NASA Technical Memorandum 4258

1989–90 NASA Space Biology Accomplishments

FEBRUARY 1991

(NASA-TM-4258) 1989-1990 NASA SPACE BIOLOGY ACCOMPLISHMENTS (NASA) 226 p
CSCL 06C
N91-19877
Unclas
H1/51 0001283

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National Aeronautics and
Space Administration
Office of Management
Scientific and Technical
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1991

PREFACE

An individual technical summary of each research task within the NASA Space Biology Program is presented in this publication. Each summary, prepared by the principal investigator, consists of a description of the research, a listing of the project's accomplishments, an explanation of the significance of the accomplishments, and a list of the publications resulting from the past year's research. Since spaceflight experiments, submitted in response to the Space Biology Dear Colleague letter, have become an integral part of the Program, reports on the activites of this related research are integrated in the report. Accomplishments of the scientists in the NASA Space Biology Research Associates Program, which provides opportunities for postdoctoral scientists to conduct research in the fields of gravitational and space biology, and the NASA Graduate Student Researchers Program, which supports promising students pursuing advanced degrees in science and engineering, are also included. The participants in these programs have been outstanding and merit independent recognition.

This publication has two objectives: first, to provide the scientific community and NASA with an annual summary of the accomplishments of the research pursued under the auspices of the Space Biology Program, and second, to stimulate an exchange of information and ideas among scientists working in the fields of gravitational and space biology.

Thanks are due to the Program participants and postdoctoral and graduate student scientists whose research and cooperative response to our requests for information made this report possible. Editorial support provided by Janet V. Powers, Katherine J. Dickson, and Elizabeth L. Hess is gratefully acknowledged and appreciated, as well as the technical assistance provided by April Commodore Roy.

Additional information about this report of the Space Biology Program can be obtained by writing to me at the following address:

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INTRODUCTION

THE NASA SPACE BIOLOGY PROGRAM

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Introduction

One of the major features of the physical environment of the surface of Earth is the constant presence of the force of gravity. The phenomenon of near-weightlessness encountered on spacecraft provides a unique biological research opportunity to study the importance of gravity to life on Earth. Access to space provides an opportunity to manipulate gravity from its normal value down to almost zero, effectively providing the full spectrum of gravitational research capability for the first time. This capability, combined with the stability and pervasiveness of gravity on Earth, its obvious impact on biological evolution, and its continuing effect on the morphology, physiology, and behavior of living organisms, has led the Space Biology Program to concentrate its efforts and resources on investigating the biological significance of gravity and advancing knowledge in the biological sciences through the use of the microgravity environment of spaceflight.

Program Goals

The Space Biology Program includes both plant and animal research, and is dedicated to: understanding the role of gravity and the effects of microgravity on biological processes; determining the effects of the interaction of gravity and other environmental factors on biological systems; and using the space environment as a tool to advance knowledge in the biological sciences to improve the quality of life on Earth.

Program Scope

Space Biology includes the following research areas:

1. Plant Biology. The primary areas of plant biology now demanding study are: a) gravity perception and transduction, b) reproduction, c) growth and development, d) transport, and e) metabolism and photosynthesis.

   All tropic responses of plants to gravity begin with the perception of the gravity vector. How ***gravity is perceived and transmitted*** to responsive plant parts is just beginning to be understood. Research is directed to the molecular level to determine the mechanism involved in gravitropism and its relationship to phototropism.

   Both sexual and asexual reproduction involve the mechanism of cell division and the transmission of the genetic code through DNA-bearing chromosomes. Aberrations in cell division and chromosomes have been observed in space, and a careful study of the entire ***reproductive process*** of plants needs to be conducted from germination through flowering, seed setting, and senescence.

   During growth, plants undergo a variety of qualitative changes. Their development is controlled by hormones, which receive signals from the

environment that influence all aspects of their metabolism. Research is encouraged to determine the effect of microgravity on the ***growth and development*** of plants, and to determine how the plants respond to gravity during their life cycles.

Conduction of water, ions, and nutrients through the bodies of plants is necessary for survival. Gravity can affect the polarization of cells and tissues and thereby affect ***transport***. The operations of membranes involved in transport and their responses to gravity need to be understood.

Evidence from spaceflight suggests that ***metabolism and photosynthesis*** are affected by the absence of gravity; amyloplasts and chloroplasts have been reported to be depleted of starch in space-grown plants. Flight experiments suggest that the structural molecules of lignin and cellulose are gravity sensitive. These topics, as well as carbohydrate metabolism in general, are of special interest, although total metabolic studies and investigations on the effects of gravity and microgravity on photosynthesis are needed.

2. Gravity Sensing. Animals have developed gravity-sensing systems that facilitate orientation and locomotion within Earth's environment. The near-weightless environment of space provides a unique research opportunity to understand how gravity-sensing systems of different organisms have developed, and how they process and transmit information. Specific objectives are:
    a. To identify gravity-sensing organs and mechanisms, and to define how they function on Earth and adapt to weightlessness;
    b. To understand how gravitational information is transduced, processed, transmitted, and integrated into a response; and
    c. To understand the role of gravity in the development and evolution of animal gravity sensing systems.

3. Cell and Developmental Biology.
Cell Biology: Cells that are building blocks of systems (e.g., plant root caps), individually functioning units of certain tissues (e.g., blood cells), and unicellular organisms (e.g., paramecia) have been shown to be sensitive to gravity. Research focuses on how gravitational loading influences cell functions and the molecular mechanisms regulating them. The objective is to determine at what level gravity affects cells and where and how they are affected. Specific objectives are:
    a. To investigate the role of gravity in maintaining normal cellular and molecular function;
    b. To determine how and where gravity affects cells;
    c. To distinguish direct from indirect, extracellular, or systemic, gravitational effects on cells;
    d. To discriminate among the influences of cosmic rays, micro-gravity, and other spaceflight environmental factors; and
    e. To assess the permanence of effects on cells exposed to micro-gravity.

Developmental Biology: Research in this area examines the influence of gravity and weightlessness on genetic integrity, reproduction, growth, development, life span, senescence, and subsequent generations of animals. Specific objectives are:

a. To determine if organisms and multiple generations of organisms can develop normally in microgravity;
b. To identify gravity-sensitive developmental stages, systems, and mechanisms; and
c. To understand the effects of gravity and weightlessness on gravity-sensitive developmental stages, systems, and mechanisms.

4. **Biological Adaptation**. All biological species on Earth have evolved under the influence of a gravity level of 1 g. In response to this force, organisms have developed structures to withstand gravity loads, as well as regulatory systems to function optimally. The objective is to understand how gravity affects and controls the physiology, morphology, and behavior of organisms; how gravity and other environmental stimuli and stresses interact in this control; and the biological mechanisms by which living systems can respond and adapt to an altered gravity environment, particularly the environment found during spaceflight. Research also includes the use or removal of gravity's physiological effects to explore biological problems. Specific objectives are:
   a. To understand the influence of gravity on the composition, regulation, and function of biological support structures, with special emphasis on biominerals;
   b. To determine the role of gravity in regulating metabolism, metabolic rate and products, fluid dynamics, and biorhythms;
   c. To understand basic mechanisms of mineral and hormonal homeostasis and the role of calcium as a mediator of gravitational effects; and
   d. To identify the effects on organisms of the interaction of environmental factors (e.g., stress) with gravity, and determine the mechanisms involved.

Focus of Program

The Program is focused on basic research that contributes to both NASA applied goals and to improving the quality of life on Earth. Understanding how plants develop, metabolize, and grow in space is essential for a space based Controlled Ecological Life Support System (CELSS). Biomineralization and the mechanisms controlling the structural integrity of bone and bone turnover are important to understanding osteoporosis on Earth and the calcium loss and bone changes of spaceflight. The reconstruction and modeling of the functional organization of mammalian gravity sensors are expected to lead to increased understanding of how information is processed by biological systems.

Research Opportunities

While the research supported and encompassed by the Space Biology Program is primarily ground-based, spaceflight experiments are an essential component of the program. Spaceflight provides the validation for experimental hypotheses developed in ground-based research, while gravitational experiments on Earth hone the questions, provide the necessary baseline data, and develop spaceflight experimental protocol.

The experimental approach of the ground-based studies is to manipulate gravity on Earth and develop weightless simulation models to: a) develop and test gravitational hypotheses, b) identify gravity-sensitive biological systems and interacting environmental response mechanisms, c) analyze biological systems and mechanisms known to be gravity-sensitive,

d) analyze flight experiment data and iteratively expand ground research capability, and e) plan and design future space experiments.

The research of the Space Biology Program is dependent upon several dynamic factors: the requirements of NASA, the characteristics of flight experiment opportunities, the sensitivity of specific biological systems to gravity, the scientific value of the research, the state of knowledge and technology in the specific scientific areas, the interest of scientists in studying the biological questions, and the availability of funds to support the research. Of these factors, scientific interest in this field of biological science is paramount; for without outspoken support from the scientific community, opportunities for space biological research will remain static.

ACCOMPLISHMENT HIGHLIGHTS

SPACE BIOLOGY
ACCOMPLISHMENTS HIGHLIGHTS

PLANT

Gravitropism: Sensing

- Nature of the Receptor
  - The first demonstration that the hydrostatic sensing mechanism may be involved with gravity perception was made during a study of cytoplasmic streaming in Characean algal cells. (Leopold)
  - There is a severe reduction in gravitropic sensitivity in the stems of a tobacco mutant with starch-deficient amyloplasts (starch-containing organelles thought to be the gravity sensor). Although gravitropism is not entirely eliminated by the lack of starch, this demonstrates that starch is necessary for complete graviperception in tobacco and that amyloplasts function in gravitropic sensing hypocotyls. (Sack)
  - Mutants of *Arabidopsis* that do not respond to gravistimulation have been identified. One of the mutants is deficient in starch and one is not, thereby providing evidence that a loss of starch does not necessarily lead to a loss of the ability to perceive gravity. (Poff)
  - Phytochrome mRNA is spatially closely associated with columella cells in the corn root cap, cells that are involved with graviperception, suggesting that the phytochrome protein mediates some early steps in the sensing and processing of the gravity stimulus. (Feldman)
  - One of the first attempts to use the technique of freeze-substitution on an entire plant organ to avoid ice crystal damage has been successfully used to study cells believed to be responsible for gravisensing in plant roots. (Sack)
  - The root cap is not necessary for the detection of mechanical and electrical stimuli, which suggests that the cap may not be the only site for the perception of the gravitational stimulus. (Evans)
  - Studies have revealed that root caps are unnecessary for root gravitropism in the plant *Tristerix aphyllus,* the only advanced plant believed to have gravity-perceiving cells located next to the root elongation zone instead of in the root cap. (Moore)
- Electrical Potential
  - An endogenous electric voltage was measured across the stele-cortex junction of corn shoots, which indicates that an electric potential exists across this region. (Bandurski)
  - The electric voltage between stele and cortex tissues changes rapidly following change in shoot orientation with respect to gravity. (Bandurski)

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- For seedlings turned horizontally, the lower side of the stele-cortex junction is at a different potential than the upper side. This supports the hypothesis of a voltage asymmetry between the upper and lower stele-cortex junctions involved in regulation of gravitropism. (Bandurski)

Gravitropism: Transduction

- The role of plant kinase proteins in transduction of the gravity stimulus has been examined for the first time. One kinase, structurally homologous to animal and microbial kinases involved in the transduction of many environmental stimuli, has been identified. This kinase is located in the root cap and is not affected by light. (Feldman)

- Role of Calcium

  - Calcium gradients are thought to develop at the tip of roots during gravitropism and to be essential for the gravitropic response. The first demonstration of a calcium gradient *in vivo* was made with calcium microelectrodes. (Cleland)

  - The successful incorporation of the calcium-sensitive fluorescent dye *fura*-2 into intact fern spores and germinating gametophytes will allow changes to be measured in the level of cytosolic free calcium after phytochrome activation or gravistimulation. This system can serve as a model for testing whether these stimuli can induce changes in cytosolic free calcium. (Roux)

  - A calcium-binding calpactin-like protein from peas has been purified and is being characterized. This protein appears to play a role in calcium-dependent cell secretion, a process which is likely to be a major control point in plant growth regulation. (Roux)

  - Enhanced levels of calmodulin mRNA and calcium-dependent protein phosphorylation have been observed in tobacco protoplasts transformed with a cloned plant calmodulin cDNA. (Poovaiah)

  - After transformation with sense constructs of calmodulin cDNA, protoplasts from tobacco plants reveal up to a 50-fold increase in calmodulin mRNA; however, the amount of calmodulin protein never increases more than 2-fold. This indicates that the amount of calmodulin in the plant cell is regulated stringently during or after translation of mRNA. (Poovaiah)

  - More calmodulin and calmodulin-binding proteins are found in the tip than in the base of corn roots, suggesting a coordinated regulation of calmodulin expression and its target peptides. (Poovaiah)

  - Application of gadolinium, which inhibits calcium channels, and other agents that block the functioning of calcium inhibits gravitropism in the fungus *Phycomyces*. (Edwards)

  - Based on an analysis of calcium and pH in shoot cell walls, cell wall calcium appears to play no part in gravitropic curvature. (Cleland)

- Role of IAA

  - Different concentrations of Ca^{2+} applied internally or externally to isolated zucchini plasma membrane vesicles had no significant effect on the transport of IAA across the vesicles; this observation provides evidence against the mechanism of coupled Ca^{2+}/IAA flux. (Lomax)

  - Auxin-binding proteins were identified in isolated plasma membrane vesicles from zucchini hypocotyls with the use of azido-IAA labeled polypeptides. The lack of azido-IAA binding in membrane proteins of the agravitropic mutant of tomato *diageotropica (dgt)* identifies an absent or altered auxin-binding protein that is necessary for plant graviresponse. (Lomax)

  - cDNA clones encoding a protein believed to bind with the auxin IAA in tomato plants are being developed. These clones will be used to study this protein and its role in plant shoot elongation. (Rayle)

  - The enzyme indole-3-acetic acid oxygenase is stimulated by a lipid-soluble cofactor which can be replaced by unsaturated fatty acids in the irreversible oxidation of IAA. (Reinecke)

