly taken up by carrot suspension cells reaching a maximum absorption within one minute.

In view of the wide fluctuations in amine levels over the culture period already found together with high amine turnover rates, it is perhaps unsurprising that the artificial imposition of constant elevated amine concentrations may lead to growth reductions, since the pattern of elevated amine levels for brief periods and far lower amine levels over the majority of the cycle may be of fundamental physiological significance. The supplementation of growth media as carried out in this study, must of necessity be a relatively crude technique to employ in investigations into amine involvement in growth - but does give a general impression of the significance of physiological levels of amines within the cell in relation to culture growth. Other studies involving the use of inhibitors (above) and cell mutants deficient in amine synthetic enzymes (Malmberg, 1983) offer a more closely controlled system for manipulation

of cellular amine content, and it is on the basis of results showing halted growth in amine deficient prokaryotic mutants that many of the claims for amine growth involvement have been based. In the absence of availability of such mutants of carrot cells, "amine-feeding" is a viable method of general cell metabolic investigation.

4.4 Amine precursors

Introduction

Ornithine and arginine

Amine metabolism in carrot cells is initiated with the decarboxylation of the amino acids ornithine and arginine, with the decarboxylation of arginine predominating (Smith, 1985b; Feirer et al, 1984). It will be apparent that if concentrations of these amino acids within the cell are not optimal, then amine synthesis will be limited by this, provided other factors are not limiting amine production. If, in addition growth is dependent to some degree on amine levels - then it may be affected by any decrease in ornithine or arginine concentration below optimal levels.

To investigate the possibility that amine synthesis and hence possibly growth also was being regulated by changes in levels of the precursor amino acids ornithine and arginine, an investigation was carried out into the effect on amine levels and final fresh weight of changes in the media concentrations of these two precursors.
To manipulate cellular levels of ornithine and arginine, cells were grown in media supplemented with these amino acids at two concentrations, 1 and 10 mM.

Results and Discussion

The effect of increased media arginine and ornithine levels on amine contents 10 d after subculture are shown (Fig. 13b). While there is little difference between amine levels in control cells (unsupplemented medium) and cells incubated in media supplemented with mM arginine and ornithine, at 10 mM arginine and ornithine there is a doubling in spermine content. This may indicate that sub-optimal levels of these precursors reduce spermine synthesis rates in control cells.

In terms of growth, other studies have found a severe inhibition of growth in arginine and ornithine-supplemented media (Walton et al, 1988). Preliminary results indicated that ornithine and arginine completely inhibited growth when supplied at 2.5 mM. In the present study, growth data (Fig. 13a) shows no significant difference in final fresh weight compared to controls in cultures incubated in arginine and ornithine supplemented medium at either concentration tested. These results indicate that neither amine precursor has any individual growth promoting properties. To summarise therefore, spermine accumulation is increased at high precursor levels, while growth as fresh weight remains unaffected.

Figure 13a Growth of undepleted cells in arginine and ornithine-supplemented media at 10 days growth

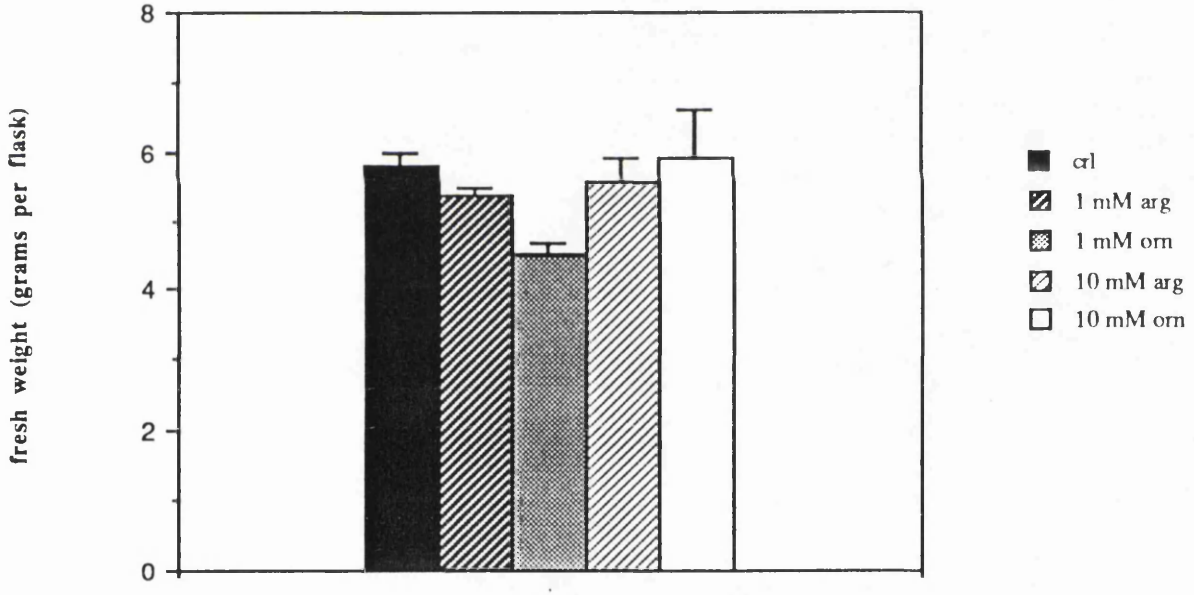
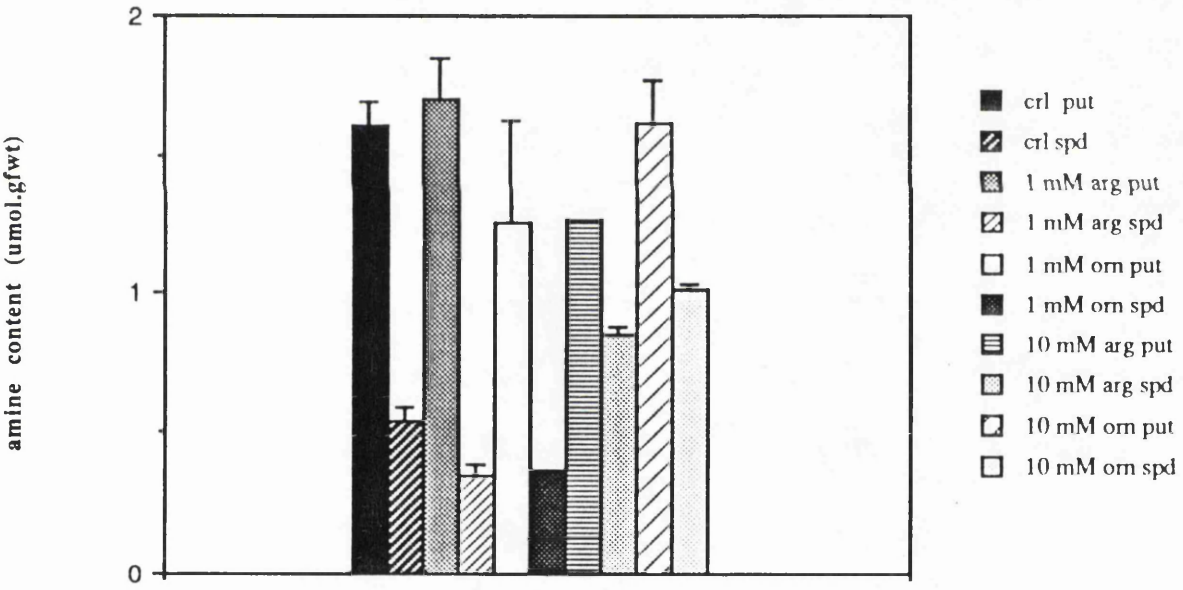


Figure 13b Amine content of cells in arginine and ornithine-supplemented media at 10 days growth



4.5 Available nitrogen and growth

Introduction

Altering the levels of the immediate amine-precursor amino acids is one technique for learning more about the points of metabolic regulation in carrot culture amine metabolism. One limitation of this method however, is the lack of a reliable means of reducing arginine and ornithine concentrations directly, without disrupting general metabolism as a result. It is possible though, to indirectly modulate amino acid levels through changes in the balance and quantity of the nitrogen source with which the medium is supplemented. The effect of changes in the balance of the nitrogen source (the ratio of ammonium to nitrate ion) on cellular amine levels was determined using a range of ratios of ammonium to nitrate ions and the standard techniques already described.

Results and Discussion

The ratio of ammonium to nitrate ions in the nitrogen source was without effect on fresh weights after 10 d in culture (Fig. 14a), though increasing amounts of ammonium ion appeared to reduce the putrescine, spermidine and spermine content (Fig. 14b). Interestingly, it has been previously reported that increasing proportions of ammonium ion in the nitrogen source may lead to a build up in cellular proton levels, resulting in a drop in cellular pH (Slocum et al, 1984) This has in turn been reported to result in increased levels of putrescine and some other amines (Priebe et al, 1978; Klein et al, 1979; Le Rudelier and Goas, 1975) a phenomenon which has been found to accompany exposure to acidic

Figure 14a Effect of variation in NH4+/NO3- balance on growth at 10 days growth

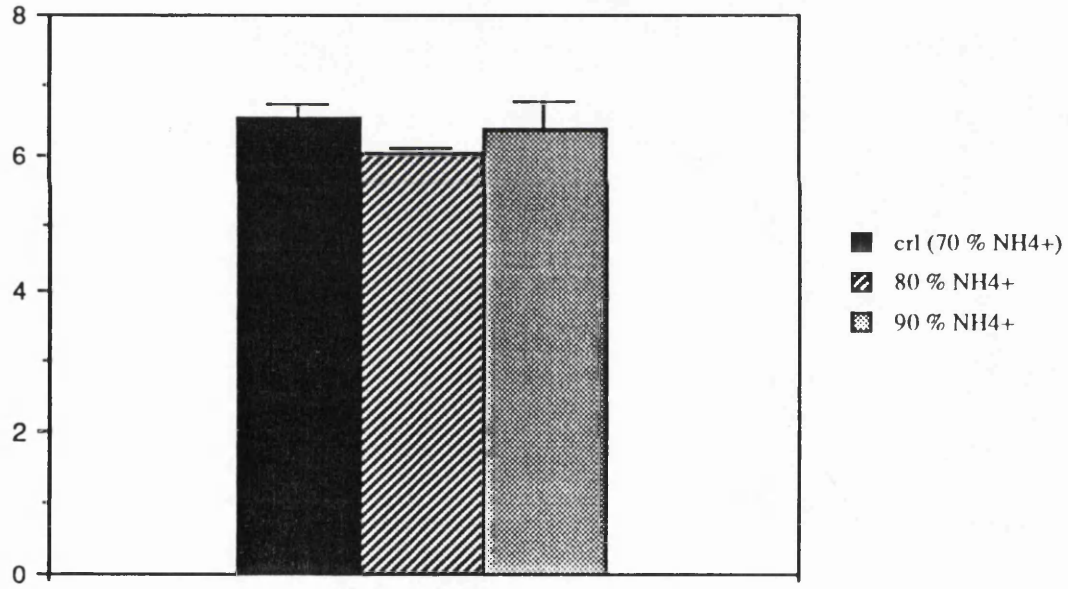


Figure 14b Effect of variation in NH4+/NO3- balance on amine content at 10 days growth

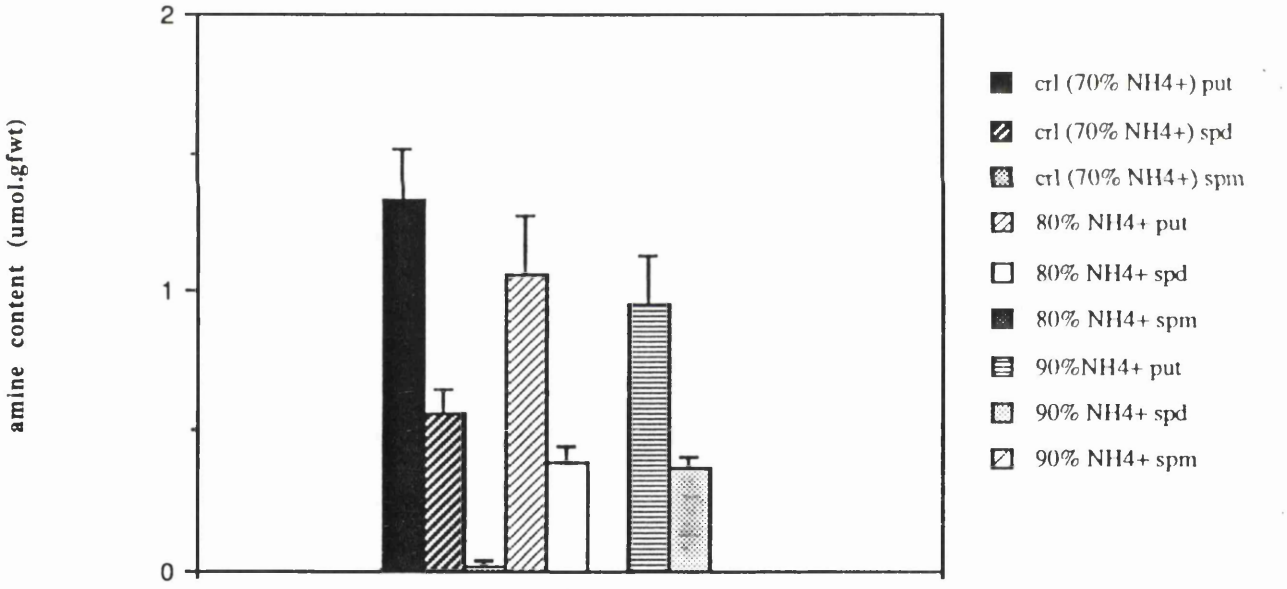
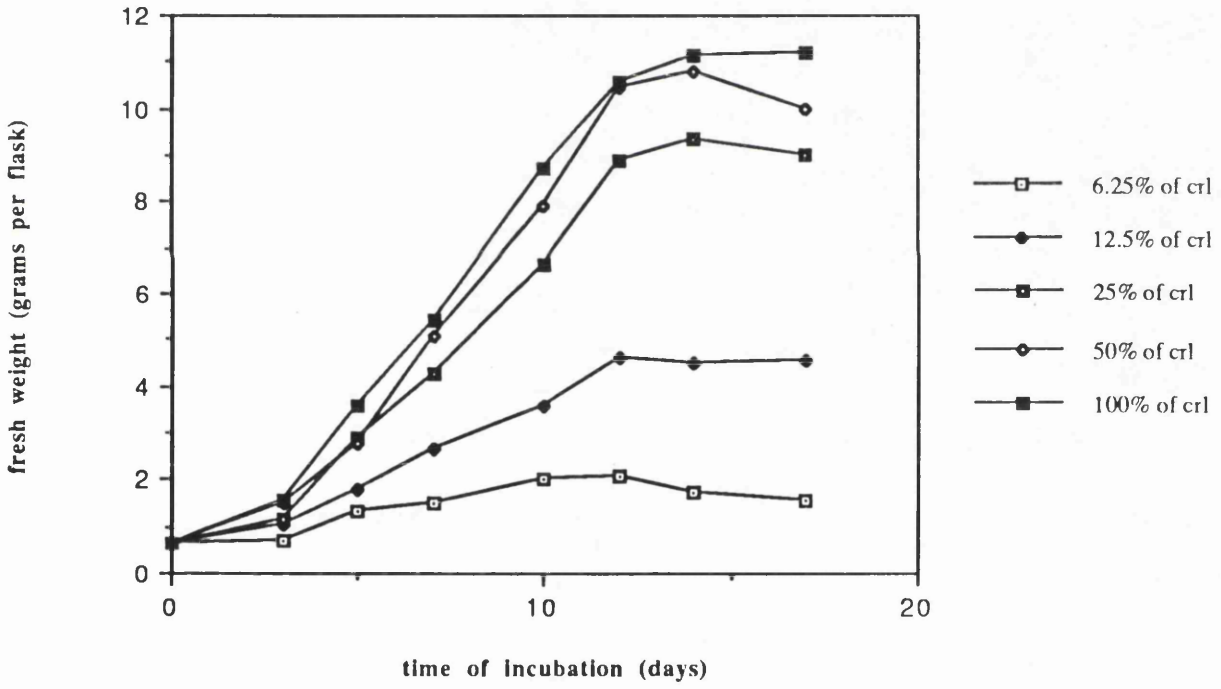


Figure 15 Effect of variation in nitrogen content on growth



reached, with final fresh weights halved on reducing nitrogen content from 25 to 12.5% of control levels.

The implication of these results is that optimal growth may be achieved at nitrogen levels of around 50% of those normally included in the culture medium. Since fresh weights alone were determined however, it cannot be confirmed that other parameters are also optimal at these levels.

The dramatic decline in growth as levels of nitrogen are reduced below 50% shows nitrogen supplementation to be an essential requirement for growth, as would be expected with the importance of nitrogen-containing molecules in general cell metabolism.

4.6 Potassium levels in relation to amine content and growth

Introduction

The final part of this study into the results of changes in media constituents concerned the importance of potassium as a component of the standard medium. Several reports have also highlighted the dramatic effect changes in potassium level may have on amine levels under some conditions (Smith, 1984), and the study thus included an investigation into changes in amine concentration in cells cultured under various potassium regimes.

Stationary-phase cells were inoculated into especially prepared "MDK" potassium-free medium supplemented with the amount of potassium in the standard media and media supplemented with only 10% of this amount.

Figure 16a Effect of variation in media potassium level on growth at 10 days growth

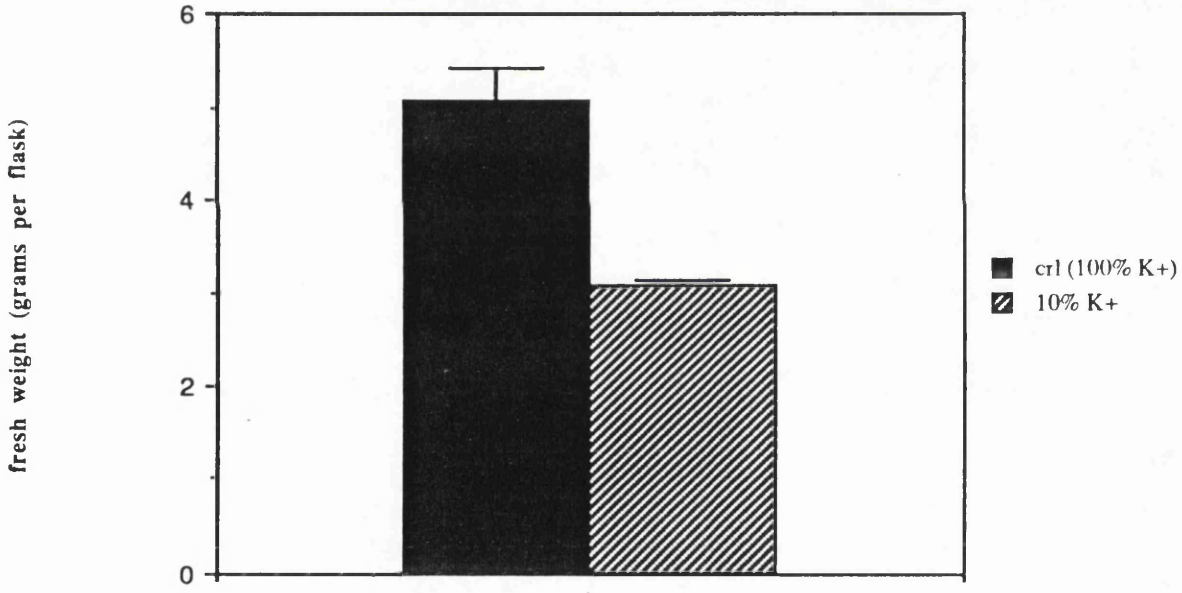
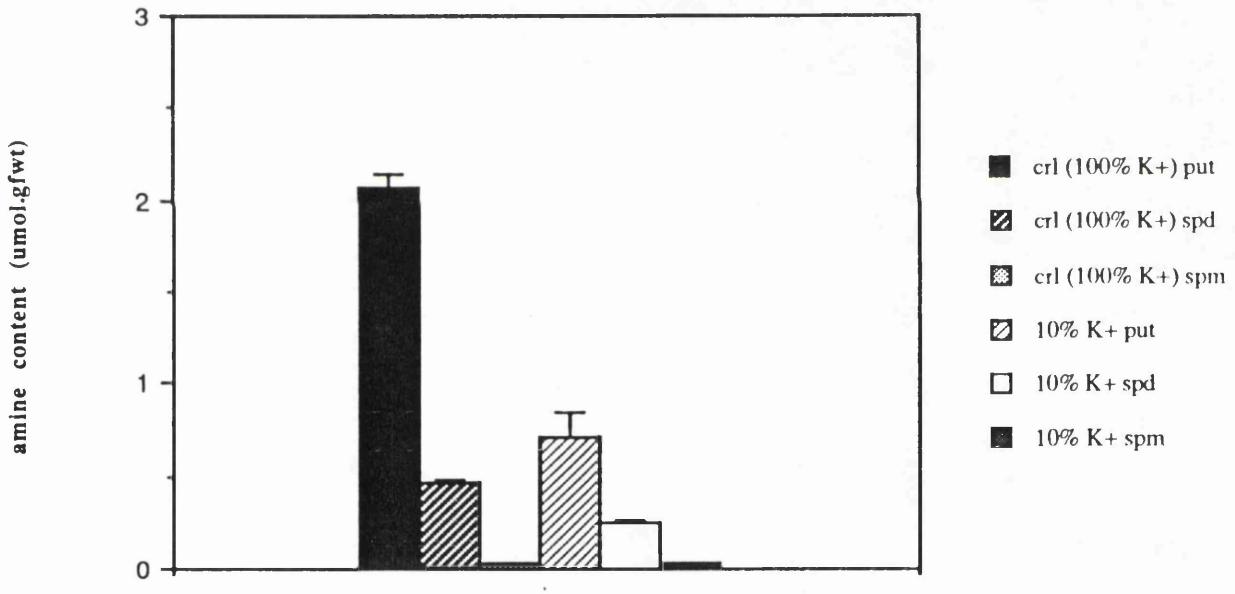


Figure 16b Effect of media potassium level on amine content at 10 days growth



(PAGING ERROR)

Results and Discussion

The results show a dramatic fall in both growth (Fig. 16a) and amine level (Fig. 16b) compared to controls after 10 d growth in low potassium media. It is immediately apparent that potassium is a key constituent of the medium which must be included if normal levels of growth are to be maintained. These results do not correspond with a number of studies showing increased putrescine levels under conditions of reduced potassium availability (Richards and Coleman, 1952; Smith, 1970; Klein et al, 1979; Young and Galston, 1984), and do not give weight to suggestions that putrescine may be an indicator of potassium deficient conditions (Smith, 1984).

In concluding this investigation of the relationship between growth and amine metabolism a few points should be noted. In studying this relationship it is a formidable task to determine which section of metabolism is affected by changing polyamine levels. A multitude of cellular processes may be modulated by changes in growth rates and establishing a direct link between polyamines and a single metabolic sequence is not a simple task. Tabor and Tabor (1984) have noted that growth inhibition affects DNA, RNA and protein synthesis and that many steps may exist between the primary effect of the polyamines and synthesis of these macromolecules. In addition studies involving polyamine deprivation are particularly difficult to interpret because such deprivation does not occur immediately. When endogenous levels are depleted other changes may also have taken place. These problems apply to those studies which attempt to show that growth stimulation directly induces increases in polyamine biosynthetic enzymes and that this increase initiates DNA and RNA synthesis and growth by the action of the

synthesized polyamines.

CHAPTER FIVE

POLYAMINES AND STRESS

5.1 Water stress

Introduction

The physiological reponses of plants to water-stress have long been of interest mainly because of the need to understand better the effects on economically important crop plants when water is a limiting factor. Kramer (1980) has estimated that losses in production due to lack of water exceed those of all other factors combined. One mechanism of resistance to water-stress is drought tolerance (Levitt, 1980), which involves the reduction of cell water potential through intracellular solute accumulation, allowing the cell to retain turgor and enhancing survival in an environment of fluctuating osmotic potential (Turner and Jones, 1980).

Research into drought stress has until now mainly been directed towards whole plants with particular emphasis on stomatal behaviour. However, suspension cultured plant cells offer a homogeneous and experimentally controllable alternative to study cellular responses to water stress. Heyser and Nabors (1979) first reported the selection of cultured cell lines resistant to the stress induced by polyethylene glycol (PEG). Adaptation procedures and growth responses have since been examined (Bressan, 1981) and physiological changes associated with water-stress adapted cells investigated in detail (Handa et al, 1983).

Two components of the response of plant cells to decreased water potential may be identified; long-term changes in cells adapted to continuous growth in low water potential, here referred to as stress-adaptation, and stress-shock responses occuring in the short-term in cells transferred abruptly to lower water potential environments.

