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Metabolic Engineering of Plant Cells in a Space Environment

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Introduction

Metabolic engineering aims to optimize yields of commercially valuable products by controlling enzymatic, transport, and cell regulatory functions by indirect or direct genetic intervention. Many natural products from plants on earth are now being metabolically engineered (eg Grotewold *et al.*, 1998; Knauf, 1998; Gruys *et al.*, 1998). While large-scale culture is technically feasible (Verpoorte *et al.*, 1998), plant cell bioreactors have historically given economically low and unreliable yields (Alfermann and Petersen, 1995; Fowler, 1988). Shikonin and berberine (Fujita, 1988) raised hopes for products that were eventually not commercially sustainable. The recovery of products from vacuoles requires harvesting and disruption of cells. Processes are sought where products are released directly into the culture medium. Slow growth and low product levels are major constraints.

Space environments offer new opportunities for the metabolic engineering of plant cells. Bioreactor process controls will differ significantly from production facilities on earth because of constraints in gravitational fields, the increased use of small modular designs, and physical limitations to downstream processing capabilities. For the International Space Station (ISS), spacecraft construction and maintenance of crew support systems will initially limit the time for basic and applied research. This review evaluates factors for the development and testing of models for drug producing plant cells, given the constraints of space environments. Mission priorities are given

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Abbreviations: RWV, rotating wall culture vessel; HARV, high aspect rotating culture vessel; MEMS, micro-electro-mechanical systems; NEMS, nano-electro-mechanical systems; ISS, International Space Station; DNA, deoxyribonucleic acid; HHT, homoharringtonine; NIH, National Institutes of Health; CaM, calmodulin; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP-biotin nick-end labelling; FITC, fluorescein-5-isothiocyanate; PCNA, proliferating cell nuclear antigen; LPA, lysophosphatidic acid; LBPA, lysobisphosphatidic acid; ELISA, enzyme-linked immunosorbent assay; AANBA, alpha amino-n-butyric acid; SEM, scanning electron microscopy.

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to enhancing human survival, fitness, and to the commercialization of space. Appended to this are ground-based studies for the development of fundamental systems biology and counter-measures for adaptive compensatory mechanism for processes in space environments.

The development of micro- and nano-electromechanical systems (MEMS and NEMS) promises to provide new models abased on individual cells by controlling gravitational forces and space environments at micron levels (Amato, 1998; Montemagno *et al.*, 1998; González *et al.*, 1998). Modular and smaller lightweight systems also are available for forensic and clinical work. While systems miniaturization does not solve the problem of large extraction and culture, it provides better economies of scale, novel hardware, and sensors for specialized bioreactors for counter-measures and leading to the eventual commercialization of space (Anon., 1998a).

Concepts of metabolic engineering (Bailey, 1991; Stephanopolous and Vallino, 1991) and metabolic control theory (Sauro *et al.*, 1987) for simple organisms offer applications for plants capable of producing a wide range of useful secondary products. Plants produce over 23,000 individual isoprenoids that comprise hormones, defence agents, visual pigments, membrane constituents, mating pheromones, photoprotective agents, components of signal transduction networks, and useful anticancer drugs (Sacchettini and Poulter, 1997). Between March 1992 to September 1994, 101 new taxoids (taxane diterpenoids) were discovered, bringing the total to nearly 300 compounds (Appendino, 1995; Kingston *et al.*, 1993). Conifers were designated of high interest by the National Cancer Institute survey (Barclay and Perdue, 1976) as having significant anticancer activity. Conifers also offer complications for metabolic engineering not always found in simpler and more recently evolved plants such as *Arabidopsis*. Over 200 million years, conifers have coupled the diversification of secondary products with ontogenetic programs for longevity, adaptive plasticity, secondary xylem, and reaction (or compression wood) in response to gravitational and mechanical forces well before angiosperms evolved (Havel and Durzan, 1996a).

Paclitaxel (Taxol™, Suffness, 1995) and homoharringtonine (HHT), (Delfel, 1980; Wickremesinhe and Arteca, 1993) are effective anti-cancer drugs (CancerNet, 1996). Paclitaxel from *Taxus* sp. inhibits mitosis and has a market value of c. US$600 per gram. Other taxane derivatives whose utility remains untested are valued to US$6 million (Hauser Chemicals, Colorado). The demand for paclitaxel will increase for the treatment of breast, ovarian, lung, and silent cancers, in treating congenital polycystic kidney disease (Woo *et al.*, 1994), and in controlling rheumatoid arthritis (Brahn *et al.*, 1994; Angiogenesis Technologies, 1996). While paclitaxel has been chemically synthesized (Holton *et al.*, 1994), the process is lengthy and economically unfeasible. Semi-synthesis from precursors is a preferred alternative (Suffness, 1995). Bioassays are needed to ensure that the products have the required properties.

HHT and harringtonine from *Cephalotaxus* sp. inhibit the initiation of protein synthesis (Huang, 1975; Parry *et al.*, 1980), DNA synthesis, and cell cycling (Xue *et al.*, 1984). HHT is currently in clinical trials for the treatment of leukaemia. HHT is partially synthesized from cephalotaxine (Zhao *et al.*, 1980). Yet unidentified lignans that show anti-leukaemic properties occur in the endangered *Fitzroya* sp. (G. Cragg, NIH, personal communication).

What is the import of all of this for space? Innovations in technology for counter-

measures in space exploration should improve life on earth and in space (Eckhart, 1996). What are the cutting edge technologies, processes, techniques, and engineering capabilities that must yet be developed? Can bioreactors provide biological materials that support long-duration space travel? Are there new probes using living cells or subcellular components to survey the impact of extreme environments on survival and crew health? Can plant cells provide models for the development of space-based industrial processes? What are the effects of weightlessness and accelerations on plant cells? Can gravitational forces increase the number of drug producing cells, metabolic flux, and decrease catabolism? These are some of many questions that do not yet have clear answers.

Gravitational forces: problems and opportunities

Gravity is a force of attraction between all matter. It affects virtually all physical, chemical, and biological processes in cells. In microgravity, adaptive plasticity, longevity, utility, and counter-measures are sought for long-term missions. On earth, zero gravity or weightlessness is virtually impossible to achieve because of the influence of earth's mass (Vogt and Wargo, 1995). Orbiting spacecraft can provide $10^{-6} \times$ g, while clinostats and bioreactors on earth can simulate microgravity to c. $10^{-4} \times$ g. In a reduced gravity environment, relative motion is slowed in direct proportion to the reduction in net acceleration. Mass forces occur between cells, among organelles and particles greater than 10 microns (Nechitailo and Mashinsky, 1993).

Low gravity can reduce by orders of magnitude the effects of buoyancy, sedimentation and hydrostatic pressure. Microgravity may allow a better understanding of large-scale colloidal aggregations or clustering without the complications of different sedimentation rates due to size and particle distortions in unit gravity. Capillary forces and surface-tension-driven flows become increasingly dominant for the interactions and coalescence of drops and bubbles as gravity is reduced. We cannot yet predict the effects of magneto/electrohydrodynamics, multiphase flows, diffusive transport, heat transfer, polymer membrane processing, and phase changes, eg boiling and condensation. The random accelerations and vibrational environments within a manned spacecraft raise issues of what are the tolerable levels for biotechnological processing. Jumping exercises on space craft introduce forces up to 9×10^{-2} g. The docking of one station with another may introduce up to 5×10^{-1} g (Nechitailo and Mashinsky, 1993). For a payload of 1,000 kg in the free-flying EURECA satellite, microgravity will range from < 1 Hz (10^{-5} g) to > 100 Hz (10^{-3} g).

A change in the vector created by gravity represents an 'orientation error' referenced to unit gravity or some other standard. The error path describes the order of preceding points or events (Buerger and Dollase, 1964). Microgravity introduces opportunities for the metabolic conversion of food and downstream processing where biofilms, foams, particles and droplets do not settle, stratify, and alter phases or redistribute products. Gravity-dependent biomaterial processing strategies will be needed for the establishment of habitats on the Moon using *in situ* resources.

PLANT RESPONSES TO GRAVITY

Plants respond to gravity with a tropic curvature that results from signal transduction

and an asymmetric auxin distribution for differential cell elongation (Fukaki *et al.*, 1996). Mutants show that gene products act on gravity perception or the signal transduction to regulate auxin distribution. Auxins and cytokinins affect the cytoskeleton by activating signalling cascades altering pH, pCa^{2+}, and producing lipophilic and ionic second messengers that directly alter the tension of actin networks (Grabski and Schindler, 1996). An auxin-binding protein is secreted from the endoplasmic reticulum to the plasma membrane and cell wall (Jones and Herman, 1993). Auxins activate phospholipase A on the plasma membrane to generate a free fatty acid and a corresponding lysophosphatidic acid (Chandra *et al.*, 1996). Auxin-stimulated phospholipase A activates a protein kinase that phosphorylates an ATPase for proton pumping, a plasma membrane NADH oxidase, and releases membrane-bound phosphatidylinositol kinase. Phospholipase A products and their metabolites are also involved in other plant defence responses.

Gravity sensing has evolved several times in plants through gravitropism of organs, gravimorphism, gravitaxis and through cytoplasmic streaming (Sack, 1997). Subcellular architecture and prestress provide a direct path through molecular connections along nuclear and cytoplasmic scaffolds for mechanical signal transfer (Ingber, 1997). For cells in aquatic environments, Wayne and Staves (1997) have challenged the statolith model for plastids, and offer a testable gravitational pressure hypothesis for total cell mass. Plastids are a primary source for the biosynthesis of isoprenoids. Gaps in knowledge require a better understanding of threshold determinations in graviperception and response (Ruyters and Scott, 1997). Gravity-induced changes in the cytoskeleton will need physico-chemical quantification of 'memory effects', phase changes in life histories, and adaptive plasticity.