Gravitropism: Growth Response

- A detailed analysis of growth patterns during the root graviresponse shows a shifting pattern of localized, time-dependent, distinct growth rate changes, suggesting that root curvature involves more than simply growth inhibition along the lower side. (Evans)

- Studies of cell wall autolysis in isolated walls, growing walls, and non-growing walls suggest that cell wall autolysis may be independent of the process(es) that cause cell wall loosening, and that it does not necessarily lead to wall expansion. (Cosgrove)

- Cell wall autolysis is qualitatively the same for epidermal (outer) and cortical (inner) tissue walls, which is evidence against any distinctive autolytic processes occurring in the epidermis. (Cosgrove)

- Gravistimulated oat pulvini treated with gibberellic acid show significantly higher glucan synthase activity than gravistimulated pulvini that are not given hormone treatment. The increase in glucan synthase activity correlates well with levels of β-d-glucan (which is associated with cell elongation) found in the lower halves of graviresponding oat pulvini. (Kaufman)

- A yeast invertase DNA that can be used to detect the invertase gene in oat pulvinus tissue has been obtained. (Kaufman)

- A polyclonal anti-yeast invertase antibody and four monoclonal antibody cell lines bind soluble oat acid invertase. These antibody preparations are being examined for specificity, and the specific probe will be used to localize invertase at the subcellular level of oat pulvini and to probe the pathway of invertase transport during graviresponse. (Kaufman)

- Plant growth in shoots appears to consist of two components: a rapid acid-mediated response and a slower non-acid response. (Cleland)

- Experiments with pH buffers and roots have cast doubt on the idea that acid-mediated growth plays a role in gravitropism. (Cleland)

- Binding and blocking the activity of a 90kD wall isoperoxidase with a monoclonal antibody inhibits the growth rate of coleoptile segments of corn seedlings. Whether this indicates that peroxidase is not responsible for growth rate inhibition but is needed for normal cell wall extension, or if the antibody-antigen complex creates steric problems that nonspecifically inhibit growth, is not known. (Roux)

- Certain peroxidase isozymes (PODs) respond to short-term gravistimulation and are believed to regulate tropistic bending. Two cationic PODs are expressed on the upper side and a weakly anionic POD is expressed on the lower side of corn coleoptiles within 30 minutes of gravistimulation. Secretion of this anionic POD appears to be dependent on calcium. (Slocum)

- In graviresponding pea stems, cell wall polymers decrease in size on the lower side and increase in size on the upper side of the bending stem. These changes in polysaccharide size occur primarily in stem epidermal tissue. (Talbott)

- Most of the polysaccharides that change size during cell wall curvature are xyloglucan polymers. As indicated by electron microscopy, the size increases of xyloglucan are due to the formation of a crosslinked network, which may be dependent on ion and auxin-induced pH levels, although enzymatic action is not excluded. (Talbott)

- There is an initial lag period after gravistimulation of plant dicot hypocotyls at different IAA concentrations before the growth rate of the bottom surface increases sharply and upward curvature begins. As auxin concentration increases, the lag time shortens until there is no detectable lag but a burst of rapid growth of both upper and lower surfaces. (Salisbury)

Plant Metabolism

- The exact bonding patterns of phenylpropanoid polymers into lignin in vascular plants was determined with nuclear magnetic resonance, providing the first direct evidence for lignin-carbohydrate bonding. (Lewis)

Environmental Factors

- Roots of corn seedlings respond not only to gravity and light, but also to temperature. (Poff)

- The rapid reduction in growth rate caused by mechanical stress in pea and soybean seedlings suggests that the initial events are biophysical, possibly involving reduction of turgor within the cell elongation zone. (Mitchell)

Circadian Rhythms

- *Neurospora* cultures flown on STS-32 in January 1990 were examined for changes in circadian rhythms during flight. The findings strongly support the theory that these daily oscillations are endogenously generated — that is, not generated by environmental factors. (Ferraro)

- Exposure to microgravity diminshed the period, phase, and amplitude of the endogenous circadian rhythms of BND and CSP cultures of *Neurospora*. (Ferraro)

Plant Cell & Developmental Biology

- The somatic embryos in a carrot tissue culture system that form first multiply initially as globular stage embryos only when the pH of the medium is allowed to fall during the culture period. This is the first demonstration that morphology of a higher plant system is reversibly influenced by changes in external pH. (Krikorian)

- At least six new proteins are produced by the spores of the sensitive fern *Onoclea sensibilis* after the plant has been exposed to hypergravity through centrifugation. A stronger gravity force results in a higher rate of synthesis of these "shock" proteins. These findings suggest that specific changes in gene expression occur in the plant spores as a response to hypergravity stress. (Raghavan)

- A cDNA library of mRNA sequences from *Onoclea sensibilis* spores has been constructed. From this library cDNA clones specific for hypergravity stress are now being selected. (Raghavan)

ANIMAL

Gravity Receptors and Neurophysiology

- Chick embryos grown in space on the shuttle had elevated vestibular thresholds when compared with Earth controls; they required stimulus intensities nearly twice those as the controls to elicit vestibular responses. This supports the hypothesis that gravity may play a role in determining vestibular sensitivity. (Jones)

- Studies of discharge patterns of otolith afferent in neonatal rats showed that they have a high level of spontaneous discharge and show sensitivities to sinusoidal and velocity steps similar to the adult. (Blanks)

- In studies using the American bullfrog, the rate of change of the projection of the gravity vector onto the utricular surface was found to be the stimulus for the faithful representation of rotation velocity followed for small-amplitude, low-frequency rotations about horizontal axes. (Lewis)

- Three-dimensional reconstructions of terminal/receptive fields and of small portions of the macular neural network in rats have identified four network organizations that differ from one another in the kinds of nerve fibers supplying the area, in terminal fields, and degree of collateralization. This finding indicates that the intrinsic collaterals are significant in information processing. (Ross)

- Labelling of impulse initiation zones in rat vestibular nerve fibers revealed that each vestibular nerve has but one impulse initiation zone, which is located just distal to the heminode. (Ross)

- The effect of sine wave linear motion on vestibular responses was studied in rats using a linear sled. Linear motion was found to produce a small phase-dependent modulation of vestibular response thresholds, latencies, and amplitudes. (Jones)

- The sequential development of graviceptor structures, particularly formation of the touch-plate (part of the rhopalium which is involved in gravity reception in the adult) was determined using *Aurelia* (jellyfish) ephyrae. (Spangenberg)

- Studies of guinea pigs maintained on a high Na^+/low K^+ diet and injected with aldosterone provided evidence of a treatment capable of increasing levels of inner-ear ATPase, and suggest that endolymphatic fluid/electrolyte balance may be altered, at least transiently, by alterations of circulating aldosterone induced by exposure to microgravity. (Kerr)

- In chinchilla, there are differences in the afferent bouton innervation of type II hair cells in the central and peripheral zones of the crista. A higher convergence ratio of hair cell synapses to afferent boutons in the central zone of the crista implies that, anatomically the central zone appears to be specialized for increased sensitivity. (Lysakowski)

- The amino acid sequence of the 22kD otoconial protein from *Xenopus laevis* was determined. (Pote)

- Carbohydrate analysis of the *Xenopus laevis* 22kD otoconial protein revealed that it is a glycoprotein, and not a glycosaminoglycan as was commonly assumed. (Pote)

Development

- Studies of synapse development in a slow clinostat focusing on the distribution of cytoskeletal proteins that may affect acetylcholine receptor distribution reveal that the ratio of vinculin (a cytoskeletal protein marker) to acetylcholine receptors is larger than in control cells. This implies that cytoskeletal organization in response to nerve contact takes place at least up to the level of vinculin. (Gruener)

- Study of fertilized amphibian eggs revealed that spawnings of eggs differ in cytoplasmic rigidity which can be quantified. Eggs with a more rigid cytoplasm display a higher survival rate in response to centrifugation, and a lower rate of twinning. (Malacinski)

- The neurotransmitters neuropeptide Y and dynorphin were localized in the brain and pituitary gland of immature and mature swordtail fish, *Xiphophorus maculatus*. The results suggest that these neurotransmitters play a role in sexual maturation, and growth and differentiation. (Schreibman)

- Signals which induce the amphibian ectoderm to become neural are traveling from the blastopore lip area (the cells which initiate the cell rearrangements at gastrulation) through the plane of the ectoderm. This differs considerably from the old paradigm of neural induction and patterning. (Phillips)

- A recombinant cDNA library was constructed of all active genes (RNA) in amphibian cells previously shown to be sending signals for neural induction. These will enable the study of the mechanisms for neural induction at the molecular level. (Phillips)

- Normal patterns of expression of subsets of the *hsp90* and *hsp70* members of the heat shock protein gene family have unique cellular and temporal patterns of expression in the developing mouse testis and the midgestation embryo. (Wolgemuth)

- The particular pattern of expression of the stress-inducible member of the HSP 70 family raises the possibility that cells in early stages of spermatogenesis may be especially vulnerable to change in their environment. (Wolgemuth)

Bone and Muscle

- In a study of long bones of rats flown on Cosmos 1887, vascular changes were observed in the subperiosteal regions. This suggests that the cardiovascular changes occurring during space flight may have a skeletal component that significantly alters bone function. In the areas of bone where severe vascular changes were seen, osteocytic death and significant histochemical changes in the perivascular cells were also observed. (Doty)

- Growth plates of rats flown on Cosmos 2044 showed increased height and cell number in the proliferative zone, and reduced height and cell number in the hypertrophy/calcification zone compared with unloaded control groups. There was evidence that these changes may be dependent on age of animal during exposure to microgravity. (Duke)

- Rats flown on Cosmos 2044 showed minimal bone growth during the mission even though the body mass gain was similar to that of rats flown on Cosmos 1887. Cosmos 2044 rats were analyzed more quickly postflight and were three weeks older than the Cosmos 1887 animals. (Morey-Holton)

- Minimal bone changes occurred in adult female rats after two or four weeks of unloading, while muscle changes were similar to those reported in growing rats. (Morey-Holton)

- Rats exposed to simulated weightlessness for 3 days showed a 52% increase in osteoblast progenitor cells and a 47% decrease in preosteoblast cells in the tibial metaphysis. These results are similar to those seen in the periodontal ligaments of animals exposed to both actual and simulated weightlessness. (Roberts)

- Muscle atrophy induced by hypokinesia in rats shows that fibers display "anti-grouping" behavior: fiber types tend to avoid themselves and to seek adjacencies with other fiber types, the grouping behavior seen in normal muscular tissue. Atrophies caused by denervation show other types of fiber grouping, suggesting that the atrophy produced by skeletal muscle unloading is not neurologically mediated, but seems solely due to myogenic variables. (Kasper)

- Osteoblast-derived factor(s) stimulate bone resorption by maintaining the viability of osteoclast precursors rather than by activating mature osteoclasts. (Greenfield)

- Evidence suggests that in muscle atrophy in rats due to unloading, membrane receptor proteins are unaffected, although membrane enzymes show differential activities. (Tischler)

- Accelerated protein breakdown in unloaded muscle occurs mostly in the cytoplasm and seems to be linked to increases in calcium-dependent proteolysis. (Tischler)

- New evidence shows that unloading results in an increased isoproterenol (beta-adrenergic agonist) response to glycogen synthesis and cyclic AMP production. (Tischler)

- Neither IGF-1 nor IGF-2 (insulin-like growth factors, apparently produced by bone through stimulation by growth hormone) prevent loss of bone mass due to unweighting in rats. (Bikle)

- In hypophysectomized rats, growth hormone sustains growth of bone, but skeletal unweighting blocks this response. (Bikle)

- In rats, parathyroid hormone regulates collagenase gene expression, in part by a transcriptional mechanism (Partridge).

- A gene, p NG 3/7, that encodes for a spicule matrix protein in the sea urchin *S. purpuratus* was isolated and begun to be characterized. A cue from the extracellular matrix in the sea urchin's blastocoel is necessary to instruct the primary mesenchyme cells to express this gene. (Wilt)

- The rapid down regulation of protein synthesis that occurs at the onset of non-weightbearing is accompanied by a shift of polysome size toward larger polysomes (more ribosomal subunits per mRNA). This indicates there is a direct coupling of mechanotransduction to the control of gene expression in muscle at the translation level in rats. (Thomason).

- Micromass cultures of differentiating cartilage cells centrifuged at 2.9 g developed cartilage 24 hrs prior to the appearance of cartilage in control cultures. (Duke)

- Cloned osteoblast cells, treated with dexamethasone to simulate the effects of increased plasma cortisols, showed a decrease in new protein synthesis and demonstrated that collagen type I accounts for approximately 97% of the total collagen synthesis in this cell line. (Hughes-Fulford)

- In addition, studies using the same cells mentioned above revealed that glucocorticoids interfere with the cytoskeleton formation and assembly of collagen matrix during mineralization. (Hughes-Fulford)

- Results of studies of crosslink formation and collagen turnover in cultured bone cells derived from osteoblasts of chicken calvaria indicates that the presence of remodeling or collagenase activity appears to occur in culture, affecting newly synthesized collagen molecules. (Landis)

- Other evidence from these studies suggest that chemical crosslinks unique to collagen are responsible for the aggregation of smaller fibrils to larger ones and for the assembly of the extracellular collagen matrix as a whole. (Landis)

Regulatory Biology

- Paraventricular lesions in rats decrease circulating angiotensinogen. For the first time, it has been demonstrated that there is a neuroendocrine mechanism regulating the circulating level of this component of the renin angiotensin system. It appears that the lesions decrease secretion of the brain hormone that stimulates the pituitary to secrete the hormone that, in turn, stimulates thyroid hormone secretion. (Ganong)

- The sympathetic nervous system plays a major role in the increase in renin secretion produced by a low sodium diet. (Ganong)

- Serotonin was effective in modulating the amplitude of one neural response in rats exposed to hypergravity and control rats. This indicates that the decrease in the number of receptors observed under hypergravic conditions is insufficient to abolish a primary modulatory action of serotonin. (Horowitz)

- Rats born and reared in hypergravity were able to maintain their core temperature when cold exposed, compared with 1 g reared animals exposed to

cold in a 2 g environment. This shows that animals acclimated to hypergravic fields have an altered thermo-control system. (Horowitz)

Cell Biology

- Demonstrated that fibronectin controls cell growth based on its ability to support changes of cell and nuclear shape and that the effects of fibronectin on cell form and function are mediated by specific binding interactions with cell surface matrix receptors. These results suggest that cells may utilize a biomechanical signal transduction system during growth control. (Ingber)

- Showed that actin assembly is necessary for progression through G-1 phase of cell cycle. Demonstrated that fibronectin regulates actin filament assembly both by binding to cell surface receptors and by physically resisting cell-generated loads that are applied to those receptors. (Ingber)

PLANT PROJECTS

AN ATTEMPT TO LOCALIZE THE GRAVITY SENSING MECHANISM OF PLANTS: EFFECT OF GRAVITY ON THE ELECTRICAL PARAMETERS OF THE STELE-CORTEX JUNCTION IN CORN SHOOTS

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Description of Research

The first response of a plant to a gravity stimulus is membrane depolarization. This response occurs within seconds and is a fundamental characteristic of a plant's response to a stimulus, not only to gravity, but also to light. The second response is the development of an asymmetric distribution of the plant growth hormone, indole-3-acetic acid (IAA). The development of this chemical asymmetry appears to be the chemical transduction of the bioelectric response. The hormone asymmetry then leads to an appropriate growth response such that the plant grows back into its normal orientation with respect to gravity.