Short-term stress

Though the changes in plant amine metabolism in response to environmental stress have been actively studied for some time (Altman et al, 1982), many of these studies have dealt with long term stress during acidification (Young and Galston, 1983), salinity (Strogonov, 1964) and drought (Turner and Stewart, 1986). One of the most dramatic changes in plant amine levels under stress was reported under shock stress conditions: The putrescine content of oat leaf cells and protoplasts increased up to 60-fold within 6 h with an osmotic stress due to 0.4-0.6 M sorbitol (Flores and Galston, 1982b). This study however, was initially concerned with the response of amine metabolism to short-term stress imposed on the unadapted carrot cultures during incubation. Cells were firstly monitored on exposure to osmotic stress in media supplemented with mannitol, sodium chloride and polyethylene glycol, and in later investigations responses to heat stress and auxin were studied. Cultured plant cell systems possess a number of features which make them especially suitable for studies of plant stress metabolism when compared to whole plant systems: The stress regime to which each plant cell is exposed may be tightly controlled, and the level of stress may be precisely determined. The level of stress is uniform across the whole cell population, whereas in whole plant systems only a small fraction of the total cell population is directly exposed to the

stress solution. The variation in response between the cell populations of the various plant organs to the stress environment is not present when uniform cell culture is used. It may be concluded that cell culture is uniquely suited for certain stress metabolism studies such as those described below.

5.1.1 Sodium chloride stress

Increasing use of irrigation in many arid areas has resulted in an increasing problem with soil salinization, prompting the search for an underlying mechanism whereby some plant species are resistant to such conditions while others are adversely affected. With reports of large cellular amine accumulations in response to water-stress, mineral balance and other environmental factors, several studies have attempted to show a relationship between amine levels and environmental salinity (Smith, 1984). A number of reports have indeed presented data showing increasing amine levels in plants exposed to high salt levels (Strogonov, 1964; Prikhod'ko and Klyshev, 1964; Shevyakova et al, 1981). Despite such findings however, the evidence remains inconclusive as others have failed to find significant amine accumulation under similar circumstances (Anderson and Martin, 1973; Priebe and Jager, 1978).

In the light of such apparently contradictory findings and as a comparative exercise with the previous mannitol stress study, an investigation was carried out in which amine levels were monitored in cells subjected to a brief period of incubation in sodium chloride supplemented medium.

The experimental procedure used involved the supplementation of MDK media with 0.4 M sodium chloride. The sodium chloride was added to the standard medium prior to sterilisation and stationary phase cells inoculated as previously described into supplemented and control media and incubated under standard conditions for a set time period. A range of incubation periods were tested and amine levels determined in each case.

Results and Discussion

On comparing the amine levels present in cells incubated in sodium chloride supplemented medium with those in control cells incubated in unsupplemented medium, it is apparent that there is no significant difference between them when the effects of dehydration through water loss to the high osmotic potential medium are taken into account (Table 2). It should be noted however, that the lack of any significant amine accumulation after 8 h does not preclude a longer-term rise in amine content on prolonged exposure to saline conditions. While the lack of response appears not to be in agreement with the results of Shevyakova and gonov(1985) who found significant accumulation of polyamines under saline conditions, closer consideration reveals that the increases reported are expressed on a fresh weight basis alone. Due to the considerable water losses which occur on incubation in media of low water potential, an increase in solute concentration would be expected in the absence of any solute accumulation. Since water contents were not determined in the study mentioned above it is difficult to conclude whether an accumulation did take place but it may be assumed that a large proportion of the 6-fold increase described might possibly be accounted for in terms of water loss alone. Indeed, if all the studies carried out on the relationship between amine levels and changes in salinisation are considered together it is apparent that evidence for a link between changes in amine levels and salinization is far from conclusive.

Table 2 Stress and amine content in carrot cells

Treatment	Hours of incubation									
	0		1		2		4		8	
	nmol.gfwt									
	Put	Spd	Put	Spd	Put	Spd	Put	Spd	Put	Spd
Control (Unadapted Cells to Standard medium)	533 (45)	498 (69)	-	-	1062 (143)	196 (44)	1298 (239)	267 (81)	833 (127)	257[a] (92)
Unadapted cells to 7.5% mannitol	380 (47)	225 (40)	1203 (101)	335 (24)	1542 (133)	280 (26)	1169 (85)	250 (37)	1273 (108)	87 (16)
Cells after 1 passage in mannitol supplemented medium	533 (44)	291 (25)	1179 (82)	344 (26)	1891 (173)	298 (30)	1083 (97)	358 (22)	257 (20)	121 (18)
Cells after 4 passages in mannitol supplemented medium	492 (67)	283 (25)	518 (77)	245 (34)	1095 (89)	750 (66)	1796 (144)	1612 (138)	1948 (223)	1012 (81)
Unadapted cells to 40°C medium	463 (61)	322 (24)	-	-	-	-	-	-	1012 (212)	343 (38)
Unadapted cells to anoxic medium	623 (53)	434 (41)	-	-	1118 (149)	789 (81)	1102 (119)	727 (65)	1217 (94)	870 (63)
Unadapted cells to 25% PEG	629 (82)	437 (66)	778 (107)	429 (131)	1172 (189)	881 (128)	1996 (338)	1032 (183)	3862 (441)	2355 (271)
Unadapted cells to 0.4 M NaCl	447 (68)	280 (72)	931 (118)	389 (56)	1231 (126)	465 (62)	1554 (134)	606 (49)	1729 (208)	643 (78)

[a] Means(± standard errors)

Table 2a Stress and amine content in carrot cells continued

Treatment	Mannitol concentration														
	Control			0.1 M			0.2 M			0.3 M			0.4 M		
	nmol.gfwt														
	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm
Unadapted cells to mannitol 8 hours incubation	579 (83)	330 (45)	62 (17)	871 (126)	280 (51)	84 (31)	1176 (189)	550 (77)	80 (25)	1093 (163)	630 (104)	36 (15)	1561 (190)	1110 (148)	64[a] (23)

[a] Means(± standard errors)

5.1.2 Mannitol stress

Introduction

Mannitol, when added to the standard culture medium prior to autoclaving, may be used to lower the water potential of the cellular environment to a predetermined level. It may therefore be used to impose water stress conditions on the cell culture, which may be determined prior to incubation through osmometer measurements. The water stress is effected through the loss of cellular water down the diffusion gradient from cell to culture solution, and leads to an increase in the concentration of cell solutes, among them the amines.

In order to determine whether amine levels increased beyond those expected due to water loss, the concentrations of amines were determined both during and after exposure to mannitol-supplemented media for various time periods. A preliminary study was initially carried out, in which stationary phase cells were inoculated into standard medium supplemented with 7.5% mannitol. Cultures were maintained on this for 8 h and amine contents determined at intervals on harvested cells as previously described. It was thus possible to follow the initial changes in cellular amine contents which occured as a result of this rapid exposure to severe water-stress conditions.

Results and Discussion

When considering the changes in amine concentrations found immediately after exposure to the stress-medium, some significant trends are evident. The most obvious initial change is a large rise in putrescine concentration after 1 h incubation in 7.5% mannitol (Table 2). Cellular putrescine concentration continued to increase up until 2 h after subculture, when levels peaked. The levels then showed a decline to approximately the 1 h level, about which it fluctuated for the remainder of the 8 h period.

In addition to such studies with 7.5% mannitol the response of cells was also monitored upon exposure to a range of different mannitol concentrations. Once again stationary-phase cells were incubated for 8 h in standard medium supplemented with mannitol at 0.1 - 0.4 M concentrations, and amine contents determined in harvested cells.
Both putrescine and spermidine levels showed a clear increase with increasing mannitol concentration (Table 2a), which would be expected due to the concentrating effects of water-loss. Spermine levels however did not show such a clear trend with levels fluctuating about a comparatively low level and the final spermine concentration barely greater than the control (unsupplemented medium) value. With an increase expected due to water-loss effects alone, there must be a real decrease in spermine content. This may possibly be due to reduced synthesis from spermidine during spermidine and putrescine accumulation.

To summarise the response of carrot cultures to mannitol osmotic stress-shock: There is little net accumulation of putrescine and

spermidine after 8 h incubation, when water-loss effects are taken into account, and a net decrease in spermine concentration after 8 h at higher mannitol concentrations.

When the time course of amine accumulation is considered however, it is clear that the amine content after 8 h incubation is not representative of that over the whole period. There is, in fact a massive increase in putrescine content immediately after subculture, an increase which has disappeared by 8 h after inoculation. This increase however is not stimulated by the mannitol medium alone, it is a characteristic of post-inoculation cultures. It would appear therefore that a short period of incubation in mannitol supplemented medium does result in increased concentrations of some amines - but that this is due mainly to dehydration.

These results contrast with those of Flores and Galston (1984) who found a more than 20-fold increase in putrescine content after 4 h incubation of oat leaf segments in 0.4 M mannitol. This is obviously a massive change in amine levels and at first sight seems difficult to equate with the amine levels found in the present study. However, the situation was clarified somewhat by Turner and Stewart (1988), who found that the increase in putrescine level appeared great partly due to tissue water loss resulting in a concentration in cell solutes and partly to a fall in control putrescine levels over the course of the experiment. They concluded that the increase compared to endogenous levels was relatively uniform at approximately 3 to 4-fold and that the enhanced accumulation of putrescine reported was largely artefactual.

It would appear therefore that the changes in amine levels found in the present study, though far smaller than the gross increases found elsewhere, are in fact of the same order of magnitude in real terms as those of Flores and Galston (1984) when distorting factors are accounted for. Turner and Stewart (1988) suggest that the way in which the results of previous studies were presented may have resulted in an exaggeration of the influence of osmotic stress on cellular polyamine contents.

5.1.3 Polyethyleneglycol (PEG) and short-term stress

Introduction

Both mannitol and sodium chloride may be used to lower the water potential of the culture medium, resulting in water loss from the cell and the consequent onset of water stress. Though both osmotica are satisfactory for this purpose and sodium chloride uniquely so in the study of salt-stress, they do have an inherent disadvantage in work on general water-stress metabolism. This disadvantage lies in the ability of the cultured cells to take up these compounds and thus possibly incorporate them into general metabolism, making it difficult to distinguish between the possible effects of such uptake and true water-stress effects.
One solution to the problem of osmoticum uptake is the use of an osmoticum compound such as PEG 4000. Due to its large molecular size this compound cannot be taken up by the culture thus avoiding any unspecific effects. It has been widely used in work with whole plants (Slavic, 1974), while its use in tissue culture has not been so extensive but is increasing as stress-metabolism studies make use of the cell culture system (Handa et al, 1986).

Purified PEG was used in these studies in a similar fashion to mannitol and sodium chloride osmotica. Standard medium was supplemented with PEG (25% w/v) prior to sterilization, after which it was inoculated with stationary phase tissue and incubated for 8 h when amine levels were determined.

Results and Discussion

If amine levels over the first 8 h of culture are considered then an increase above control values for both putrescine and spermidine is noted (Table 2). This increase is greater than that noted for any of the other stress osmotica. Putrescine levels increase 6-fold between 0 and 8 h, while spermidine levels increase by a similar amount. The peak in amine concentration over the period studied occured at 8 h, with the rate of increase in concentration showing no sign of decreasing at this point. As we have seen when considering polyamine accumulations with other stress solutes, a progressive increase in solute concentration would be expected on incubation in PEG-supplemented media due to water-loss alone. Since percentage water-losses were not determined in this short study it is difficult to determine in exact terms which proportion of the polyamine increase arises from this effect though subsequent studies have found a doubling in polyamine concentration due to dehydration to be a realistic figure. This suggests that a 3-fold real increase in putrescine and spermidine content occurs after 8 h exposure to PEG, which is very similar to the 3 to 4-fold increase in putrescine concentration observed in peeled barley leaf sections after 4 h incubation in a 400 mM sorbitol solution (Turner and Stewart, 1988) and to the response in droughted plants (Turner and Stewart, 1986).

It remains unclear what significance if any this response to rapid stress has. It would seem unlikely that adaptive changes should take place in the short time period investigated and it is uncertain on the basis of this study whether similar responses are likely to take place under prolonged stress conditions. These questions were however addressed when shock and prolonged stress were examined together.

5.2 Short and long-term stress

Introduction

We have already considered the distinction between short-term responses to stress which may have little or no adaptive significance and those longer term changes which may take place over a prolonged period of stress and involve measures enabling the plant to withstand lengthy periods of sub-optimal conditions. Since plants are routinely exposed to non-lethal levels of stress (Slocum et al, 1984) and must complete their life-cycle within the constraints these impose, such adaptive developments are of great importance. The distinction between the responses of cultured plant cells to these two forms of stress formed the basis of the remainder of this work with PEG.

Both responses were examined in carrot suspension cells transferred to fresh medium of the same or lower water potential for a period of 10 h. For stress-shock changes, cells of a line adapted to growth in a standard tissue culture medium (control cells) were transferred directly to media containing various concentrations up to 25% PEG. For stress-adaptation, cell lines previously adapted to similar PEG concentrations were transferred to fresh medium with appropriate PEG levels. Changes in growth rate, water potential and solute content for both control and adapted cells were determined.

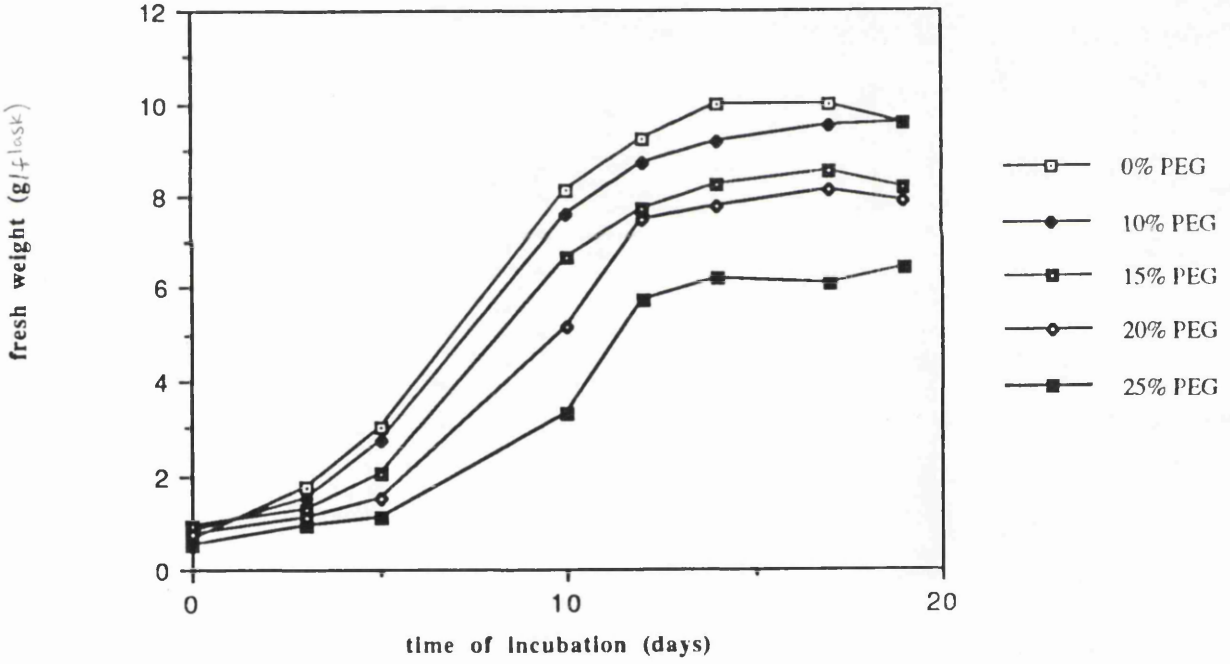
Results and Discussion

On inoculation into fresh medium both adapted and unadapted cells showed a characteristic sigmoidal growth pattern over an 18 d period, at all concentrations of PEG (Fig. 17a, 17b). In unadapted cells (Fig. 17b) increasing PEG concentration progressively increased the length of the lag period, and decreased the rate of fresh weight increase. The lag time was extended from 2-3 days in control medium to 10 days with 25% PEG, and the rate of fresh weight increase reduced from 0.75g/day to 0.25g/day. Final fresh weight attained was reduced at all PEG concentrations, from a 15% reduction at 10% PEG to a 63% reduction at 25% PEG.

In adapted cells the effects of PEG on growth are less severe (Fig. 17a). There is an effect on lag phase only at the two highest concentrations, extending it from 3 days in the control to 5 days at 25% PEG, and little change in the rate of fresh weight accumulation was observed. Final fresh weight was however affected with increasing PEG concentrations, though to a lesser degree than in unadapted cells.

The distinct responses of adapted and unadapted cells to water stress are illustrated when final fresh and dry weights are compared after 18 d growth in media of varying PEG content (Fig. 18). Fresh weight yields fell with decreasing osmotic potential, the decline being greater in unadapted than adapted cells. Dry weight yields, on the other hand, were reduced only in unadapted cells with a fall of approximately 40% at 25% PEG. Clearly, adapted cells have an enhanced capacity for both water retention and dry weight accumulation over unadapted cells. Reductions in water content relative to control cells of up to 36% for adapted and 54% for unadapted cells were observed.

Figure 17a Growth of adapted cells in media supplemented with 0-25% PEG



Note on standard deviation in Figures 17-20

Figures 17,18: standard deviation of three replicates was within 5%

Figures 19,20: standard deviation of three replicates was within 25%, and is indicated for each value in first publication in Appendix

Figure 17b Growth of unadapted cells in media supplemented with 0-25% PEG

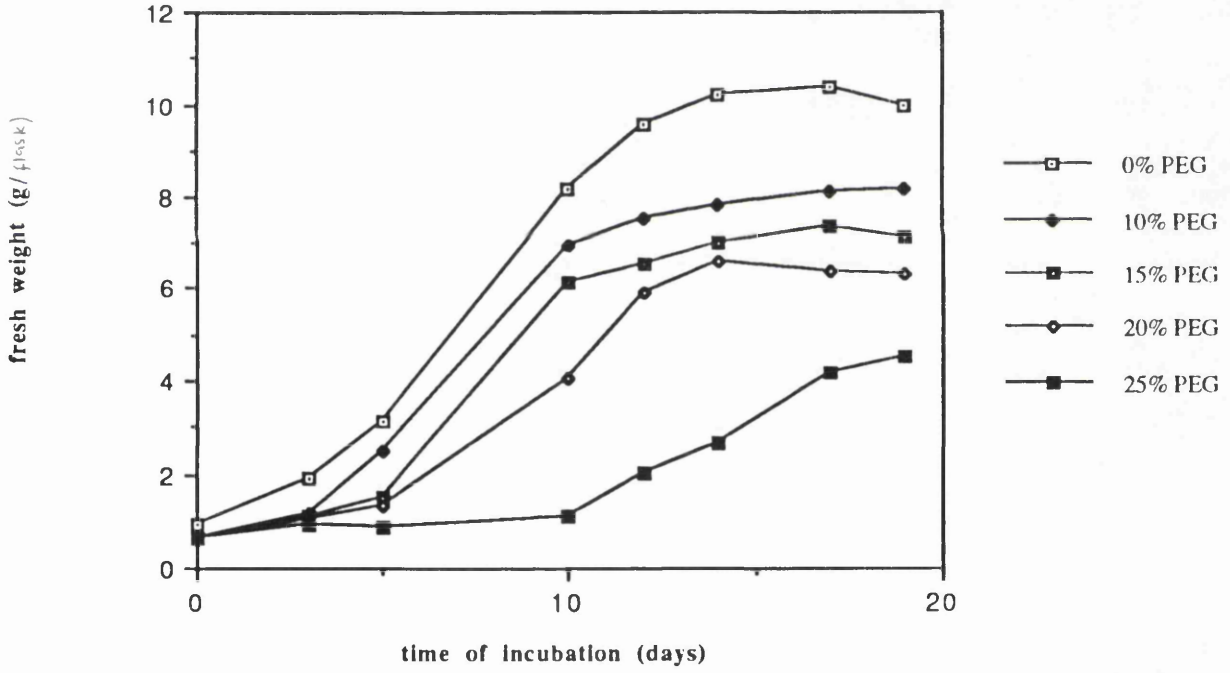
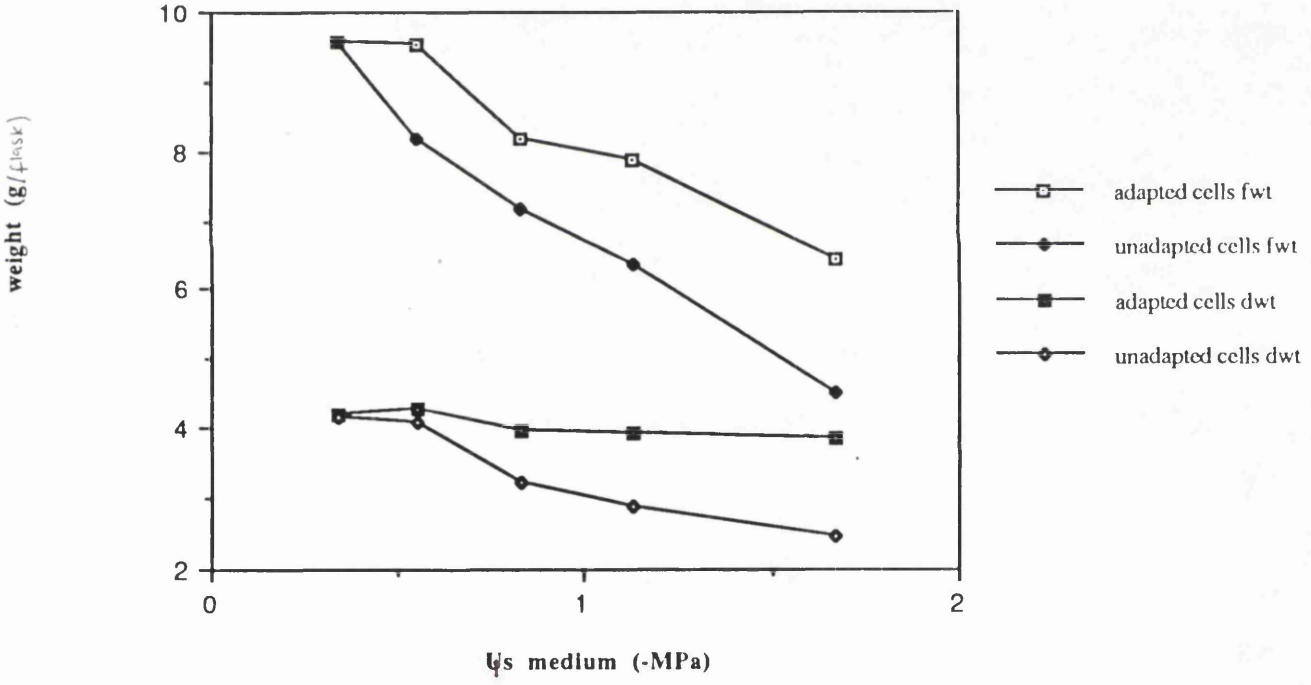


Figure 18 Relative growth of adapted and unadapted cells in PEG-supplemented medium at 20 days growth



The solute potentials of media containing 0, 10, 15, 20 and 25% PEG were found to be -0.34, -0.55, -0.83, -1.13 and -1.67 MPa respectively. The osmotic potential of adapted cells was maintained at a substantially lower value than that of the corresponding medium, the deficit actually increasing at higher PEG concentrations, such that in the line adapted to 25% PEG the solute potential decreased 4-fold relative to control cells (Fig. 19). Unadapted cells were in equilibrium with the medium (and were usually plasmolysed) at 15% PEG and above.

Putrescine and spermidine were the major polyamines present, with spermine as a minor component (Table 3). The total polyamine concentration increased with PEG concentration in an approximately linear fashion; adapted cells showed larger increases than unadapted cells at higher solute potentials, while levels fluctuated at lower values. Increased total concentrations were primarily due to increases in putrescine , and the rather small increases in concentration (approx. x2) can be accounted for by concentration due to water loss, (c.f. Fig. 18).

The cellular concentration of proline rose significantly in both normal and adapted cells grown in media of decreasing solute potential (Fig. 20b). Proline concentration increased 12-fold in unadapted cells, and over 40-fold in adapted cells as compared with controls. Proline levels continued to increase in adapted cells up to the highest concentration tested while in unadapted cells no increase occured beyond 20% PEG.

The combined cellular concentration of the major free amino acids shows a strong correlation with medium solute potential in adapted cells

Table 3 Free amine concentrations in unadapted (U) and adapted cell lines at 10 hours growth

Mean of three replicate determinations.