Only indirect evidence supports the role of calcium in graviperception (Ruyters and Scott, 1997). Calcium binding to calmodulin (CaM) depends on interactions with target proteins (Zielinski, 1998). This may determine how Ca^{2+} signals are transduced to give physiological responses. It remains unclear how gene families encoding CaM and CaM-like proteins function in response to gravitational and mechanical forces. Genetic strategies for the expression of target proteins are being pursued over a wide range of physiological responses, as in the case of the touch (*TCH*) family of genes (Antosiewicz *et al.*, 1997; Johnson *et al.*, 1998). *TCH1* encoded calmodulin, *TCH2* and *TCH3*, and several CaM-like proteins. *TCH4* encoded a xyloglucan endotransglycosylase that breaks and joins xyloglucans in hemicellulose for adding new xyloglucans to growing and mechanically stressed cell walls (*Figure 13.1*).

SPACE SYNDROMES

Bodily adjustments to space by astronauts typically take four days. Thereafter, the adaptation continues to decline in water-electrolyte regulation, blood-cell regeneration, and bone changes (Scano and Strollo, 1996). Plant organs are thought to perceive 10^{-3} to 10^{-4} g. Cells can perceive g signals lasting 0.5 sec, but we do not yet know the long-term sequence of events for adaptive plasticity in space because conditions vary and flights are of comparatively short duration. For whole plants, changes in gravitropism, circumnutation, circadian phase shifts, phototropism, hyponastry, enzyme activity, thigmomorphogenesis and genomic abnormalities occur well within 90 days (eg Krikorian and Levine, 1991).

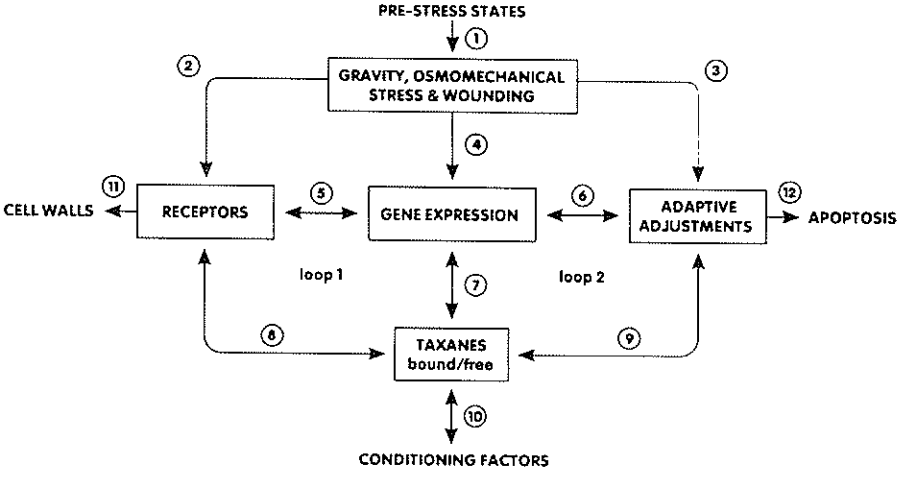

Figure 13.1. Open-architected model shows the relations among twelve propositions involving GENE EXPRESSION for touch *(TCH)* and taxane early-response *(TER)* gene families over a wide range of gravitational forces. Pre-stress physiological states comprise a memory that conditions the responses to GRAVITY, OSMOMECHANICAL STRESS and WOUNDING. Signals are transduced via RECEPTORS in cells and at cell walls. Thresholds for ADAPTIVE ADJUSTMENTS by organelles and subcellular structures are evident as induction, maturation, maintenance, gain, loss, attunement, and facilitation. Taxane production by cells under stress inter-operates with system parameters to create *TER* stress syndromes. Free and bound taxanes, and other CONDITIONING FACTORS are released into the culture medium by exocytosis, physical expulsion, and by diffusion. Metabolic and lytic activities (eg xylosidases, apoptosis) further elaborate the range of taxanes recovered from cell suspensions. Loops 1 and 2 comprise TCH activities that alter cell walls. Other proteins, eg expansins, also offer adaptive adjustments to gravity, osmolality, stress, and wounding. Failure to adapt predisposes p53-independent apoptosis in cells (Durzan, 1999).

Gravitational forces have led to 'space syndromes' and genomic decay (Krikorian, 1998, 1996a,b). Completely developed and morphologically complex plants were less likely to show stress than cells during growth. The nature and larger sizes of genomes were important with polyploids being more stress tolerant in space environments. Multiple factors and interactions brought about aberrant karylogical responses. A single space variable was inadequate to produce a direct significant adverse response, unless the stress was severe. Severe stresses resulted in genomic decay (micronuclei, bridges, breaks, ring chromosomes, etc.). Chromosomal damage in plants depended on many factors that could be eliminated by the appropriate 'countermeasures' (Krikorian, 1998). Among these are better control over the delivery of water, nutrients, and gases. The process-controlled bioreactors, such as the low shear Rotating Wall Vessels (RWVs) with cell suspensions, offer some advantages here (Unsworth and Lelkes, 1998).

Hypergravity predisposed programmed cell death (apoptosis) and led to the overproduction of taxanes (Durzan *et al.*, 1998). At unit gravity, the uptake of taxanes induced the expression of 'taxane early response genes' (Moos and Fitzpatrick, 1998). In animal cells, this led to the induction of transcriptional regulators, inflammation, and apoptosis. For plants in simulated microgravity, products from stressed cells were released that, when assimilated by other cells, recalibrated and promoted cell divisions.

ADAPTIVE AGEING, LONGEVITY, AND APOPTOSIS

Adaptation is a 'special and onerous' concept that cannot be applied lightly (Rose and Lauder, 1996). Most experiments with eukaryotic cells consistently show that prolonged exposure to true microgravity has detrimental effects on cells (Albrecht-Beuhler, 1992). For plants failing to adapt, a model for programmed cell death (apoptosis) has been formulated (Havel and Durzan, 1996a).

Ageing is arbitrarily defined as the consequence of *genetic* lesions that accumulate over time, but by themselves do not necessarily cause death. The word 'ageing' is avoided by Finch (1990) because many age-related changes have little or no adverse effect on vitality or life span. *Genetic ageing* comprises a wide array of degenerative processes, driven primarily by exogenous factors. *Adaptive ageing* is deterioration (phenotypic and genotypic) due to adaptation to stressful exogenous factors while the genome is attempting to maintain genetic fitness. *Accelerated ageing* is a conserved mechanism related to nucleolar fragmentation in yeast (Sinclair *et al.*, 1997).

At unit gravity and under stress, cells accumulate unfolded proteins in the endoplasmic reticulum (ER) by increasing the transcription of genes encoding ER resident proteins (Chapman *et al.*, 1998). This information is transmitted from the ER lumen to the nucleus by an intracellular signalling pathway called the unfolded protein response. The pathway uses translational attenuation and a regulated mRNA splicing step. In the absence of chaperones, heat shock proteins, or betaines to maintain protein structures, this situation would lead to the turnover of unfolded proteins by ubiquitination (Bonifacino and Weissman, 1998). The result would change the physiological states of cells as an expression of the adaptive plasticity of regulatory proteins in cells. We do not know if, or how, the misfolding of plant proteins contributes to adaptive ageing and space syndromes.

The best examples of longevity are shown by conifers, which are among the tallest and most ancient and longest-lived organisms (Finch, 1990). The potential for limitless life-cycle extension is based on cloning complete plants from single culture cells ('*totipotency*') by Steward (1968). The concept was extended (Durzan and Steward, 1984) to the molecular level and to cycles of nuclear-cytoplasmic determination. Totipotency was then related to latent apomictic plasticity with palingenic, cenogenic, and progenic expressions (Durzan, 1996b), and to adaptive programmes in apoptosis (Havel and Durzan, 1996a). Conifer genomes were expressed millions of years ago when days were less than 24 hours, and gravitational forces were less than $1 \times g$ (Nechitailo and Mashinsky, 1993; Dott and Prothero, 1994). Gene expression under simulated Cretaceous conditions of high temperatures and carbon dioxide (Crowley and North, 1991) has revealed latent apomictic features under today's conditions (Durzan, 1996b).

Apoptosis (Gk *apo* away from, *ptosis* falling) is a phenotypically distinct form of genotypically programmed cell death. It plays a major role during development and homeostasis, and in the expression of many diseases (Gilchrist, 1997). Apoptosis originally referred to the loss of petals from flowers, or leaves from trees (Havel and Durzan, 1996a). Endonuclease-mediated apoptosis of nuclei and of the scattered nucleoids (Gillham, 1994) in Kalanchoë chloroplasts was detected cytochemically (Pedroso and Durzan, 2000a). Nucleoids degenerated in response to changes in gravitational forces. Cut ends of DNA were detected by a terminal deoxynucleotidyl

transferase (TdT)-mediated dUTP-biotin nick-end labelling (TUNEL reaction, Havel and Durzan, 1996b). TdT labelled the 3'OH ends of DNA, which were formed by the endonuclease. The assay used a secondary system with cy3, rhodamine, FITC or peroxidase and a substrate to detect individual apoptotic nuclei and fragments. Calcium-activated endonuclease activity was also detected in nuclear fragments in cells exposed to free taxanes and paclitaxel (Durzan *et al.*, 1998). At 150 × g for 4 months without subculture, most cells were wounded, terminally differentiated, or apoptotic. Slightly injured amyloplasts and chloroplasts curled and moved to the cell wall (Wildman *et al.* 1974). Amyloplasts become vesicular and their membranes fuse with the endoplasmic reticulum Golgi and plasmalemma (Durzan, 1999). In hypergravity, nuclei were slightly flattened perpendicular to the applied force.

Proliferating cell nuclear antigen (PCNA) for DNA processivity (Kellman, 1997) and anti-RAG-1,2 (recombination activating proteins) are markers for the recovery from genomic decay, and for the adaptive benefits of apoptosis. In latent diploid parthenogenesis (Durzan *et al.*, 1994), apoptotic cells released material with anti-PCNA activity (eg Durzan *et al.*, 1999). PCNA was found bound to chromosomes and, as cycling continued into G1, the PCNA recalibrated the rate of DNA synthesis such that the divisional cycles maintained rates typical of embryonic cells.