The objective of this research is to link the bioelectric response to gravity to the chemical response, that is, the asymmetric distribution of growth hormone. How is a bioelectric response transduced into a chemical asymmetry? We have attempted to explain this by means of our potential-gating theory. ***Our measurements reported here of the changes in potential difference between stele and cortex following a gravity stimulus were predictable by the potential gating theory.***

Our working hypothesis, the potential gating theory, is that the plant's internal bioelectrical gradients serve as the sensors and transducers of the gravity response of plants. Shifts in the plant's orientation with respect to gravity are followed by corresponding shifts in the plant's internal bioelectric fields. The internal bioelectric fields control the voltage-sensitive IAA-transporting channels between the plant's vascular tissue (stele) and the surrounding cortical tissue (cortex) such that IAA accumulates on the lower side of the shoot. This increased concentration of IAA on the lower side of the shoot leads to increased growth of that side resulting in curvature of the shoot back to the vertical.

In previous years we tested this hypothesis by examining the effect of externally applied potentials on growth, endogenous IAA concentration, and IAA transport from the seed kernel to the shoot. A small, externally applied potential affected the growth rate of the shoots in a polarity-dependent manner. The changes in potential-induced growth rate of the shoots corresponded to changes in the endogenous IAA concentration in the plant shoot but not to the transport of IAA from the seed kernel to shoot.

We also showed that an apoplastic transported dye would not penetrate the stele-cortex barrier in response to the same externally applied potentials even though the electrical resistance across the stele-cortex junction of the active growing regions was low. These experiments suggest that the plasmodesmatal channels across the stele-cortex junction are the controlling points for the transport of IAA from the vascular tissue to the cortical tissue in the shoot and the controlling factor for the stele-cortex junction is the potential difference across it.

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Accomplishments

We have measured the endogenous potential across the stele-cortex junction at three different regions of the shoot (the cell division region, the cell extension region, and a mature region) as a function of the orientation of the shoot with respect to gravity.

(1) ***An endogenous voltage is measurable across the stele-cortex junction.***

(2) ***The endogenous voltage changes in response to a shift in the orientation of the plant with respect to gravity.*** This is illustrated in Figure 1.

(3) For both vertical-to-horizontal and horizontal-to-vertical positional changes the shift in the voltage across the stele-cortex barrier at the division region of the shoot was significantly different from the other two regions of the shoot in both magnitude and direction.

(4) ***For vertical-to-horizontal positional changes in the orientation of the shoot, the voltage change across the lower stele-cortex barrier is different from the voltage change across the upper stele-cortex barrier in the regions of the shoot which show curvature*** (Figure 1).

(5) For a horizontal-to-vertical orientation change of the shoot, there was no asymmetric potential difference between the upper and lower junctions between the stele and the cortex (Figure 1).

(6) The electrical resistance of the stele-cortex junction is lowest in the regions of the shoot where curvature occurs.

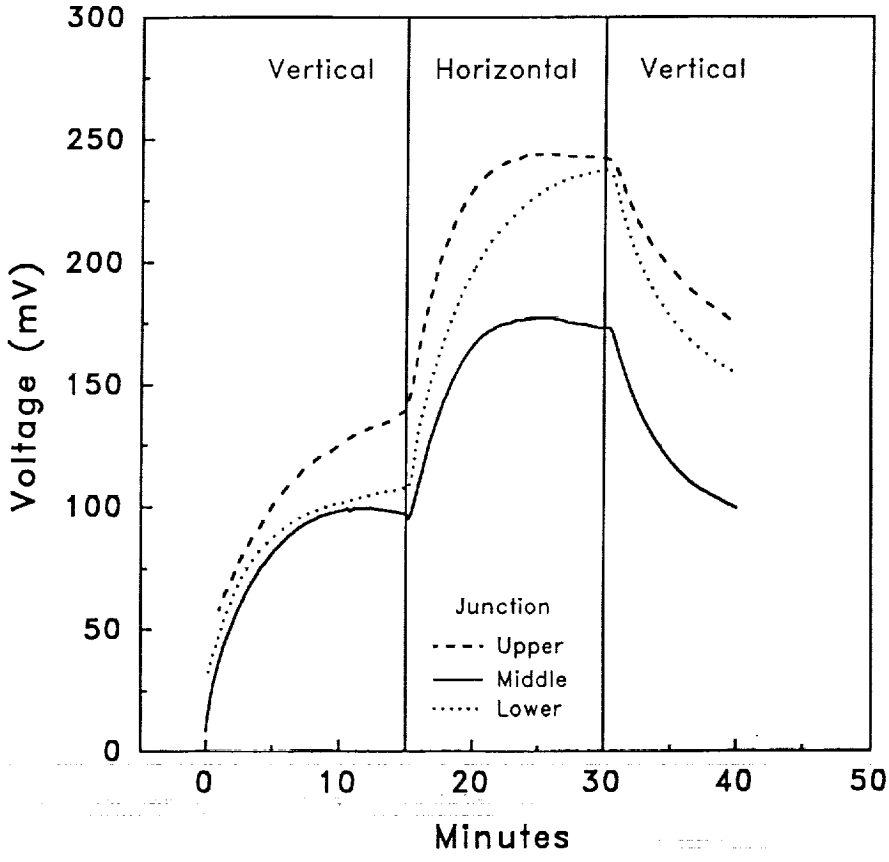

Figure 1. Changes in the electrical potential between the stele and cortex in the mesocotyl of *Zea mays* seedlings. Vertical lines indicate a change in orientation of the tissue.

Significance of the Accomplishments

Finding #1: A potential difference exists between the stele and cortex tissues.

Finding #2: The potential between the stele and cortex tissues changes within seconds following a change in the sample's orientation with respect to gravity and continues to change over 15 minutes. This potential change occurs throughout the length of the shoot.

Finding #3: The cell division region of the shoot behaves electrically in a manner different from the extension and mature regions of the shoot.

Finding #4: The lower side of the stele-cortex junction is at a different potential than the upper side of the stele-cortex junction. This supports the hypothesis of a voltage asymmetry between the upper and lower stele-cortex junctions,when the shoot is in a horizontal position, as being the controlling factor in regulating IAA transport out of the vascular stele into the cortical tissues.

Finding #5: The lateral electrical asymmetry does not develop when the gravity vector is aligned with the shoot.

Finding #6: The region of the shoot that curves in response to a lateral gravity stimulus has a lower resistance between the stele and cortex tissues than the region of the shoot which does not curve.

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THE ROLE OF CALCIUM IN GRAVITY SENSING

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Description of Research

The overall goal of this research is to determine how gravity is sensed in plants, and how this information is transduced into changes in the growth rates of plant organs leading to gravicurvature. There is convincing evidence that calcium might be required at two steps: at the site of perception of gravity and at the site of the unequal rates of elongation growth which actually results in the curvature. A current hypothesis states that a lateral redistribution of calcium occurs at both sites in horizontal stems and roots, and is essential for the response. At the site of perception the calcium redistribution is believed to be directly involved in the redistribution of the actual growth-controlling hormone. It has been suggested that at the site of curvature an accumulation of calcium in the walls of the future concave side stiffens the cell walls and inhibits acid-mediated wall extension. Direct evidence for both of these ideas is limited. Our objective is to provide evidence as to whether changes in extracellular calcium are directly involved in either the perception or the reaction stages of gravitropism.

Our research during the past year has been directed along three main lines. The first is to determine the amount and timing of the gradient of apoplastic calcium that develops at the tip (the site of perception) of maize roots. This is followed by studies in which the gradient has been eliminated, in order to determine whether it is needed for graviperception.

The second direction has been to determine the relationship between the wall-bound calcium, free apoplastic calcium, wall pH and wall extensibility in soybean hypocotyl tissues. In the first phase of this work the role of wall-bound calcium as a stiffening agent in cell walls has been determined by measuring the effect of calcium removal on the facilitated creep of isolated cell walls. In the second phase, the apoplastic calcium concentration has been measured with calcium microelectrodes, and then the pH that must have existed in the wall in order for that apoplastic calcium concentration to be in equilibrium with the bound calcium has been determined.

The third project is a reassessment of the role of apoplastic acidity in the control of cell elongation. Current theories, backed by considerable evidence, indicate that in the curvature zone differential rates of auxin-induced proton excretion lead to different rates of acid-induced wall loosening and thus growth. We have begun to reassess this concept, with the goal of determining to what extent auxin-induced growth is acid-mediated and to what extent a second, acid-insensitive process is involved.

Accomplishments

(1) A calcium microelectrode has been used to demonstrate that a gradient of apoplastic calcium does develop at the tip of corn roots during graviperception. The apoplastic calcium concentration is about 5 mM at the tips of vertical corn roots, but begins to change within 10 min after roots are placed in a horizontal position. The calcium concentration on the upper side decreases to one-half while on the lower side it increases two-fold. The result is a four-fold gradient of calcium across the gravisensing region. These data demonstrate directly, for the first time, that ***there is a gradient in***

apoplastic calcium that develops across the graviperception zone of horizontal roots.

(2) The role in graviperception of gradients of calcium, indoleacetic acid and hydrogen ions across the root apex has been examined by fixing small lengths of capillary tubing onto the tips and filling them with solutions. When the tubes are empty or filled with water, gravicurvature is normal. When filled with a solution of the weak calcium chelator dinitro-BAPTA and 1 mM Ca^{2+} so as to equalize the apoplastic calcium concentration across the tip, curvature is severely inhibited. This demonstrates that the ***calcium gradient that develops across the perception zone of horizontal roots is essential for the gravitropic response.***

When the tubes are filled with 10 μM IAA, gravitropic curvature is also blocked. This provides direct evidence that a ***gradient of auxin across the graviperception zone is necessary for curvature.*** On the other hand, addition of either a pH 6.5 buffer, to maintain a constant neutral pH, or fusicoccin, to maintain a constant acidic apoplastic pH, has no effect on curvature. ***A gradient of acidity, if it occurs, is not important in the initial steps of gravitropism.***

(3) Once an asymmetrical distribution of growth regulators occurs in the graviperception zone, it must be transmitted back to the reaction zone in roots. Evans and co-workers have argued that this transmission occurs in the epidermal layers from experiments where removing a girdle of epidermal cells seemed to prevent curvature beyond the girdle. We have reinvestigated this, and have shown that even when the whole epidermis, from the cap through the elongation zone is removed, gravicurvature still occurs. Our data demonstrate that the ***epidermal cells are not required for root gravitropism, and that the growth regulator does not have to pass through this cell layer.***

(4) Calcium microelectrodes have been used to measure the apoplastic free calcium activity in soybean hypocotyls. The values obtained vary from hypocotyl to hypocotyl, but the average value is about 10 μM. There are no comparable published values for apoplastic free calcium from other systems.

(5) A study has been made as to the conditions of pH and free Ca^{2+} which are in equilibrium with the wall-bound calcium of soybean hypocotyl walls. It has been shown that if the free Ca^{2+} is 150 μM, the apoplastic solution must have a pH of about 3.5. This pH is considerably lower than that normally accepted for the apoplastic solution, i.e., a pH of 5.0 to 6.5. There are two possible explanations. The first is to assume that the wall-bound calcium is not in equilibrium with the apoplastic free calcium. There is no reason to believe that this could be true. The second is to assume that the uronic acids exist as part of the Donnan Free Space (DFS), whose pH is different from that of the Water Free Space (WFS). Theoretical studies have suggested that the pH of the DFS can be as much as 2.5 pH units lower than that of the WFS, due to protons trapped in this space to neutralize the uronic acids.

(6) If the pH of the DFS is significantly lower than that of the WFS, addition of exogenous Ca^{2+} would be expected to raise the pH of the DFS and lower that of the WFS, since Ca^{2+} would substitute for H^+ as the ion balancing the fixed negative charges. This, in turn, would inhibit acid-mediated cell elongation. To provide a partial test of this idea, the effect of exogenous calcium on the WFS pH of soybean hypocotyl sections was determined using a newly developed assay in which the rate of uptake of a weak acid, benzoic acid, is used to measure the pH at the external face of the plasma membrane (the

WFS pH). We have shown that exogenous Ca^{2+} causes a decrease in WFS pH of almost 1 pH unit. While this does not prove the DFS concept, it provides strong evidence in its favor.