Amine		PEG concentration				
		0%	10%	15%	20%	25%
		mM				
Putrescine	U	1·36	1·55	2·36	3·99	3·86
	A	1·36	2·51	2·97	3·20	3·64
Spermidine	U	1·34	1·83	1·88	2·03	1·36
	A	1·34	1·81	1·75	1·71	1·69
Spermine	U	0·09	0·17	0·24	0·23	0·38
	A	0·09	0·08	0·11	0·32	0·43
Total	U	2·79	3·55	4·48	6·25	5·60
	A	2·79	4·40	4·83	5·23	5·76

Figure 19 Water potentials of adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

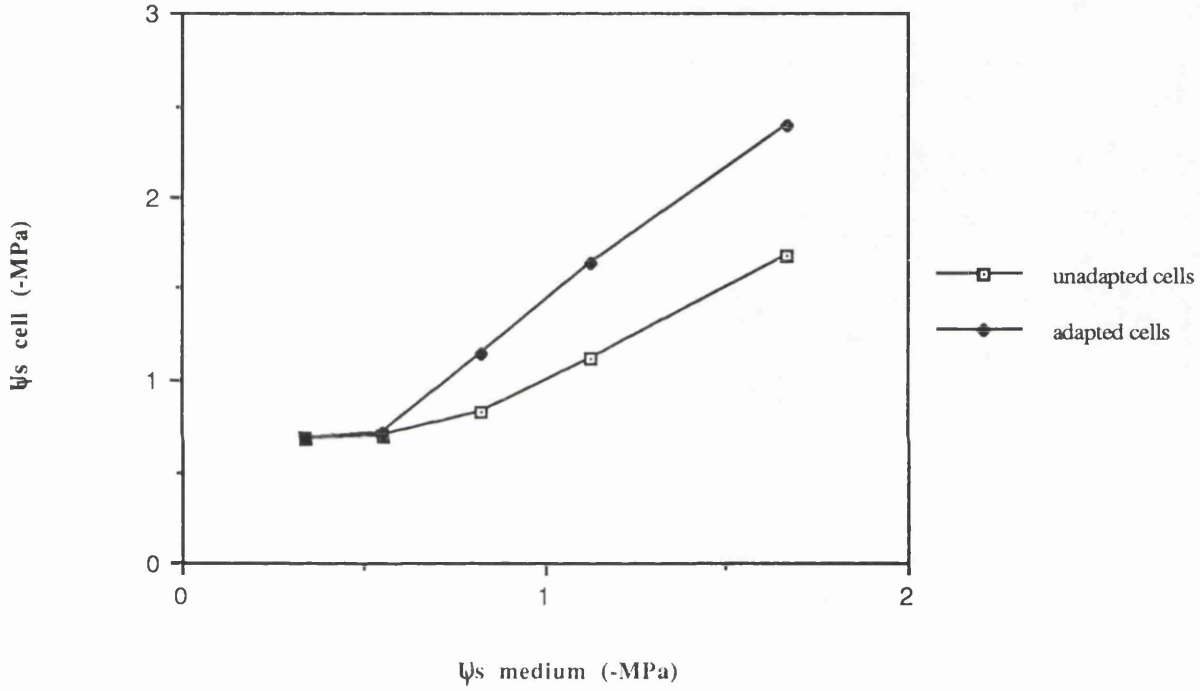


Figure 20a Total amino acids in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

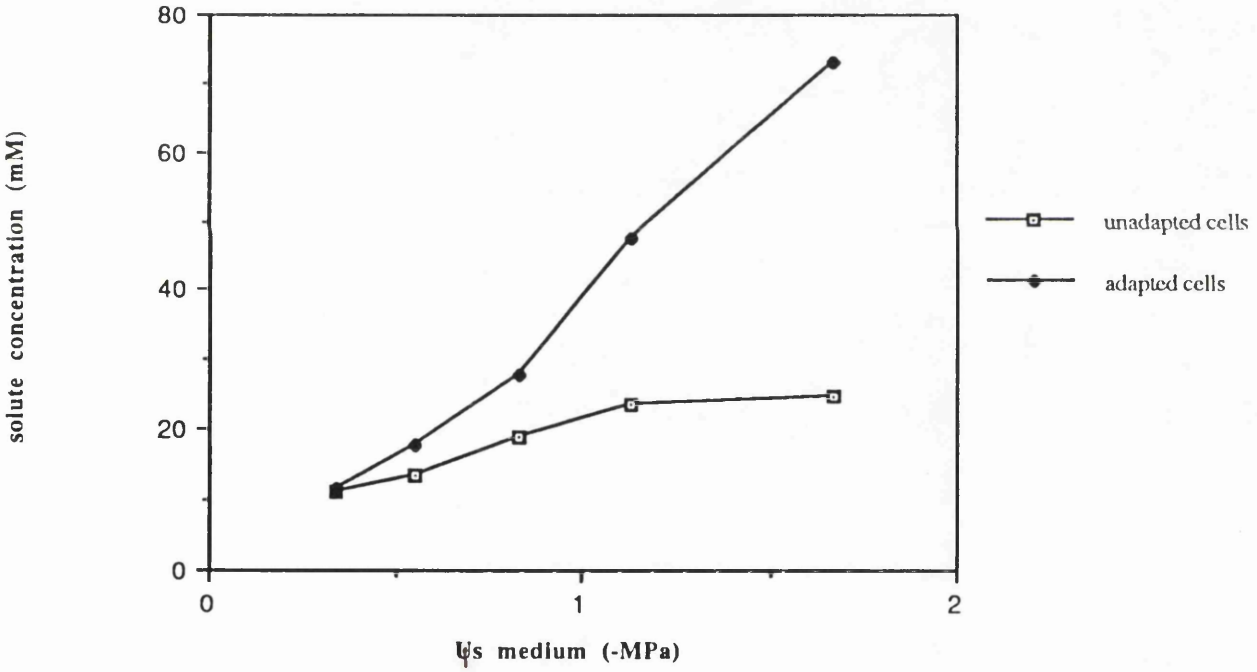
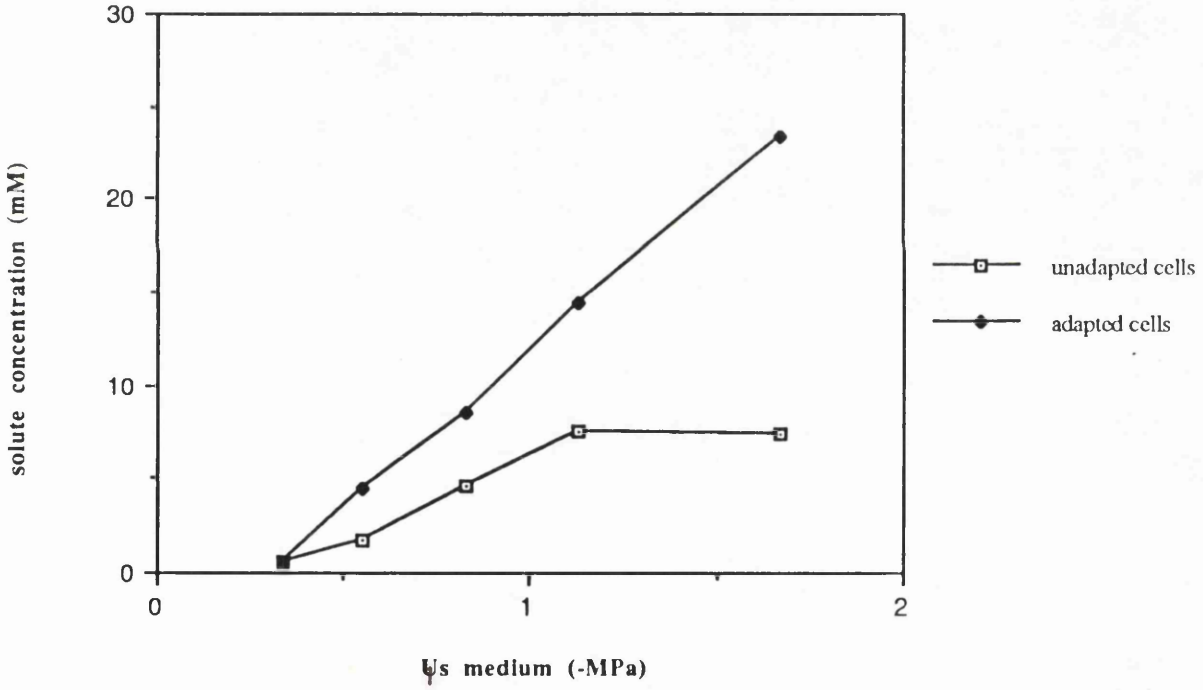


Figure 20b Proline concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth



(Fig. 20a). The response was approximately linear at lower solute potentials and was not saturated at the highest PEG concentration employed. In unadapted cells however the response was far less pronounced with a doubling in concentration over the control, as opposed to an 8-fold increase in adapted cells. While the response of individual non-proline amino acids varies considerably (Table 4), higher concentrations are generally found in adapted cells rather than unadapted. Alanine concentration increased up to 18-fold over control values (adapted cells), but the highest concentrations observed were those of histidine, arginine and valine, which were relatively abundant in control cells. With the exception of serine, all amino acids were present at higher concentration in adapted than unadapted cells at the lowest solute potential. However, proline was the most abundant amino acid by a factor of 2 and constituted 30% of the total amino acid concentration.

Betaine concentration changed little in either cell line in response to increasing water stress (Table 4).

Cellular sucrose concentration increased approximately 3-fold up to 120 mM in adapted cells under water stress and doubled in non-adapted cells (Fig. 21a). Concentrations in adapted cells were significantly higher than in unadapted at all solute potentials.

Glucose concentrations rose 4.5-fold in adapted cells at the lowest solute potential, reaching a cellular concentration of nearly 350 mM (Fig. 21c). Levels also rose 3.5-fold in unadapted cells to a concentration exceeding 240 mM . Concentrations of fructose increased 5-fold in adapted and 3-fold in unadapted cells, reaching a peak

Table 4 Free amino acid and glycinebetaine concentrations in unadapted (U) and adapted (A) cell lines at 10 hours growth

Mean of 3 replicate determinations.

Amine		PEG concentrations				
		0%	10%	15%	20%	25%
	mM					
Asp	U	0·57	0·50	0·36	0·65	0·64
	A	0·57	0·87	0·85	1·06	1·57
Gln	U	0·71	0·72	0·79	0·74	0·71
	A	0·71	0·99	1·11	1·12	1·11
Ser	U	1·67	1·82	2·11	2·41	2·45
	A	1·67	1·19	1·87	4·20	1·95
His	U	2·16	2·14	2·57	2·50	2·74
	A	2·16	3·13	3·73	6·60	12·69
Gly	U	0·33	0·28	0·39	0·45	0·58
	A	0·33	0·36	1·18	0·83	1·12
Thr	U	0·89	0·85	0·99	1·05	1·09
	A	0·89	0·76	1·53	1·78	2·29
Arg	U	1·32	1·52	1·89	1·95	1·94
	A	1·32	1·81	2·68	3·53	8·66
Ala	U	0·29	0·34	0·83	1·11	1·09
	A	0·29	0·24	0·40	5·32	5·48
Tyr	U	0·17	0·17	0·18	0·21	0·24
	A	0·17	0·28	0·34	0·36	0·58
Met	U	0·25	0·34	0·35	0·37	0·37
	A	0·25	0·32	0·53	0·58	0·67
Val	U	0·83	1·24	1·44	1·86	1·90
	A	0·83	1·86	2·59	4·63	7·07
Phe	U	0·86	1·14	1·20	1·44	1·88
	A	0·86	0·65	1·03	1·44	1·73
Ile	U	0·41	0·49	0·64	0·80	1·06
	A	0·41	0·58	0·82	1·12	2·88
Leu	U	0·22	0·29	0·37	0·48	0·59
	A	0·22	0·40	0·54	0·62	1·78
Betaine	U	0·73	0·78	1·01	0·85	0·93
	A	0·73	0·66	0·80	0·82	0·77

Figure 21a Sucrose concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

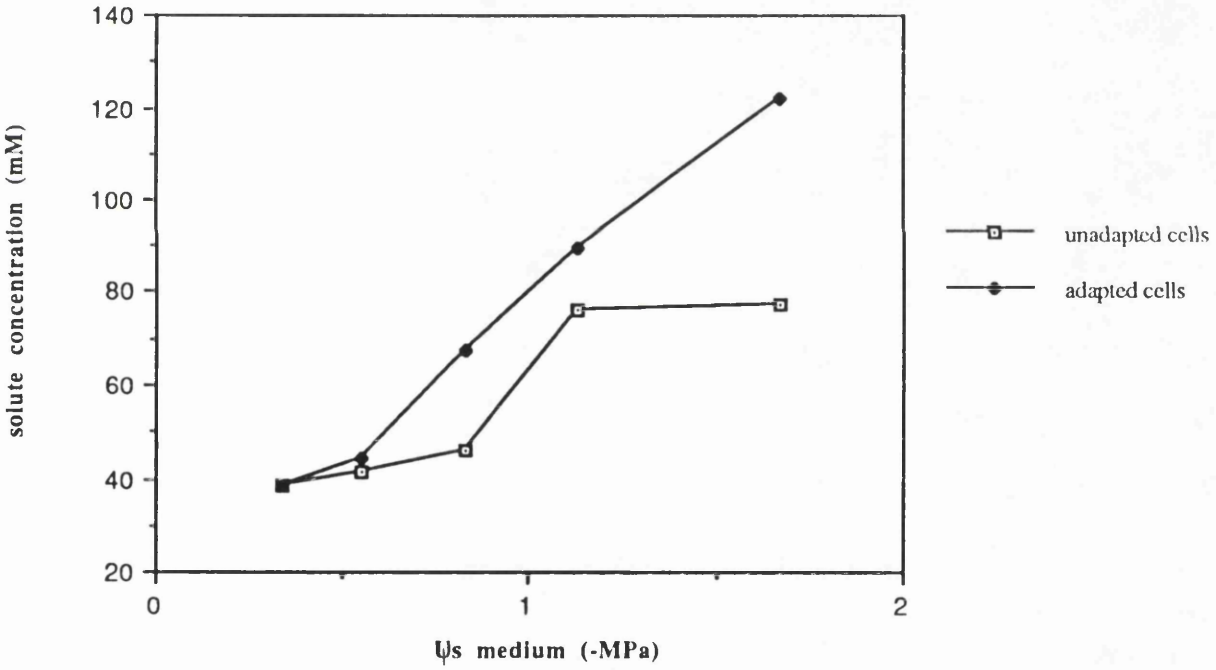


Figure 21b Fructose concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth

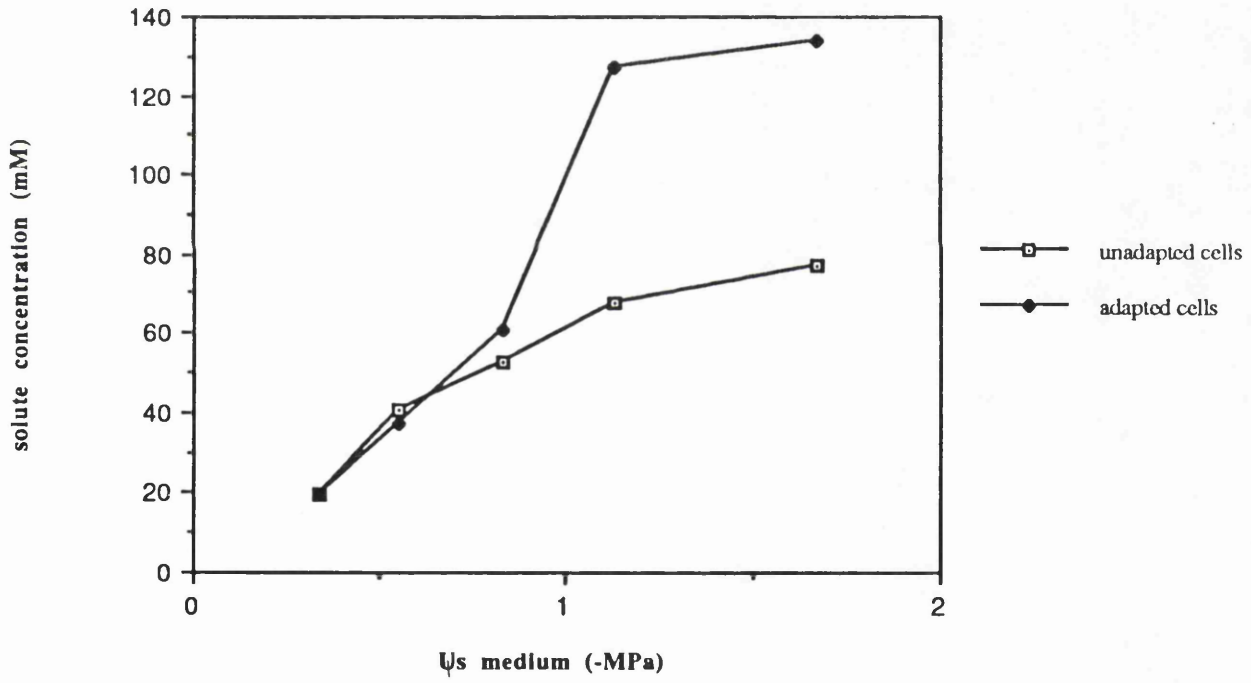
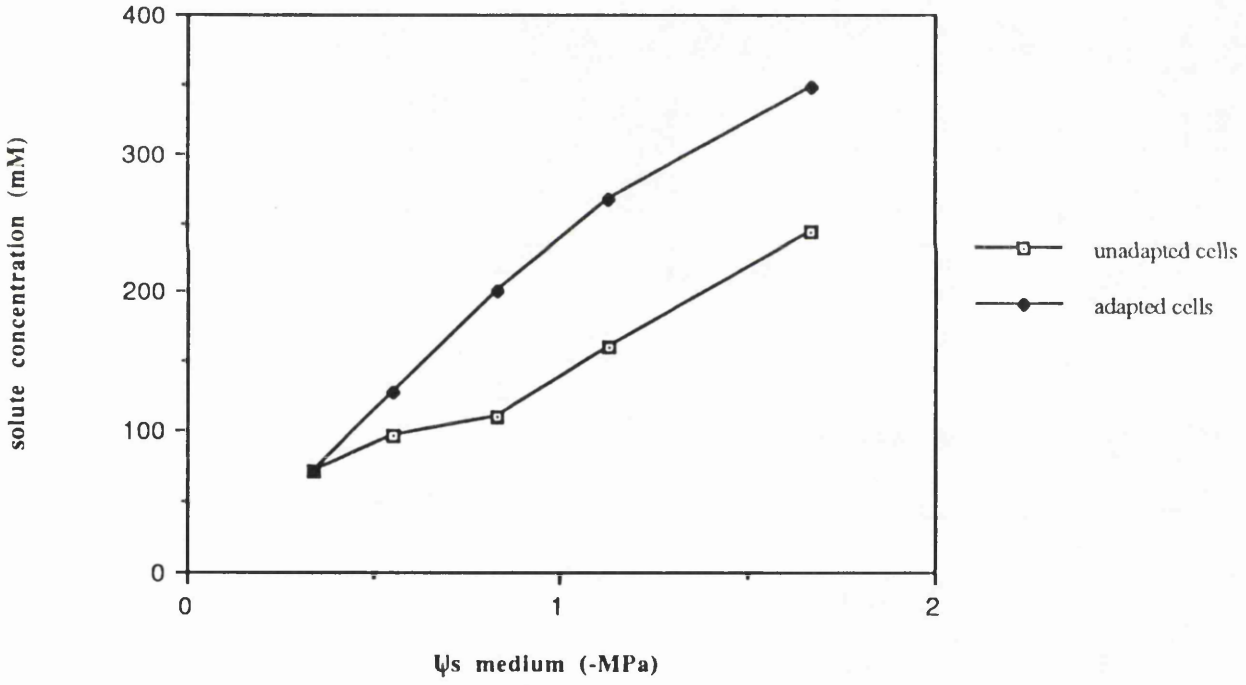


Figure 21c Glucose concentration in adapted and unadapted cells in PEG-supplemented medium at 10 hours growth



of more than 130 mM in adapted cells (Fig. 21b). Glucose, fructose and sucrose are major contributors to solute concentration, contributing over 60% of the osmolarity of stressed cells (Table 5).

To illustrate the contribution of each solute investigated to the overall cell water potentials, values for medium and cell water potentials and solute concentrations have been calculated in milliosmoles (mOsm), and the percentage contribution of each expressed (Table 5). Glucose, fructose and sucrose were the principal osmotic agents, contributing 61.5% towards the cell water potential of adapted cells in 25% PEG. Under the same conditions, proline contributes 2.4% and other amino acids 5.1%. Polyamines and betaine total only 0.5% in osmotic contribution. The organic solutes investigated contribute 69.5% to the total; potassium and other ionic species have not been determined.

The adaptation of cultured plant cells to both water and NaCl stress has been reported (Bressan et al, 1981; Ben-Hayyim, 1986). Adapted cells characteristically show a shorter lag phase and increased growth rate as compared with non-adapted cells exposed to similar stress conditions. Comparable responses with carrot suspension cultures were obtained in this study. This adaptation involves a decrease in cell solute potential (Fig. 19) which is substantially greater in adapted than in unadapted cells and directly related to a reduction in cell water content (Fig. 18), and an accumulation of intracellular solutes compared to controls. This is similar to the response of sorghum (Bhaskaran et al, 1985) and tomato (Handa et al, 1983) cells to a low water potential environment.

Table 5 Medium and cell osmolarity and percentage contribution of each solute in unadapted (U) and adapted (A) cell lines at 10 hours growth

Medium and cell osmolarity and percentage contribution of each solute in unadapted (U) and adapted (A) cell lines

All solute concentrations were recalculated in terms of mOsm and expressed as a percentage of the corresponding cell osmotic potential. AA = total amino acids excluding proline.

PEG (%)	Ψmed (mOs)	Ψcells (mOs)		Contribution of solutes to Ψcell (%)															
				Glucose		Fructose		Sucrose		AA		Proline		Polyamine		Betaine		Total	
		U	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A
0	140	278	278	25·6	25·6	6·8	6·8	14·0	14·0	3·9	3·9	0·2	0·2	1·0	1·0	0·2	0·2	51·5	51·5
10	225	288	293	33·4	43·5	13·9	12·7	14·6	15·1	4·2	4·4	0·6	1·5	1·2	1·5	0·3	0·2	62·8	78·9
15	340	341	467	32·5	43·2	15·5	13·1	13·5	14·5	4·1	4·1	1·4	1·8	1·3	1·0	0·3	0·2	68·6	77·9
20	465	456	674	35·3	39·5	14·9	18·8	16·7	13·2	3·5	4·9	1·6	2·1	1·4	0·8	0·2	0·1	73·6	79·4
25	685	687	983	35·4	35·5	11·2	13·6	11·2	12·4	2·5	5·1	1·1	2·4	0·8	0·6	0·1	0·1	62·3	69·7

When considering changes in solute concentrations, it should be noted that the water contents of cells of either line transferred to medium of low water potential are sharply reduced as compared with control cells (Fig. 18). This is due to water loss to the medium, and will result in increased solute concentrations even in the absence of any absolute rise in solute content. For example, the water content of unadapted cells transferred to 25% PEG falls by 54% (Fig. 18); concentrations of all solutes will thus double without any net accumulation. This point has been considered by some investigators, (Turner and Stewart, 1988) and should be noted, particularly when concentration changes of the order of 1-2 x are considered.

The accumulation of solutes has been widely reported under stress conditions (Hanson and Hitz, 1982; Stewart and Hanson, 1980) with polyamine accumulation in particular receiving increased attention in the last decade (Smith, 1984). Large increases in polyamines (50- 60-fold) have been reported in detatched oat leaves and protoplasts after 6 h exposure to osmotic stress (Flores and Galston, 1982). However, in similar experiments with barley leaf sections Turner and Stewart (1988) found only a 3- 4-fold increase in putrescine levels over intact leaf controls, and suggested that the massive increases reported by Flores and Galston (1982; 1984) were artefactual and due in part to low control values.

In this study, no increase in total polyamines and only a small increase in putrescine, over that dictated by concentration due to water loss, was detected. This may be due to the high levels of putrescine found in control cultures, which has been reported previously (see above). It may be that putrescine accumulation is saturated at the control values of

approximately 1.3 mM , but in any case no significant increase in putrescine was detected either in adapted or stress-shocked cells. The minor component spermine increased 4- 5-fold in concentration, suggesting rather more than a doubling in net content. Spermine has not been previously identified as a stress-indicator.

Large increases in proline concentration were observed. Relative to control values, proline in cells exposed to 25% PEG increased by 12-fold in stress-shock conditions and 40-fold in adapted cells, to a maximum of 23 mM . Handa et al (1983) reported a 100-fold increase in proline in tomato cells adapted to 25% PEG up to a similar maximum concentration. Comparable increases in proline content have been found in drought-stressed barley (Hanson and Nelsen, 1978) and sorghum (Stewart and Hanson, 1980; Blum and Ebercon, 1976), and thus the response of carrot cells is in line with that widely reported for both cell and whole-plant systems subjected to water-stress.

A significant difference in the degree of response to adaptation and stress-shock was observed; proline accumulation apparently continues to increase in adapted cells to substantially higher levels than produced by a 10 h stress-shock period. In salt-stressed barley leaves, proline accumulation increased over a 16 h period (Voetberg and Stewart, 1984) but did not increase further with osmotic adjustment. Handa et al (1983) have discussed the possibility that proline is derived from glutamate under stress conditions. These observations are similar to theirs, in that glutamate levels decline somewhat in low water potential medium when water-loss is taken into account, but that the observed values in control cells are not high enough to account for the increase in proline.

The varied response of the individual non-proline amino acids in adapted cells supports speculation (Handa et al, 1983) that water-stress adaptation involves changes in rates of assimilation, synthesis, utilisation and interconversion of amino acids; certainly no common mechanism can be evoked. While proline is clearly unique in the extent of its response, it may be noted that the increase in osmotic contribution due to non-proline amino acids is nearly twice that of proline itself. In stress-shocked cells on the other hand, the concentration of non-proline amino acids only increases by 70%. This means that the net content falls, when concentration effects are taken into account. Adaptation and stress-shock processes do not therefore seem closely related for these amino acids.