In human cells, the tumour necrosis factor, p53, implements cell cycle arrest in response to DNA damage and activates the p21 gene for p53-dependent cell death (Gorospe *et al.*, 1997). The p21 product of the *WAF1* gene binds to PCNA and stops DNA synthesis. Loss of both p53, which suppresses cell growth, and of the retinoblastoma protein lead to cell proliferation. Over a wide range of gravitational forces, two paths to cell death were detected in *Taxus* cells. A few cells (< 0.001%) expressed proteins reactive to specific epitopes of monoclonal p53 (amino acids 371–380 of human p53) and monoclonal p21 (amino acids 58–77 of human p21), presumably when DNA could not be repaired. Levels of p53 were predicted to increase in response to DNA damage or other cellular distress signals. Presumably, overexpression of the p53 gene's transcription factor induced either cell cycle arrest or apoptosis by regulating other genes such as the cell cycle inhibitor p21 (Evan and Littlewood, 1998). p21 was not needed for the induction of p53. Using cDNA arrays, p53 was found to regulate the stress-induced secretion of serine proteinases and other components that either attenuated or inhibited the growth of adjacent cells (Hill and Diatchenko, 1998).

Most *Taxus* cells exposed to paclitaxel and taxane died by a p53 and p21 *independent* pathway. In animal cells, paclitaxel also caused p53-independent apoptosis (Lanni *et al.*, 1997; Wahl *et al.*, 1996). Mechanical and hypo-osmotic stresses created an oxidative burst (Cazalé *et al.*, 1998) that could widen the range of taxane metabolites by hydroxylation. The assimilation of lipophilic taxanes by membrane vesicles with an ATP-dependent cassette family transporter was responsible for conferring multidrug resistance to cells (Paul *et al.*, 1998).

'Secreted apoptosis related proteins' (SARPs) were recovered from atrophying *Taxus* cells. In populations of cells showing p53-independent death, a SARP with anti-PCNA (proliferating nuclear cell antigen) reactivity was bound to all chromosomes. Binding of PCNA to chromatin has the potential to recalibrate and enhance rates of divisional cycling. In *Cupressus* callus at unit gravity, PCNA activity was found in rapidly cycling nuclei, but not in cells during xylogenesis (Havel *et al.*,

1997). Microtubule depolymerization early in divisional cycles is sufficient to initiate DNA synthesis (Crossin and Carney, 1981). 'Apoptotic chaperones' activate proenzymes for programmed cell death (Hengartner, 1998).

1-Acyl-2-lyso-*sn*-glycero-3-phosphate (lysophosphatidic acid, LPA) is nature's simplest glycerophospholipid. In plants, LPA is a product of the auxin-stimulated phospholipase defence mechanism (Chandra *et al.*, 1996). In animal cells, it suppressed apoptosis in cells after gamma radiation (Guo *et al.*, 1996). Extracellular LPA activates second messenger pathways to elicit diverse cellular responses in many types of cells by coupling to guanine nucleotide regulatory (G) proteins (Fukushima *et al.*, 1998). G proteins also activate phospholipase A (Chandra *et al.*, 1996). Lysobisphosphatidic acid (LBPA) is a marker for the antiphospholipid syndrome (Kobayashi *et al.*, 1998). LBPA regulates endosome multivesicular and multilamellar structure and function during the formation of lysosomes. It may also be involved in protein and drug sorting during the early response to taxanes in stressed plant cells.

Autophagy is the bulk delivery of cytoplasmic materials to the lysosomes for cell survival during starvation and differentiation (Mizushima *et al.*, 1998). In conifers, cannibalism serves the same function in brood reduction (Haig, 1992). Re-use of plant cells is a response to sucrose starvation where proteins are degraded non-selectively (Moriyasu and Ohsumi, 1996; Haig, 1992). Autophagy in yeast and mammalian cells employs a large tag, unrelated to ubiquitin, to mark proteins for structures called autophagosomes, with double membranes. The latter deliver bulk proteins to lysosomes or vacuoles for degradation. In microgravity, autophagy and the build-up of metabolic products can lead to the build-up of toxic metabolites around cells (Albrecht-Beuhler, 1992).

Metabolic engineering

MODEL PLANT SYSTEMS

Ground-based studies have demonstrated some practical aspects of plant metabolic engineering using direct genetic intervention. This required accessibility to the gene encoding the desired enzyme or protein, ideally from several gene sources; a technique for transforming the host organism; cDNA cloning, and making of promoter cassettes; co-cultivation of target cells with the vector; and adequate expression of the gene. In theory, this is accomplished by controlling the pathways, prosthetic groups, enzyme properties, protein sequences and folding, substrate availability, inhibitory environments, and predicted side reactions. Genetic engineering of gymnosperms has been confined to conifers (Ellis, 1995). Despite few successes, most conifers have yet to be stably transformed. The main limitation to genetic engineering has been the inability to regenerate plants from transformed cells. Two recent U.S. patents have addressed the problem of regeneration from cell suspensions (**5**,821,126, 1998; **5**,840,567, 1998).

With angiosperms, a recombinant bacterial antigen produced in transgenic agronomic plants now appears feasible for human oral immunization. This illustrates the rapid progress with plants as pharmaceutical processors (Tacket *et al.*, 1998; Arntzen, 1998). Potatoes expressing the antigen have protected mice and humans against the *E. coli* heat-labile enterotoxin causing diarrhoea. This technology represents a milestone

in creating vaccines that are useful in immunizing people, eg against hepatitis and diarrhoea, where high cost and logistical issues thwart vaccination programmes.

Regulatory genes have been used to engineer two separate but overlapping pathways of flavanoid metabolism (Grotewold *et al.*, 1998). Pathways accumulate two cyanidin derivatives in differentiated maize tissues. Phenylpropanoids and green fluorescent compounds were targeted to different subcellular compartments by vesicle trafficking pathways that sorted metabolites into separate subcellular compartments. This work showed how a gene might act as a quantitative trait locus controlling levels of insecticidal C-glycosyl flavone in maize silks.

Biodegradable plastics have been produced by multigene pathways in transgenic plants (Gruys *et al.*, 1998). As an alternative to fermentation by *Ralstonia*, plants generated the appropriate intermediates for polyhydroxyalanoates (PHAs) from common carbon metabolite pools. This strategy employed carbon from different developmental stages, and targeted seeds and leaves as sites for biosynthesis in a 2-step process.

The engineering of fatty acid ratios in major oilseed crops has created novel vegetable oils (Ohlrogge, 1994). Oils have been engineered with enhanced levels of capric, stearic, ricinoleic, and nervonic fatty acids (Knauf, 1998). This work demonstrated how the 'superficial simplicity' of making a single gene change in a strong pathway affected the timing and production of fatty acids. Genes were introduced into oilseed crops from fruits having little trans fatty acids. Stress tolerance was improved by introducing properties of one single stress factor, eg salt stress tolerance in transgenic tobacco expressing a bacterial choline dehydrogenase (Lilius *et al.*, 1996). By contrast, cholesterol-lowering plant products are available in bulk from other processes without genetic engineering (Hicks, 1998). For example, pine oil and resin by-products, or 'tall oil', of the pulp and paper industry are a source of micronized sterols, sitostanol fatty acid esters, etc. These have led to the development of products for margarine and their chlolesterol-lowering properties are being reaffirmed by clinical studies in several countries.

Using gene overexpression, antisense, or co-suppression technologies, plants have been tailored to produce alkaloids and condensed tannins by introducing side pathways, eliminating side pathways, or accumulating biosynthetic intermediates (Kutchan, 1995; Robbins *et al.*, 1998). For isoprenoids, the cloning of genes for synthases and better understanding of secondary transformations still leaves significant gaps (McCaskill and Croteau, 1997).

Seedlings of *Taxus brevifolia* genetically transformed by *Agrobacterium rhizogenes* produced hairy roots with very rapid growth but with little or no paclitaxel being reported (Flaut-Carcasson *et al.*, 1993). With *T. brevifolia*, and *T. baccata* transformed by *A. tumefaciens*, paclitaxel production detected by a monoclonal antibody immunoassay method (ELISA) varied from 0.08 to 4.0 μg/g dry weight (Han *et al.*, 1994). Untransformed callus contained 8.0 μg/dry weight. Paclitaxel recovery was not significantly correlated with cell growth ($R^2 = 0.19$). The benefits cited were based on the notions that the phytohormone-free and fast-growing transgenic lines can serve as a 'bioreactor' for paclitaxel by feeding precursors. The system is also useful for studying biosynthesis and, in the long run, for the direct manipulation of rate-limiting enzymes that could be used to increase paclitaxel formation. This notion was based on the model for overproducing amines by genetic transformation (Berlin *et al.*, 1992).

GENETIC AND PHYSIOLOGICAL REQUIREMENTS FOR TAXANE BIOSYNTHESIS

Paclitaxel has a complex 4-member taxane ring with an oxetane moiety and an N-phenylisoserine side chain attached at the carbon 13 of the ring (Kingston *et al.*, 1993). The N-phenylisoserine side chain is a product of phenylpropanoid metabolism that becomes N-benzoylated during the final assembly of the drug. The taxane ring of paclitaxel is derived from geranylgeranyl pyrophosphate and baccatin III, or a related metabolite in chloroplasts. Ring formation is rate limiting in some but not all systems (Hezari and Croteau, 1997; Srinivasan *et al.*, 1996). The biosynthetic pathway(s) appear not of mevalonate origin, but have features of the alternative pathways for isoprenoids seen in some eubacteria (Eisenreich *et al.*, 1996). Procedures for the isolation and analysis of all 11 intermediates of the mevalonic acid pathway for acetyl-CoA through geranylgeranyl pyrophosphate have been developed (McCaskill and Croteau, 1997). Antibodies with fluorescent tags were available for both parts (ring and side chain) of paclitaxel, and for other taxanes used in immunocytochemical studies and in competitive inhibition enzyme immunoassays (CIEIA) (Grothaus *et al.*, 1995).