(7) It is well established that in tissues in which growth is promoted by auxin, auxin promotes proton excretion into the apoplast, and exogenous acid has the ability to cause these cells to elongate in the absence of auxin. The acid-growth theory states that auxin-induced elongation requires this apoplastic acidification, and that wall loosening only occurs under acidic conditions. The most significant evidence against this theory is that growth induced by exogenous acid only persists for about 2 hours while auxin-induced growth can persist at a constant rate for many hours. Several investigators have suggested, without direct evidence, that auxin-induced growth consists of two sequential reactions: initially, via an acid-growth mechanism and, subsequently, via a completely different auxin-mediated reaction. If so, it should be possible to show that auxin-induced growth should require an acidic apoplast during the first 2 hours but then proceed equally well at more neutral pHs thereafter. Amazingly, this simple test has never been carried out. We have now tested this with *Avena* coleoptiles, and find that auxin-induced growth can best be explained as consisting of two simultaneous processes: the acid-growth response during the first 2 hours and a second process whose pH optimum is 5.5-6.5 and which persists for up to 24 hours. These results are being refined and confirmed; if they are correct, the ideas concerning the mechanism of auxin-induced growth, including gravitropism, will have to be modified accordingly.

Significance of the Accomplishments

Three major conclusions have arisen from this year's research. The first is that gradients of calcium do develop across the graviperception zone of the root during normal gravitropism and are essential for the gravitropic response. It has been shown previously that applied gradients of calcium cause curvature, but this is the first demonstration of an *in vivo* calcium gradient.

The second conclusion is that calcium does not act as a major wall stiffening agent in hypocotyl walls, and that increases or decreases in wall-bound calcium cannot be a part of any mechanism of gravitropism. There is simply no evidence for either an *in vivo* redistribution of calcium in the actual zone of curvature, or a role of extracellular calcium in the curvature itself.

The third conclusion, which is still somewhat tentative but potentially of great importance, is that auxin-induced acidification is only involved in the initiation of growth and that a second, acid-insensitive process is responsible for prolonged auxin-induced growth. This has considerable implications for gravitropism, which occurs over short time periods. Of particular interest will be a determination as to whether the gravitropic curvature is due to the acid-mediated growth or to the non-acid growth mechanism.

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MECHANISM OF DIFFERENTIAL GROWTH DURING STEM GRAVITROPISM

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Description of Research

Gravitropism offers us a valuable opportunity to learn about the interaction of gravity with growth processes and to gain insight into the cellular, physical, and molecular processes that contribute to and govern plant growth. When plants are placed in a horizontal position, their stems typically grow upright and their roots grow downwards. This gravitropic bending occurs because of a growth redistribution on the upper and lower sides of the organ. We are using young cucumber (*Cucumis sativus* L.) seedlings to investigate the mechanism of gravitropism because they show a very vigorous response: after a lag of about 10 min the upper stem surface ceases growth entirely whereas the lower surface doubles or triples its expansion rate. Subsequently, this growth asymmetry reverses itself. Since the stem diameter is only 1.5 mm, this means that cells very close to one another respond oppositely to gravity. Elucidation of this rapid growth response will likely tell us a great deal about how plants integrate sensory stimuli and use this information to control their growth.

When intracellular pressure, or turgor pressure, was measured with the pressure probe, we found only a negligible, passive change on the two sides of the stem. This demonstrated that cell expansion was not modulated by altering cell turgor or other related hydraulic properties of the growing cells. By stress-relaxation analysis on growing tissues and on isolated cell walls, we found that the wall on the upper stem was not physically stiffened during the period when its growth was inhibited. Rather, the active process of wall loosening was inhibited. At present the nature of this loosening process is uncertain.

We are focusing on the working hypothesis that enzymatic modification of the wall regulates wall loosening, and that the activity of these hypothetical enzymes is regulated during the gravitropic response, perhaps through changes in the ionic (Ca^{2+}, H^{+}) environment of the wall or through some covalent modification. In this past year we have attempted to characterize some wall enzymes in the cucumber seedling which may be responsible for wall relaxation. Attention was focused on enzyme activities that break down the polysaccharides of the wall matrix.

Accomplishments

(1) Cell walls from the growing region of cucumber seedlings were found to exhibit autolytic activities. Release of monosaccharides, oligosaccharides and uronic acids from wall fragments was measured by anion exchange chromatography and pulsed amperometry. A number of sugars were released in a time-dependent fashion.

(2) ***The pH-dependence of wall autolytic activities was found to vary among the sugars released. The maximum rates of autolysis were found to be at pH 5.5 or 6.5. In comparison, long-term extension of isolated cucumber walls had a much lower pH optimum (about 4).***

(3) ***The pattern of autolytically released sugars was the same from epidermal walls as for inner cortical tissue walls.***

(4) ***The autolytic process was relatively insensitive to most added cations (Al^{+3}, Cu^{+2}, Ca^{+2}, K^{-1} and others), with the exception of H^{+} and Hg^{+2}. In contrast, extension of isolated walls and growing segments were sensitive to some of these ions.***

(5) Basal wall fragments also exhibited autolytic activity, but the pattern of sugar release differed from that of the apical growing walls.

(6) A large number of proteins are associated by ionic and hydrophobic bonding to isolated cell walls. This was demonstrated by extracting isolated walls with 3M LiCl or with phenol/acetic acid/water, and separating the solubilized proteins by SDS gel electrophoresis.

Significance of the Accomplishments

Finding #1 confirms and enlarges upon previous studies, showing that walls from growing plant tissues indeed contain substantial autolytic activities. Our analytical technique has high sensitivity (10 pmoles of glucose can be measured) and can resolve nearly all of the wall fragments released by autolytic enzymes. Thus we have a powerful system for examining wall autolysis and how it may be affected during gravitropic responses.

Finding #2 suggests that wall autolysis shows a distinctly different pH dependence than (a) endogenous growth during gravitropism, and (b) extension of isolated walls. Finding #4 extends this observation to several cations. The implication is that growth and wall extension *in vitro* are not rate-limited by wall autolytic reactions. It may be that wall autolysis is independent of the process(es) causing wall loosening and relaxation. This is reinforced by Finding #5, showing high autolytic activity in nongrowing walls. This shows that wall autolysis does not necessarily lead to wall expansion.

Finding #3 shows that autolysis is qualitatively the same for epidermal and inner tissues. This is important to know because the epidermal wall is hypothesized to be the major restraint to stem growth and the major site of action of auxin and gravitropic growth responses. We find no evidence for distinctive autolytic processes in the epidermis.

Finding #6 indicates that plant walls may be enzymatically complex mixtures. The results also provide a basis for further investigations into possible alterations of wall enzymes during gravitropic responses.

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THE GRAVITATIONAL RESPONSE OF *PHYCOMYCES* WITH RESPECT TO GADOLINIUM AND FACTORS AFFECTING CALCIUM FUNCTION

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Description of Research

One of the simplest systems which detects and responds to gravity is the large single-celled, upright, reproductive sporangiophore of the terrestrial fungus, *Phycomyces*. As a single cell it contains all mechanisms for the detection of gravity to the asymmetrical cell wall growth response. This system is being considered because of the appealing possibility that a number of molecular mechanisms for gravitropism may be evolutionarily preserved and be common to a wide variety of cell wall-bound organisms, including the higher plants.

Preliminary studies indicated that the lanthanide gadolinium, which has been shown to inhibit gravitropism but not linear growth in higher plants, has a similar effect on *Phycomyces* gravitropism. The focus during the last year has been to describe the uptake, movement, and location of gadolinium in the sporangiophore to more closely describe the effect of gadolinium on the kinetics of gravitropism, and to preliminarily examine the effects of calcium modulators on gravitropism. Much of this work has been done in conjunction with two Honors Research students, Bill Joiner and Tawny Stecker.

Accomplishments

(1) The *Phycomyces* sporangiophore has a chitinous cell wall covered with a cuticle to reduce water loss. These structures are minimalized in the growing zone at the apex just beneath the sporangium. Radiolabelled gadolinium applied in the agar medium on which the fungal mycelium is grown or applied as a thin layer of liquid to the top of the agar medium moves slowly (days) into the sporangiophore itself. Gadolinium is readily taken up into sporangiophores when applied to them directly, either by: (a) inverting intact sporangiophores and submerging them in solution or (b) applying the gadolinium to the apical region by agar block.

(2) Radiolabelled gadolinium is incorporated largely into cytosolic and membrane fractions of the sporangiophore and is tightly though not covalently bound. ***Gadolinium is associated with precipitable proteins in both of these fractions and to one class of large molecular weight proteins in the cytosol.*** Since gadolinium is highly charged it is known to be generally "sticky," but we find that tightly held gadolinium appears to be associated with specific proteins. The nature of these proteins is not known. Further characterization is underway.

(3) The sporangiophore displays a large number of tropistic movements of which gravitropism is one of the 'weaker' responses. It is over-ridden by phototropism with adequate light, for example. Examination of sporangiophore kinetics requires careful technique under infrared conditions, yet among individuals there is considerable variability, which is enhanced by many physical disturbances (e.g., air currents, touch, heat). For these reasons we initially wanted to avoid physically dipping sporangiophores into gadolinium chloride solutions to study its effects on the kinetics of gravitropism. Instead, vertical sporangiophores produced from 5-7 day-old mycelia grown on agar containing

low, 1-7 μm, gadolinium were oriented horizontally, and the kinetics of gravitropism were examined. Gadolinium, even at 1 μM, consistently extended the latent period prior to the onset of asymmetric upward curvature by 2.5 hr. Controls initiated gravicurvature after 30 min reorientation to gravity while gadolinium experimentals began responding after 3 hr. The curvature rate was reduced 20-40% in the presence of gadolinium, as was the linear growth rate. Thus, ***long term exposure to gadolinium affects both gravity detection/transduction/growth and linear growth.*** Short-term exposure of sporangiophores to gadolinium may, as in higher plants, similarly eliminate the effect of linear growth rate. This possibility is being examined. However, it is clear that gadolinium does affect gravity perception/transduction.

(4) Preliminary study of various calcium effectors on sporangiophore gravitropism was pursued by application in small 1-3 mm agar blocks to one side of the sporangiophore apex. ***Blocks containing gadolinium chloride, EGTA (external calcium chelator), BAPTA/AM (internal calcium chelator), and Compound 48/80 (calmodulin inhibitor) induced curvature towards the block when applied to vertical sporangiophores;*** they were not affected significantly by control agar blocks. BAPTA/AM and C48/80 were the most effective agents and were able to override upward gravitropism when applied to the lower surface of horizontal sporangiophores. We suggest that internal calcium and calmodulin may serve in stimulating wall growth and asymmetric curvature.

Significance of the Accomplishments

Phycomyces sporangiophores grown on medium containing gadolinium at low micromolar concentrations show reduced ability to detect and transduce gravitational signals and have a slower curvature rate. Gadolinium has been shown to bind to proteins in the cytosol and membranes of the sporangiophore. These proteins may be involved in both sensing/transduction and growth. The sensing/transduction mechanism theoretically could entail stretch-activated ion channels or voltage-gated calcium channels which have been shown to be inhibited by gadolinium in frog oocytes (Yang and Sachs, 1989) and hybrid neuroblastoma-glioma cells (Docherty, 1988), respectively. Asymmetric growth could be realized through the involvement of voltage-gated calcium channels and calmodulin, for which gadolinium competes with calcium, resulting in inhibition (Buccigross et al., 1986). Calmodulin is present in *Phycomyces* and is known to be similar to that in animals. The results from the agar block experiments suggest involvement of calmodulin and internal calcium, in at least the regulation of growth. We are presently undertaking the characterization of stretch-activated and calcium ion channels in sporangiophore tonoplast and plasma membrane vesicles to better our understanding of gravity detection and signal transduction.

ROLE OF CALCIUM IN SIGNAL TRANSDUCTION IN ROOT GRAVITROPISM

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Description of Research

This research is directed toward understanding the influence of gravity on plant growth — in particular how roots become oriented with respect to gravity (gravitropism). The detection of gravity occurs at the tip of the root while adjustments in growth rate occur in the growing region about 0.5 cm behind the tip. There is evidence that calcium within the cap plays a role in linking gravity detection to asymmetric distribution of the growth-inhibiting hormone, auxin, in the growing region of the root. We are interested in the pathway of movement of signal from the cap to the elongation zone. We are also interested in characterizing the signal detection function of the root cap for signals other than gravity, e.g., pressure or electrical gradients. It is likely that these detection mechanisms have features in common, and the elucidation of these features will help in the understanding of stimulus/response coupling in plants.

Our research has centered on the following: (1) Measurement of calcium levels in protoplasts from cells of the elongation zone and cap of maize roots, and testing whether treatments known to affect gravitropic sensitivity also affect cell calcium; (2) Testing the effects of antibodies to auxin and abscisic acid (a second candidate for the hormonal mediator of gravitropism) on the longitudinal transmission of the gravitropic signal. In these experiments we remove entire rings of cortical tissue from around the root at various positions between the cap and elongation zone, fill the gap with agar or with agar containing antibody, and then determine the effect of these treatments on gravitropism. In related experiments, we measure the relative responsiveness of different cell layers to auxin using longitudinally bisected roots; (3) Use of a computerized video analysis system to determine the detailed kinetics of growth changes during gravitropic curvature; and (4) Analysis of the effects of two different stimuli (mechanical impedance of root elongation and imposition of an electrical field across the root tip) on root growth patterns. The objective is to compare detection and response to these signals with the graviresponse. It is likely that these detection/response systems have features in common.

Accomplishments

(1) We isolated protoplasts from both the cap and the elongation zone of maize roots, loaded them with the calcium indicator dyes indo-1 and fura-2 and measured cytoplasmic free calcium. The cytoplasmic free calcium level is much higher in protoplasts from the elongation zone than in protoplasts from the root cap. Preliminary results indicate that auxin has no effect on the cytoplasmic calcium content of protoplasts from root cap cells. We also found that ***transient plasmolysis of the root cap prevents gravity-induced calcium redistribution across the cap but does not prevent gravitropic curvature.***

(2) Earlier, we found that when we remove a narrow (0.5 to 1 mm) ring of cortical tissue from the root within the apical portion of the elongation zone, gravitropic curvature occurs apical to the girdle but not behind it. The signal will pass a girdled zone filled with a

diffusion medium such as agar. We have obtained antibodies to free auxin and abscisic acid and plan to test their ability to block signal movement through an agar-filled girdle.