Reducing sugars, particularly glucose, together with sucrose, provide the major contribution to solute accumulation in adapted cell lines, in agreement with findings in stressed tomato cells by Handa et al (1983), who suggest that such increases might be due to a reduction in cell wall synthesis mediated by reduced expansion. It should be noted, however, that the culture medium initially contains 78 mM sucrose, and that high levels of sugars may well be due to uptake and catabolism from this source; in sorghum and sunflower leaves, levels of sucrose and reducing sugars were approximately an order of magnitude lower than in the present study (Jones et al, 1980). It would be of interest to investigate sucrose uptake rates to clarify this point.

This study reveals substantial differences in cellular responses to stress-shock as compared with long term adaptation to water-stress. When concentration effects are discounted, stress-shocked cells show major

increases in proline, and to a lesser extent reducing sugars. Long-term adaptation is accompanied by enhanced levels of these solutes as well as sucrose, and other amino acids. Whether this is solely a function of the time of exposure to water-stress, or whether qualitatively different mechanisms are involved is not clear.

Adaptation involves a substantial reduction in cellular water potential allowing turgor to be maintained. The organic solutes investigated here account for about 70% of the osmotic potential change observed between control and adapted cells. The nature of the remaining 30% must be speculative, although inorganic solutes (mostly as potassium ions) made up 20% of the osmotic potential in adapted tomato cells (Handa et al, 1983). Part of the short-fall might be due to underestimation of the cell osmotic potential by mixing of extracellular water with cellular contents during osmometer measurements. In a major study with adapted tomato cells, Handa et al (1983) were able to account for only 65% of the observed osmotic potential, including inorganic solutes, although others (Jones et al, 1980; Meyer and Boyer, 1981) accounted for close to 100% in whole plant organs, with the major contribution from sugars and amino acids.

Other stress conditions

5.3 Anoxia

Introduction

Under some circumstances plants and plant cells may be subject to an environment in which there is low partial pressure of oxygen. This may be due to such factors as reduced oxygen uptake and excessive respiration. In the case of cultured cells such conditions may occur due to insufficient aeration coupled with a culture with an oxygen demand too great to be met by media oxygen levels. It would appear possible that amines, like ethylene can accumulate under anoxic conditions in some systems since their metabolisms are related, and since the well-characterised carrot cell culture system is ideal for determining the consequences of changes in the extracellular environment, an experiment was developed to assess the effects on cellular amine concentrations of decreased media oxygen.

Results and Discussion

If the levels of amines in anoxic and control cultures are compared then it is clear that incubation for this period in anoxic conditions has no significant effect on amine accumulation (Table 2). Since anoxic conditions if prolonged would lead to necrosis it is impossible to determine whether amine accumulation might take place in the longer term. Indeed since amine accumulation is a characteristic of necrotic tissue, and the lack of elevated amine levels after 8 h incubation suggests that cell death is not at an advanced stage at this time. In addition the

similar amine levels in control and anoxic cultures indicates that lack of oxygen alone has no major short-term effects on amine metabolism.

5.4 Heat stress

Introduction

Under natural conditions plants are faced with large diurnal changes in temperature, with day temperatures far above night temperatures especially in arid regions. Responses to such extremes of temperature may involve changes in transpiration rate and foliage attitude. Metabolic changes may also occur, with the balance of cell solutes altering during temperature variations. Some solutes may accumulate, among them the amines, and it has been suggested that elevated amine concentrations may serve to stabilise heat labile molecules within the cell (Slocum et al, 1984). To establish whether amines accumulate during periods of high temperature, cells were incubated after subculture as previously described in an incubator at 40°C. After 8 h the cells were harvested and amine concentrations determined.

Results and Discussion

Table 2 shows the effects of 8 h high temperature on amine concentrations. No alteration in levels is apparent for any of the amines studied.

This lack of response does not however indicate that polyamines do not have a role in the response of plants to heat stress. Both the short period of exposure to heat and the temperature used may be important in this respect. Once again it should be noted that short-term studies such as this do not necessarily provide a guide to any long term changes which may take place as a result of progressive adaptations to the stress

stimulus. Oshima (1983) found a number of unique tetraamines and pentaamines in thermophilic bacteria which protected in vitro protein synthesis from the effects of heat while Galston and Kaur-Sawhney (1982) found no relationship between polyamine content and temperature stress. Though this study does not indicate that a relationship between the two exists in carrot cells, further investigation of amine synthesis under thermal stress conditions might prove interesting.

CHAPTER SIX

DISCUSSION

In a recent review, Evans and Malmberg (1989) have suggested that the polyamines have not fulfilled their early promise, as important mediators of growth, development, and stress metabolism in plant cells. The purpose of this study has been to assess their role in the growth of higher plants, and also in their response to various stress factors in their culture environment. These two aspects, growth and stress, have been addressed separately within this report, but they are closely linked since stress stimuli have an effect on growth. Also, the state of growth affects the response to stress. This distinction may thus be somewhat artificial. However, a discrete treatment of these two phases of the investigation was preferable at the results stage in terms of clarity and readability. They will now be discussed together to give a better description of how polyamine metabolism changes during growth, under various regimes.

The possible involvement of polyamines as regulating compounds in culture growth was the initial subject of interest. Subsequent investigation immediately revealed a striking lack of correlation, between levels of the major amines putrescine and spermidine, and the level of growth activity. Inevitably, the absence of a clear relationship between the two was insufficient evidence to rule out the involvement of polyamines in cellular growth. However, it did show that elevated levels during active cell growth are inessential. A less direct link remains a possibility. Further data was provided by a study of polyamine biosynthetic and catabolic enzymes. Their activities were manipulated using specific inhibitors, and accompanying measurements showed the pattern of polyamine accumulation over the culture period.

In the first period, (inoculation to 6 h after subculture), polyamine concentrations were at stationary phase levels. With high cell density and depletion in the original medium, and nutrient and waste product levels markedly different from those of the original medium medium, cell metabolism was strongly affected. The strong increase in amine accumulation detected could be accounted for by increased putrescine biosynthesis via ADC, which doubled in activity, and similarly increased decarboxylation of ornithine. A similar report (Montague, 1979), characterised the changes during this period as the 'fresh medium effect'.

The fresh medium effect is not only restricted to synthetic activity, since the levels of the catabolic enzyme DAO rose significantly at the same time. This indicated that polyamine turnover was becoming more rapid. There is little comparable work involving DAO in the literature. One may only speculate on the reasons for such an increase in turnover. Perhaps a requirement for polyamine breakdown products exists. Perhaps there is a need to maintain polyamine levels within closely controlled levels, through active oxidation of excess molecules. Possibly there is a requirement for freshly synthesised polyamine molecules. Or, perhaps there is a need for the rapid conversion of putrescine to spermidine and spermine, to prevent toxic amine accumulations. Without further experimentation, these possibilities cannot be properly assessed; the stimulus which leads to the raised activity of these enzymes remains unclear.

In the next apparent stage in the pattern of polyamine metabolic activity - between 6 and 12 h after inoculation - the continued polyamine accumulation could not be accounted for by continuing synthetic activity, since biosynthetic enzyme activities fell at this time, and the rise in polyamine concentration is more likely to result from the drop in the rate of oxidation and the release of free-amines from bound forms.

In the next period considered from 12 to 24 h after inoculation continued accumulation of spermidine and spermine could be accounted for by increased synthesis via SAMDC though the accumulation of putrescine was more problematic. A likely explanation is the conversion of conjugated to free-putrescine which may be detected during analysis. It is known that polyamines are present in plants in both free and bound forms (Smith, 1981; Smith et al, 1983) and putrescine conjugates have been shown to form 64 to 86% and spermidine conjugates 8 to 20% of total polyamines in tobacco callus (Tiburcio et al, 1986), and so a large sink of undetected polyamines may be present, permitting the fluctuation of free-polyamine levels without the modulation of biosynthesis or breakdown. During this period, then, falling putrescine biosynthesis and rapid putrescine conversion to spermidine and spermine may be counterbalanced by the release of putrescine from bound forms. It would be interesting to determine the level of polyamine conjugates within suspension cultured carrot cells as has been accomplished in tobacco (see above) and to monitor the balance of free:conjugated forms during growth to determine whether the concentration of conjugated putrescine declines after 12 h in culture. If a large proportion of the polyamines within the cell exist as conjugates, then measurement of free-polyamine levels would provide only a partial picture of total polyamine accumulation during growth. If this

were the case then conjugated polyamine measurements and rates of binding and release should be carried out to support determinations of free-polyamine content and synthesis.

During the next 24 h of culture, levels of putrescine and spermine corresponded to levels of activity of their biosynthetic enzymes. In the case of spermidine, however, the situation is less clear. Rapid increase in SAMDC activity, is accompanied by no significant change in free-spermidine levels. This indicates that a sink of conjugated amines may be present. Excess free-spermidine may be actively converted to bound forms over this period, and a small part of this free-spermidine may be converted to spermine, via enhanced spermine synthase activity. On the other hand, the increase in SAMDC activity, and the consequent increase in spermidine synthesis may not result in increased spermidine concentrations. The spermidine thus produced may be rapidly broken down by the oxidation activity of DAO. DAO levels have been shown to be far greater than levels of the main polyamine biosynthetic enzymes. A situation might thus occur of rapid spermidine flux unaccompanied by significant spermidine accumulation. Thus, observed changes in free-amine concentrations may not always be directly related to biosynthetic activities alone. The pattern of change is more likely to be understood in terms of a mixture of biosynthesis, conjugation, breakdown and interconversion of polyamines. A determination of conversion rates to and from bound forms of these amines would provide a valuable addition to the present study. These would suggest at which points in the growth period, and under what conditions, any changes in the rates of interconversion between bound and free forms of these polyamines occur.

The results of the inhibitor studies correlate well with the enzyme studies, and with polyamine accumulation data during normal growth. However, the lack of a growth inhibitory effect accompanying polyamine biosynthetic inhibitor feeding, is not conclusive proof of a lack of polyamine involvement. A valuable addition to this part of the study would be a simultaneous set of polyamine determinations during the inhibitor studies. These would be particularly interesting, if taken during the initial phase of growth, since there is no indication of the speed with which polyamine biosynthetic activity is affected by the inhibitor. It may be the case that initial rapid amine accumulation is necessary to trigger growth. This initial burst of polyamine biosynthesis and accumulation may be unaffected by inhibitor feeding from the time of subculture. This could be the case either if the uptake of the inhibitors was slow, or if their action on enzyme activity was not sufficiently rapid. Again, a clearer picture might emerge with a more detailed investigation. The results of studies involving the feeding of polyamine precursors support the conclusions above. They emphasise the general impression of a polyamine metabolism with little connection to any mechanism of growth regulation.

One may contrast the identification of polyamines as a new class of growth regulators by some authors (Galston and Kaur-Sawhney, 1982), with both the conclusion by other authors that much of the evidence on which such hypotheses are based is inconclusive (Phillips et al, 1987), and the results of the present investigation . It is clear that despite an increasing number of studies on this question, no consensus has been reached. This differs from the situation in animal cells and bacteria, where convincing conclusions may be drawn (Tabor and Tabor, 1984).

So far, the present results have been considered solely in terms of their relation to the growth status of the suspension, and consequent growth activity. It may be useful in addition to consider the relationship of polyamine metabolism, to the stress status of the carrot cells, since this will also vary during subculture. Both the chemical balance of the fresh medium and its osmotic potential will be significantly different from the chemistry and osmolarity of the spent medium, in which the stationary phase cells are present, prior to subculture. The fresh medium will be higher in sucrose, hormones and nitrogen than the spent medium, and lower in amino acids and CO_2. Thus, the stationary phase cells will be subjected to both osmotic stress on subculture, and also the stress of adjustment to a medium with an unfamiliar balance of constituents.

The initial pattern of polyamine biosynthesis and accumulation may now be considered in relation to the cellular environment. The initial burst of polyamine biosynthetic activity, and the post-subculture accumulation of putrescine and spermidine, might be a response to such stress as well as growth potentiating phenomena. The decline in primary biosynthesis, which occurs after this initial burst of activity, may be connected to any adjustment to the fresh medium which is taking place at this point. If polyamines were implicated only in the initial response to stress, polyamine biosynthesis might decline, and accumulation of amines might be reversed. Also during active growth and cell expansion, the cell must maintain an intracellular osmotic equilibrium and adjust to the stress which this process involves. It is evident that growth and cellular stress are intimately related, and may be considered in conjunction.

Studies of polyamine biosynthesis and growth were followed by detailed investigations of the relationship between polyamines and stress. Cells are exposed to stress on transfer to fresh medium, and accumulate polyamines thereafter (a 'fresh medium effect' which does not occur when cells are subcultured into spent medium (Montague et al, 1979)). However, a clearer picture of the importance of the polyamines in plant stress physiology, was given by detailed investigation of the effects of different levels of stress on amine accumulation. Once again growth was an important parameter in the response to stress, with a clear negative correlation between growth activity and level of stress, most clearly in cells exposed to various levels of water stress via PEG. In spite of the large peturbations in growth, however, amine levels were largely unaffected, and polyamines rose in concentration only as a result of water-loss effects. The PEG-stress investigation thus provided a system in which cells could be subjected to both significant growth changes, and to high levels of osmotic stress, while free-amine determinations show no significant accumulation of polyamines.

The large increase in the stress indicator proline, and in levels of some amino acids, suggests cell metabolism is stress-affected. These data form a valuable reference, to which the polyamine changes may be compared.

The investigation of amine metabolism under PEG-stress formed part of an extensive study of free-amine accumulation under a wide range of stress conditions. All of these forms of stress, excepting anoxia, had been previously identified as modulators of free-amine accumulation. Yet, in every case, a study of carrot suspensions under carefully controlled and thoroughly replicated conditions, revealed no increase in polyamine

levels in response to stress. Indeed, in every case where some increase in free-amine level was detected, this could either be ascribed to water-loss effects, or shown to be statistically insignificant. With such data then, the question of the basis for suggestions of amine involvement in stress-adaptation (Flores and Galston, 1984), requires careful consideration. The importance of water-loss, and inappropriate controls in the production of artefactual data, has already been mentioned (Stewart and Turner, 1988), as has the unspecific effects on cell metabolism caused by penetration of solutes through the cell wall (Kandpal and Rao, 1985). It is clear therefore that difficulties exist in the interpretation of such data, and this in turn suggests that a highly-controlled system would be required for meaningful conclusions to be reached. The study of PEG-induced water stress carried out in the present study is not ideal in this respect. The water stress is not imposed by lack of environmental water, but through the action of an osmoticum in preventing uptake. The behaviour of the cells in vitro, while it cannot necessarily be taken to be that of the whole plant tisues under similar conditions, does provide some valuable information on cellular responses to water stress.

The study has shown, in particular, that water stress does have profound effects on cell metabolism. The cell undergoes a process of adaptation to the stimulus, with some metabolites present at very different concentrations in adapted, as opposed to unadapted cells. The range of stress-related changes found may be due to the modification of normal membrane functions, according to some authors (Altman et al, 1982), with the location of the membranes governing the type of response. Reports of the modulation of such responses by amines, led to the hypothesis that they might increase membrane stability. Stress

conditions also lead to a deterioration in protein and nucleic acid synthesis, and reports of amines binding to acidic areas of membrane phospholipids and nucleic acids, have prompted suggestions that amines may have a stabilising role. Such reports have been strengthened by results indicating that spermidine may promote DNA replication (Morris and Lockshon, 1981). Potential modulating qualities such as these are important, if amines are to be shown to have a role in the overall control of metabolic responses to water deficits. This control is probably achieved through changes in fluxes and concentrations, of substrates and regulatory substances, within and between cells, tissues and organs (Hanson, 1982).

It is worth noting that only a small number of regulating metabolic pathways, modulated by a form of stress, may provoke changes through a large part of general metabolism, producing changes in metabolic areas not in themselves directly affected by such forms of stress. This present study provides some indications of where stress adaptation takes place, such as in sugar metabolism, with the strong accumulation of simple sugars. Little indication exists that amine metabolism may be similarly involved. Despite these findings, however, other studies indicating that amines can promote physiological responses similar to auxin, coupled with the universal occurence of putrescine, spermidine and spermine in higher plants, and their association with changes in growth rates, suggests further research should be undertaken, to explore their role more fully.

In conclusion, the main achievements of this study may be described as follows:

The growth pattern of the carrot suspension has been characterised, and a

system developed which is suitable for a systematic investigation of cell physiology. This growth pattern was thus available for comparison with the pattern of amine biosynthesis, accumulation and breakdown over the culture period. This provided insights into the behaviour of amine metabolism, during the different phases of culture growth. This has in turn been supplemented with a range of inhibitor studies, which have established the efficacy of the inhibitory compounds in relation to higher plant cell metabolism. They have also demonstrated that amine metabolism may be severely perturbed during the culture growth cycle, without any diminution of growth being detected. This, while not confirming that amines are unnecessary in terms of culture growth, has confirmed that amine synthesis is not necessary during the process of active growth. It may, however, be of importance prior to increased growth activity. Also, polyamines may be active as hormonal second messengers (Galston, 1983). They may be involved through pools of S-adenosylmethionine in ethylene biosynthesis (Evans and Malmberg, 1989) and thus through ethylene modulate development and growth. Their function may indeed be expressed through the triggering of biochemical pathways, and the catalysis of reactions in cellular metabolism. Moreover, the large flucuations in amine synthesis and accumulation during the lag phase immediately after subculture, have raised interesting questions concerning the importance of amine synthesis, during acclimation to the plant cell environment. Evidently, both stress and growth components, are involved in the behaviour of the plant cell during this period.

This stress component was investigated in further detail later in the study when a range of further stress conditions were imposed on the cell

culture. The close control of the cellular environment possible with the use of the suspension culture system provided convincing evidence that, while they led to changes in certain areas of metabolism in some cases, the forms of stress employed had no significant effects on amine accumulation. Also the controlled conditions employed allowed such data to be contrasted with some confidence with previous studies, in which large changes in amine levels were reported in response to similar stress conditions. Indeed, the role of amines in relation to stress was possible to define to some extent, within the general metabolic pattern of the cell culture during growth - an approach which had not previously been attempted. This, in turn, then allowed a more revealing analysis of the interaction of growth and stress responses in the carrot culture, and an insight into the intimate relationship between these two components. It is now clear that study of either growth or stress alone, within the cell culture system, will of necessity involve aspects of the other, which in turn will require consideration, to produce a meaningful set of conclusions. This leads to consideration of the approaches which might be adopted in future studies, in order to further clarify the position of amines within the plant growth/stress framework:

It is evident that the carrot suspension culture system would be a powerful tool to be employed in any future investigation. In addition, a further use of amine biosynthesis inhibitors would be likely to yield still more useful data, on the impact of an artificial modulation of amine metabolism. EMGBG, in particular, might enjoy wider use in metabolic studies, with its potency and specificity - qualities which have led to the widespread use of DFMO and DFMA. The growth of cultures for several passages in DFMA, should also yield interesting data, since the initial

amine synthesis 'burst' after subculture should be suppressed with such treatment. If this phenomenon is indeed stress-related (see above), then such an investigation might shed light on the position of amines, in relation to the stress-response of plant cells. Such a treatment, coupled with regular determinations of the activity of synthetic and catabolic enzymes, might help to provide a more complete picture than inhibitor studies alone.

In terms of the growth cycle of the carrot suspension, the initial few hours after subculture appear to form the most interesting period, in terms of rapid change in amine metabolism. In terms of stress responses, this study has only considered the adaptive response in one case - that of water-stress induced by PEG. It would be interesting to follow the effects, on general and amine metabolism, of adaptation to other forms of stress, such as heat, low pH, low potassium, low nitrate to ammonium ion ratio, anoxia etc. These would also provide data on amine metabolism, during the periods of reduced growth activity produced by such treatment. In addition, it would be useful to extend the scope of the PEG-adaptation study, to include regular amine determinations throughout the growth cycle, in both adapted and unadapted cells. This might determine whether there were specific phases of the amine metabolic pattern affected by stress, during the growth period. Lastly, a thorough investigation into the levels of conjugated amines in carrot culture could be carried out, using acid hydrolysis to release bound amines for detection. If significant levels were found, then this method could be incorporated into routine amine analyses, for a clearer representation of true amine accumulation.

The conclusions of Evans and Malmberg (1989), regarding the lack of evidence for an important role for the polyamines in relation to growth and stress is thus strongly supported by these studies, which also satisfy the majority of their recommendations for standards in polyamine investigations.

APPENDIX

BIBLIOGRAPHY

AHMAD N., WYN JONES R.G, 1979. Glycinebetaine, proline and inorganic ion levels in barley seedlings following transient stress. Plant Sci Lett 15, 231-237

ALTMAN A, 1980. Inhibition of senescence of leaves and other plant organs by polyamines: possible modes of action. Israel J Bot 28, 55

ALTMAN A, FRIEDMAN R, AMIR D, LEVIN N, 1982. Polyamine effects and metabolism in plants under stress conditions In: Plant Growth Substances-1982. Wareing PF Ed. pp483-494

ALTMAN A, KAUR-SAWHNEY R, GALSTON AW, 1977. Stabilization of oat leaf protoplasts through polyamine mediated inhibition of senesence. Plant Physiol 60, 570-574

ANDERSON JN, MARTIN RO, 1973. Identification of cadaverine in Pisum sativum. Phytochem 12, 443-446

ANGUILLESI MC, FLORIS C, GRILLI I, MELETTI P, 1978. Metabolism in wheat seed dormancy: Polyamine content during early germination. Biochem Physiol Pflanzen 173, 340-346

APELBAUM A, YANG SF, 1981. Biosynthesis of stress ethylene induced by water deficit. Plant Physiol 68, 594-596

ARNOTT SM, 1982. PhD. Univ of London.

ASHOUR NI, THALOUTH AT, 1971. Effect of putrescine on growth and photosynthetic pigments of broad bean plants grown under chloride salinization conditions. Biochem Physiol Pflanz 162, 203-208

AUDISIO S, BAGNI N, SERAFINI-FRACASSINI D, 1976. Polyamines during growth in vitro of Nicotiana glauca R.Grah habituated tissue. Z.Pflanzenphysiol 77 146-151

BACHRACH U 1973 The function of naturally occuring polyamines. Ac Press, New York.