In space environments, the taxane early response (*TER*) and 'touch' (*TCH*) gene families need consideration. *TER* genes (Moos and Fitzpatrick, 1998) appear partially responsible for gravitationally induced space syndromes in *Taxus*. They lead to altered physiological states and apoptosis. The induced gene activities may have impacted the acid pH induced loosening of cell walls by expansins (Cosgrove, 1998), and mediated the pleiotropic effects of *TCH* genes (Johnson *et al.*, 1998). TCH4 activity exposes xylan residues for the formation of xylosyl 7-OH taxane derivatives (Durzan, 1999). A recombinant brassinosteroid protein also acted as a functional xyloglucan endotransglycosylase (Clouse and Sasse, 1998). The recovery of taxanes from wood and cell cultures is described by U.S. Patents No. **5**,019,504; **5**,407,816; **5**,547,866 and **5**,670,663.

In unit gravity, the control over biosynthesis and discharge of taxanes into culture media requires a different substrate supply management strategy involving membranes. Amyloplast substrates are processed for ring and side chain moieties for final paclitaxel assembly. Processing may be achieved by altering paths of vesicular trafficking, enzymatic modifications, docking, and fusion. Directional organelle movement, microtubule–microtubule sliding at the mitotic spindle, phragmoplast or, in the interphase cytoplasm transport, were powered along microtubules by kinesin proteins (Kashina *et al.*, 1996; Liu *et al.*, 1996). Plasma membrane H^+-ATPases responsible for the gradient of electrochemical potential in active solute transport were coupled to metabolic energy in the taxane early response. External signals penetrate into the lipid bilayer of membranes (Casey, 1995). Signals on the outer surface of membranes are dependent on covalent modification of proteins by specific lipids. Lipids have farnesyl or geranylgeranyl moieties that become linked to proteins by S-prenylation. Apoptosis in human prostrate cancer cells was partially prevented by farnesyl-pyrophosphate and geranylgeranyl pyrophosphate (Danesi *et al.*, 1995).

The cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene (Koepp *et al.*, 1995; Lin *et al.*, 1996), followed by the cytochrome P450-catalyzed hydroxylation of the diene, is the first step in paclitaxel biosynthesis (Hefner *et al.*, 1996). This suggested that the seven remaining oxygenation steps in paclitaxel biosynthesis may

involve similar catalysts. The first oxygenation step is slow, relative to downstream metabolic transformations. Cytochrome P450 enzymes introduced multiple hydroxylation steps in brassinosteroid biosynthesis (Choe *et al.*, 1998) and in paclitaxel metabolism (Vuilhorgne *et al.*, 1995). These relations have the potential to further integrate the effects of gravity, amyloplast osmoregulation, to taxane production at cell walls. In murine macrophages, paclitaxel increased the steady-state levels of lipopolysaccharide inducible genes and protein-tyrosine phosphorylation (Manthey *et al.*, 1992). Simulated microgravity would suppresses convective currents inside cells and around the surfaces of cells responsible for the products sought (Albrecht-Buehler, 1992). Vibrations, Coriolis mixing, and convective currents would reduce the microgravity significantly and foster the release of taxanes and taxane-bound materials.

Ingber (1997), like Krikorian (1998), places molecular signals within the context of the whole cell. Unit gravity limits our approach to metabolic engineering as a function of cytoskeletal tensional integrity in four ways. First, the adaptive plasticity and resilience of cells as a function of homeostasis has not been fully defined. Knowledge of current feedback and feed-forward process controls is not enough. Second, metabolic pathway performance is dependent on many factors. At the gene level, expression of a given trait may represent pleiototropy (one gene affects many traits), polygenic expression (one trait depends on many genes), or be dominantly phenetic. The third requirement is the evaluation of the interaction of genes and environment over many environments to reveal latent and unexpected expressions. This is especially true for genetically engineered cells. Fourth, the pathway must be compatible with and controllable over growth, differentiation, morphogenesis, and the life history of cells in each defined (constant or changing) environment. Wounding of cells and the expression of the taxane early response genes could have compromised biosynthetic events by the ATP-dependent uptake and transport of free paclitaxel by membrane vesicles (Paul *et al.*, 1996).

Other target organisms for taxane production are endophytic fungi (Stierle *et al.*, 1993, 1995; Strobel *et al.*, 1993). Taxadiene synthase coverts the universal precursor of diterpenoids, geranylgeranyl diphosphate to taxadiene, a key intermediate in paclitaxel biosynthesis. The gene encoding this enzyme was cloned and its cDNA was heterologously overexpressed in *Escherichia coli* (Huang *et al.*, 1998). The transformation of target cells and organelles to overproduce or down-regulate enzymes by antisense mRNA also promises to be a useful tool.

CELL LINES AND BIOREACTORS

At unit gravity, one strategy has been to select, for high paclitaxel producing, cells with strong cell walls that endure hydrodynamic shear in bioreactors. Cells experience mechanical shear when they bump into the side of the vessel or are hit by other cells or by propellers that stir the medium. Paclitaxel, under the trade name Genexol™ produced under unit gravity conditions, is available from Samyang Genex in Korea (Annual Report 1997). While this strategy may improve cell fitness to mechanical forces in bioreactors, it is not clear how cell walls contributed to the release of taxanes into the culture medium, nor to the increased liability of contaminated cultures from callus where microorganisms can remain inaccessible and latent.

Robust cells with pioneering genotypes are needed for long episodes in space bioreactor environments. An ideal stirred tank chemostat has perfectly mixed continuous-flow (Shuler and Kargi, 1992). Bioreactors simulating microgravity offer low shear (< 2–4 dynes/cm^2), which promotes spatial co-localization of cells. Randomized gravitational vectors *directly* affect gene expression and *indirectly* facilitate intercellular signalling (Unsworth and Lelkes, 1998; Hammond, 1998). Weightlessness of cells in RWVs is approximated because inertial forces required for mixing no longer oppose the forces of gravity. Weightlessness (zero gravity) is the condition where the acceleration of a cell is independent of its mass (Albrecht-Buehler, 1992). The absolute sum of all mass-dependent accelerations does not exceed a 'noise' level, eg 10^{-2} to $10^{-4} \times g$.

Collisions in the bioreactor generate forces that accelerate the motion of subcellular organelles. During prolonged exposure to microgravity, poor mixing leads to the build-up of toxic products around cells. Fluidic motions create a surface pressure gradient that removes products from the surface of cells. Other forces, due to electromagnetic and gravitational potentials, act on the entire mass of cells (Wayne and Staves, 1997). Microgravity minimizes the effects caused by gravitational acceleration with virtually unlimited capillary rise and increased interfacial tension. Density-driven thermal convection and sedimentation are suppressed. These may be useful for drug overproduction, but troublesome for separation technologies.

Genomic complexity and history of the explant obfuscates predictions of yields. Explants are a function of position, local environments that precondition drug production, and phasic development in the donor tree. For drug production at unit gravity by trees, harringtonine, and probably homoharringtonine (HHT), content varies with the seasons, parts, and age of the tree (Pan, 1983). The same is true for paclitaxel (Vance *et al.*, 1994). The cytotoxicity of harringtonine to leukaemia cells is potentiated by calcium antagonists (Yamamoto *et al.*, 1989). Clearly, we do not have full control of divisional cycles and biosynthetic enzymes for the optimization of pathways. Krikorian (1996a) reported that uninucleate plant cells in flight produced more than 10% binucleate cells spread throughout embryonic tissues. Ground samples were uniformly uninucleate. In unpublished work (Durzan and Havel), maintenance of the protoplast condition by low levels of cell wall lytic enzymes produced multinucleate cells. The free nuclear condition is a function not only of gravitational forces and ontogenetic programmes, but also of stress-induced enzymatic activities at newly forming cell walls.

The photoautotrophic capabilities of cells are useful to enhance taxane production. However, the depletion of cytosolic free calcium during photosynthesis is a complicating factor (Miller and Sanders, 1987). High light energy and heat removal would add to instrumentation and flight costs. Low light signals to control phytochrome may prove useful to synchronize and hold cells at G1. Other photoreceptors, such as cryptochromes, absorb UV-A-blue light that sets the daily cycles of plants (Christie *et al.*, 1998). These, and other proteins that regulate seasonal changes in woody perennials, are added factors in the use of light and temperature in bioreactors.

The NASA programme aims for the selective removal of molecules from bioreactors such as valuable metabolites, inhibitors, and waste products (Gonda, 1998). Separation processes depend on subtle differences between molecules and solvents, the volume fractions of the component phases, bioreactor geometry, interfacial tension, relative

phase viscosities, and surface tension interactions among aqueous phases and bioreactor walls. The system must be miniaturized to meet volume, power, and safety constraints in microgravity. Culture media flow through a perfusion loop into cartridges, each of which is packed with an adsorbent that binds, separates, and retains bioproducts. Adsorbents may contain specific-affinity materials for the recovery of products such as the taxanes.

SELECTION AND INTERVENTION IN BIOSYNTHESIS

Stabilizing selection in a gravitational field favours a single optimum in the population. The main effect is to eliminate peripheral variants that arise by mutation, or altered physiological states. The existing state of adaptation is maintained. Parental genes controlling abnormal phenotypes, or which render developmental paths sensitive to potentially disturbing and drug elicitation stresses, will tend to be eliminated unless stabilized by selection. *Directional selection* also favours a single optimum, but results in a systematic shift in gene expression for the pathways sought. It operates in a progressively changing environment and leads to a state of adaptation. *Disruptive selection* favours more than one optimum in a population when the bioreactor environment becomes heterogeneous. Here, each genotype may be adapted to one parameter in the bioreactor. *Recurrent selection* is based on the repeated recovery of various genotypes over an array of environments. 'Molecular phenogenetics', or the study of relationships between the genotype and its phenotypic manifestations, will continue to become a useful tool in developing process controls (Durzan and Durzan, 1991).

The primary aim is to block, enhance, and control points in the pathways of metabolism, either reversibly or irreversibly. Blocking leads to the inability to produce certain compounds that are normal metabolites, and to the accumulation of precursors normally subject to conversion into other compounds in a reaction sequence. Loss or gain of precursors can be of use for the design alternative adaptive pathways for drug overproduction, and to change surface properties for the isolation of specific cells. With tissues, the problem remains that cells producing the product may be few. Other cells maintain correlations among and within tissue and organ types. The reduction of plants to cells or protoplasts enables the assessment, identification, and sorting out of special developmental factors in drug overproduction. Direct genetic intervention may be required to control special structures, alter organelle genomes, or transport processes to favour the exocytosis. Better control over exocytosis may avoid the need to disrupt cells to recover products.