(3) We have made progress in the reliability and accuracy of a computer-based video digitizer system for automated analysis of growth during gravitropism. We used the system to obtain a detailed characterization of localized growth rate changes in graviresponding maize roots (Figure 1). The results reveal ***large time-dependent changes in localized growth rates during the gravity response. In particular, the pattern of growth rate distribution changes dramatically during the response from an early phase with rapid growth on the top and zero growth on the bottom to a later phase with the opposite pattern*** (rapid growth on the bottom and zero growth on the top). The analysis also reveals a shift in the growth zone toward the apex, especially along the upper side.

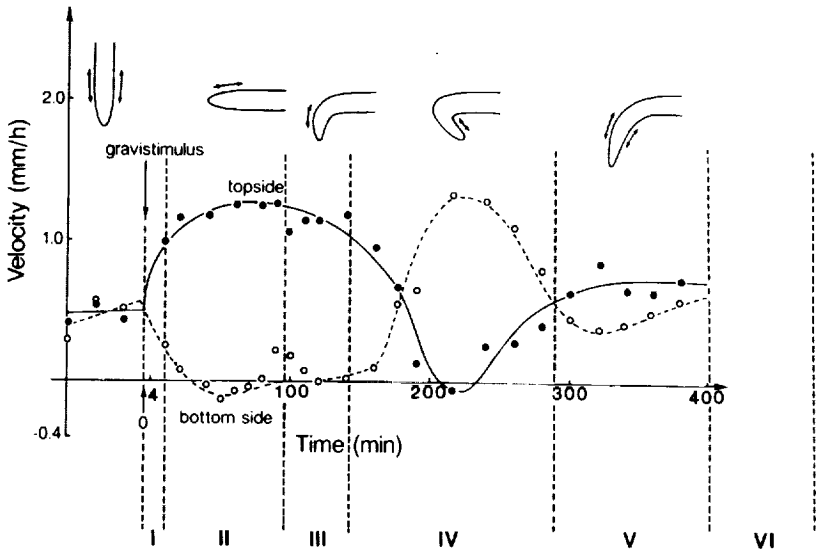

Figure 1. Time-dependent variation of total rate of extension (velocity, mm h^{-1}) on the upper (convex surface of bend) and lower (concave surface of bend) side of a gravistimulated maize root. Convex surface of bend = solid line, concave surface of bend = dashed line. The curvature response is arbitrarily divided into six phases indicated I-VI.

(4) We have tested the ability of roots to respond to mechanical pressure and to electrical gradients and compared these responses to the gravitropic response. Roots respond to an electrical gradient by curving strongly toward the positive (Figure 2). ***They respond to mechanical resistance by increasing their growth rate and, if the resistance is great enough, initiating curvature*** resulting in growth around the obstacle. ***Both the responses to mechanical resistance and to electrical fields occur even when the cap is removed from the root.*** The response to gravity requires the cap.

RESPONSE OF MAIZE ROOTS TO AN ELECTRICAL GRADIENT

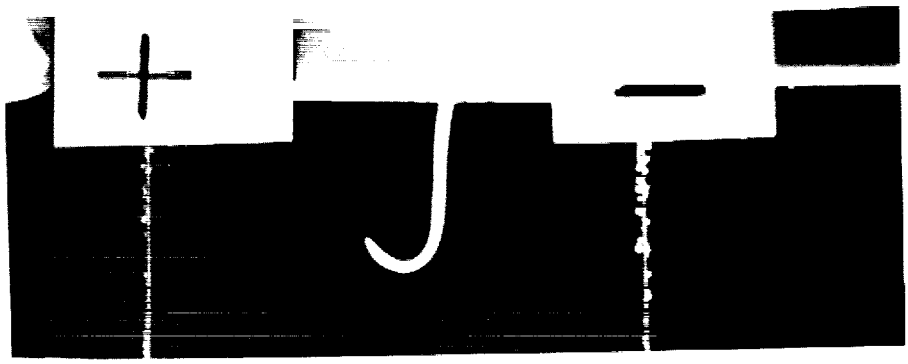

Figure 2. Electrotropic curvature of the primary root of a maize seedling (cv. Merit). Curvature is toward the positive electrode. Roots with the cap removed respond strongly to electrical fields but do not respond to gravity stimulation.

Significance of the Accomplishments

Finding #1: Calcium has been proposed to mediate transduction of the gravitropic signal. The finding that the cytoplasmic calcium level is unusually low in root cap protoplasts suggests that these cells may be especially sensitive to calcium as a signaling ion. Our observation that transient plasmolysis prevents gravity-induced calcium redistribution but not curvature indicates that, although calcium is necessary for the gravitropic response, a gradient of calcium may not be critical.

Finding #2: Last year we reported that the gravitropic signal can pass across a region of the elongation zone from which a ring of cortex has been removed, provided the gap is filled with a diffusion medium such as agar. This system provides a convenient system for testing the nature of the signal transmitted from the cap to the elongation zone. The planned experiments in which antibodies to auxin and abscisic acid will be incorporated into the agar should provide a specific test of whether the signal moves in the form of one of these two hormones.

Finding #3: The detailed analysis of growth patterns during the gravitropic response shows that curvature cannot be attributed to a simple inhibition of growth along the lower side as proposed by some models. Not only does the growth zone itself shift, but the gradient of growth rates reverses completely at least once during the gravitropic response. This information is critical to evaluation of models of gravitropism. Our data indicate that multiple factors control growth rate distribution during the response. This makes it especially important to investigate the role of changes in sensitivity to hormones (or to the gravity stimulus itself) during the response.

Finding #4: The fact that the root is sensitive both to electrical stimulation and to mechanical resistance provides two related stimuli that can alter root growth performance. In both cases growth rates are affected and curvature is induced (only above a certain threshold for mechanical impedance). It is particularly significant that both of these responses also occur in decapped roots, i.e., the root cap is not necessary for the detection of either of these stimuli. The fact that cells of the root other than from the cap are highly sensitive to environmental stimuli suggests that they may also participate in gravity sensing.

This raises the possibility that, although the cap is necessary for the gravity response, it may not be the sole site of gravity perception. Characterization of the response to electrical fields and mechanical impedance may provide important clues concerning the nature of gravity detection by plants.

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PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR PROCESSES ASSOCIATED WITH GRAVITROPISM IN ROOTS OF MAIZE

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Description of Research

On Earth roots typically respond to gravity by growing downward. Our research focuses on the physiological, biochemical, and molecular steps involved with converting the stimulus of gravity into a biological response.

For this effort we would like to be able to "turn gravity off" at will and establish some base line with regard to biochemical and physiological processes, and after having done this, "turn gravity back on" and observe what new processes are initiated. Unfortunately, being able to turn gravity on or off at will is not yet available to us, especially for Earth-based materials. Hence we use systems in which the response of the tissues to gravity, even in the presence of gravity, is incomplete unless some other requirement is also satisfied. For our work, that other requirement is light. Roots of certain varieties of corn will not respond completely to gravity unless the root is also provided with some light. Light therefore initiates some biochemical processes which are necessary for complete response of the roots to gravity. Our hope was that by learning about the processes initiated by light, we might gain an understanding of steps involved in converting the gravity stimulus into a growth response. Our efforts for this project have concentrated on defining the role of a particular pigment, phytochrome, which we earlier showed was responsible for mediating the light/gravity response in roots. Our efforts have shown that messenger RNA (mRNA) for phytochrome (the compound coding for the phytochrome protein) is located preferentially in the root cap where gravity is perceived and processed in roots. Moreover, the phytochrome mRNA is highly expressed in specific cells within the root cap, the so-called columella cells, where we now believe gravity is first detected by the root. The intimate spatial association between these cells and the phytochrome mRNA makes it likely that the phytochrome protein mediates very early steps in the processing of the gravity stimulus. Our best guess right now is that probably the phytochrome protein affects receptors involved with gravity perception. Other evidence showing how rapidly (kinetics) phytochrome can affect a response also supports our notion of this molecule being involved in early steps of gravity processing. The challenge now will be to determine which events are phytochrome-mediated and, in particular, how they are associated with gravity perception and/or processing.

The second focus of our work has been on understanding the relatively later steps in the processing of the gravity stimulus. These steps occur after the involvement of phytochrome and are believed to make use of particular proteins known as kinases. These proteins function by phosphorylating (by putting a phosphate onto other proteins), thereby changing their structure and allowing for the transduction of an environmental signal. Based on analogies with animal and microbial systems, it is hypothesized that the processing of the gravity signal involves a kinase. Unfortunately, little is known about plant kinases. Indeed, none has ever been completely purified. Thus, we have taken a molecular approach and looked for kinases which, based on their characteristics, may be involved with processing various environmental signals, such as gravity. To date this effort has resulted in the positive identification of a kinase, which is structurally highly homologous to other animal kinases involved in the transduction of many environmental

stimuli. The kinase is located in the root cap and is not affected by light. The challenge ahead will now be to further characterize this kinase with regard to its possible role in mediating the transduction of the gravity signal in roots.

Accomplishments

The major findings from these studies are:

(1) ***There is an intimate association between the phytochrome mRNA and the cells in the root cap believed to be associated with gravity perception.***

(2) ***Light leads to very rapid changes in the level of the mRNA for phytochrome and the kinetics of this light-induced change correlate very strongly with the light-induced gravity response.***

(3) ***A kinase homologous to those involved with environmental signal transduction in animals and microbial systems is present in the root cap. This kinase is not light-regulated, therefore implying that it works late in the signal transduction chain.***

Significance of the Accomplishments

Virtually nothing is known about the biochemistry involved with converting the gravity stimulus into a developmental response in plants. Our recent work has shown an intimate spatial association between the phytochrome mRNA and those cells involved with the perception of gravity, thus making it likely that phytochrome mediates some early steps in processing gravity. Knowing this, we can now concentrate on looking for those steps which are phytochrome-mediated, and hence which may be involved with the perception of gravity. Our work with kinases is the first to look at the role of this important group of proteins specifically in the transduction of the gravity stimulus. We have demonstrated the presence in the root cap of a kinase protein which is similar to proteins in animals and microbes already shown to transduce a variety of environmental signals, thus making it likely that the root kinase functions in a similar way (although not necessarily for gravity) in roots of maize.

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CIRCADIAN RHYTHMS PERSIST IN SPACE WITH A DECREASED FREQUENCY: PRELIMINARY RESULTS FROM STS-32 LIFE SCIENCE EXPERIMENT "CHARACTERIZATION OF *NEUROSPORA* CIRCADIAN RHYTHMS IN SPACE"

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Description of Research

An experiment utilizing the fungus *Neurospora crassa* was conducted on NASA space shuttle flight STS-9 in late 1983 to examine the function of circadian rhythms (i.e., biological oscillations with periods approximately 24 hours in length) when removed from the daily geophysical rhythms induced by the Earth's rotation. The STS-9 experiment reexamined whether the drive of circadian oscillations is generated endogenously or imposed exogenously via environmental influences. While it is known that environmental oscillations such as light provide time cues which influence diurnal oscillations in the physiology of most organisms, it is also known that many physiological rhythms persist in the absence of these geophysical times cues. The STS-9 results did not conclusively support the endogenous generation of biological rhythms. The results demonstrated a significant reduction in the clarity of conidial banding (rhythm of asexual spore formation) which has been suggested to be a reflection of circadian rhythm amplitude. This damping of the conidiation rhythm was sufficient to obliterate the distinction of daily cycles, resulting in apparent arrhythmicity in 25% of the cultures. The damping and arrhythmicity in the conidiation rhythm, while unexpected, was reversed by the inflight marking procedure. Following this procedure, in which the cultures were exposed to light (and acceleration via handling), rhythmicity was reinstated and persisted for the remainder of the flight, suggesting that endogenous biological clocks can function in space. Other findings included an increase in variability of the free-running period and growth rate. This increase in variability of the period may have obscured an apparent decreased frequency of the rhythm in space.

Postflight studies at the University of California-Davis Acceleration Laboratories supported a hypothesis that the hypergravity of launch (centrifugation at 3 g for 15 min) could have been responsible for the damped circadian rhythm. Furthermore, the damping effect of hypergravity was eliminated by a light pulse following centrifugation. These studies suggested a gravity sensing system in the pacemaker of *Neurospora.*

To test the hypothesis that *Neurospora* possesses a circadian rhythm of conidiation that is endogenously generated and that *Neurospora* has a gravity sensing system which can affect aspects of circadian function (e.g., period, phase and amplitude), we conducted an experiment on shuttle flight STS-32 in January of 1990. Substantial alterations in the design of the STS-9 package were implemented to greatly increase further delineation of the timekeeping function of organisms in space. These changes consisted of: doubling the sample size; adding a second strain of *Neurospora* (CSP; which possesses a more robust rhythm and appears less susceptible to hypergravity); subdividing the package into three sections {one package treated similarly to the original STS-9 experiment (Blue), a second package of tubes wrapped in a red filter which allowed the tubes to be marked without exposure to white light (Red), and a third package left undisturbed during the first marking procedure (White)}; marking tubes earlier inflight (Figure 1); increasing inflight marks; and

adding gas sampling syringes. One flight package (G) and four 25°C synchronous ground controls (B1-B4) were compared. Two asynchronous Orbital Environmental Simulator (OES) controls and one 25°C asynchronous ground control were run at KSC in May 1990, to discern the effects of variations in orbiter ambient temperatures, humidity, and carbon dioxide from the effects of microgravity. The OES data is currently under analysis.

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Figure 1. STS-32 mission specialist Marsha Ivins performs the first inflight marking procedure nine hours after launch. She is holding one of the 25 "race" tubes in the "Blue" package up to the mid-deck light and marking the growth front. Only the "Red" and "Blue" packages were marked at MET L+00/09:00. Gas samples were also taken at this time.

Accomplishments

While there was some damping in flight, the amplitude of the conidiation rhythm of Neurospora flown on STS-32 was much more robust than that seen on STS-9. Only two flight cultures became arrhythmic in space. The conidiation rhythm in these tubes was quickly reinstated by a marking procedure late on flight day 5. Therefore, the results of the flight experiment support the hypothesis that while influenced by environmental perturbations in light, temperature, and gravity, ***circadian rhythms appear to be endogenously generated diurnal oscillations.***

The rhythm amplitude of the BND cultures in the "Red" flight package was significantly more damped than in only one of the four "Red" ground control packages. The BND cultures in the "Blue" flight package were, however, significantly more damped than three of the four "Blue" ground control packages. There was no significant difference in rhythm amplitude between the "White" flight or any of the "White" ground packages.