APELBAUM A, BURGOON AC, ANDERSON JD, LIEBERMAN M 1981 Plant Physiol **68**, 453

BAGNI N, CALDARERA CM, MORUZZI G 1967 Experientia **23**, 139-140

BAGNI N, 1966. Aliphatic amines and a growth factor of coconut as stimulating cellular proliferation of Helianthus tuberosus (Jerusalem artichoke) in vitro. Experientia 22, 732-733

BAGNI N, 1970. Metabolic changes of polyamines during the germination of Phaseolus New Phytol 69, 159-164

BAGNI N, 1983. Biosynthetic enzymes related to polyamines and their inhibition in Helianthus tuber. Giorn Bot Ital 117, 61-62

BAGNI N, CALDARERA CM, MORUZZI G, 1973. Spermine and spermidine distribution during wheat growth. Experientia 23, 139-140

BAGNI N, SERAFINI-FRACASSINI D, 1974. The role of polyamines as growth factors in higher plants and their mechanism of action. In: Plant Growth Substances-1973 Proc 8th Inter Conf Plant Gth Subs p1205-1217. Hirosawa Pub Co, Tokyo

BAGNI N, SERAFINI-FRACASSINI D, CORSINI E 1972 Tumours of Scorzonera hispanica: their content in polyamines. Z.Pflanzenphysiol 67:19-23

BAGNI N, MALUCELLI C, TORRIGIANI P, 1980. Polyamines, storage substances and abscisic acid-like inhibitors during dormancy and very early activation of Helianthus tuberosus tuber tissues. Physiol Plant 49, 341-345

BAGNI N, SERAFINI-FRACASSINI D, TORRIGIANI P, 1982. Polyamines and cellular growth processes in higher plants. In PF Wareing, ed, Plant growth substances. Ac. Press, London pp 473-482

BAGNI N, BARBIERI P, TORRIGIANI P, 1983. Polyamine titer and biosynthetic enzymes during tuber formation of Helianthus tuberosus J Plant Gth Reg 2, 177-184

BARBIERI P, TORRIGIANI P, BAGNI N, 1983. Biosynthetic enzymes related to polyamines and their inhibition in Helianthus tuber. Giorn Bot Ital 117,

61-62

BAXTER C, COSCIA CJ, 1973. In vitro synthesis of spermidine in the higher plant. Biochem Biophys Res Commun 54, 147-154

BEHE M, FELSENFELD G, 1981. Effects of methylation on a synthetic polynucleotide: The B-Z transition in poly(dG-m5dC).poly(dG-m5dC). Proc Natl Acad Sci USA 78, 1619-1623

BEN-HAYYIM G, 1986. The relationship between salt tolerance and resistance to polyethylene glycol induced water stress in cultured citrus cells. Plant physiol 85, 430-433

BERLIN J, FORCHE E, 1981. DL-a-difluoromethylornithine causes enlargement of cultured tobacco cells. Z.Pflanzenphysiol 101, 277-282

BERTOSSI F, BAGNI N, MORUZZI G, CALDARERA CM, 1965. Spermine as a new growth promoting substance for Helianthus tuberosus (Jerusalem artichoke) in vitro Experientia 21 80-81

BHASKARAN S., SMITH R.H., NEWTON R.J, 1985. Physiological changes in cultured sorghum cells in response to induced water stress. Plant physiol 79, 266-269

BLUM A, EBERCON A, 1976. Genotypic responses in sorghum to drought stress III. Free-proline accumulation and drought resistance. Crop Sci 16, 428-431

BRESSAN R.A., HASEGAWA P.M., HANDA A.K, 1981. Resistance of cultured higher plant cells to polyethylene glycol-induced water stress. Plant Sci Lett 21, 23-30

BROWN R, RICKLESS P, 1949. A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and nutrients. Pro Roy Soc Ser B 136, 110-125

CASTELLI A, ROSSONI C, 1968. Polyamines and nucleic acids in the growing yeast. Experientia 24, 1119-1120

BIRECKA H, BITONTI AJ, Mc CANN PP 1985a Assaying ornithine and arginine decarboxylases in some plant species. Plant Physiol **79,** 509-514

BIRECKA H, BITONTI AJ, MC CANN PP 1985b Activities of arginine and ornithine decarboxylases in various plant species. Plant Physiol 79, 515-519

CHAUDHURI MM, GHOSH B, 1984. Purification and characterisation of diamino oxidase from rice embryos. Phytochem 23, 241-243

CHIU JF, SUNG SC, 1970. DNA nucleotidyltransferase activity of the developing rat brain. Biochem Biophys Acta 209, 34-42

CHO SC, 1983. Enhancement by putrescine of giberellin-induced elongation in hypocotyls of lettuce seedlings. Plant Cell Physiol 24, 305-308

CHRIQUI D, D'ORAZI D, BAGNI N, 1986. Ornithine decarboxylase and arginine decarboxylase and polyamine involvement during in vivo differentiation and in vitro dedifferentiation of Datura inoxia explants. Physiol Plant 68, 589-596

COHEN SS, 1971. Introduction to the polyamines. Prentice-Hall, Englewood Cliffs, New Jersey

COHEN AS, POPOVIC RB, ZALIC S, 1979. Effect of polyamines on chlorophyll and protein content, phytochemical activity and chloroplast ultrastructure of barley leaf discs during senescence. Plant Physiol 64, 717-720

COHEN SS, BALINT R, SINDHU RK, 1981. The synthesis of polyamines from methionine in intact and disrupted leaf protoplasts of virus-infected chinese cabbage. Plant Physiol 68, 1150-1155

COHEN E, MALIS ARAD S, HEIMER YM, MIZRAHI Y, 1982. Participation of ornithine decarboxylase in early stages of tomato fruit development. Plant Physiol 70, 540-543

COHEN SS, GREENBERG ML, 1981. Spermidine, an intrinsic component of turnip yellow mosaic virus. Proc Natl Acad Sci USA 78, 5470-5474

COHEN E, ARAD SM, HEIMER YH, MIZRAHI Y, 1984. Polyamine biosynthetic enzymes in the cell cycle of Chlorella. Plant Physiol 74, 385-388

COHEN AS, ZALIK S, 1978. Magnesium replacement by polyamines in higher plant cell-free polyphenylalanine synthesis. Phytochem 17, 113-118

COSTA G, BAGNI N, 1983. Effect of polyamines on fruit set of apple. Hort Sci 18, 59-61

COSTA G, BARALDI R, BAGNI N, 1984. Influence of putrescine on fruit set of apple (cv. Ruby Spur) Acta Hortic 149, 189-195

DUMORTIER FM, GALSTON AW, 1984. The effect of osmotic stress on polyamine biosynthesis as related to protoplast viability. Fed Eur Soc Plant Physiol (abstr) 1984 meeting. Strasbourg, France.

ELO H, MUTIKAINEN I, ALHONEN-HONGISTO L, LAINE R, JÄNNE J, PAAVO L, 1986. Biochemical and crystal structure of ethylmethylglyoxal Bis (guanylhydrazone) Sulfate - an extremely powerful novel inhibitor of adenosylmethionine decarboxylase. Z Naturforsch 41c, 851-855

EVANS PT, MAMBERG RT, 1989. Do polyamines have roles in plant development? Ann Rev Plant Physiol Plant Mol Biol 40, 235-269

FEDERICO R, ANGELINI R, 1986. Occurence of diamino oxidase in the apoplasts of pea epicotyls. Planta 167, 300-302

FEIRER RP, MIGNON G, LITVAY JD, 1984. Arginine decarboxylase and polyamines required for embryogenesis in the wild carrot. Science 223, 1433-1435

FIENBERG AA, CHOI JH, LUBICH WP, SUNG ZR, 1984. Developmental regulation of polyamine metabolism in growth and differentiation of carrot culture. Planta 162, 532-539

FILLINGAME RH, JORSTAD CM, MORRIS DR, 1975. Increased cellular levels of spermidine or spermine are required for optimal DNA synthesis in lymphocytes activated by concanavalin A. Proc Natl Acad Sci USA 72, 4042-4045

FLORES HE, GALSTON AW, 1982a. Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol 69, 701-706

FLORES HE. GALSTON AW, 1982b. Polyamines and plant stress: Activation of putrescine biosynthesis by osmotic shock. Science **217**, 1259-1261

FELIX H, HARR J 1987 Association of polyamines to different parts of various plant species. Physiol Plant **71**, 245-250

FLORES H.E., GALSTON A.W, 1984. Osmotic stress induced polyamine accumulation in cereal leaves I. Physiological parameters of the response. Plant physiol 75, 102-109

FLORIS G, GIARTOSIO A, RINALDI A, 1983. Diamino oxidase from Lens esculentia seedlings: purification and properties. Phytochem 22, 1871-1874

FOLK JE, 1980. Transglutaminases. Ann Rev Biochem 49, 517-531

FOZARD JR, PART ML, PRAKASH MN, GROVE J, SCHECHTER PJ, SJOERDSMA A, KOCH-WESER J, 1980. L-ornithine decarboxylase: An essential role in early mammalian embryogenesis. Science 208, 505-509

GALSKY A, KULPA J, 1983. Spermidine and plant tumour growth. Plant Physiol 72(suppl), 160(Abstr 909)

GALSTON AW, 1983. Polyamines as modulators of plant development. Bioscience 33, 382-388

GALSTON AW, ALTMAN A, KAUR-SAWHNEY R, 1978. Polyamines, ribonuclease and the improvement of oat leaf protoplasts. Plant Sci Lett 11, 69-79

GALSTON AW, KAUR-SAWHNEY R, ALTMAN A, FLORES M, 1982. In: Adv Prot Res, Proc 5th Inter Prot Symp p485. Akademiai Kiado, Budapest

GALSTON AW, KAUR-SAWHNEY R, 1982. Polyamines - are they a new class of plant growth regulators? In: Wareing PF Ed. Plant growth substances-1982 pp 451-461. Ac Press, NY

GALSKY A, KULPA J, 1983. Spermidine and plant tumour growth. Plant Physiol 72(suppl), 160(abstr 909)

GEIGER LE, MORRIS DR, 1978. Polyamine deficiency reduces the rate of DNA replication fork movement in Escherichia coli. Nature(Lon) 272, 730-732

HANDA S, BRESSAN RA, HANDA AK, CARPITA NC, HASEGAWA PM, 1983. Solutes contributing to osmotic adjustment in cultured plant cells adapted to water stress. Plant Physiol **73**, 834-843

GALSTON AW, KAUR-SAWHNEY R, ALTMAN A, FLORES M 1979. In: Advances in Prot Res. Proc 5th Int Prot Symp p485. Acad Kiado, Budapest

HANDA S, HANDA AK, HASEGAWA PM, BRESSAN RA, 1986. Proline accumulation and the adaptation of cultured plant cells to water stress. Plant Physiol 80, 938-945

HANSON AD, 1982. Metabolic responses of mesophytes to plant water deficits. Ann Rev Plant Physiol 33, 163-203

HANSON AD, HITZ WD, 1982. Metabolic responses of mesophytes to plant water deficits. Annu Rev Plant Physiol 33, 163-203

HANSON AD, NELSEN CE, 1978. Betaine accumulation and formate metabolism in water stressed barley leaves. Plant physiol 62, 305-312

HEBY O, 1981. Role of polyamines in the control of cell proliferation and differentiation. Differentiation 19, 1-20

HEIMER YM, MIZRAHI Y, BACHRACH U, 1979. Ornithine decarboxylase activity in rapidly proliferating plant cells. FEBS Lett 104, 146-148

HERBST EJ, SNELL EE, 1948. Putrescine as a growth factor for Haemophilus influenzae. J Biol Chem 176, 986-990

HEYSER JW, NABORS MW, 1979. Osmotic adjustment of tobacco cells and plants to penetrating and non-penetrating solutes. Plant Physiol suppl 63, 77

HIRASAWA E, SUZUKI Y, 1983. Biosynthesis of spermidine in maize seedlings. Phytochem 22, 103-106

HOLLIGAN PM, 1971. Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. New Phytol, 70, 239-269

HUHTINEN O, HONKANEN J, SIMOLA LK, 1982. Ornithine and putrescine supported divisions and cell colony formation in leaf protoplasts of alders (Alnus glutinosa and Alnus incana) Plant Sci Lett 28, 3-9

HUNG DT, MORTON LJ, DEEN DF, SCHAFER RH, 1983. Depletion of intracellular polyamines may alter DNA conformation in 9L rat brain tumour cells.

Science 221, 368-370

JARRETT H.W., COOKSY K.D., ELLIS B., ANDERSON J.M, 1986. The separation of o-phthalaldehyde derivatives of amino acids by reversed-phase chromatography on octylsilica columns. Anal Biochem 153, 189-198

JONES MM, OSMOND CB, TURNER NC, 1980. Accumulation of solutes in leaves of sorghum and sunflower in response to water deficits. Aust J Plant Physiol 7, 193-205

KALLIO A, McCANN PP, BEY P, 1981. DL-alpha-(difluoromethyl)arginine-a potent enzyme activated irreversible inhibitor of bacterial arginine decarboxylase. Biochem 20, 3163-3166

KANDPAL RP, RAO NA, 1985. Changes in levels of polyamines in Ragi seedlings during water stress. Biochem Int 11, 365-370

KATOH Y, HASEGAWA T, SUZUKI T, FUJII T, 1985. Changes in amounts of putrescine, spermidine and spermine in hiproly barley callus after auxin withdrawal. Agric Biol Chem 49, 1027-1032

KAUR-SAWHNEY R, ALTMAN A, GALSTON AW, 1978. Dual mechanisms in polyamine mediated control of ribonuclease activity in oat leaf protoplasts. Plant Physiol 62, 158-160

KAUR-SAWHNEY R, FLORES HE, GALSTON AW, 1980. Polyamine induction of polyamine synthesis and mitosis in oat leaf protoplasts. Plant Physiol 65, 368-371

KAUR-SAWHNEY R, GALSTON AW, 1979. Interaction of polyamines and light on biochemical processes involved in leaf senescence. Plant Cell Env 2, 189-196

KAUR-SAWHNEY R, SHIH LM, FLORES HE, GALSTON AW, 1982a. Relation of polyamine synthesis and titer to ageing and senescence in oat leaves. Plant Physiol 69, 405-410

KAUR-SAWHNEY R, SHIH LM, GALSTON AW, 1982b. Relation of polyamine

biosynthesis to the initiation of sprouting in potato tubers. Plant Physiol 69, 411-415

KAUR-SAWHNEY R, SHEKHAWAT NS, GALSTON AW, 1984. Induction of differentiation in tissue cultures by inhibition of polyamine biosynthesis. Plant Physiol 75(suppl)15 (abstr)79

KING PJ, 1984. Induction and maintenance of cell suspension cultures. In: Cell Culture and Somatic Cell Genetics of Plants Vol 1 pp 130-138. Ac Press, NY

KLEIN H, PRIEBE A, JAGER HJ, 1979. Putrescine and spermidine in peas: effect of nitrogen source and potassium supply. Physiol Plant 45, 497-499

KRAMER P.J, 1980. Drought stress and the origin of adaptations. In: Adaptation of plants to water and high temperature stress. (NC Turner and PJ Kramer Eds) pp 7-20. J Wiley and Sons, NY.

KUEHN GD, AFFOLTER HU, ATMAR VJ, SEEBECK T, GUBLER U, BRAUN R, 1979. Polyamine-mediated phosphorylation of a nucleolar protein from Physarum polycephalum that stimulates rRNA synthesis. Proc Natl Acad Sci USA 76, 2541-2545

KUEHN GD, ATMAR VJ, 1982. Post-translational control of ornithine decarboxylase by polyamine-dependent protein kinase. Fed Proc 41, 3078-3083

KUNG WS, WU PL, CROCOME OJ, SHARP WR, 1973. Ann J Bot 60(suppl), 8

KYRIAKIDIS DA, PANAGIOTIDIS CA, GERGATSOS JG, 1983. Ornithine decarboxylase (germinated barley seeds). In H Tabor, CW Tabor, eds, Methods in enzymology, Vol 94. Ac Press, NY, pp 162-166

LE RUDELIER D, GOAS G, 1975. Influence des ions ammonium et potassium sur l'accumulation de la putrescine chez les jeunes plantes de Soja hispida Moench. privées de leurs cotyledons. Physiol Veg 13, 125-136

LEVITT J, 1980. Responses of plants to environmental stresses, 2nd ed. Academic Press, NY.

LIQUORI AM, CONSTANTINO L, GRESCENZI V, ELIA A, GIGLIO E, PULITI R, DE SANTIS S, SAVINO M, VITAGLIANO V, 1967. Complexes between DNA and polyamines: a molecular model. J Mol Biol 24, 113-122

LOFTFIELD RB, EIGNER EA, PASTUSZYN A, 1981. The role of spermine in preventing misacylation by phenylalanyl tRNA synthetase. J Biol Chem 256, 6729-6735

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ, 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265-275

MALMBERG RL, 1983. Mutants of Nicotiana tabacum that alter polyamine synthesis. In: Current Topics in Plant Biochemistry ed. DD Randall Vol 2 pp211-221. Columbia Univ Press, Missouri

MALMBERG RL, Mc INDOO J, 1983. Abnormal floral development of a tobacco mutant with elevated polyamine levels. Nature 305, 623-625

MAMONT PS, BEY P, KOCHWESER J, 1980. Biochemical consequences of drug-induced polyamine deficiency in mammalian cells. In: Polyamines in Biomedical Res ed. Gangus JM Ch 10 pp 147-165. Wiley, UK.

McANULTY PA, WILLIAMS JPG, 1977. Polyamines and their biosynthetic decarboxylases in various tissues of the young rat during recovery from undernutrition. Biochem J 162, 109-121

McGOWAN RE, MUIR RM, 1971. Purification and properties of amine oxidase from epicotyls of Pisum sativum. Plant Physiol 47, 644-648

MENGOLI M, BAGNI N, BIONDI S, 1987. Effect of alpha-difluoromethylornithine on carrot cell cultures. J Plant Physiol 129, 479-485

METCALF BW, BEY P,DANZIN C, JUNG MJ, CASARA P, VEVERT JP, 1978. Catalytic irreversible inhibition of mammalian ornithine decarboxylase (EC

4.1.1.17) by substrate and product analogues. J Am Chem Soc 100, 2551-2553

MEXAL J, FISHER JT, OSTERYOUNG J, REID CPP, 1975. Oxygen availability in polyethylene glycol solutions and its implications in plant water relations. Plant physiol 55, 20-24

MEYER RF, BOYER JS, 1981. Osmoregulation, solute distribution and growth in soybean seedlings having low water potentials. Planta 151, 482-489

MONTAGUE MJ, ARMSTRONG TA, JAWORSKI EG, 1978. Polyamine metabolism in embryogenic cells of Daucus carota I. Plant Physiol 62, 430-433

MONTAGUE MJ, ARMSTRONG TA, JAWORSKI EG, 1979. Polyamine metabolism in embryogenic cells of Daucus carota II. Plant Physiol 63, 341-345

MORRIS DR, HANSEN MT, 1973. Influence of polyamine limitation on the chain growth rates of beta-galactosidase and of its messenger ribonucleic acid. J Bacteriol 116, 588-592

MORRIS DR, JORSTAD CM, SEYFRIED CG, 1977. Inhibition of the synthesis of polyamines and DNA in activated lymphocytes by a combination of alpha-methylornithine and methyylglyoxal-Bis(guanylhydrazone). Cancer Res 37, 3169-3172

MORRIS DR, LOCKSHON D, 1981. Polyamines, chromosome conformation and macramolecular synthesis in prokaryotes: a hypothesis. In: Eds. CM Caldarera, U Zappia, U Bachrach. Advances in Polyamine Research Vol 3 pp 299-307. Raven Press, NY

MURASHIGE T, SKOOG F, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15, 473-479

MURTY KS, SMITH TA, BOULD C, 1971. The relation between the putrescine content and potassium status of blackcurrant leaves. Ann Bot 35, 687-695

NADEAU P, DELANEY S, CHOUINARD L, 1987. Effect of cold hardening on the regulation of polyamine levels in wheat (Triticum aestivum L) and alfalfa

(Medicago sativa L) Plant Physiol 84, 73-77

NAKAMURA S, KAZIM AL, WANG HP, CHU JW, KIMURA T, 1972. Polyamine effect on NADPH oxidation catalyzed by spinach and adrenal flavoprotein iron-sulphur-protein complexes and a possible regulatory mechanism. FEBS Lett 28, 209-212

O'BRIEN TG, SIMSIMAN RC, BOUTWELL RK, 1975. Induction of the polyamine biosynthetic enzymes in mouse epidermis and their specificity for tumour protection. Cancer Res 35, 2426-2433

OSHIMA T, 1983. Unusual polyamines in the extreme thermophile Thermus thermophilus. Adv Pol Res 4, 479-487 ed RA Campbell. Raven Press, NY

OSHMARINA VI, SHEVYAKOVA NI, SHAMINA ZB, 1982. Dynamics of free amino acids and amides in Nicotiana sylvestris cell cultures at different concentrations of putrescine in the medium. Sov Plant Physiol 29, 477-481

OSMAMA E-SM, PETER EK, 1984. The influence of exogenous hormones on the growth of habituated cotton callus. Plant Physiol 78, abstr(78) suppl(4)

OTANI S, MIZOGUCHI Y, MATSUI I, MORISAWA S, 1974. Inhibition of DNA synthesis by MGBG during lymphocyte transformation. Mol Biol Rep 1, 431-436

PALAVAN N, GALSTON AW, 1982. Polyamine biosynthesis and titer during various developmental stages of Phaseolus vulgaris. Physiol Plant 55, 438-444

PEGG AE, 1983. Inhibitors of SAMDC. Methods in Enzymology 94, 239-247

PEGG AE, McCANN PP 1982. Polyamine metabolism and function. Amer J Physiol 245, c212-c221

PEGG AE, McGILL SM, 1978. Inhibition of diamino oxidase by 1,1-(methylethanediylidene)-dinitrilo -bis-(3-aminoguanidine) and 1,1'-(methylethanediylidene)-dinitrilo -diguanidine. Biochem Pharmacol 27, 1625-1629

PHILLIPS R, PRESS MC, BINGHAM L, GRIMMER C, 1988. Polyamines in cultured artichoke explants; effects are primarily on xylogenesis rather than cell division. J Exp Bot 39, 473-480

PHILLIPS R, PRESS MC, EASON A, 1987. Polyamines in relation to cell division and xylogenesis in cultured explants of Helianthus tuberosus: Lack of evidence for growth regulatory action. J Exp Bot 38, 164-172

PISTOCCHI R, BAGNI N, CREUS JA, 1987. Polyamine uptake in carrot cell cultures. Plant Physiol 84, 374-380

POHJANPELTO P, VIRTANEN I, HOLTTA E, 1981. Depletion of polyamines results in disappearance of actin filaments and microtubules in a polyamine auxotrophic Chinese hamster ovary cell line. Nature 293, 475-477

PRIEBE A, KLEIN H, JAGER HJ, 1978. Role of polyamines in sulphur dioxide polluted pea plants. J Exp Bot 29, 1045-1050

PRIEBE A, JAGER HJ, 1978. Effect of sodium chloride on the levels of putrescine and related polyamines of plants differing in salt tolerance. Plant Sci Lett 12, 365-369

PRIKHOD'KO LS, KLYSHEV LK, 1964. Nitrogen metabolism of pea shoots at different substrate salinity. Tr Inst Botan Akad Nauk SSR 20, 166-182 (Chem Abst 62, 9474b)

PROUTY WF, 1976. Ornithine decarboxylase inactivation in HeLa cells. J Cell Physiol 89, 65-76

RAMAKRISHNA S, ADIGA PR, 1975. Amine levels in Lathyrus sativus seedlings during development. Phytochem 14, 63-68

RICHARDS FJ, COLEMAN RG, 1952. Occurence of putrescine in potassium-deficient barley. Nature 170, 460

ROLLE I, PAYER R, SOEDER CJ, 1971. Uber die amine einzelliger Grunalgen I. Mitteilung. Der Spermiding enhalt von Scenedesmus acutus and Chlorella fusca. Arch Mikrobiol 77, 185-195

RUSSELL DH 1981. Post-translational modification of ODC by its product putrescine. Biochem Biophys Res Commun 99, 1167-1172

RUSSELL WC, PRECIOUS B, MARTIN SR, 1983. Differential promotion and suppression of Z-B transitions in poly[d(G-C)] by histone subclasses, polyamino acids and polyamines. EMBO J 2, 1647-1653

SAKAI TT, COHEN S, 1976. Effects of polyamines on the structure and reactivity of tRNA. Prog Nuc Acid Res 17, 15-42

SERAFINI-FRACASSINI D, ALESSANDRI M, 1983. Polyamines and morphogenesis in Helianthus tuberosus explants. Adv Pol Res 4, 419-426

SERAFINI-FRACASSINI D, BAGNI N, GIORGIO CIONINI P, BENINCI A, 1980. Polyamines and nucleic acids during the first cell cycle of Helianthus tuberosus after dormancy break. Planta 148, 332-337

SHEVYAKOVA NI, ARUTYONOVA NV, STROGONOV BP, 1981. Disturbance in arginine and putrescine metabolism in cotton leaves in the presence of excessive sodium bisulphate. Fiziol Rast(Moscow) 19, 1098-1104

SHEVYAKOVA NI, STROGONOV BP, 1985. Metabolism of polyamines in sodium chloride resistant cell lines from Nicotiana sylvestris. Plant Growth Regn 3, 365-369

SINDHU RK, COHEN SS, 1984. Propylamine transferases in Chinese cabbage leaves. Plant Physiol 74, 645-649

SINDHU RK, DESAI HV, SRIVASTAVA SK, 1979. Purification and properties of agmatine iminohydralase from groundnut cotyledons. Phytochem 18, 1937-1938

SINGH TN, PALEG LG, ASPINALL D, 1973. Stress metabolism. I. Nitrogen metabolism and growth in the barley plant during water stress. Aust J Biol Sci 26, 45-56

SLAVIC B, 1974. Methods of studying plant water relations. Akademia, Prague and Springer Verlag, Berlin.

SLOCUM RD, KAUR-SAWHNEY R, GALSTON AW, 1984. The physiology and biochemistry of polyamines in higher plants. Arch Biochem Biophys 235, 283-303

SMITH TA, 1963. L-arginine carboxy-lyase of higher plants and its relation to potassium nutrition. Phytochem 2, 241-252

SMITH TA, 1970. Putrescine, spermidine and spermine in higher plants. Phytochem 9, 1479-1486

SMITH TA, 1979. Arginine decarboxylase of oat seedlings. Phytochem 18, 1447-1452

SMITH TA, 1981. Amines. In: The Biochemistry of Plants - A Comprehensive Treatise Vol 7. Eds PH Stumpf, EE Cohn pp 249-268. Ac Press, NY

SMITH TA, 1984. Putrescine and inorganic ions. In: Recent Advances in Phytochemistry. Eds BN Timmermann, C Steelink, FA Loewus vol 18 pp 7-54

SMITH TA, 1985a. The di- and polyamines of higher plants. Biochem Soc Trans 13, 319-322

SMITH TA, 1985b. Polyamines. Ann Rev Plant Physiol 36, 117-143

SMITH TA, BEST GR, 1977. Polyamines in barley seedlings. Phytochem 16, 841-843

SMITH TA, BEST GR, ABBOTT J, CLEMENTS ED, 1978. Polyamines in Paul's Scarlet Rose suspension cultures. Planta 144, 63-68

SMITH TA, NEGREL J, BIRD CR, 1983. The cinnamic acid amides of the di- and polyamines In: U Bachrach, A Kaye, R Chayen Eds. Advances in Polyamine Research Vol 4 pp 347-370. Raven Press, NY

SMITH TA, SINCLAIR C, 1967. The effect of acid feeding on amine formation in barley. Ann Bot 31, 103-111

SMITH TA, WILSHIRE G, 1975. Distribution of cadaverine and other amines in higher plants. Phytochem 14, 2341-2346

SNEATH PHA, 1955. Putrescine as an essential growth factor for a mutant of Aspergillus nidulans Nature 175, 818

SMITH TA, MARSHALL JHA 1988 Oxidation of amino acids by manganous ions and pyridoxal phosphate. Phytochem **27**, 1611-1613

SPERANZA A, BAGNI N, 1977. Putrescine biosynthesis in Agrobacterium tumefascens and in normal and crown gall tumours of Scorzonera hispanica. Z. Pflanz 81, 226-233

SPRAGG BP, HUTCHINGS AD, 1983. High performance liquid chromatographic determination of putrescine, spermidine and spermine after derivatization with 4-fluoro-3-nitrobenzotrifluoride. J. Chrom 258, 289-291

STALKER DM, MOSBAUGH DW, MEYER RR, 1976. Novikoff hepatoma DNA polymerase. Purification and properties of a homogeneous beta polymerase. Biochemistry (Washington) 15, 3114

STEVENS L, STEVENS E, 1980. Inhibitors of the biosynthesis of putrescine, spermidine and spermine. In: Polyamines in Biomedical Research Ch 11 pp 167-173 Wiley.