Limitations in metabolic engineering depend on the accessibility of the gene encoding the desired protein, donor organisms, techniques for transforming the host organism, adequate expression of the gene(s), protein folding and proteolysis, prosthetic groups, cellular and subcellular targets, substrate availability, inhibitory environments, and side reactions due to genetic intervention, and side reactions of new compounds. In practice, other factors emerge, such as the need for osmotic adjustments, turnover and repair, protection, storage, adjuvants, and release into a suitable matrix for downstream processing.

Paclitaxel in *T. chinensis* was essentially of plastidic origin (Srinivasan *et al.*, 1996). Biosynthesis was limited by the ability of cells to convert phenylalanine to

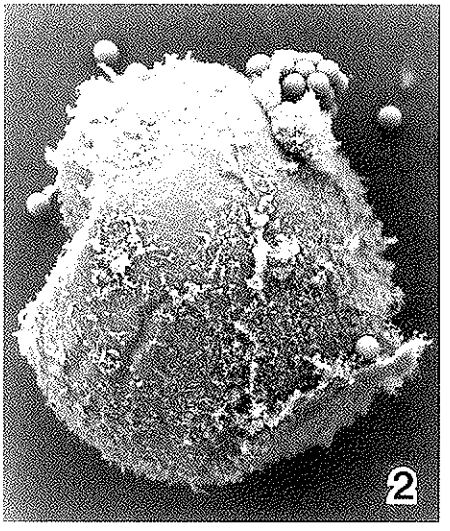

Figure 13.2. Recovery of a taxane-producing cell of *Taxus cuspidata* using anti-taxane labelled Dynal magnetic beads (4.3 μ dia.). This process separates drug and non-drug producing cells to prepare more homogenous populations. Uniform cell suspensions enable the application of more predictable process controls for drug overproduction. The anticancer drug, paclitaxel, is water insoluble and generally make up less than 0.1% of dried plant material. Other metabolites are produced by *Taxus* sp. Some are known for their toxic and medicinal properties (Appendino, 1995). Extracts show hormonal activity in insects and mammals. Some taxanes are inhibitors of P-glycoprotein-mediated transport and can reverse multi-drug resistance. *Taxus* species show NGF-like activity and are reported useful for the treatment of Alzheimer's disease.

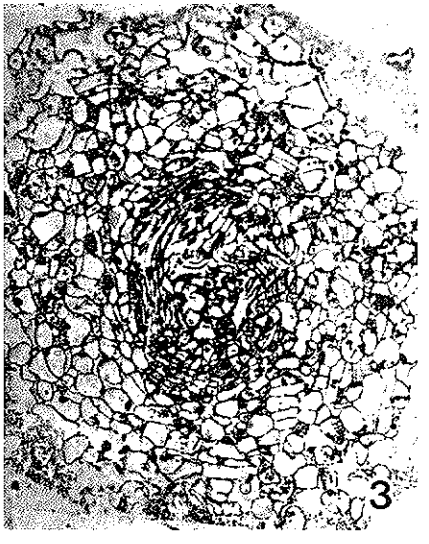

Figure 13.3. In unit and simulated microgravity for 3 months, *Taxus* cells reconstruct 3-dimensional spherical bodies (sphaeroblasts) that in cross-section mimic young branches from which tissues were explanted. The central core becomes vascularized (xylogenesis). Cells on the outer surface are programmed for cell death with the release of taxanes. *Taxus* wood is burned for incense, and the bark has antioxidant properties. Sphaeroblast morphology is altered by the addition of auxins and cytokinins that regulate growth, development, and morphogenesis. Their sizes can approximate 1 cm in diameter, but at this stage they may initiate the formation of multiple sphaeroblasts.

phenylisoserine, rather than by the branch-point acyl transferase. Translocators on the plastid envelope membranes were postulated as importing isopentenyl pyrophosphate for isoprenoid biosynthesis. Conceptual models offered for the flow of carbon in taxane and paclitaxel production were based on: (1) the selective inhibition of baccatin III by cycloheximide without a corresponding effect on the yield of paclitaxel; (2) the unrelated response to 1-aminobenzotriazole, and (3) the unchanging kinetic profile for baccatin in terms of paclitaxel kinetics. All implied that baccatin III need not be a precursor of paclitaxel. Arachidonic acid stimulated paclitaxel production but did not have an effect on the yield of baccatin III.

The cyclization of geranylgeranyl diphosphate to taxa-4(5), 11(12)-diene is the first committed, but not slowest, step for paclitaxel biosynthesis in plastids of *Taxus canadensis* (Hezari *et al.*, 1997). A rate-limiting step was found farther down the pathway and not identified. Paclitaxel accumulated after day 11 following subculture. By day 27 it reached a maximum of c. 19 mg/litre. One chemical signal for these events may be octadecanoid 12-oxo-phytodienoic acid (OPDA). In amyloplasts, OPDA accumulated after mechanical stimulation (Stelmach *et al.*, 1998). It was also an intermediate in the biosynthesis of jasmonic acid (Blée and Joyard, 1996). Addition of methyl jasmonate to the culture medium significantly increased paclitaxel recovery from cells of several *Taxus* species (Mirjalili and Linden, 1996; Yukimune *et al.*, 1996; Pezzuto, 1996).

In *T. brevifolia* and *T. cuspidata*, gravitationally induced stresses caused plastids producing baccatin III to bleb (Durzan, 1999). The resultant taxane-bearing vesicles fused with the endoplasmic reticulum and Golgi before docking at the plasmalemma, where paclitaxel, baccatin III, and other taxanes with a C13 side chain were released into the culture medium (Durzan *et al.*, 1998). This process also released membranes and fragments having paclitaxel and other taxanes.

The selective recovery of drug-producing membranes, organelles, protoplasts, and cells with antibodies to magnetic Dynal beads (*Figure 13.2*) improves homogeneity and the synchrony of events. The loss of diversity may occur at a cost, since stressed and apoptotic cells impact positively and negatively other cells that sustain or inhibit biomass growth and differentiation (*Figure 13. 3*). In practice, it is simpler to scale up the cell biomass first, then block divisional cycling so that more substrates can be directed into drug production rather than to growth. Organelles can also be isolated, purified, and studied for their role in biosynthesis using magnetic beads (Lueers *et al.*, 1998). Selection determines the control of metabolic nodes and may avoid complex metabolic interlocks, while maintaining rigid and predictable relations among pathways. New genomic and pharmacogenomic interactions introduced by protoplast fusion and culture in specific environments complement strategies for direct genetic intervention and cell sorting.

DEFINE SYSTEM CONFIGURATION AND PROCESS CONTROLS

For bioreactor design and the determination of operating set points, no detailed dynamic model is needed. Models mainly consider physical properties and global parameters for culture survival, growth, and differentiation. Controls for the automatic diagnosis of conditions in plant-growth chambers deal with monitoring, lighting, pressure, logistics, nutrient delivery, atmospheric control, heating, ventilating,

air conditioning, condensation, and recovery systems (Clinger and Damiano, 1996).

An accurate model of the biological system is needed. Metabolic networks are not always optimized in practical applications. Questions that must be addressed are: to what parts of the environment is the genotype adapting? How does the environment act upon the adapting, developing, and metabolite overproducing system? What structures and mechanisms are responsible for metabolite production? What part of the life history of the genotype reacts to process controls and is retained in subsequent operations? What are the limits to the metabolic engineering process? What are the critical, useful, and different process controls and system configurations for a wide gravitational spectrum? In the simplest model, increased metabolite flux and drug-producing cells, and a decrease in catabolism of the desired products, are sought (Verpoorte *et al.*, 1998).

An important task is to control the process. Control implementations are not always designed by using models but by practical experience based on improvements in operational stability and productivity. Recently, cascades of highly conserved protein kinases have become central elements of signal transduction pathways. These activate transcription factors in the nucleus and other effectors throughout the cell (Elion, 1998). The sharing of cascades determines how various signals are routed for substrate management to prevent cross talk among pathways. Routing now appears to

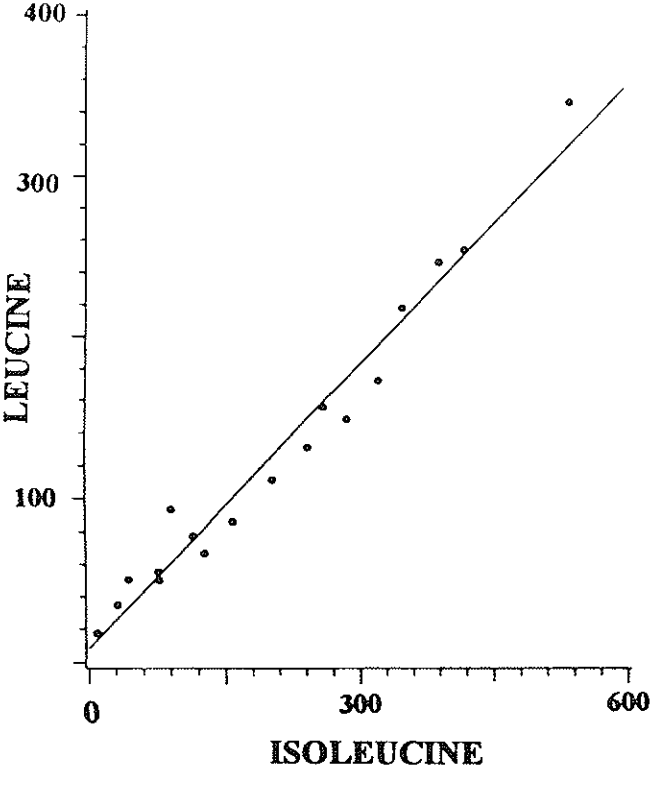

Figure 13.4. Metabolic rigidity and linear relations among substrates enable better process controls. Levels of leucine and isoleucine retain strong linear correlations over a wide range of chlorsulphuron concentrations aimed at maintaining cells at interphase.

be enabled by scaffolding proteins that form multienzyme complexes with kinases for shared and multiple pathway routing (Whitmarsh *et al.*, 1998). Rate-limiting enzymes, feedback inhibition and competitive pathways need to be controlled.