The CSP cultures in the "Red" and "Blue" flight packages had a significantly higher percentage of tubes with a damped amplitude of the circadian rhythm of conidiation (88% and 100%, respectively) than any of the four ground control "Red" and "Blue" packages (80% of the "Red" and "Blue" ground cultures demonstrated no rhythm damping). Again there was no significant difference between the CSP cultures in the "White" flight vs. the "White" ground control packages.

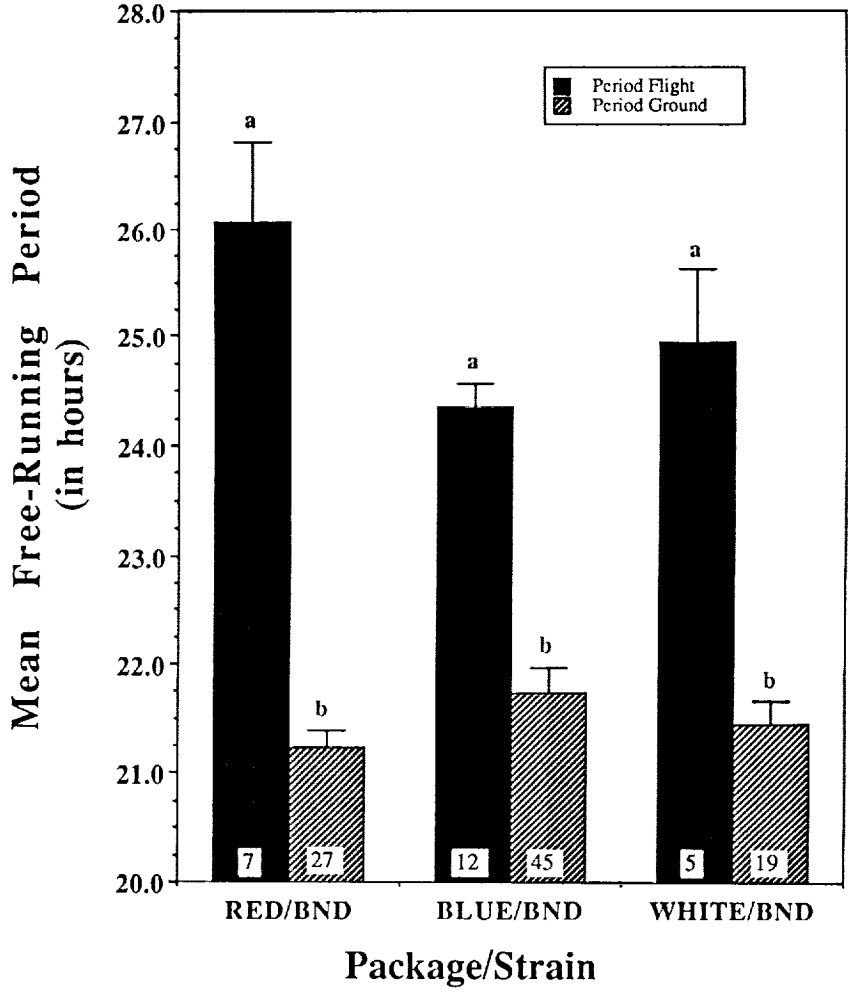

Figure 2. Mean ± SEM free-running period (in hours) of the circadian rhythm of conidiation in the BND strain of *Neurospora crassa* for each of the three flight packages ("Red", "Blue", and "White") and each of the three packages of the mean of the four sets of ground controls. The number at the lower portion of the bar represents the number of cultures in each mean. Bars with dissimilar superscripts are significantly different ($p<0.01$).

To some extent in the BND strain, but to a greater extent in the CSP strain of Neurospora, in both the "Red" and "Blue" packages, there was a decrease in the amplitude of the conidiation rhythm in space. The rhythm in the "White" flight package, however, was similar in amplitude to "White" ground control packages for both strains. While the UC-Davis studies demonstrated that the hypergravity of launch could cause damping of the circadian conidiation in *Neurospora*, the flight data suggest that ***microgravity can also induce a diminution of the rhythm of conidiation.*** Some aspect (e.g., light exposure, acceleration) of the ***marking procedure can reinstate high amplitude rhythmic conidiation.***

The period length of the circadian rhythm of conidiation in the BND cultures in the "Red" flight package (26.1±0.7 hr) was significantly longer than any of the "Red" ground packages (21.6±0.6, 21.1±0.3, 21.3±0.1 and 20.9±0.1 hr; $p<0.01$) which were not significantly different from one another. The BND cultures in the "White" flight package had a mean period length (24.9±0.7 hr) which was also significantly longer than any of the "White" ground control packages (20.9±0.1, 21.5±0.2, 21.8±0.6 and 21.7±0.6 hr; $p<0.01$). The "White" ground packages were not significantly different from one another. While the BND cultures in the "Blue" flight package had significantly longer free-running periods (24±0.2 hr) than the BND cultures in the "Blue" ground packages (22.2±0.7, 22.9±0.3, 21.3±0.3 and 20.6±0.1 hr; G vs. B2, $p<0.05$; G vs. B1, B3, or B4, $p<0.01$), there were also smaller, yet significant differences in the average period of BND cultures among the four ground control packages (see Figure 2). The overall trend, however, was that ***spaceflight was correlated with tremendous increases in the length of the free-running period of the circadian oscillator in the BND strain of Neurospora.***

The effect of gravity on the period length of the circadian rhythm of conidiation was more attenuated in the CSP strain. Spaceflight, however, did significantly increase the free-running periods of the rhythm in the "Red" and "White" flight packages when compared to ground controls (Red G vs. Red B1, $p<0.05$; Red G vs. B2, B3, or B4, $p<0.01$). The CSP cultures in the "Blue" flight package, however, had an average free-running period that was not significantly different from the "Blue" B1 ground control package. In fact, CSP cultures in the "Blue" flight package had an average period length that was significantly shorter than three of the four "Blue" ground control packages ($p<0.05$; see Figure 3).

Significance of the Accomplishments

The findings of STS-32 Life Science Experiment CNCR-01 strongly suggest that circadian rhythms can persist in space and support the hypothesis of endogenous generation of these daily oscillations (Figure 4). The results also suggest that gravity, like light, is an environmental influence that can affect the period and perhaps the phase and amplitude of the daily rhythms generated by the circadian pacemaker. The suggestion that gravity (or its absence) can be an environmental Zeitgeber (time cue) is unique and profound. The circadian pacemaker has been suggested as being an endogenous time-keeping mechanism that is very accurate over long periods of time without external input. The system has an inertial component that resists alterations in frequency and an environmental sampling system which corrects for temporal discrepancies between internal time and diurnal oscillations of the environment. The environmental sampling system is restricted to a limited number of inputs which are almost exclusively confined to highly predictable geophysical rhythms. Environmental factors that are not valuable as reliable indicators of diurnal and/or seasonal time are substantially ignored by the pacemaker (e.g., the pacemaker is relatively temperature compensated; i.e., Q_{10} of approximately 1); however,

they may induce temporary alterations in observed overt rhythms. This brief modification in an observable rhythm is termed "masking" and usually has little, if any, impact on subsequent circadian phase or period length. Therefore, the discovery of another geophysical force that provides temporal information to the pacemaker is of substantial importance. The suggestion that organisms can receive temporal information from minor fluctuations in gravitational force will have substantial impact on the study of biological rhythms on Earth and could have significant consequences for the health and performance of astronauts, as well as for life science experiments using plants and animals during long-term space travel.

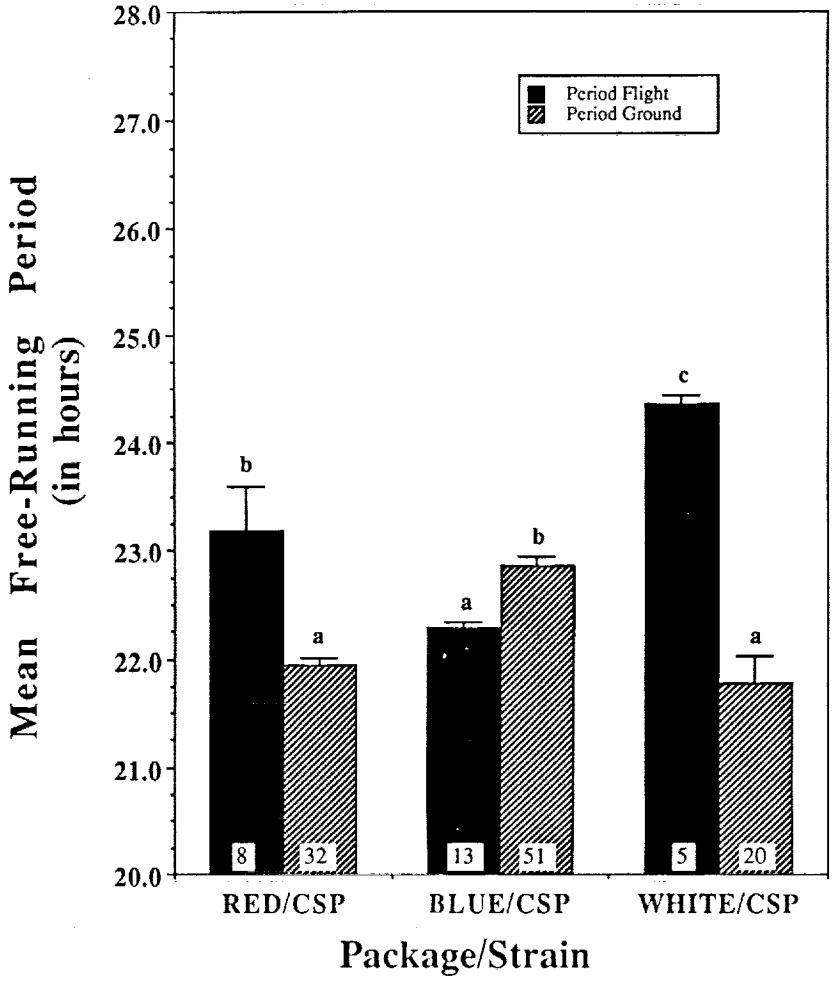

Figure 3. Mean ± SEM free-running period (in hours) of the circadian rhythm of conidiation in the CSP strain of *Neurospora crassa* for each of the three flight packages ("Red", "Blue", and "White") and each of the three packages of the mean of the four sets of ground controls. The number at the lower portion of the bar represents the number of cultures in each mean. Bars with dissimilar superscripts are significantly different ($p<0.01$).

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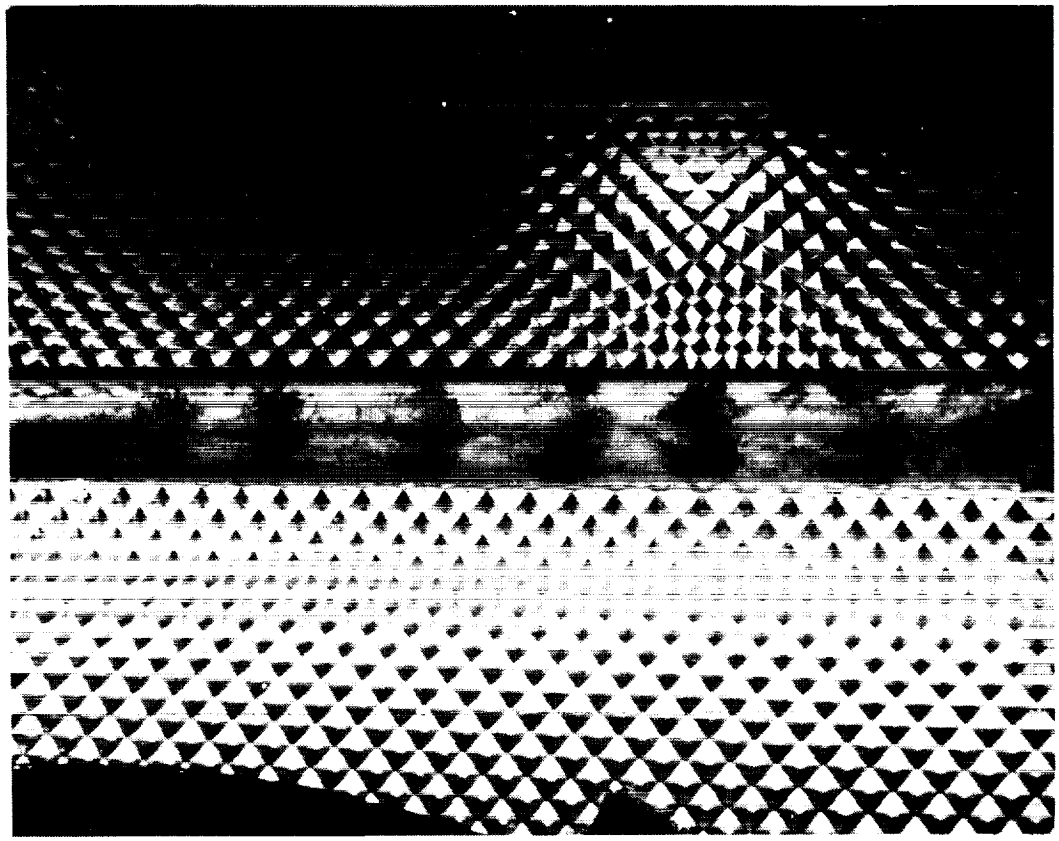

Figure 4. Inflight photograph taken by STS-32 mission specialist Marsha Ivins of a "race" tube from the "Blue" package during the second marking procedure at MET L+05/22:15. The preflight mark (L-00/15:50) and the first inflight mark (MET L+00/09:00) are visible in the lower portion of the tube. It is readily apparent that the banding pattern of the *Neurospora crassa* conidiation rhythm is quite regular and of high amplitude. The gross observation suggest clock functions similar to those found normally on the ground.