STEWART CR, HANSON AD, 1980. Proline accumulation as a metabolic response to water stress. In: Adaptation of plants to water and high temperature stress, ed. NC Turner, PJ Kramer pp 155-172 New York, Wiley.

STREET HE(Ed.), 1977. Plant tissue and cell culture. Botanical monographs vol 11 2nd ed. Oxford Blackwell Scientific 614 pp.

STREETER JG, THOMPSON JF, 1972. Anaerobic accumulation of gamma-aminobutyric acid and alanine in radish leaves (Raphanus sativus L.). Plant physiol 49, 572-578

STROGONOV BP, 1964. Physiological basis of salt tolerance of plants. Pub Acad Sci USSR

STUMPF D.K, 1984. Quantitation and purification of quaternary ammonium compounds from halophyte tissue. Plant physiol 75, 273-274

SRIVASTAVA SK, SMITH TA, 1982. The effect of a series of oligoamines and guanidines on membrane permeability in higher plants. Phytochem 21, 997-1008

SUNG ZR, DUDITS D, 1981. Carrot somatic cell genetics. In: Genetic engineering in the plant sciences pp 11-37, Panopoulos NJ ed. Praeger, NY

SUNG ZR, JACQUES S, 1980. 5-Fluorouracil resistance in carrot cell culture: its use in studying the interaction of pyrimidine and arginine pathways. Planta 148, 386-389

SUNG ZR, OKIMOTO R, 1981. Embryonic proteins in somatic embryos of carrot. Proc Nat Acad Sci 78, 3683-3687

SURESH MR, ADIGA PR, 1977. Putrescine-sensitive (artefactual) and insensitive (biosynthetic) S-adenosyl-L-methionine decarboxylase activities of Lathyrus sativus seedlings. Eur J Biochem 79, 511-518<

SURESH MR, RAMAKRISHNA S, ADIGA PR, 1978. Regulation of arginine decarboxylase and putrescine levels in Cucumis sativus cotyledons. Phytochem 17, 57-63

SUZUKI Y, HIRASAWA E, 1980. S-adenosylmethionine decarboxylase of oat seedlings. Plant Physiol 66, 1091-1094

SWEELEY CC, BENTLEY R, MAKITA M, WELLS WW, 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J Am Chem Soc 85, 2497

TIBURCIO AF, KAUR-SAWHNEY R, INGERSOLL RB, GALSTON AW, 1985. Correlation between polyamines and pyrrolidine alkaloids in developing tobacco callus. Plant Physiol 78, 323-326

TIBURCIO AF, KAUR-SAWHNEY R, GALSTON AW, 1987. Effect of polyamine biosynthetic inhibitors on alkaloids and organogenesis in tobacco callus cultures. Plant Cell Tissue and Organ Culture 9, 111-120

TABOR CW, TABOR H, TYAGI AK, COHN MS, 1982. The biochemistry genetics and regulation of polyamine biosynthesis in Saccharomyces cerevisae. Fed Proc 41, 3084-3088

TABOR CW, TABOR H, 1984. Polyamines. Ann Rev Biochem 53, 749-790

TABOR CW, TABOR H 1976 Ann Rev Biochem **45**, 285-306

TABOR CW, TABOR H, TYAGI AK 1983 In: Bachrach U, Kaye A, Chayen R eds. Adv in Polyamine Res Vol 4 pp 467-478 Raven, NY

TABOR H, TABOR CW, 1983. Polyamine biosynthesis and function in Escherichia coli Adv Polyamine Res 4, 455-465

TIBURCIO AF, MASDEU MA, DUMORTIER FM, GALSTON AW, 1986. Polyamine metabolism and osmotic stress I. Relation to protoplast viability. Plant physiol 82, 369-374

TORRIGIANI P, SERAFINI-FRACASSINI D, BAGNI N, 1987a. Polyamine biosynthesis and effects of dichlorohexylamine during the cell cycle of Helianthus tuberosus tuber. Plant Physiol 84, 148-152

TORRIGIANI P, SERAFINI-FRACASSINI D, BIONDI S, BAGNI N, 1986. Evidence for the subcellular localization of polyamines and their biosynthetic enzymes in plant cells. J Plant Physiol 124, 23-29

TORRIGIANI P, SERAFINI-FRACASSINI D, FARA A , 1987b. Evidence for the presence of a diamino oxidase activity in different physiological stages of Jerusalem artichoke tuber. In: The Phytochemical Society of Europe Symposium on Amines in Plants. University College London, UK.

TROLL W, LINDSLEY J., 1955. A photometric method for the determination of proline. J Biol Chem 215, 655-660

TURNER NC, JONES MM, 1980. Turgor maintenance by osmotic adjustment: A review and evaluation . In: Adaptation of plants to water and high temperature stress (NC Turner and PJ Kramer, eds.) pp 87-104. John Wiley and Sons, NY.

TURNER LB, STEWART GR, 1986. The effect of water stress upon polyamine levels in barley leaves. J Exp Bot 37, 170-177

TURNER LB, STEWART GR, 1988. Factors affecting polyamine accumulation in barley (Hordeum vulgare L.) leaf sections during osmotic stress. J Exp Bot 39, 311-316

VILLANUEVA VR, ADLAKHA RC, CANTERN-SOLER AM, 1978. Changes in polyamine concentration during seed germination. Phytochem 17, 1245

VOETBERG G, STEWART CR, 1984. Steady state proline levels in salt-shocked barley leaves. Plant physiol 76, 567-576

WALTERS D, 1987. Polyamines: The cinderellas of cell biology. Biologist 34, 73-76

WALTON NJ, ROBINS RJ, RHODES MJ, 1988. Peturbation of alkaloid production by cadaverine in hairy root cultures of Nicotiana rustica. Plant Science 54, 125-131

WU WH, MORRIS DR, 1963. Biosynthetic arginine decarboxylase from Escheri chia coli: Purification and properties. J Biol Chem 248, 1687-1695

WYN JONES RG, STOREY R, 1978. Salt stress and comparative physiology in the Graminae II. Glycinebetaine and proline accumulation in two salt and water-stressed barley cultivars. Aust J Plant Physiol 5, 817-829

YAMASAKI Y, ICHIHARA A, 1976. Induction of ornithine decarboxylase in cultured mouse L-cells I. Effects of cellular growth, hormones and actinomycin D. J Biochem 80, 557-562

YAMASAKI Y, ICHIHARA A, 1977. Induction of ornithine decarboxylase in cultured mouse L-cells II. Effects of additions of amino acids and serum. J Biochem 81, 461-465

YANAGISAWA H, HIRASAWA E, SUZUKI Y, 1981. Purification and properties of diamino oxidase from pea epicotyls. Phytochem 20, 2105-2108

YANAGISAWA H, SUZUKI Y, 1982. Purification and properties of N-carbamyl putrescine amidohydrolase from maize shoots. Phytochem 21, 2201-2203

YANAGISAWA H, KATO A, HOSHIAI S, KAMIYA A, TORII N, 1987. Polyamine oxidase from water hyacinth. Plant Physiol 85, 906-909

YOUNG ND, GALSTON AW, 1983. Putrescine and acid stress. Plant Physiol 71, 767-771

YOUNG ND, GALSTON AW, 1984. Physiological control of arginine

decarboxylase activity in potassium deficient oat shoots. Plant Physiol 76, 331-335

Journal of Experimental Botany, Vol. 40, No. 215, pp. 681–687, June 1989

Responses to Water Stress in Adapted and Unadapted Carrot Cell Suspension Cultures

K. M. FALLON and R. PHILLIPS[1]

Biology Department (Darwin Building), University College London, Gower Street, London WC1E 6BT, UK

Received 21 September 1988

ABSTRACT

The responses of suspension-cultured cells of carrot to polyethylene glycol (PEG)-induced water stress were studied after transfer to culture medium containing PEG at concentrations between 0% and 25%. Growth characteristics, cellular osmotic potential and organic solute concentration changes were followed in unadapted cells and in cell lines adapted to growth in various PEG concentrations. A decline in fresh and dry weight increase occurred in unadapted cells with decreasing water potential, while dry weight gain was unaffected in adapted lines. Substantial osmotic adjustment was observed in adapted lines, due mainly to increased glucose, fructose and sucrose. Proline concentration increased up to 40-fold in adapted and 12-fold in unadapted cells and other amino acids including alanine, histidine and arginine showed similar, though smaller, responses. Polyamines and glycinebetaine did not increase significantly in either adapted or unadapted cells. Changes leading to long-term adaptation to water stress are discussed in relation to short-term stress-shock responses.

Key words: Water stress, cell culture, *Daucus carota*, osmotic adaptation, solute accumulation.

INTRODUCTION

The physiological responses of plants to water-stress have long been of interest, mainly because of the need to understand better the effects on economically important crop plants when water is a limiting factor. Kramer (1980) has estimated that losses in production due to lack of water exceed those of all other factors combined. One mechanism of resistance to water-stress is drought tolerance (Levitt, 1980), which involves the reduction of cell water potential through intracellular solute accumulation, allowing the cell to retain turgor and enhancing survival in an environment of fluctuating osmotic potential (Turner and Jones, 1980).

Research into drought stress has until now been mainly directed towards whole plants, with particular emphasis on stomatal behaviour. However, suspension-cultured plant cells offer a relatively homogeneous and experimentally controllable alternative for the study of cellular responses to water stress. Heyser and Nabors (1979) first reported the selection of cultured cell lines resistant to the stress induced by polyethylene glycol (PEG). Adaptation procedures and growth responses have been examined (Bressan, Hasegawa, and Handa, 1981) and physiological changes associated with water-stress adapted cells investigated in detail (Handa, Bressan, Handa, Carpita, and Hasegawa, 1983). Harms and Oertli (1985) employed mannitol-adapted carrot cell suspension cultures to study the interaction of osmotic and ionic stresses.

Two components of the response of plant cells to decreased water potential may be identified; long-term changes in cells adapted to continuous growth at low water potential, here referred to as stress-adaptation, and stress-shock responses occurring in the short-term in cells transferred abruptly to lower water potential environments. In this study, both responses were examined in carrot suspension cells transferred to fresh medium of the same or lower water potential for a period of 10 h. For stress-shock changes, cells of a line adapted to growth in a standard tissue culture medium (control cells) were transferred directly to media containing various concentrations up to 25% PEG. For stress-adaptation, cell lines previously adapted to similar PEG concentrations were transferred to fresh medium with appropriate PEG levels. Changes in growth rate, water potential and solute content for both control and adapted cells were determined.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Cell culture

Suspension cultures of *Daucus carota* (cv. Chantenay) were maintained on Murashige and Skoog (1962) medium, and fresh and dry weights determined as previously described (Fallon and Phillips, 1988). Five cell-lines were used; unadapted cells were grown in standard culture medium without PEG, while four cell-lines were adapted to growth in medium containing 10, 15, 20, and 25% (w/v) PEG (BDH-4000 mol. wt., purified by ion-exchange with BDH-Duolite MB5113 mixed resin) by repeated subculture until consistent final fresh weights were obtained in consecutive passages; the adaptation period was for a minimum of eight subcultures. For the experimental treatments, adapted cells were transferred to fresh medium of corresponding PEG concentration. Unadapted cells were transferred both to standard medium without PEG (control treatment) and to media containing each PEG concentration. For each cell-line, cells from stationary phase cultures were collected by nylon mesh filtration, allowed to drain, and resuspended in the same volume of fresh medium. Water potential and solute determinations were made 10 h after transfer.

Determination of cellular and media solute potential

The osmotic potential of cell contents and media was determined by the use of a vapour pressure osmometer (Wescor 5100C). Samples of fresh and spent media were sampled directly, while for the determination of cellular solute potential, collected, filtered and spin-dried tissue (Fallon and Phillips, 1988) was stored frozen at −20 °C, and allowed to reach room temperature prior to determination.

Estimation of solutes

Free amines were determined by HPLC, as described previously (Fallon and Phillips, 1988). Proline was determined by the ninhydrin technique of Troll and Lindsley (1955), and glycinebetaine by the colorimetric method of Stumpf (1984). Free amino acids were determined by HPLC following extraction with sulphosalicylic acid and OPA/mercaptoethanol derivatization (Jarrett, Cooksy, Ellis, and Anderson, 1986). Sugars were determined by the method of Sweeley, Bentley, Makita, and Wells (1963) with the modifications recommended by Holligan (1971) on a Shimadzu GC Mini 3/CR3A gas chromatograph with an SE30 packed column.

RESULTS

Growth of culture

On inoculation into fresh medium both adapted and unadapted cells showed a characteristic sigmoidal growth pattern over an 18 d period, at all concentrations of PEG (Fig. 1). In unadapted cells (Fig. 1b), increasing PEG concentration progressively increased the length of the lag period, and decreased the rate of fresh weight increase. The lag time was extended from 2–3 d in control medium to 10 d with 25% PEG, and the rate of fresh weight increase reduced from 0·75 g d^{-1} to 0·25 g d^{-1}. Final fresh weight attained was reduced at all PEG concentrations, from a 15% reduction in 10% PEG to a 63% reduction in 25% PEG.

In adapted cells the effects of PEG on growth are less severe (Fig. 1a). There is an effect on lag phase only at the two highest concentrations, extending it from 3 d in the control to 5 d with 25% PEG, and little change in the rate of fresh weight accumulation was observed. However, final fresh weight was affected by increasing PEG concentration, though to a lesser degree than in unadapted cells.

The distinct responses of adapted and unadapted cells to water stress are illustrated by comparison of final fresh and dry weights after 18 d of growth in media of varying

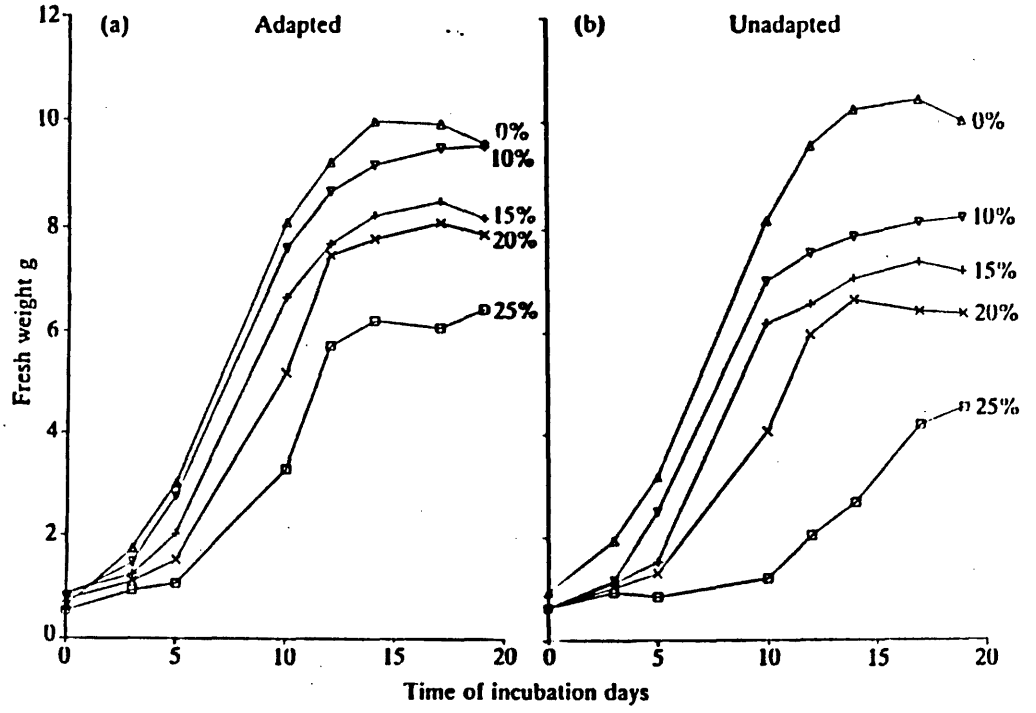


FIG. 1. Changes in fresh weight of adapted (a) and unadapted (b) cells over the culture period at a range of PEG concentrations; means of three replicates.

PEG content (Fig. 2). Fresh weight yields declined with decreasing osmotic potential, the decline being greater in unadapted than adapted cells. Dry weight yields, on the other hand, were reduced only in unadapted cells, with a decrease of approximately 40% in 25% PEG. Clearly, adapted cells have an enhanced capacity for both water retention and dry weight accumulation compared to unadapted cells. A reduction in water content relative to control cells of up to 36% for adapted and 54% for unadapted cells was observed (Fig. 2).

Osmotic potential

The solute potentials of media containing 0, 10, 15, 20 and 25% PEG were found to be −0·34, −0·55, −0·83, −1·13 and −1·67 MPa, respectively. The osmotic potential of adapted cells was maintained at a substantially lower value than that of the corresponding medium, the deficit actually increasing at higher PEG concentrations, such that in the line adapted to 25% PEG the solute potential decreased 4-fold relative to control cells (Fig. 3). Unadapted cells were in equilibrium with the medium (and were usually plasmolysed) at 15% PEG and above.

Free polyamines

Putrescine (put) and spermidine (spd) were the major PAs present, with spermine (spm) as a minor component

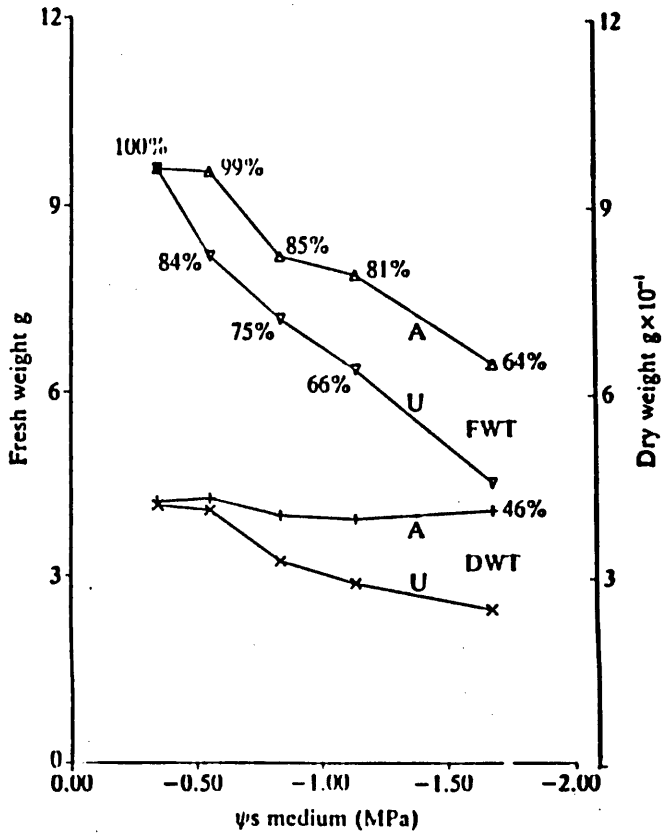


FIG. 2. Changes in fresh weight, dry weight and water content in unadapted (U) and adapted (A) cells after 19 d incubation at a range of PEG concentrations; means of three replicates. Water content is expressed as a percentage of that of control cells.

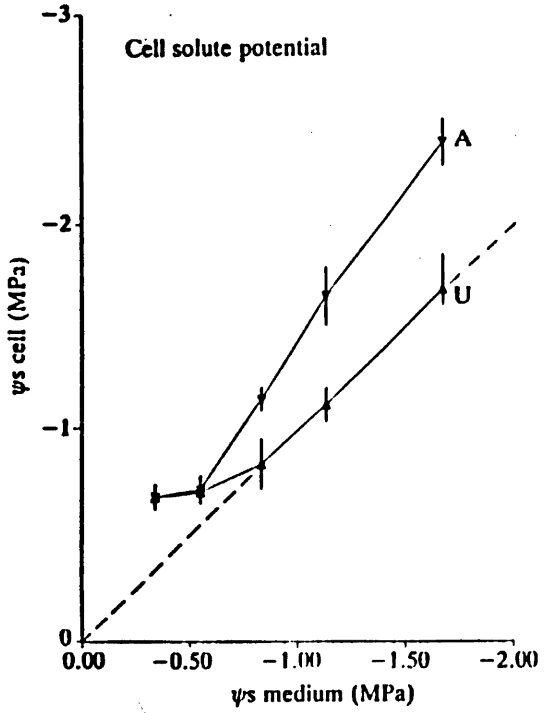


FIG. 3. Solute potentials of unadapted (U) and adapted (A) cells after 10 h incubation at a range of PEG concentrations; means and standard deviation of three replicates. The broken line indicates the equivalence of cell and medium potentials.

Free amine concentrations in unadapted (U) and adapted (A) cells lines

Mean of three replicate determinations.

Amine		PEG concentration				
		0%	10%	15%	20%	25%
		mol m^{-3}				
Putrescine	U	1·36	1·55	2·36	3·99	3·86
	A	1·36	2·51	2·97	3·20	3·64
Spermidine	U	1·34	1·83	1·88	2·03	1·36
	A	1·34	1·81	1·75	1·71	1·69
Spermine	U	0·09	0·17	0·24	0·23	0·38
	A	0·09	0·08	0·11	0·32	0·43
Total	U	2·79	3·55	4·48	6·25	5·60
	A	2·79	4·40	4·83	5·23	5·76

(Table 1). The total PA concentration increased with PEG concentration in an approximately linear fashion; any differences between the adapted and unadapted lines were small and showed no clear trends. Increased total concentrations were primarily due to increases in putrescine and the rather small increases in concentration (approximately 2-fold) can be accounted for by concentration due to water loss (cf. Fig. 2).

Proline, free amino acids and glycinebetaine

The cellular concentration of proline rose significantly in both normal and adapted cells grown in media of decreasing solute potential (Fig. 4b). Proline concentration increased 12-fold in unadapted cells, and over 40-fold

in adapted cells compared with controls. Proline levels continued to increase in adapted cells up to the highest concentration tested, while in unadapted cells no increase occurred beyond 20% PEG.

The combined cellular concentration of the major free amino acids shows a strong correlation with medium solute potential in adapted cells (Fig. 4a). The response was approximately linear at lower solute potentials and was not saturated at the highest PEG concentration employed. In unadapted cells, however, the response was far less pronounced, with a doubling in concentration over the control, as opposed to an 8-fold increase in adapted cells. While the response of individual non-proline amino acids varies considerably (Table 2), higher concentrations are generally found in adapted rather than unadapted cells. Alanine concentration increased up to 18-fold over control values (adapted cells), but the highest concentrations observed were those of histidine, arginine, and valine, which were relatively abundant in control cells. With the exception of serine, all amino acids were present at higher concentration in adapted than unadapted cells at the lowest solute potential. However, proline was the most abundant amino acid by a factor of 2 and constituted 30% of the total amino acid concentration.

Betaine concentration changed little in either cell line in response to increasing water stress (Table 2).

Sucrose

The cellular sucrose concentration increased approximately 3-fold up to 120 mol m^{-3} in adapted cells under

Free amino acid and glycinebetaine concentrations in unadapted (U) and adapted (A) cell lines

Mean of 3 replicate determinations.