Multistep reactions often have multiple feedbacks at different overall concentrations (Sauro *et al.*, 1987). Randomly perturbed substrate concentrations have been used to model pathways near a steady state but far from equilibrium (Arkin *et al.*, 1997). State-network maps diagnosed changes in N flux of amino acids of spruce cells in bioreactors, and in trees (control and disease symptomatic) under field conditions (Durzan, 1995; Durzan and Durzan, 1991; Durzan and Kester, 1997). Mismatches in flux behaviour revealed adaptive roles of specific amino acid families in physiological fluids that characterized a bud failure syndrome. Kinetics with signal time delays for genetic networks with tens to hundreds of genes have been simulated for the *in vivo* behaviour of phage lambda (McAdams and Shapiro, 1995). These approaches identify critically responsible pathways, their stability, and opportunities to control rate-determining steps in biosynthesis. To simplify the control problem, the process is usually represented by a hierarchical order of subsystems, each with a narrow range of couplings to other subsystems.

Metabolic regulation at a local level is observed by the actual rate of the pathway, while couplings and interactions are related to a master coordinating reaction. A 'metabolic regulator' tracks and determines the activities of associated pathways and reaction rates. This is a key point in the simplification of models. Chlorsulphuron blocks acetolactate synthase responsible for the biosynthesis of branched chain amino acids in all plant tissues (Singh and Shaner, 1995; Zhu-Shimoni *et al.*, 1997). It also reversibly holds cells at G1 and G2 (Rost, 1984). Maintenance of cells at interphase has advantages to explore their ability to direct activities towards more rigidly controlled taxane overproduction rather than to cell replication. A chlorsulphuron block of acetolactate synthase limits the availability of branched chain amino acids required for viability of cells and for the biosynthesis of ubiquitin. Ubiquitin comprises c. 25% of branch chain amino acids and is involved in the turnover of cell regulatory proteins (Pollmann and Wettern, 1989; Rechsteiner, 1987), and terminal differentiation of axial tiers of conifer embryos (Durzan 1996a). Linear ratios for branched chain amino acids in the soluble N pool are maintained over a wide range of chlorsulphuron concentrations (*Figure 13.4*).

Blocked acetolactate synthase can be characterized by rigid linear relationships among metabolites in the branched chain pathway, and by the predicted accumulation of α-amino-n-butyric acid (AANBA) (*Figure 13.5*). Linear, rather than nonlinear, relationships among metabolites are useful in predicting metabolite overproduction. A reversal of the chlorsulphuron-induced block would require that glutamine provide carbon and nitrogen by transamination for the renewed synthesis of branched chain amino acids. Reversal would not occur if ratios between AANBA and valine started to fall after peaking. Apoptosis would have already been initiated. Exocytosis and secretion of drugs are induced by sulphonylureas like chlorsulphuron (Eliasson *et al.*, 1996). Branched chain amino acid biosynthesis in plastids (Singh and Shaner, 1995) may yet relate to gravitropic competence (cf. Sack, 1997).

Lipid rafts in biological membranes are implicated in function in secretory and endocytic pathways (Brown and London, 1998). These detergent-resistant membrane fragments can be isolated from cells as a liquid-ordered phase. Raft associations aid

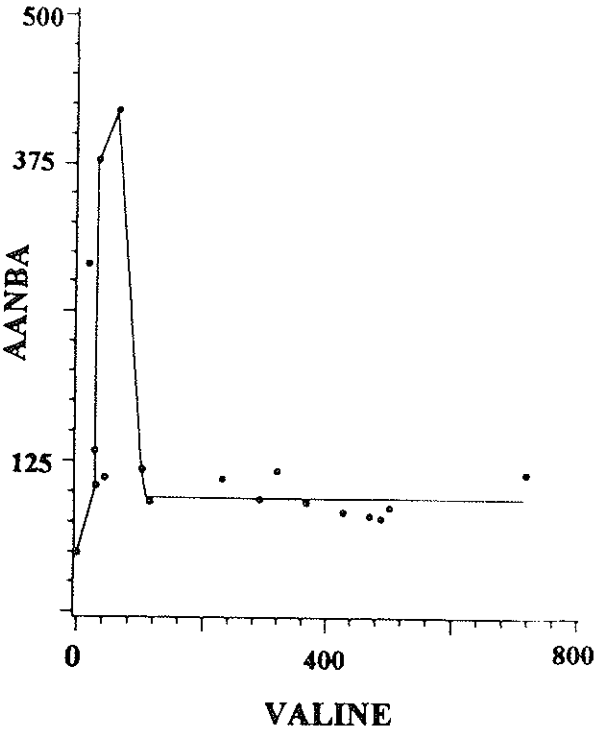

Figure 13.5. Cells become spent or apoptotic under the influence of chlorsulphuron when the relation between α-amino-n-butyric acid (AANBA) and valine drops from a maximum to a minimum of 50 at zero valine. At this stage, drug production becomes dependent on lytic events during apoptosis.

in signalling through proteins anchored by glycosylphosphatidylinositol. Numerous phosphoinositide-binding proteins are potential effectors for phosphoinositide signals. Localized signal generation and recruitment of effector proteins appear to underlie mechanisms for signal transduction, cytoskeletal, and membrane trafficking events (Martin, 1998). The fate of proteins in the secretory and endocytic pathways is also controlled by ubiquitination (Bonifacino and Weissman, 1998).

A blueprint for a hybrid organic/inorganic molecular machine that acts as a pump and biological motor was described by Montemagno *et al.* (1998). The motor is an F_1-ATPase (Wang and Oster, 1998) that was integrated with a nano-electro-mechanical system (NEMS) that was capable of generating a force of > 100 pN. The surface of the pump, c. 50 atoms across, was attached to artificial components for controlling the delivery of toxins or monitoring purposes. The device could be programmed to target specific cells, bind on to them, and deliver chemicals that would be too toxic to tissues.

Re-engineering genomes, and even molecular machines, requires the stabilization of the cell's life history for new artificial space environments. Light, temperature, osmolality, pH cannot always be applied predictably. In conifers, colchicine stimulates free-nuclear genomic replication (Durzan *et al.*, 1994). Like paclitaxel, colchicine binds to microtubules. Three specific binding sites on tubulin are the colchicine, *Vinca*, and paclitaxel binding sites (Han *et al.*, 1998). Most ligands binding to the

'colchicine site' on tubulin associate with the protein before polymerization to inhibit microtubule assembly. Ligands binding preferentially to tubulin polymers at the 'paclitaxel site' stimulate microtubule assembly. Ligands binding to the '*Vinca* site' inhibit tubulin assembly and induce at higher levels tubulin self-association into paracrystals. Since metabolic pathways are sensitive to endogenous and exogenous ligands and effectors, tubulin becomes an important mechanical target for chemotherapy and for process controls.

Nodal rigidity, metabolic blocks, and turnover of cell regulatory proteins are important process control considerations (Stephanopoulos and Vallino, 1991). Controls aim to define how information circulates around set points or reference values, comparators, compensation algorithms, elicitors and actuators, disturbances, and measurements to compare yields with the predictions. Simple lumped-parameter models require testing over a wide range of gravitational forces. An important task becomes determining which assumptions in unit gravity can validly be made in microgravity and hypergravity. Systems that are nonlinear may need linear approximations for process control.

TAXANE YIELDS FROM CELL SUSPENSIONS

Natural and domesticated populations of *Taxus* sp. show a wide range of paclitaxel and taxane content that is dependent on location, season and explant sources (Wheeler *et al.*, 1992; Fett-Neto and DiCosmo, 1996). Rao *et al.* (1996) have developed a large-scale process for the recovery of taxanes from needles. Ground-based studies have shown biomass dry weight doubling times of cells varies between 7 to 20 d. Yields of paclitaxel vary between 0.001% to 0.6% dry weight or up to 300 mg/litre in 2 weeks (Yukimune *et al.*, 1996; Shuler, 1996). Strobel *et al.* (1996) reported 60 to 70 μg/litre with the fungus *Pestalotiopsis microspora*. Cells of *Taxus baccata* have been encapsulated for a continuous *in situ* extraction of paclitaxel (Worlitschek *et al.*, 1998). Encapsulation of cell clusters with calcium alginate gave yields of 0.3 mg/g dry cell weight per day under continuous production for 40 days (Seki *et al.*, 1997). Srinivasan *et al.* (1996) report a yield of 8 μM paclitaxel in 25 days. In some cases, these values exceed that recovered from the bark of trees under field conditions. While yields were not reported, Cseke *et al.* (1995) found that clinostats with *Taxus* sp. increased paclitaxel yields 2- to 5-fold. By contrast, hypergravity at 3 × g decreased paclitaxel yields.

With cell suspensions of *Taxus cuspidata*, nearly two-fold more biomass was produced in simulated microgravity over 14 days than in unit to 24 × g. In simulated microgravity, the percent contribution of paclitaxel to total taxanes remained significantly higher than in all other treatments, even though not much paclitaxel was formed. Membranes with taxanes were released at amyloplast docking and fusion sites at the plasma membrane and cell wall (Durzan, 1999). Cells were also more hydrated and spherical. In hypergravity, cells had a greater fresh weight/dry weight ratio and produced a 100-fold more taxanes (Durzan *et al.*, 1998). Syneresis was a major factor in taxane overproduction. Yields of paclitaxel varied between 0.01 to 0.10% (w/w) air-dry biomass.

Taxanes were bound at the 7-OH of the ring to xylan residues in extracellular material. This partially accounted for the immunocytochemical and SEM detection of

taxanes on cell walls. At least six xylosyl derivatives of paclitaxel were already known (Kingston *et al.*, 1993). After solvent extraction of taxanes, gold-labelled antibodies reaffirmed the occurrence of residual covalently bound taxanes on cell walls. Xylanase treatment released a number of taxanes that were recovered from the digest (Durzan and Ventimiglia, 1994). Chromatographic evidence with digests reaffirmed the occurrence of bound baccatin III, 10-deacetyl baccatin III, 10-deacetyl-paclitaxel, several unidentified taxanes, but little or no paclitaxel. 10-Deacetyl derivatives were already recovered bound to xylose, viz. 7-xylosyl-10-deacetyl-paclitaxel, 7-(β-xylosyl)-10-deacetylcephalomannine (Kingston *et al.*, 1993).