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GRAVITROPIC RESPONSE MECHANISM IN CEREAL GRASS SHOOTS

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Description of Research

The primary goal of our research is to determine the mechanism by which gravistimulation causes negative gravitropic curvature (upward bending) in cereal grass shoots. The locus of this response is the swollen leaf-sheath pulvinus located next to a node at the base of each leaf. Gravitropic curvature in the pulvinus involves differential cell elongation, with zero elongation at the top and maximal elongation at the bottom. In a given pulvinus, such differential elongation has the potential to cause upward bending in the shoot portion above the pulvinus 90^{o} to an upright position.

The gravitropic response mechanism has three basic components: graviperception, transduction, and asymmetric growth response. In our earlier work, we focused on the graviperception component and showed unequivocally that starch statoliths (starch-containing chloroplasts) are the gravisensor organelles in the cereal grass pulvinus system. When they are rendered starch-free by pretreatment in the dark, no graviresponse occurs. When starch-free statoliths resynthesize starch by sucrose feeding, the graviresponse is restored. We have postulated that starch statoliths may act as membrane pressure probes to open ion/hormone channels, serve as information carriers (e.g., serve as a source of hormone deconjugating enzymes or a source of Ca^{2+}), or provide substrate (as D-glucose) for cell wall biosynthesis that occurs during gravity-induced cell elongation.

More recently, we have probed into the nature of the transduction process, where key effector hormones, IAA (indole-3-acetic acid) and gibberellins, become asymmetrically distributed in the graviresponding pulvinus. Our current model of transduction portrays the pulvinus storing inactive conjugates of IAA and GAs (gibberellic acids) when it is in an upright, non-gravistimulated position. During the course of gravistimulation, the free IAA and GAs become differentially released from their respective conjugates and differentially synthesized so as to give a 1:1.5-2.0 top/bottom asymmetry of the free hormones. Such ratios have been experimentally determined for both IAA and GAs in the pulvinus system. Currently, we are testing the model to determine precisely how and when these hormone asymmetries are established in relation to the kinetics for the upward bending response.

The asymmetric growth response component of gravitropic curvature in the cereal grass pulvinus involves cell wall loosening, cell wall biosynthesis, starch degradation, changes in β-D-glucan composition in the cell wall, and differential enhancement of sucrose hydrolysis.

Accomplishments

(1) ***Invertase Gene Expression in Oat Pulvinus Tissue:*** The pBR 322 plasmid containing yeast invertase gene in *E. coli* HB101 was obtained from Carlson et al., Department of Human Genetics and Development, Columbia University, NY. *E. coli* was grown overnight in LB medium in our laboratory and the plasmid DNA was isolated.

Qualitative agarose gel electrophoresis indicated plasmid purity and expected size (ca. 23 kb). The plasmid DNA thus obtained was initially probed with biotin-labelled ATP (a nonradioactive label) for Southern Blot analysis. Total genomic DNA was isolated from oat pulvini and then partially digested with restriction enzyme (BAMH1). Following DNA agarose gel electrophoresis and transfer onto HyBond-N hybridization membrane, the DNA was hybridized with the biotin-labelled probe and detected with a Streptavidin-alkaline phosphatase conjugate system. Results obtained were quite satisfactory. The experiment was repeated with a radioactive ^{32}P probe to increase sensitivity. ***Results indicated that the yeast invertase DNA could be used to detect the invertase gene in oat pulvinus tissue.*** The next stage of the present study will be to construct an oat recombinant plasmid library in an effort to isolate and clone the invertase gene and sequence it. Simultaneously, employing the same probe, invertase mRNA levels will be monitored following the effects of gravity and plant hormone treatments.

(2) ***Invertase Localization in Oat Pulvinus Tissue:*** It has been demonstrated that acid invertase activity increases in the bottom halves of gravistimulated oat and barley pulvini. In addition, two forms of acid invertase (soluble and cell wall-bound) have been detected in this tissue system. They exhibit different K_m's and pH optima. To more accurately determine the subcellular locations of these enzymes, an immunolocalization study has been undertaken.

We will attempt to localize invertase in oat (*Avena sativa* cv. 'Victory') at the cellular and subcellular levels via light and transmission electron microscopy utilizing histo/cytochemical and immunochemical techniques. We hope to be able to determine (from a time-course series of studies on gravistimulated tissue) the specific tissue and cellular location(s) where invertase production is turned on, where invertase is acting inside graviresponding cells, and the cellular transport pathway that invertase follows on its way to being secreted into the cell wall.

Polyclonal antibodies, raised against soluble acid invertase from barley, bound both invertase and several other barley proteins, as determined by protein A-Sepharose affinity chromatography and gel electrophoresis. Thus, a second attempt at raising invertase specific polyclonal antibodies (this time raised against yeast invertase) by the Kaufman laboratory was undertaken. Also attempted was raising monoclonal antibodies against soluble oat invertase by the University of Michigan's Hybridoma Facility, located on the medical campus.

The polyclonal anti-yeast invertase antibody does bind soluble oat acid invertase as determined by ELISA. Four monoclonal antibody cell lines also bind soluble oat acid invertase, again determined by ELISA. We are currently checking the specificity of both the polyclonal and monoclonal antibodies with western blotting assays. Preliminary results indicate that the polyclonal and three out of four monoclonal antibody preparations may be specifically binding oat acid invertase. We are now carefully checking for antibody specificity before we continue with the next step of monoclonal antibody production or begin our immunolocalization work using the polyclonal antibody preparation. Once we have a specific antibody probe, it will be used in conjunction with an immunogold conjugated second antibody to localize invertase at the subcellular level, when increased invertase expression is induced by gravity and hormone treatments.

(3) ***Glucan Synthase Activity in Graviresponding Oat Pulvini:*** The leaf-sheath pulvinus is the primary gravisensor organ in cereal grass shoots. During gravistimulation, we find asymmetric growth in an upward-bending pulvinus. There are

two mechanisms involved in this phenomenon: cell wall loosening and cell wall elongation. In cell walls of cereal grasses, a mixed-linkage (β-1,3, β-1,4) -D-Glucan is synthesized only when cells expand and turnover of this polymer is coincident with cell elongation. A gravistimulation time-chase experiment was done to check how β-D-glucan synthase activity changes in top and bottom halves of oat pulvini. When pulvinus-containing stem segments are gravistimulated in the dark at 25°C and fed with 0.1 M sucrose from the bases, they start to bend upward by 3 hr. Upper and lower halves of pulvini were collected and assayed for glucan synthase activity after being gravistimulated for 0, 3, 6, 12, and 24 hr. Glucan synthase activity increased noticeably after 12 hr of gravistimulation as compared with that in pulvini of upright control segments. Lower halves of pulvini had higher enzyme activity than upper halves (Figure 1). ***Oat pulvini gravistimulated for 24 hr and treated with 30 μM GA_3 had significantly higher glucan synthase activity than those pulvini without any gravistimulation and hormone treatment.***

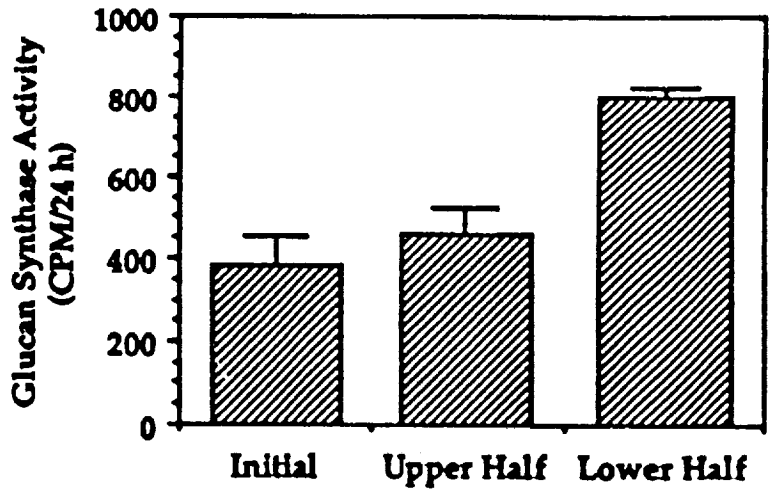

Figure 1. Glucan synthase activity of upper and lower halves of oat pulvini after 24 hrs of gravistimulation.

(4) ***Analysis of IAA/IAA-Conjugates in Gravistimulated Oat Pulvini:*** Experiments were conducted to analyze the asymmetric distribution of IAA and its conjugation products in p-1 (next-to-last) pulvini of oat shoots during gravistimulation. P-1 segment of oats were placed horizontally and incubated in a dark growth chamber at room temperature for gravistimulation. At 0, 3, 6, 9, 12, and 24 hr after gravistimulation, segments were harvested and partitioned into "top" and "bottom" halves. Analysis of IAA and its amide-linked and myo-inositol conjugates in oat p-1 pulvini have been carried out according to the methods of Chen (1987) with a little bit of modification.

Samples were prepared for free IAA analysis. Now free IAA in these samples is being purified by using HPLC (with reverse phase C18 columns packed with Whatman Partosil ODS-3) and quantitated by using mass spectrometry (ms).

Significance of the Accomplishments

Finding #1: Invertase Gene Expression: Yeast invertase DNA will be used as a probe to detect the invertase gene in oat pulvinus tissue. An oat recombinant plasmid will be

constructed in pBR322. Following transformation in *E. coli*, the colonies will be screened with the yeast invertase DNA probe to isolate, clone, and sequence the invertase gene. Once this is achieved, we can assess how oat pulvinus invertase gene expression is regulated by gravity and by the two hormones, IAA and GAs, that are involved in the transduction process. This will allow us to couple gravity transduction with one of the early steps in the asymmetric growth response mechanism.

Finding #2: Invertase Localization: Once an antibody specific to invertase is produced, we will have a very powerful tool capable of detecting invertase at the subcellular level. The antibody probe will allow us to: (a) produce visual data (micrographs) corroborating the biochemical data on the invertase asymmetry at the tissue level; (b) extend our understanding of where invertase asymmetry occurs at the subcellular level; (c) probe the pathway of invertase transport as the gravity response proceeds; and (d) probe the pathway of invertase transport after hormone treatment.

Finding #3: Glucan Synthase Activity Changes Induced by Gravistimulation: Our data on differential expression of glucan synthase activity in graviresponding pulvini indicate that this enzyme may be a primary regulatory site involved in differential enhancement of cell wall synthesis in our pulvinus system. The significant increase in glucan synthase activity in lower halves of pulvini, as compared with upper halves, correlates well with enhanced levels of β-D-glucan found in cell walls of tissues located in the lower halves of pulvini that are responding to gravistimulation.

Finding #4: IAA and IAA-Conjugate Analyses: By analyzing the distribution of IAA and its conjugates in gravistimulated oat pulvini, as compared with that in upright control pulvini, we can provide evidence for our current model of signal transduction during gravistimulation, in which free IAA is differentially released from inactive conjugates of IAA stored in the pulvinus, and/or is differentially synthesized during gravistimulation.

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CELLS, EMBRYOS, AND DEVELOPMENT IN SPACE

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Description of Research

It is now generally recognized that the problems of development constitute a major part of the objectives of modern biology. The ultimate aim of our research plan is to furnish systems at different levels of initial organization that will enable the effect of microgravity in the space environment to be tested on the behavior of plants in contrast or comparison to their performance at 1 g and in ground controls. While the main focus is aimed towards the broad effects of near zero gravity that operate on systems as they grow and develop, the systems in being are or will be adaptable to a variety of more specific tests. For example, any one of our test systems is capable of being used to ascertain whether there might be differences in the normal rate, frequency, and patterning of cell division, or in the fidelity of partitioning of the chromosomes of their cells during or after exposure to spaceflight.

The thrust of the investigations deal with: (a) the induction of active growth, cell proliferation, and metabolism in otherwise mature quiescent cells as they exist *in situ*. This is a problem that has involved and still involves the identity and mode of action of relatively simple growth regulating substances of low molecular weight, their synergists and cofactors; (b) the obtaining and multiplication in culture of free cells and their contrasted development into unorganized callus masses, on the one hand, and as somatic (non-zygotic) embryos into plantlets, on the other; (c) the growth, morphogenesis, and metabolism of intact plantlets and tissue-culture-derived propagules with their established growing regions of shoot and root, in response to interacting factors which are both environmental (i.e., different regimes of photoperiodicity and changing temperature) and nutritional; (d) the development of protocols which have a high level of reliability for establishing chromosomal characteristics and profiles for the plant species we are working with, while at the same time seeking to extend the principles so gained to a still broader range of species; and (e) the management of cultured systems from the perspective of being able to use them effectively and with a minimum of human intervention in a space environment setting.

Accomplishments

Work has focused largely on characterizing further our initial finding that somatic embryos can be produced from two tissues of cultivated carrot on hormone-free medium. We had learned that mericarps ("seeds"), after germination of their zygotic embryos, reliably yield somatic embryos. Mechanically wounded zygotic embryos also yielded somatic embryos, but were a much poorer source of somatic embryo production.

(1) Attempts to increase somatic embryo production were realized. Experiments showed that hormone-free nutrient medium, containing 1 mM NH_4^+ as the sole nitrogen source, fostered production of somatic embryos from wounded zygotic embryos to the same high degree initially found with mericarps.

(2) Growth on 1 mM NH_4^+-containing nutrient medium prevents the initially formed somatic embryos from developing into later stages, but does not prevent cell multiplication. The apparent inability to continue development resulted in cultures consisting entirely of preglobular stage proembryos.

(3) The histological characterization of production or initiation of somatic embryos from wounded zygotic embryos was carried out. The data suggested that preglobular stage proembryos could be maintained as such on 1 mM NH_4^+-containing medium due to a drop in pH.

(4) The first-formed somatic embryos will continue development into later embryo stages, without continued secondary embryo formation, if the medium pH is maintained above 4.5 (tested at pH 4.5, 5, 5.5, and 6).