Amine		PEG concentrations				
		0%	10%	15%	20%	25%
		mol m^{-3}				
Asp	U	0·57	0·50	0·36	0·65	0·64
	A	0·57	0·87	0·85	1·06	1·57
Gln	U	0·71	0·72	0·79	0·74	0·71
	A	0·71	0·99	1·11	1·12	1·11
Ser	U	1·67	1·82	2·11	2·41	2·45
	A	1·67	1·19	1·87	4·20	1·95
His	U	2·16	2·14	2·57	2·50	2·74
	A	2·16	3·13	3·73	6·60	12·69
Gly	U	0·33	0·28	0·39	0·45	0·58
	A	0·33	0·36	1·18	0·83	1·12
Thr	U	0·89	0·85	0·99	1·05	1·09
	A	0·89	0·76	1·53	1·78	2·29
Arg	U	1·32	1·52	1·89	1·95	1·94
	A	1·32	1·81	2·68	3·53	8·66
Ala	U	0·29	0·34	0·83	1·11	1·09
	A	0·29	0·24	0·40	5·32	5·48
Tyr	U	0·17	0·17	0·18	0·21	0·24
	A	0·17	0·28	0·34	0·36	0·58
Met	U	0·25	0·34	0·35	0·37	0·37
	A	0·25	0·32	0·53	0·58	0·67
Val	U	0·83	1·24	1·44	1·86	1·90
	A	0·83	1·86	2·59	4·63	7·07
Phe	U	0·86	1·14	1·20	1·44	1·88
	A	0·86	0·65	1·03	1·44	1·73
Ile	U	0·41	0·49	0·64	0·80	1·06
	A	0·41	0·58	0·82	1·12	2·88
Leu	U	0·22	0·29	0·37	0·48	0·59
	A	0·22	0·40	0·54	0·62	1·78
Betaine	U	0·73	0·78	1·01	0·85	0·93
	A	0·73	0·66	0·80	0·82	0·77

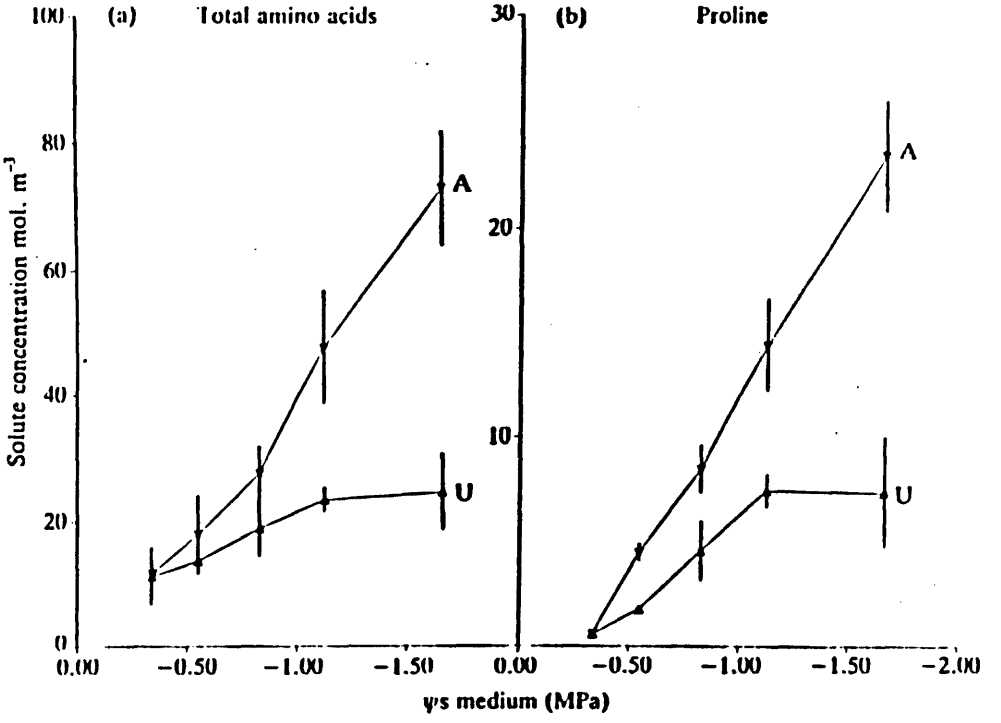


FIG. 4. Changes in total amino acids (a) and proline (b) in unadapted (U) and adapted (A) cells after 10 h incubation at a range of PEG concentrations; means and standard deviation of three replicates.

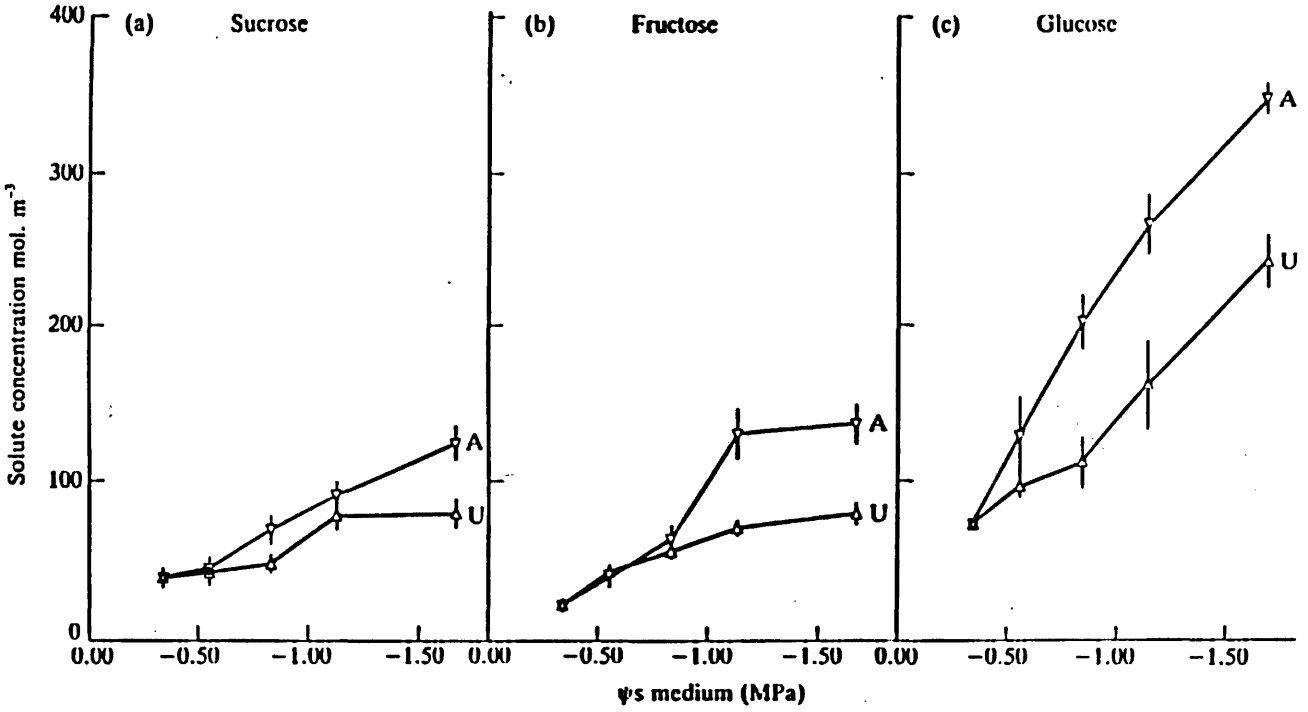


FIG. 5. Changes in sucrose (a), fructose (b) and glucose (c) concentrations in unadapted (U) and adapted (A) cells after 10 h incubation at a range of PEG concentrations; means and standard deviation of three replicates.

water stress, and doubled in non-adapted cells (Fig. 5a). Concentrations in adapted cells were significantly higher than in unadapted cells at all solute potentials.

Glucose and fructose

Glucose concentrations rose 4·5-fold in adapted cells at the lowest solute potential, reaching a cellular concentration of nearly 350 mol m^{-3} (Fig. 5c). levels also rose 3·5-fold in unadapted cells to a concentration exceeding 240 mol m^{-3}. Concentrations of fructose increased 5-fold in adapted and 3-fold in unadapted cells, reaching a peak of more than 130 mol m^{-3} in adapted cells (Fig. 5b). Glucose, fructose and sucrose are major contributors to solute concentration, contributing over 60% of the osmolarity of stressed cells (Table 3).

To illustrate the contribution of each solute investigated to the overall cell water potentials, values for medium and cell water potentials and solute concentrations have been calculated in milliosmoles (mOsm), and the percentage contribution of each expressed in Table 3. Glucose, fructose, and sucrose were the principal osmotic agents, contributing 61·5% of the cell water potential of adapted cells in 25% PEG. Under the same conditions, proline contributed 2·4% and other amino acids 5·1%. Polyamines and betaine represented an osmotic contribution of only 0·5%. The organic solutes investigated contribute 69·5% of the total; potassium and other ionic species have not been determined.

DISCUSSION

The adaptation of cultured plant cells to both water and NaCl stress has been reported (Bressan *et al.*, 1981; Ben-Hayyim, 1986; Harms & Oertli, 1985). Adapted cells characteristically show a shorter lag phase and increased growth rate compared to non-adapted cells exposed to similar stress conditions. Comparable responses were obtained with carrot suspension cultures in this study. This adaptation involves a decrease in cell solute potential (Fig. 3) which is substantially greater in adapted than in unadapted cells and directly related to a reduction in cell water content (Fig. 2), and an accumulation of intracellular solutes compared to controls. This is similar to the response of sorghum (Bhaskaran, Smith, and Newton, 1985) and tomato (Handa *et al.*, 1983) cells to a low water potential environment.

When considering changes in solute concentration, it should be noted that the water contents of cells of either line transferred to medium or low water potential are sharply reduced as compared with control cells (Fig. 2). This is due to water loss to the medium, and will result in increased solute concentrations even in the absence of any absolute rise in solute content. For example, the water content of unadapted cells transferred to 25% PEG falls by 54% (Fig. 2); concentrations of all solutes will thus double without any net accumulation. This point has been considered by some investigators, (Turner and Stewart, 1988) and should be noted, particularly when concentration changes of the order of 1–2× are considered. The accumulation of solutes has been widely reported under stress conditions (Hanson and Hitz, 1982; Stewart and Hanson, 1980) with polyamine accumulation in particular

TABLE 3. *Medium and cell osmolarity and percentage contribution of each solute in unadapted (U) and adapted (A) cell lines*

All solute concentrations were recalculated in terms of mOsm and expressed as a percentage of the corresponding cell osmotic potential. AA = total amino acids excluding proline.

PEG (%)	Ψmed (mOs)	Ψcells (mOs)		Contribution of solutes to Ψcell (%)															
				Glucose		Fructose		Sucrose		AA		Proline		Polyamine		Betaine		Total	
		U	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A
0	140	278	278	25·6	25·6	6·8	6·8	14·0	14·0	3·9	3·9	0·2	0·2	1·0	1·0	0·2	0·2	51·5	51·5
10	225	288	293	33·4	43·5	13·9	12·7	14·6	15·1	4·2	4·4	0·6	1·5	1·2	1·5	0·3	0·2	62·8	78·9
15	340	341	467	32·5	43·2	15·5	13·1	13·5	14·5	4·1	4·1	1·4	1·8	1·3	1·0	0·3	0·2	68·6	77·9
20	465	456	674	35·3	39·5	14·9	18·8	16·7	13·2	3·5	4·9	1·6	2·1	1·4	0·8	0·2	0·1	73·6	79·4
25	685	687	983	35·4	35·5	11·2	13·6	11·2	12·4	2·5	5·1	1·1	2·4	0·8	0·6	0·1	0·1	62·3	69·7

receiving increased attention in the last decade (Smith, 1984). Large increases in polyamines (50- to 60-fold) have been reported in detached oat leaves and protoplasts after 6 h exposure to osmotic stress (Flores and Galston, 1982). However, in similar experiments with barley leaf sections, Turner and Stewart (1988) found only a 3- to 4-fold increase in putrescine levels over intact leaf controls, and suggested that the massive increases reported by Flores and Galston (1982, 1984) were artefactual and due in part to low control values.

In this study, no increase in total polyamines and only a small increase in putrescine over that dictated by concentration due to water loss, was detected. This may be due to the high levels of putrescine found in control cultures, reported previously (Fallon and Phillips, 1988). It may be that putrescine accumulation is saturated at the control values of approximately 1·3 mol m^{-3}, but in any case no significant increase in putrescine was detected either in adapted or stress-shocked cells. The minor component spermine increased 4- to 5-fold in concentration, suggesting rather more than a doubling in net content. Spermine has not been previously identified as a stress-indicator.

Large increases in proline concentration were observed. Relative to control values, proline in cells exposed to 25% PEG increased 12-fold under stress-shock conditions and 40-fold in adapted cells, to a maximum of 23 mol m^{-3}. Handa *et al.* (1983) reported a 100-fold increase in proline in tomato cells adapted to 25% PEG up to a similar maximum concentration. Comparable increases in proline content have been found in drought-stressed barley (Hanson and Nelsen, 1978) and sorghum (Stewart and Hanson, 1980; Blum and Ebercon, 1976), and thus the response of carrot cells is in agreement with that widely reported for both cell and whole plant systems subjected to water-stress. A significant difference in the degree of response to adaptation and stress-shock was observed; proline accumulation apparently continues to increase in adapted cells to substantially higher levels than are produced by a 10 h stress-shock period. In salt-stressed barley leaves, proline accumulation increased over a 16 h period (Voetberg and Stewart, 1984) but did not increase further with osmotic adjustment. Handa *et al.* (1983) have discussed the possibility that proline is derived from glutamate under stress conditions. Our observations are similar in that glutamate levels decline somewhat in low water potential medium when water loss is taken into account, but that the observed values in control cells are not high enough to account for the increase in proline.

The varied response of the individual non-proline amino acids in adapted cells supports speculation (Handa *et al.*, 1983) that water-stress adaptation involves changes in the rates of assimilation, synthesis, utilization and interconversion of amino acids; certainly no common mechanism can be evoked. While proline is clearly unique in the extent of its response, it may be noted that the increase in osmotic contribution due to non-proline amino acids is nearly twice that of proline itself. In stress-shocked cells, on the other hand, the concentration of non-proline amino acids only increases by 70%. This means that the net content falls, when concentration effects are taken into account. Adaptation and stress-shock processes do not, therefore, seem closely related for these amino acids.

Reducing sugars, particularly glucose, together with sucrose, provide the major contribution to solute accumulation in adapted cell lines, in agreement with findings in stressed tomato cells by Handa *et al.* (1983), who suggest that such increases might be due to a reduction in cell wall synthesis mediated by reduced expansion. It should be noted, however, that the culture medium initially contains 78 mol m^{-3} sucrose, and that high levels of sugars may well be due to uptake and catabolism from this source; in sorghum and sunflower leaves levels of sucrose and reducing sugars were approximately an order of magnitude lower than in the present study (Jones, Osmond, and Turner, 1980). It would be of interest to investigate sucrose uptake rates to clarify this point.

This study reveals substantial differences in cellular responses to stress-shock as compared to long-term adaptation to water-stress. when concentration effects are discounted, stress-shocked cells show major increases in proline and, to a lesser extent, reducing sugars. Long-term adaptation is accompanied by enhanced levels of these

solutes as well as sucrose and other amino acids. Whether this is solely a function of the duration of exposure to water-stress, or whether qualitatively different mechanisms are involved is not clear.

Adaptation involves a substantial reduction in cellular water potential, allowing turgor to be maintained. The organic solutes investigated here account for about 70% of the osmotic potential change observed between control and adapted cells. The nature of the remaining 30% must be speculative, although inorganic solutes (mostly potassium ions) represented 20% of the osmotic potential in adapted tomato cells (Handa *et al.*, 1983). Part of the short-fall might be due to underestimation of the cell osmotic potential by mixing of extracellular water with cellular contents during osmometer measurements. In a major study using adapted tomato cells, Handa *et al.* (1983) were able to account for only 65% of the observed osmotic potential, including inorganic solutes, although other workers (Jones *et al.*, 1980; Meyer and Boyer, 1981) accounted for almost 100% in whole plant organs, with the major contribution from sugars and amino acids.

ACKNOWLEDGEMENTS

We wish to thank S. Laurie for help with GC determinations, J. Graves for aid in the production of figures, and J. Pearson and G. R. Stewart for much helpful advice.

LITERATURE CITED

BEN-HAYYIM, G., 1986. The relationship between salt tolerance and resistance to polyethylene glycol-induced water stress in cultured citrus cells. *Plant Physiology*, 85, 430–3.

BLUM, A., and EBERCON, A., 1976. Genotypic responses in sorghum to drought stress. III. Free-proline accumulation and drought resistance. *Crop Science*, 16, 428–31.

BRESSAN, R. A., HASEGAWA, P. M., and HANDA, A. K., 1981. Resistance of cultured higher plant cells to polyethylene glycol-induced water stress. *Plant Science Letters*, 21, 23–30.

BHASKARAN, S., SMITH, R. H., NEWTON, R. J., 1985. Physiological changes in cultured sorghum cells in response to induced water stress. *Plant Physiology*, 79, 266–9.

FALLON, K. M., and PHILLIPS, R., 1988. Polyamines in relation to growth in carrot cell cultures. Ibid. 88, 224–7.

FLORES, H. E., and GALSTON, A. W., 1982. Polyamines and plant stress: Activation of putrescine biosynthesis by osmotic shock. *Science*, 217, 1259–61.

—— —— 1984. Osmotic stress induced polyamine accumulation in cereal leaves. I. Physiological parameters of the response. *Plant Physiology*, 75, 102–9.

HANDA, S., BRESSAN, R. A., HANDA, A. K., CARPITA, N. C., and HASEGAWA, P. M., 1983. Solutes contributing to osmotic adjustment in cultured plant cells adapted to water stress. Ibid. 73, 834–43.

HANSON, A. D., and HITZ, W. D., 1982. Metabolic responses of mesophytes to plant water defecits. *Annual Review of Plant Physiology*, 33, 163–203.

—— and NELSEN, C. E., 1978. Betaine accumulation and formate metabolism in water-stressed barley leaves. *Plant Physiology*, 62, 305–12.

HARMS, C. T., and OERTLI, J. J., 1985. The use of osmotically adapted cell cultures to study salt tolerance *in vitro*. *Journal of Plant Physiology*, 120, 29–38.

HEYSER, J. W., and NABORS, M. W., 1979. Osmotic adjustment of tobacco cells and plants to penetrating and non-penetrating solutes. *Plant Physiology* supplement, 63, 77.

HOLLIGAN, P. M., 1971. Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. *New Phytologist*, 70, 239–69.

JARRETT, H. W., COOKSY, K. D., ELLIS, B., and ANDERSON, J. M., 1986. The separation of *o*-phthalaldehyde derivatives of amino acids by reversed-phase chromatography on octylsilica columns. *Annals of Biochemistry*, 153, 189–98.

JONES, M. M., OSMOND, C. B., and TURNER, N. C., 1980. Accumulation of solutes in leaves of sorghum and sunflower in response to water deficits. *Australian Journal of Plant Physiology*, 7, 193–205.

KRAMER, P. J., 1980. Drought stress and the origin of adaptations. In *Adaptation of plants to water and high temperature stress*. Eds N. C. Turner and P. J. Kramer. J. Wiley and Sons, New York. Pp. 7–20.

LEVITT, J., 1980. *Responses of plants to environmental stress*, 2nd edition. Academic Press, New York.

MEYER, R. F., and BOYER, J. S., 1981. Osmoregulation, solute distribution and growth in soybean seedlings having low water potentials. *Planta*, 151, 482–9.

MURASHIGE, T., and SKOOG, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia plantarum*, 15, 473–9.

SMITH, T. A., 1984. Putrescine and inorganic ions. In *Phytochemical adaptations to stress*, *Volume 8*. Eds B. N. Timmermann, C. Steelink and F. A. Loewus. Plenum Press, New York, Pp. 6–54.

STEWART, C. R., and HANSON, A. D., 1980. Proline accumulation as a metabolic response to water stress. In *Adaptation of plants to water and high temperature stress*. Eds N. C. Turner and P. J. Kramer. J. Wiley and Sons, New York, Pp. 155–72.

STUMPF, D. K., 1984. Quantitation and purification of quaternary ammonium compounds from halophyte tissue. *Plant Physiology*, 75, 273–4.

SWEELEY, C. C., BENTLEY, R., MAKITA, M., and WELLS, W. W., 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *Journal of the American Chemical Society*, 85, 2497.

TROLL, W., and LINDSLEY, J., 1955. A photometric method for the determination of proline. *Journal of Biological Chemistry*, 215, 655–60.

TURNER, L. B., and STEWART, G. R., 1988. Factors affecting polyamine accumulation in barley (*Hordeum vulgare* L.) leaf sections during osmotic stress. *Journal of Experimental Botany*, 39, 311–16.

—— and JONES, M. M., 1980. Turgor maintainance by osmotic adjustment: A review and evaluation. In *Adaptation of plants to water and high temperature stress*. Eds N. C. Turner and P. J. Kramer. J. Wiley and Sons, New York, Pp. 87–104.

VOETBERG, G., and STEWART, C. R., 1984. Steady-state proline levels in salt-shocked barley leaves. *Plant Physiology*, 76, 567–76.

Plant Physiol. (1988) 88, 224–227
0032-0889/88/88/0224/04/$01.00/0

Polyamines in Relation to Growth in Carrot Cell Cultures[1]

Received for publication October 26, 1987 and in revised form May 5, 1988

KEVIN M. FALLON AND RICHARD PHILLIPS*
Department of Botany and Microbiology, University College, London, WC1E 6BT, United Kingdom

ABSTRACT

Changes in polyamine metabolism were investigated in relation to growth of cell suspension cultures of carrot (*Daucus carota*, cv Chantenay). Changes in levels of the major amines putrescine and spermidine throughout the culture period correlated poorly with changes in fresh weight, but a closer correlation with the minor component spermine was observed. The arginine decarboxylase (ADC) inhibitor difluoromethylarginine (DFMA) strongly and specifically inhibited ADC activity in the supernatant, reduced the major amine (putrescine) by 95% and the total amine content by 80%. It had no effect on cell number and stimulated fresh weight by over 25% through increased cell expansion. Spermine content, in contrast, increased with DFMA concentration in parallel with fresh weight increases. Difluoromethylornithine strongly inhibited ornithine decarboxylase activity in the pellet, but had little effect on either polyamine levels or culture growth. It was concluded that little evidence for a correlation between free polyamines and cell number in carrot cultures could be detected, but that a possible correlation between spermine content and cell expansion was observed.

The polyamines occur ubiquitously in plants, animals, and prokaryotes, and their role in growth, development, and stress metabolism has received active investigation (4, 21 24). Although, in both animal cells and bacteria, changes in polyamine concentration and in the activity of their biosynthetic enzymes have been shown to accompany growth (13, 29), in plants similar correlations require clarification, particularly with regard to the relationship between these compounds and mitotic activity. Due to the complexity of higher plants, difficulties may arise in separating effects on mitosis and growth from other physiological responses involving polyamines. The use of tissue culture, and particularly cell suspension culture, should help to resolve these difficulties.

Studies using callus, explant, or suspension culture have produced conflicting results. For example, studies using inhibitors of polyamine metabolism have shown either an increase in fresh weight accumulation with inhibitor treatment (5), no significant change (23), or a reduction in final fresh weight (3). Similarly, determinations of endogenous polyamine content over the culture period have shown a strong positive correlation between growth rates and amine content (26), while in other studies none has been found (10). In addition, the activities of the polyamine biosynthetic enzymes are reported to correlate with rates of growth (2), while in other studies these correlations have been less conclusive (19).

In the present study, cell suspension cultures of carrot (*Daucus carota*) have been used to investigate the relationship between growth rate and free polyamines, both by measurement of endogenous levels over the growth period and by the use of inhibitors (15) that lead to large alterations in polyamine content.

MATERIALS AND METHODS

Cell Culture. Suspension cultures of *Daucus carota* (cv Chantenay) isolated 3 years previously were routinely maintained on Murashige and Skoog (20) medium (Flow Labs, Irvine, Scotland), supplemented with 2,4-dichlorophenoxyacetic acid (0.2 mg/L), kinetin (0.2 mg/L), and sucrose (2%), in 250-mL Erlenmeyer flasks at 25°C in diffuse fluorescent light at a shaker speed of 90 rpm and subcultured at 14-d intervals by inoculating 5 mL of suspension into 85 mL of fresh medium. For determination of fresh weight, aliquots of suspension were filtered through preweighed plastic syringe barrels fitted with sintered polypropylene discs (40-μm pore size). After centrifugation at 200*g* for 5 min to remove surface liquid, the tubes were re-weighed. The pad of weighed tissue was removed from the tube by air pressure and immediately used for amine and enzyme activity determinations.

Cell counts were made on weighed tissue by a modification of the method of Brown and Rickless (6). Portions (100 mg) of tissue were macerated in 5% chromium trioxide in 5% HCl, separated by drawing through a hypodermic needle, and counted with a hemocytometer after appropriate dilution.

Estimation of Free Amines. For amine determination, triplicate samples of 1 g fresh weight tissue were extracted overnight at 0°C in 5 mL of 5% perchloric acid to which were added 50 μL of 10 mM diaminooctane as internal standard, after Flores and Galston (11). After centrifugation, the supernatants were neutralized with 1.0 N KOH and were freeze-dried, and the dry sample was reconstituted in 500 μL of Na_2CO_3. Aliquots of 150 μL were then derivatized with fluoronitrobenzotrifluoride following the procedure of Spragg and Hutchings (28). The bright yellow polyamine derivatives were quantified by HPLC against authentic standards (Sigma) and processed as above.

The separation was performed isocratically on a Varian 5040 liquid chromatograph with a UV50 variable wavelength detector set at 254 nm and equipped with a 25-cm reverse phase C18 column (Spherisorb ODS2, 5 μm particle size) and a 20-μL injection loop. The mobile phase was 85% acetonitrile (HPLC grade, Fisons)/15% double-distilled water at a flow rate of 1.5 mL/min.

Assay of Arginine Decarboxylase and Ornithine Decarboxylase Activities. An aliquot of weighed tissue (0.5 g) was homogenized with 1.5 mL of 0.1 M Hepes, pH 7, containing 5 mM dithiothrietol, with 30 strokes of a glass homogenizer at 0°C. After centrifugation at 12,000*g* for 15 min, the supernatant was frozen and stored at −20°C for ADC[2] assay, while the pellet was

[1] K. M. F. was supported by a Science and Engineering Research Council/Cooperative Award in Science and Engineering with Shell Research, Sittingbourne, UK.