Acetyl-CoA:10-deacetylbaccatin-III-10-O-acetyltransferase activity has been detected in leaves and cell suspensions of *Taxus cuspidata* (Pennington *et al.*, 1998). By contrast, in *Taxus brevifolia* trees, (1-^{14}C) acetate feeding led to traces of paclitaxel in xylem, suggesting that paclitaxel may be mobilized from sites of greatest biosynthesis to the xylem (Strobel *et al.*, 1993). Xylogenesis is an expression of terminal differentiation during wood formation and proceed by ontogenetically programmed apoptosis (Havel and Durzan, 1996a,b; Havel *et al.*, 1997). The upper yield of paclitaxel from cell suspensions, while sometimes greater than from trees in Nature, has not been determined. Recent studies continue to show progress (Pezzuto, 1996).

Direct genetic intervention

Heritable defects in biosynthesis can be followed in haploid cells with fewer complications because dominance and recessive relations tend to be removed from the response. This is one advantage for the use of haploid and haploid-derived plant cells (Durzan and Ventimiglia, 1994). The formation of homozygous diploids removes lethal recessive mutations for cloning, breeding and further hybridization.

Pharmacogenetics is concerned with the unanticipated or unusual responses to drugs that may have a hereditary basis for their action. Ground-based research is beginning to explore the metabolic and genetic control of gene expression and cell pharmacodynamics on a genomic scale (Abbott and Povey, 1995; Brody *et al.*, 1998; DeRisi *et al.*, 1997). Engineered cells can be hybridized, cybridized (cytoplasmic hybrids), backcrossed, and selected for specific retention of biosynthetic capacity. Problems are expected due to loss of viability, apoptosis, irregular divisional cycles, and loss of genetic stability during processing activities. Where product recovery has a hereditary basis, pharmacogenetic differences can be exploited by designer hybrids. Generally, pharmacogenetic differences in response to drug overproduction indicate an inherited defect that accounts for the variability in cell viability and product recovery. Where numerous metabolites are produced, changes in the magnitude or duration for product recovery may represent the effect of one drug in the presence of another.

Metabolic engineering has the potential for direct and precise control (Verpoorte *et al.*, 1998). Cloned DNA molecules in vectors are introduced into individual cells followed by genomic cloning, selection, and testing for the predicted gene expression. Metabolite flux can be increased by introducing a strong promoter to increase enzyme levels. A heterologous gene encoding for an enzyme with similar functions and from a genomic source represents another strategy. For example, a compatible gene may be introduced that is insensitive to feedback inhibition. Working with conifers is

complicated because they have a lot of DNA (eg > 30 pg per interphase nucleus) (Price *et al.*, 1973). While foreign genes were stably inserted in conifers (Dandekar *et al.*, 1987), more than ten years of effort still has not yielded useful results. An important start has been made to map and domesticate the large genomes of *Taxus* species to farm taxanes in plantations (Wheeler *et al.*, 1995).

An important new development is the heterologous overexpression of cDNA encoding taxadiene synthase in *Escherichia coli*. This was achieved using a thioredoxin fusion expression system that increased the solubility of the expression protein (Huang *et al.*, 1998). The purified recombinant protein was similar to native plant protein in steady-state kinetic parameters and mobility on gels. This is a first step in the introduction of an important gene for paclitaxel biosynthesis in a microorganism. The outcome will still depend on the total flux and interference from competitive pathways. The latter may be blocked by introducing antisense genes. Another approach is the introduction of a gene that produces antibodies against the enzyme that competes and limits product recovery. The latter requires better control over a balance or the sequence of anabolic and catabolic events, particularly in interphase.

Genetic intervention may eventually provide better control of biomass recovery, optimization of interphase repair, proteolysis, apoptosis, entrainment of substrates and products, avoidance of side reactions, acceleration and stabilization of rate-determining steps, enhanced product recovery options, and the development of simpler process controls. Comparisons of the activities of 10,000 genes of animal cells in RWVs in flight and ground cultures has revealed several genes controlling differentiation and morphogenesis (Hammond, 1998).

Tissue engineering and drug delivery

Growing cells and reconstructing three-dimensional tissues in a space environment are both neither easy, inexpensive, nor completely free of risks. Growing tissues in simulated microgravity would promote cell-cell associations, while avoiding the detrimental effects of high shear stress (Anon., 1998a; Unsworth and Lelkes, 1998). The reconstruction of 3-D tissue equivalents usually leads to differentiation, and to the restricted diffusion of nutrients and oxygen. Animal cell spheroids larger than a few mm produce hypoxic, necrotic centres surrounded by a rim of viable cells. By contrast, plant spheroids or sphaeroblasts (*Figure 13.3*) tend to overcome this by the terminal differentiation of vascular elements. These also act as 'carriers' that suspend tissues in RWVs. The recovered tissues resemble the cross-sections of tree stems. As cell aggregates grow, the initial rotational speeds of RWV bioreactors are increased to compensate for increased sedimentation and to provide low shear mass transfers of nutrients and wastes. Drug-producing 3D tissue reconstructions in simulated microgravity are also a function of temperature, hydrostatic pressure, compatible osmolytes, etc. This leads to the development of theoretical models for adaptive tissue patterning, differentiation, strength, and new life histories. Will we be some day dealing with evolutionarily ideal and engineered organisms? 'Darwinian Demons' (Durzan, 1996b; Partridge and Harvey, 1988) simultaneously maximize all, or most, aspects of biosynthetic performance.

The insolubility and delivery of paclitaxel appears to have been overcome by attachment to poly L-glutamic acid (Li *et al.*, 1997), by lipid-coated bubble technology

(Ho *et al.*, 1997), and by using co-polymers as micellar carriers for controlled release (Winternitz *et al.*, 1995). A new drug delivery system based on 'chemoembolism' and consisting of multilayered microcapsules formed under microgravity has been developed by NASA and The Institute for Research Inc. (Anon., 1998b). The liquid-filled microcapsules are several times larger than blood cells. They deliver drugs after injection into arteries leading into solid tumours and create emboli that reduce blood supply to the tumour. The microcapsules release cytotoxic drugs and radio-contrast oil to show that the drugs have reached the targeted tissues. After several Space Shuttle flights, the delivery system was reformulated for unit gravity. The Microencapsulation Electrostatic Processing System used to form the microcapsules will be available for experiments on the ISS.

Tissue engineering of cartilage in the Mir Space Station using RWVs has shown progress, but bubbles in the bioreactor were a problem (Freed *et al.*, 1997). Compared to earth, space grown constructs were more spherical, smaller, and mechanically inferior. Metabolic gases play a role in initial growth and in venous gas emboli formation in human volunteers during hypobaric exposures (Foster *et al.*, 1998).

EXTRACELLULAR TAXANES AND CONDITIONING FACTORS

Paclitaxel undergoes hydrophobic clustering in aqueous media and tends to stack in nonpolar environments (Sharma *et al.*, 1995). The stacking is stabilized by a meshwork of intermolecular hydrogen bonds or bridges that propagate multimeric structures and nucleate precipitation. The solubility of paclitaxel has been overcome by the synthesis of analogs and congeners, surfactants, emulsions, hydrophilic polymers, liposomes. Some have useful side effects, but most formulations did not improve paclitaxel's effectiveness (Sharma *et al.*, 1995). Cyclodextrins and other molecular complexing agents also increased the solubility and stability of poorly soluble drugs (guests) such as paclitaxel. However, cyclodextrins were marginally useful for administering paclitaxel and reducing the dose-limiting toxicity in patients (Sharma *et al.*, 1995).

In *Taxus* suspension cultures, taxane-bearing membranes, vesicles, fibres, chromatin, and particles were released from stressed cells during adaptation and apoptosis (Durzan, 1999). Digested components were metabolically salvaged and recycled by other cells. The recovered materials displayed antibody and gold labelling patterns that may have represented short term, past structural history, eg sensor efficacy and intrinsic membrane properties.

Small transport vesicles were involved in the formation of taxane-bearing islands on the plasma membrane. Vesicles were also expelled into the culture medium by 'exocytotic vesiculation' (Durzan, 1999). 'Osmocytosis' has been proposed as a term for the deletion of parts of the plasma membrane (Oparka *et al.*, 1996). In simulated microgravity (10^{-2} × g RWVs), cells released large dumbbell-shaped membranous bubbles with surface anti-taxane and anti-paclitaxel reactivity. These trapped air and interfered with the performance of high-aspect rotary bioreactors (HARVs, Synthecon, TX). Unfortunately, very little is known about the origins and control of factors leading to the deformation of taxane-bearing materials upon expulsion into the culture medium. Membranes or polymers are easily deformed by a sudden hydrodynamic friction that exceeds the elasticity of these materials. They become folded, kinked, or dumbbell-shaped (Smith and Chu, 1998). Bubbles (Foster *et al.*, 1998) were a

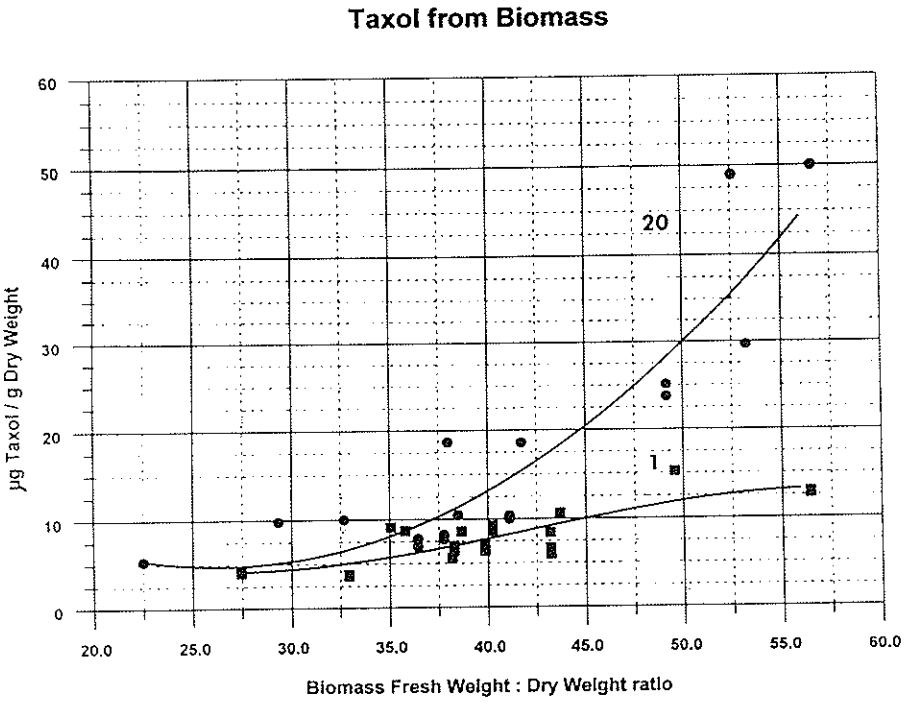

Figure 13.6. The recovery of Taxol™ from PVDF filters beneath cells in unit gravity (1) and hypergravity (20 × g) after 2 weeks in culture. The relations show that, as the ratio of fresh weight to dry weight drops, more drug is released (syneresis) into the culture medium, especially at 20 × g.

problem in RWVs for space flight and the tissue engineering of cartilage (Freed *et al.*, 1997).

Four months of continuous hypergravity (20 × g) spread the cell colonies on plastic plates laterally and perpendicular to the force vectors. Physiological fluids and subcellular particles from the compressed cells were recovered on the supporting moist substratum of cellulose and/or polyvinylidiene difluoride (PVDF) (*Figure 13.6*). A thin membranous film covered the extracellular matrix over cells. Edges of the film attached to the plate surface giving light-refraction patterns where membrane folding and stretching was evident. Hypergravity produced up to a 100-fold more total taxane than in other treatments (Durzan *et al.*, 1998).

Taxane labelling patterns on membranes showed reaction kinetics characteristic of trapping by molecular aggregates, fusion of composite materials, and charge recombination typical of some colloids (Kopelman, 1988). Variations in patterns of gold-labelled taxanes on membranes also represented fluctuations in diffusion of hydrophobic and more hydrophilic taxanes from one into another fluidic phase. Gravitational forces would limit diffusive fluctuations along a concentration gradient. At unit gravity, particles and membranes would not have time to diffuse before convection due to buoyancy would bring them back to their original position (Vailati and Giglio, 1997). In microgravity, diffusional fluctuations become more macroscopic than at unit gravity. Many aspects remain poorly understood in the context of colloidal behaviour.

The photochemical modulation of liquid crystalline phase cell membranes represents a challenge for information processing by cells under parabolic flight (Clarke *et al.*, 1997). Racemic photoresponsive organic materials are able to switch between the enantiomers in a system where molecular memory comprises a binary logic system. Dynamic control over molecular chirality was obtained by the interconversion of enantiomers of helically shaped molecules with either left or right circular polarized light (Huck *et al.*, 1996).

Vibrations in spacecraft may produce unexpected nodal patterns that redistribute catalytic enzymes in membranes. Topological variations in domains may create bottlenecks or gaps in the final assembly of complex lipophilic drugs. Effects of variable-frequency sounds on plant growth have been reported (Weinberger and Das, 1972; Weinberger and Graefe, 1973). Sound also represents a possible complicating factor in drug processing in space environments. Materials released to the culture medium create problems by plugging up membranes, channels, etc., much like the biofilms of microorganisms (Koenig *et al.*, 1993).

Cinnamoyl taxanes (Appendino, 1995) occur in extracellular materials, viz., cutin, suberin, wax, bark, and lignified cell walls (Cseke *et al.*, 1994). Cinnamate 4-hydroxylase enzymatic complexes have P-450 monooxygenase activity linked to several metabolic branch points arising from phenylalanine ammonia lyase. Phenylalanine is a substrate for the side chain of taxol (Fett-Neto and DiCosmo, 1996), and P-450 is involved in the hydroxylation of the paclitaxel ring (Yuan and Kingston, 1998; Vuilhorgne *et al.*, 1995). The control of flux of metabolites in the phenylpropanoid pathway seems possible through piperonylic acid, an inactivator of *trans*-cinnamate 4-hydroxylase (Schalk *et al.*, 1998). This, and other related reactions, may account for the recovery of taxanes in the extracellular matrix.

Perspectives

Research with cells in rotating bioreactors is currently concerned with technologies for ensuring astronaut health (Anon., 1998a; Unsworth and Lelkes, 1998). Bioreactors provide a means to culture red blood cells or skin in the event of astronaut trauma. They can also be used to culture plant cells to provide food, drugs, replenished air supplies for the spacecraft, or planetary colony (Blüm *et al.*, 1997; Gitelson *et al.*, 1995). The initial enrichment of drug-producing cells by cell sorting and magnetic antibodies improves population homogeneity, interphase biosynthesis, yields, and offers better overall process controls with bioreactors. The recovery of useful products and formation of biofilms add challenges for the downstream processing in the space programme (Gonda, 1998). Cells could be designed to respond to environments of planets or used to judge if the atmosphere and environment is supportive of life. This technology is a 'primitive forerunner' that aims to support space exploration.

Gravitational forces leave unique footprints along biosynthetic sequences. Tensions are redistributed among organelles, the cytoskeleton, and at cell walls. Capping of taxanes on the outer cell surface, syneresis, material rearrangements and forcible expulsions into the culture medium pointed to the importance of adaptive self-regulated behaviour. Membrane transformations and flows, small transport vesicles, and networks with processing enzymes became disrupted and adjusted. We now

know that plant cells and chloroplasts (*Taxus* sp., *Kalanchoë*), under extreme gravitational stresses, produce nitric oxide, which predisposes apoptosis (Garcês *et al.*, 1999; Magalhaes *et al.*, 1999; Pedroso and Durzan, 2000a,b). The stress-related production of nitric oxide and programmed cell death are manipulable and offer metabolic and ontogenetic advantages (cf. Havel and Durzan, 1996a,b). In hypo- and hyper-gravity, analogues of L-arginine, the substrate for nitric oxide synthase, reduced the production of nitric oxide in individual cells. While this approach may offer counter-measures against genomic decay in space environments, the opportunities to control specific biosynthetic pathways generated by gravitational forces through the manipulation of nitric oxide and apoptosis remains unexplored.

Prolonged exposure of conifer cells to space environments tend to lead to irreversible genomic decay, apoptosis, and terminal differentiation (xylogenesis). Apoptosis requires calcium-activated lytic enzymes, eg proteases and endonucleases that mediated cell death. For some reason, lytic enzymes are also encoded but silent in intergenic DNA regions and by retrotransposons (Kinlaw and Neale, 1997). The products of apoptosis released into the bioreactor appeared to re-calibrate the divisional cycling in other target cells. Other products were either metabolically salvageable substrates or factors that promoted autophagy and necrosis.

Genetic engineering and pharmacogenomics continue to offer the potential for rapid advances. For drug overproduction a cost-effective process is needed that does not rely on the complexity of plant cells or on the trafficking of their subcellular organelles. Relational databases about gene products and their expression profiles and profiles of protein modifications and metabolites will become increasingly available. Cell-free approaches through proteomics, protein engineering and/or partial synthesis continue to be promising, as long as products meet or exceed the effectiveness of the 'natural' drug. The realization and take-over time of new space technologies are difficult to predict because of many external factors.

The assay of thousands of genes immobilized on chips will change the approach to how plants function, develop, and evolve phenotypes in space environments (eg Eisen *et al.*, 1998). More than 1,631 out of 10,000 genes in human renal cortical cells changed at a steady state during microgravity culture on a space transportation flight STS-90 (Hammond *et al.*, 1999). The shear and turbulence in a rotating wall culture vessel (RWV) approximated zero, while co-spatial relations among cells and three-dimensional growth were almost 'perfect'. Shear-stress response elements and heat-shock proteins were not involved. Specific transcription factors such as zinc finger proteins and vitamin D receptors changed in microgravity. Growth in rotating wall vessels changed the expression of 914 genes relative to controls grown in parallel. Cell aggregate growth was influenced by residual fluid dynamic stresses and wall impacts that degraded the aggregates. Only 5 genes changed more than 300% during 3 g centrifugation selected to simulate the forces on flight launch. This work indicated that gene expression in microgravity was unique and not just an extension of the observations with the RWV.

The expression of large arrays of genes by animal cells contrasts to the work on metabolic engineering with plants where either one, or at least very few, genes are believed to determine metabolite overproduction. Microarray technology may yet show that other genes not yet detected are needed to overproduce designated metabolites. In the absence of gravity, control by temperature and pressure dominates

other factors. The selected gene expressions can be represented by mathematical relations between the probability of system failure and dose of some stressful or injurious agent (cf. Quastler, 1959). This approach may have value in modelling complex systems that overproduce metabolites, adapt to new environments, and yet survive with or without programmed cell death in some cells.

Many problems with experiments in space environments remain. Future modular payloads will require smaller instrumentation, better sensors for process controls and downstream processing, and significantly more replications to improve experimental designs and hypothesis testing. Cell cultures offer advantages over tissues or whole plants, especially for collecting replicated data sets. MEMS and NEMS should enable the evaluation of forces (mechanical, optical, fluidic, osmotic, vibrational, etc.) at the level and scale of individual cells (Amato, 1998; González *et al.*, 1998; Montemagno *et al.*, 1998; Sievers *et al.*, 1996) for comparisons with cell populations and tissue reconstructions. The rapid progress and innovations in microscale technologies make obsolescent many of the bulky (weight, volume, energy costs, etc.) designs already selected for the ISS payloads. Current commitments, the time-line and costs to engineer, test and adopt new safe technologies rule out the immediate adoption of recent advances. The need to upgrade becomes larger as flights and construction of the ISS are delayed. The space station appears ready for scientific studies in 2005.

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