[2] Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; DFMA, difluoromethylarginine; DFMO, difluoromethylornithine.

resuspended in ODC buffer (50 mM Tris [pH 8.5], 0.3 mM EDTA, 5 mM dithiothrietol, 50 mM pyridoxal-5-phosphate) and frozen as above for later ODC assay. In preliminary studies more than 90% of the total ODC activity was found in the pellet fraction, as previously reported for carrot cell cultures (19).

Enzyme reactions were carried out in glass tubes within sealed scintillation vials containing 0.5 mL of Packard Carbosorb to absorb released CO_2.

The ADC assay was initiated by injection of 2 μL of L-[U-^{14}C] arginine monohydrochloride (Amersham 0.1 μCi, 342 mCi/mmol) through a serum cap on the vial into 150 μL of extract mixed with 10 μL of 50 mM pyridoxal-5-phosphate, and the vial was incubated at 30°C for 60 min on a shaker at 100 rpm. The reaction was then terminated by addition of 50 μL of 0.2 N perchloric acid. After shaking for a further 60 min, the reaction tubes were removed, and the radioactivity of the absorbed CO_2 was determined in a scintillation counter after addition of 10 mL of scintillation fluid (Optiphase, Fisons), with a counting efficiency of 89%. Since uniformly labeled arginine was used, data were corrected to account for nonspecific labeling.

For ODC assay the same procedure was used, except that 150 μL of pellet suspension were mixed with 50 μL of ODC buffer, and the reaction was initiated by addition of 2 μL of L-[1-^{14}C] ornithine hydrochloride (Amersham 0.1 μCi, 61 mCi/mmol) as substrate. Triplicate samples were used throughout, and the data confirmed in a subsequent experiment.

The protein content of the extracts was determined by the method of Lowry *et al.* (16), using BSA as standard, after precipitating with PCA and dissolving in NaOH to avoid effects of Hepes.

RESULTS

After inoculation into fresh medium, the fresh weight of the cultures increased rapidly after a lag of approximately 2 d to give a characteristic sigmoidal growth curve (Fig. 1). Levels of putrescine, the principal amine detected, increased rapidly over the first 2 d and then declined during the most rapid phase of growth; spermidine levels showed a similar pattern of change. However, levels of the minor component spermine, after increasing approximately three-fold after 2 d, continued to rise throughout most of the culture period. Very low concentrations of cadaverine (<20 nmol) were consistently detected (data not shown); changes in concentration were small and showed no clear trends. A rapid and transient increase in all amines on subculture to fresh medium was consistently observed.

Effects of DFMO and DFMA on Growth and Amine Content. When cells were grown for 10 d in medium supplemented with filter-sterilized inhibitors, growth of the culture (as fresh weight) was not affected at 0.1 or 0.3 mM DFMA and was markedly stimulated (30% increase) at 1 mM and 3 mM DFMA (Fig. 2), while dry weight remained unaffected with a maximum value of 0.180 g/flask and a minimum value of 0.174 g. The increase in fresh weight (mean 25%) and the lack of change in dry weight were consistently observed in three experiments. A dramatic fall in putrescine content with increasing DFMA concentration was observed, such that at 1 mM DFMA putrescine was reduced by 95%; spermidine levels also declined markedly. Spermine content on the other hand, increased steadily with increasing DFMA concentration (Fig. 2).

These findings point to a lack of correlation between fresh weight changes and endogenous levels of the major amines putrescine and spermidine, but to a possible link between the minor component spermine and growth.

A similar experiment was carried out with varying concentrations of DFMO; after 10 d incubation, no significant effect on dry weight (max 0.180 g, min 0.174 g) or content of spermidine and spermine was detectable, and a much smaller reduction in putrescine level was observed (Fig. 3).

Cell counts were made on cultures incubated for 10 d with 1.0 mM DFMA, 1.0 mM DFMO, or no inhibitor. As shown in Table I, neither cell numbers nor dry weight values were significantly different in any treatment. The increase in fresh weight in the presence of DFMA is presumably due to increased cell expansion resulting from an increase in water uptake of the order of 25%.

To confirm the specificity and site of action of DFMO and DFMA, the activities of ADC in the supernatant and ODC in the pellet were determined in cells incubated for 10 d in the

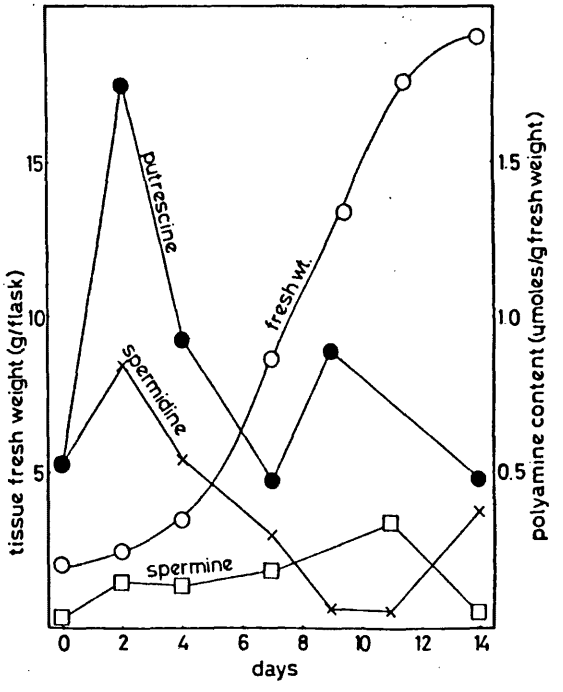


FIG. 1. Mean changes in fresh weight (O) and putrescine (●), spermidine (×), and spermine (□) contents during a 14-d culture period; standard deviation of three replicates was within 15%.

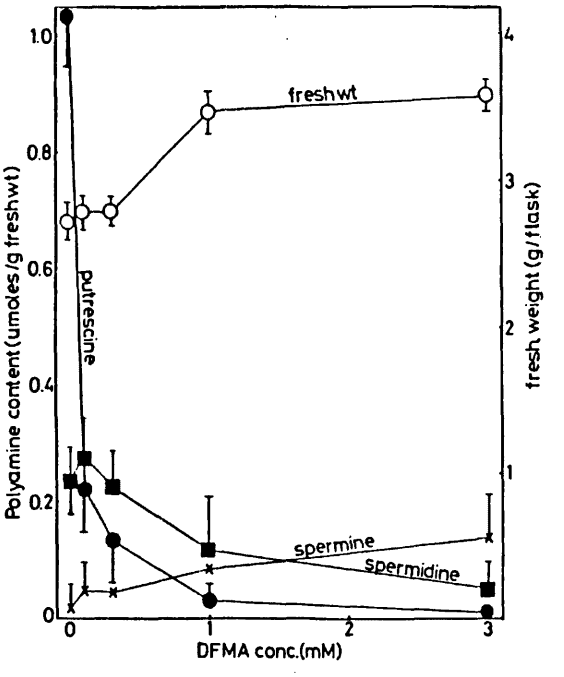


FIG. 2. Effect of DFMA on fresh weight (O) and putrescine (●), spermidine (□) and spermine (×) contents after 10 d in culture; means and standard deviation of three replicates.

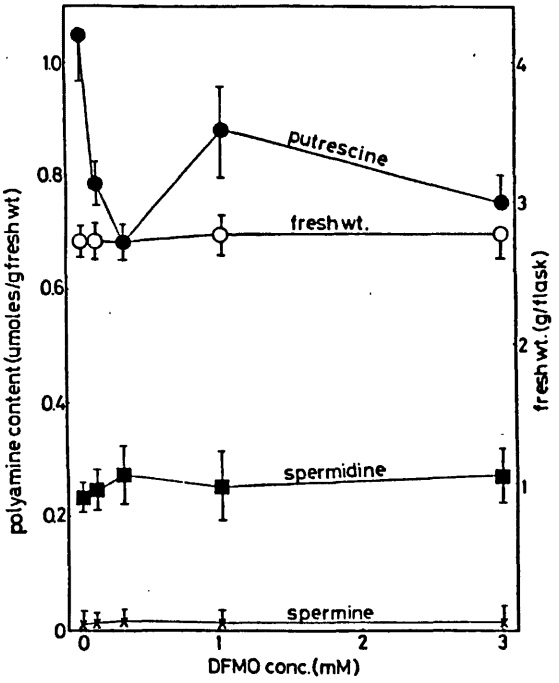


FIG. 3. Effect of DFMO on fresh weight (O) and putrescine (●), spermidine (□), and spermine (×) contents after 10 d in culture; means and standard deviation of three replicates.

Table I. *Cell Number, Fresh Weight, and Dry Weights per Culture of Carrot Cells Grown for 10 d in the Presence of 1 mM DFMA, 1 mM DFMO, or no inhibitor*

Treatment	Cell Number × 10^7	Fresh Weight	Dry Weight
		g	*g*
Control	11.9 ± 0.5[a]	2.75 ± 0.31	0.180 ± 0.006
DFMA (1 mM)	12.5 ± 0.6	3.63 ± 0.29	0.173 ± 0.004
DFMO (1 mM)	12.1 ± 0.9	2.80 ± 0.22	0.170 ± 0.009

[a] Means ± standard errors.

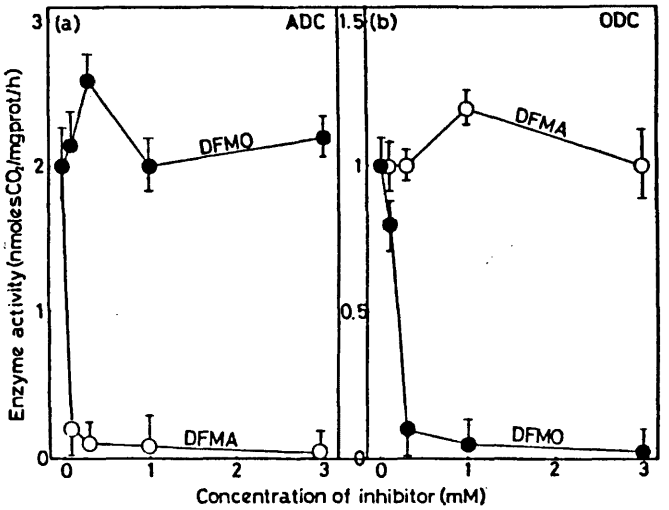


FIG. 4. Effect of DFMA (O) and DFMO (●) on activities of (a) ADC in the supernatant and (b) ODC in the pellet after 10 d in culture; means and standard deviation of three replicates.

presence of either DFMA or DFMO at varying concentrations as before. In the presence of DFMA, ADC activity was dramatically reduced at concentrations as low as 0.1 mM, and virtually no activity was detectable at 3 mM. On the other hand, ODC activity was unaffected by any concentration tested (Fig. 4a). In the complementary experiment, DFMO reduced the activity of ODC by 90% at 0.3 mM, and more than 98% at 3 mM. Again, ADC activity was unaffected by any concentration of DFMO (Fig. 4b). Both ODC and ADC activities found in this study are compatible with those in which the supernatant alone was used for enzyme assay or ADC (19), and studies in which activities of both pellet and supernatant fractions were determined for ODC and ADC assay (7).

DISCUSSION

Substantial evidence of a requirement for active synthesis of polyamines in the growth of some microorganisms and cultured mammalian cells has been reported (7, 14, 29). In the present study, suspension cultured carrot cells have been employed as a model system in which growth, principally by cell division, follows a well-defined and reproducible pattern, in order to determine whether a similar close connection exists for plant cells between polyamine content and growth rates. Changes in endogenous polyamine levels were not found to correlate well with active growth. The concentration of the major amines putrescine and spermidine declined during the period of most rapid growth between 2 and 6 d post-transfer. It is possible that the increases in these amines during the lag period (0–2 d) potentiates subsequent mitosis; however, the absence of mitotic inhibition in the presence of DFMA concentrations that markedly reduce putrescine and spermidine levels weakens this possibility. No consensus currently exists for a correlation between polyamine titers and growth rates in tissue culture. The absence of any clear correlation was reported for embryogenic (18) and nonembryogenic (10) carrot tissue and for Jerusalem artichoke explants (23), although closer correlations were found for rose suspension cultures (25), habituated *Nicotiana* callus (1), and *Scorzonera* tumor tissue (27). In this study, only the minor component spermine maintained elevated levels throughout the period of active cell growth.

The major finding of this study was that cell division was not affected under conditions causing profound alterations in polyamine concentrations and relative proportions. In the presence of 1 mM DFMA, ADC activity was reduced by 98% and the concentration of the major free amines putrescine and spermidine fell by 95% and 50%, respectively. While the minor component spermine increased 2- to 3-fold, the combined free amine titer fell by more than 80%. For changes of this magnitude to occur without discernible change in cell number indicates that no simple causal relationship exists between endogenous polyamine levels and cell division activity. In contrast with the lack of effect on cell division, cell expansion of the order of 25% was reproducibly induced by DFMA. Interestingly, spermine levels increased roughly in parallel with final fresh weights. This, together with the pattern of change occurring during the growth passage, suggests a possible correlation between spermine content and cell expansion.

The response of other cultured tissues to inhibitors of polyamine biosynthesis is again inconclusive, and in only a few studies have precise measurements of cell division activity been made. In a recent study (22) neither DFMA or DFMO at concentrations up to 10 mM affected mitotic rates in short-term Jerusalem artichoke explant tissue. Mengoli *et al.* (17) found DFMO at 5 mM to be without effect on cell numbers in carrot suspension cultures, although cell expansion was stimulated in the post-exponential phase. In contrast, cell division in tobacco cultures was completely inhibited by DFMO, while cell expansion was

stimulated to a remarkable extent (5). It was not clear whether this was due to changes in polyamine metabolism since putrescine levels were not reduced although ODC activity was completely inhibited. Reductions in fresh weight have been reported in response to DFMA in embryogenic carrot cultures (10) and Jerusalem artichoke tissue (4), although in both cases neither cell counts nor amine determinations were made. In the present study, ADC was found to be the principal route of polyamine biosynthesis in carrot cell suspensions, as indicated by the reduction in putrescine and spermidine in response to ADC but not ODC inhibition, confirming previous observations (9). The species dependence of polyamine biosynthetic routes (24) may account for some of the variation reported above in response to single inhibitors; the operation of more than one pathway for polyamine biosynthesis is well established (13, 26).

Acknowledgments—We thank Merrel Dow Research Institute for their kind gift of DFMA and DFMO, Dr. M. Kerr of Shell Research, Sittingbourne, for his help with enzyme assays, and Prof. G. R. Stewart and Dr. M. C. Press for advice and encouragement.

LITERATURE CITED

1. AUDISIO S, N BAGNI, DS FRACASSINI 1976 Polyamines during growth in vitro of *Nicotiana glauca* R. Grah habituated tissue. Z Pflanzenphysiol 77: 146–151
2. BAGNI N, DS FRACASSINI, E CORSINI 1972 Tumours of *Scorzonera hispanica*: their content in polyamines. Z Pflanzenphysiol 67: 19–23
3. BAGNI N, DS FRACASSINI, P TORRIGIANI 1982 Polyamines and cellular growth processes in higher plants. *In* PF Wareing, ed, Plant Growth Substances, Academic Press, London, pp 473–482
4. BAGNI N, B MALUCELL, P TORRIGIANI 1980 Polyamines, storage substances and abscisic acid-like inhibitors during dormancy and very early activation of *Helianthus tuberosus* tuber tissues. Physiol Plant 49: 341–345
5. BERLIN J, E FORCHE 1981 DL-α-Difluoromethylornithine causes enlargement of cultured tobacco cells. Z Pflanzenphysiol 101: 277–282
6. BROWN R, P RICKLESS 1949 A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and nutrients. Proc R Soc Ser B 136: 110–125
7. COHEN E, SM ARAD, YH HEIMER, Y MIZRAHI 1984 Polyamine biosynthetic enzymes in the cell cycle of *Chlorella*. Plant Physiol 74: 385–388
8. COHEN SS 1971 Introduction to the Polyamines, Prentice-Hall, Englewood Cliffs, NJ
9. FEIRER RP, G MIGNON, JD LITVAY 1984 Arginine decarboxylase and polyamines required for embryogenesis in the wild carrot. Science 223: 1433–1435
10. FIENBERG AA, JH CHOI, WP LUBICH, ZR SUNG 1984 Developmental regulation of polyamine metabolism in growth and differentiation of carrot culture. Planta 162: 532–539
11. FLORES HE, AW GALSTON 1982 Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol 69: 701–706
12. FOZARD JR, ML PART, NJ PRAKASH, J GROVE, PJ SCHECHTER, A SJOERDSMA, J KOCH-WESER 1980 L-Ornithine decarboxylase: an essential role in early mammalian embryogenesis. Science 208: 505–509
13. HEIMER YM, Y MIZRAHI, U BACHRACH 1979 Ornithine decarboxylase activity in rapidly proliferating plant cells. FEBS Lett 104: 146–148
14. HERBST EJ, EE SNELL 1948 Putrescine as a growth factor for *Haemoplilus influenzae*. J Biol Chem 176: 986–990
15. KALLIO A, MCCANN PP, P BEY 1981 DL-alpha-(Difluoromethyl)arginine—a potent enzyme activated irreversible inhibitor of bacterial arginine decarboxylase. Biochemistry 20: 3163–3166
16. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
17. MENGOLI M, N BAGNI, S BIONDI 1987 Effect of alpha-difluoromethylornithine on carrot cell cultures. J Plant Physiol 129: 479–485
18. MONTAGUE MJ, TA ARMSTRONG, EG JAWORSKI 1978 Polyamine metabolism in embryogenic cells of *Daucus carota* I. Plant Physiol 62: 430–433
19. MONTAGUE MJ, TA ARMSTRONG, EG JAWORSKI 1979 Polyamine metabolism in embryogenic cells of *Daucus carota* II. Plant Physiol 63: 341–345
20. MURASHIGE T, F SKOOG 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–479
21. MURTY KS, TA SMITH, C BOULD 1971 The relation between the putrescine content and potassium status of blackcurrant leaves. Ann Bot 35: 687–695
22. PHILLIPS R, MC PRESS, L BINGHAM, C GRIMMER 1988 Polyamines in cultured artichoke explants; effects are primarily on xylogenesis rather than cell division. J Exp Bot 39: 473–480
23. PHILLIPS R, MC PRESS, A EASON 1987 Polyamines in relation to cell division and xylogenesis in cultured explants of *Helianthus tuberosus*: lack of evidence for growth regulatory action. J Exp Bot 38: 164–172
24. SLOCUM RD, R KAUR-SAWHNEY, AW GALSTON 1984 The physiology and biochemistry of polyamines in higher plants. Arch Biochem Biophys 235: 283–303
25. SMITH TA 1985 Polyamines. Annu Rev Plant Physiol 36: 117–143
26. SMITH TA, GR BEST, J ABBOTT, ED CLEMENTS 1978 Polyamines in Paul's Scarlet Rose suspension cultures. Planta 144: 63–68
27. SPERANZA A, N BAGNI 1977 Putrescine biosynthesis in *Agrobacterium tumefascens* and in normal and crown gall tumours of *Scorzonera hispanica*. Z Pflanzenphysiol 81: 226–233
28. SPRAGG BP, HUTCHINGS AD 1983 High performance liquid chromatographic determination of putrescine, spermidine and spermine after derivatization with 4-fluoro-3-nitrobenzotrifluoride. J Chromatogr 258: 289–291
29. TABOR CW, H TABOR, AK TYAGI, MS COHN 1982 The biochemistry, genetics and regulation of polyamine biosynthesis in *Saccharomyces cerevisae*. Fed Proc 41: 3084–3088

in adapted cells compared with controls. Proline levels continued to increase in adapted cells up to the highest concentration tested, while in unadapted cells no increase occurred beyond 20% PEG.

The combined cellular concentration of the major free amino acids shows a strong correlation with medium solute potential in adapted cells (Fig. 4a). The response was approximately linear at lower solute potentials and was not saturated at the highest PEG concentration employed. In unadapted cells, however, the response was far less pronounced, with a doubling in concentration over the control, as opposed to an 8-fold increase in adapted cells. While the response of individual non-proline amino acids varies considerably (Table 2), higher concentrations are generally found in adapted rather than unadapted cells. Alanine concentration increased up to 18-fold over control values (adapted cells), but the highest concentrations observed were those of histidine, arginine, and valine, which were relatively abundant in control cells. With the exception of serine, all amino acids were present at higher concentration in adapted than unadapted cells at the lowest solute potential. However, proline was the most abundant amino acid by a factor of 2 and constituted 30% of the total amino acid concentration.

Betaine concentration changed little in either cell line in response to increasing water stress (Table 2).

Sucrose

The cellular sucrose concentration increased approximately 3-fold up to 120 mol m^{-3} in adapted cells under

Free amino acid and glycinebetaine concentrations in unadapted (U) and adapted (A) cell lines

Mean of 3 replicate determinations.

Amine		PEG concentrations				
		0%	10%	15%	20%	25%
		mol m^{-3}				
Asp	U	0·57	0·50	0·36	0·65	0·64
	A	0·57	0·87	0·85	1·06	1·57
Gln	U	0·71	0·72	0·79	0·74	0·71
	A	0·71	0·99	1·11	1·12	1·11
Ser	U	1·67	1·82	2·11	2·41	2·45
	A	1·67	1·19	1·87	4·20	1·95
His	U	2·16	2·14	2·57	2·50	2·74
	A	2·16	3·13	3·73	6·60	12·69
Gly	U	0·33	0·28	0·39	0·45	0·58
	A	0·33	0·36	1·18	0·83	1·12
Thr	U	0·89	0·85	0·99	1·05	1·09
	A	0·89	0·76	1·53	1·78	2·29
Arg	U	1·32	1·52	1·89	1·95	1·94
	A	1·32	1·81	2·68	3·53	8·66
Ala	U	0·29	0·34	0·83	1·11	1·09
	A	0·29	0·24	0·40	5·32	5·48
Tyr	U	0·17	0·17	0·18	0·21	0·24
	A	0·17	0·28	0·34	0·36	0·58
Met	U	0·25	0·34	0·35	0·37	0·37
	A	0·25	0·32	0·53	0·58	0·67
Val	U	0·83	1·24	1·44	1·86	1·90
	A	0·83	1·86	2·59	4·63	7·07
Phe	U	0·86	1·14	1·20	1·44	1·88
	A	0·86	0·65	1·03	1·44	1·73
Ile	U	0·41	0·49	0·64	0·80	1·06
	A	0·41	0·58	0·82	1·12	2·88
Leu	U	0·22	0·29	0·37	0·48	0·59
	A	0·22	0·40	0·54	0·62	1·78
Betaine	U	0·73	0·78	1·01	0·85	0·93
	A	0·73	0·66	0·80	0·82	0·77

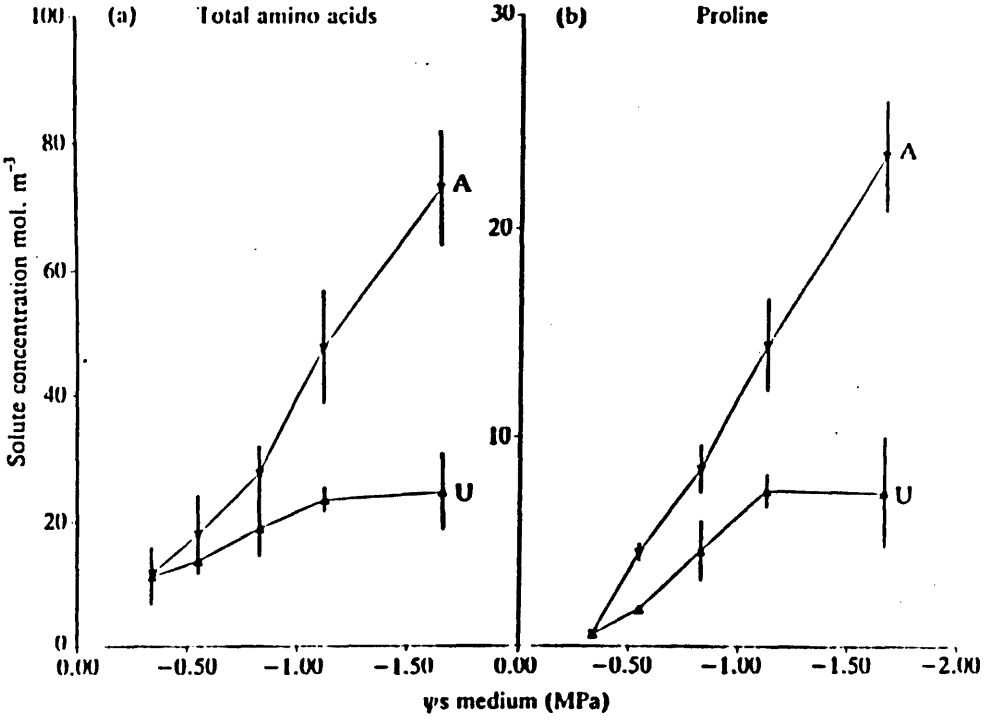


FIG. 4. Changes in total amino acids (a) and proline (b) in unadapted (U) and adapted (A) cells after 10 h incubation at a range of PEG concentrations; means and standard deviation of three replicates.