(5) The establishment of cultures consisting entirely of preglobular stage proembryos is a process, not an event. The first-formed somatic embryos multiply in the beginning as globular stage embryos only when the pH of the medium is allowed to fall during the culture period. During each successive culture period, the volume per tissue mass made up of globular stage embryos decreases while the volume of preglobular stage proembryos increases; a total of 4 to 6 transfers of the entire tissue mass after the initiation of somatic embryos is required to establish a culture consisting entirely of preglobular stage proembryos.

(6) Establishment of preglobular stage proembryos can be hastened by repeated mashing or wounding of the first formed globular stage embryos at the time transfers are made.

Significance of the Accomplishments

It has long been suspected that the mineral element composition of culture media influences virtually every aspect of *in vitro* culture including initiation, maintenance, and development of somatic embryos. Most often, though, these suspicions have been ignored and emphasis has been placed on the *extent* of somatic embryo formation, and only when exogenously added growth regulators were used to initiate and maintain the cultures. Also, special attention has been given to developing protocols that can yield somatic embryos, which in turn develop into mature plants phenotypically identical to the plant from which the original explant was derived. Moreover, the requirements for, or the ability to respond to, certain mineral elements and other media components has widely been thought to be controlled by the exogenously added growth regulator(s) and these requirements, in turn, then direct the developmental fate of those cells. Hence, there has been little incentive to investigate inorganic components in isolation from the more usual organic, hormonal additives. This work shows conclusively that the so-called classic or model carrot system, thought in the past only to be possible in a reliable way via added growth regulator(s), is achievable with high confidence without the use of any added growth regulator.

We have, as yet, no evidence of what the physical or chemical nature of the pH effect is on the morphogenetic fate of preglobular stage proembryos. Therefore, we have no direct evidence of its mechanism of action. Even so, ***this is the first demonstration that morphology of a higher plant system is reliably and reversibly influenced by changes on external pH.*** Since pH changes probably exert control indirectly via changes in internal pH, particular attention will now be paid to examining this hypothesis. The ability to promote continuation of early embryogenesis by means of pH adjustment

provides a vehicle whereby flight experimentation can easily by accomplished with minimal technological intervention.

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PERCEPTION AND TRANSDUCTION OF GRAVIRESPONSES IN PLANTS

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Description of Research

The research in this project is divided into two components: a search for the gravity perception mechanisms in plants which lack amyloplasts, and an analysis of the transduction mechanisms which may be involved in root gravitropism, where amyloplasts are directly involved.

In an effort to develop a simpler model of gravisensing than that found in the root of angiosperm seedlings, we focused our studies on the polarity of cytoplasmic streaming in internodal cells of Characean algae. Our intent is that hypotheses derived from this simple system could then be tested using the more complex systems of higher plants.

As a continuation of our research on the gravity responses of higher plants, we are seeking a linkage between the orthogravitropic response, the diagravitropic response, and the phenomenon of springback, or loss of curvature upon withdrawal of the gravity stimulus.

Accomplishments

In our studies of Characean responses to gravity, we have demonstrated that hydrostatic qualities of the external medium are a component of gravity perception. The polarity of streaming can be altered by either altering the osmolarity of the ambient medium, or by altering the density of the medium. As the density of the medium is increased to equal that of the cytoplasm, or to exceed the density of the cytoplasm, polarity is diminished, and then actually reversed. This is the ***first demonstration of a hydrostatic role in gravity-sensing in plants.***

From these results, we became concerned about a possible role of hydrostatic sensing in roots. Rice roots were employed because of their tolerance of immersion; with this material we found that ***increased density of the ambient solution could readily inhibit gravitropism in rice roots.***

Turning to the problem of the regulation of directional responses to gravity, we have evidence that the cessation of a gravity stimulus can cause a change in the direction of the curvature response. In short, we are able to convert orthogravitropic roots into diagravitropic roots by withdrawal of the gravity stimulus.

Significance of the Accomplishments

Our work with Characean cells opens an entirely new area of study of the perception of gravity in plants. Our experiments with rice roots indicate further that the hydrostatic sensing mechanism may be involved in the graviresponses of roots of angiospermous seeds as well.

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DETERMINING THE EFFECTS OF GRAVITATIONAL STRESSES ON LIGNIN FORMATION AND STRUCTURE: A NEW APPROACH

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Description of Research

Lignins are complex phenlypropanoid polymers produced by terrestrial vascular plants. One of their roles is to provide compressive strength to plant cell walls, thereby allowing such structures to stand in upright forms. There is some tentative evidence suggesting that the lignin content of plants can be affected by gravitational loads experienced during growth.

The specific objectives of this research are to determine the effect of gravitational forces on: (a) the rate of uptake and binding of specifically labelled lignin precursors; (b) the bonding patterns of lignin *in situ*; (c) the function, formation, and activities of specific peroxidase isozymes responsible for lignin formation; (d) cinnamyl alcohol dehydrogenase activity; and (e) development of vascular tissue.

In this reporting period, we have grown plants (*Leucaena leucocephala)* and loblolly pine (*Pinus taeda)* aseptically on hydroponic media, supplemented with various specifically labelled (^{14}C, ^{13}C) lignin precursors. Lignifying *P. taeda* cell suspension cultures have also been obtained in order to study initiation of the lignification process in culture. All experiments were first carried out at 1 g in order to establish appropriate controls. A similar sequence of experiments is currently underway using aseptic, hydroponically grown plants on clinostats. In addition, *L. leucocephala* plants were grown under identical conditions on soil for 10, 20, and 30 days on clinostats (horizontally rotating, vertically rotating, and vertical static). This was done to establish differences in cell-wall development (i.e., ultrastructure) and polymer composition (e.g., lignin content and monomer ratio, and alpha-cellulose and hemicellulose content), and activities of key marker enzymes.

Accomplishments

(1) ***Uptake and Binding of Isotopically Labelled Lignin Precursors into Lignin in Vascular Plant Tissue.*** [U-^{14}C] phenylalanine was demonstrated to be intactly and efficiently incorporated (>10%) into leaf, stem and root sections of *L. leucocephala*, *T. aestivum*, *N. tabacum*, and *P. taeda* plants, which were grown hydroponically under aseptic conditions. [2-^{14}C] ferulic acid, on the other hand, was only efficiently incorporated into root tissue and was not significantly translocated further. Following administration of [U-^{14}C] Phe and [2-^{14}C] ferulic acid to hydroponically grown plants, the lignin composition (i.e., ratio of monomer degradation products, *p*-hydroxybenzaldehyde (H), vanillin (V), and syringaldehyde(S)) was determined; only data for roots is reported here (Table 1). As can be seen, a significant perturbation of the H:V:S ratio occurred with ferulic acid; this was not observed with administration of phenylalanine. Similar results were obtained with stem sections.

Molar Ratio	Soil-Grown	Hydroponically Grown	
		With Ferulic Acid	With Phenylalanine
H/V	0.14 ± 0.00	0.06 ± 0.00	0.14 ± 0.00
S/V	0.56 ± 0.00	0.48 ± 0.00	0.64 ± 0.01
H:V:S	8:59:33	4:65:31	8:56:36

Where H = *p*-hydroxybenzaldehyde; V = vanillin; S = syringaldehyde

Table 1. Lignin composition of *L. leucocephala* roots, as determined by nitrobenzene oxidation

Since [U-^{14}C] Phe was a more efficient and natural precursor, as regards maintaining both lignin content and monomer composition, the distribution of radioactivity into *L. leucocephala* roots and stems was next determined (Table 2). As can be seen, approximately 90% of radioactivity in the stem sections was associated with wall-bound (i.e. lignified) components.

Fraction	Organ	
	Roots	Stems
Aqueous Solubles (%)	33.9 ± 5.09	12.7 ± 1.32
Organic Solubles (%)	1.56 ± 0.62	0.73 ± 0.04
Insoluble (%)	64.5 ± 4.48	86.6 ± 1.36
Total Recovery (%)	74.0 ± 2.57	91.3 ± 5.87

Table 2. Distribution of radioactivity in *L. leucocephala* roots and stems administered [U-^{14}C] phenylalanine

(2) ***Uptake of [1-^{13}C], [2-^{13}C], and [3-^{13}C] Phenylalanine.*** Next, *L. leucocephala* plants were grown for 28-day periods and were individually administered [1-^{13}C], [2-^{13}C], and [3-^{13}C] phenylalanine. Solid state C-13 nuclear magnetic resonance (NMR) spectroscopic analyses of the resulting tissues were then carried out. Results are shown in Figure 1a-c; assignment of the signals due to substructural units in the lignin polymer are included.

METABOLIC SPECTROSCOPIC ANALYSES OF PHENYLALANINE ADMINISTERED INTO LIGNIN IN PLANT STEMS

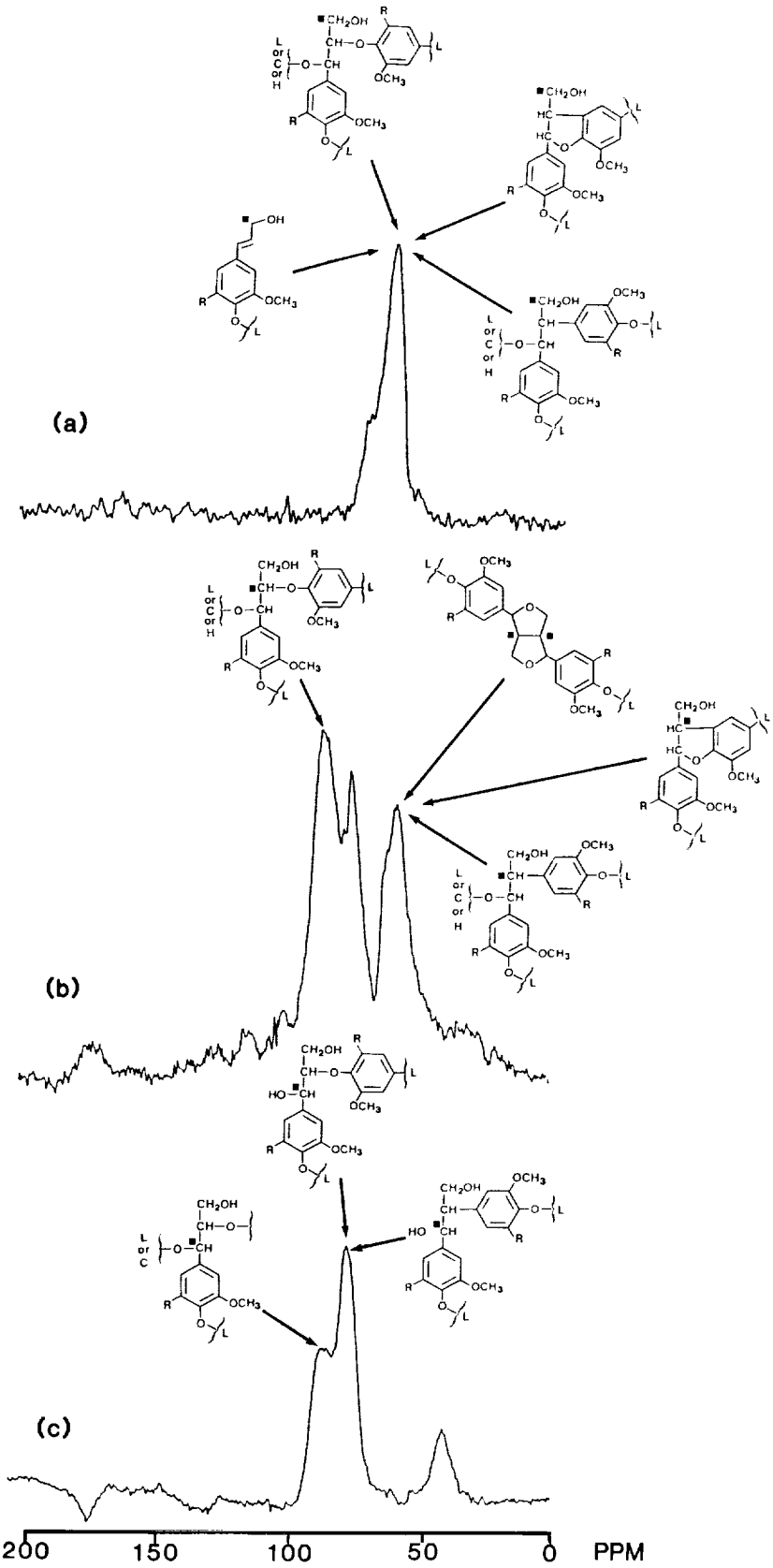

Figure 1. Incorporation of (a) [1-^{13}C], (b) [2-13C], and (c) [3-13C] phenylalanine into *L. leucocephala* stems. L=lignin; C=carbohydrate

(3) ***Design of Clinostats for Aseptic, Hydroponic Growth of Plants.*** As described in the last reporting period, clinostats were developed, each of which permitted the horizontal or vertical growth of six plants in a relatively vibration-free environment under controlled conditions. These have now been adapted to permit growth of aseptic, hydroponically grown plants on a horizontally rotating clinostat. The corresponding ^{14}C and ^{13}C experiments, previously conducted at 1 g, are now underway with this configuration; appropriate vertical rotating and vertical static control experiments will be carried out.

(4) ***Function of Specific Peroxidases in Lignin Formation.*** Cell suspension cultures of *P. taeda* have been obtained which excrete two peroxidase isozymes into the nutrient media. These have been preparatively separated, and appropriate kinetic data (for monolignol oxidation and H_2O_2 formation with NADH) is being determined. The formation of each isozyme with clinostat-grown plants and their correlation with active lignification is currently under investigation.

(5) ***Cinnamyl Alcohol Dehydrogenase.*** We consider this to be an important marker enzyme in the lignification process. Crude cinnamyl alcohol dehydrogenase was obtained from *P. taeda* suspension cell cultures (250 mg) by an extraction-grinding procedure (100 μM tris-HCl, pH 7.5 containing 10 μM DTT, 5% PEG, insoluble PVP (100 mg) and sea-sand) in a chilled mortar. The homogenate was then centrifuged (40,000 g) and the crude enzyme desalted on a Sephadex G-25 column. Isoenzymes were separated by PAGE (8% polyacrylamide), using a stacking gel, and desalted with Coomassie Blue and coniferyl alcohol/NADH with an appropriate azo dye. The appearance and correlation of these isozymes in clinostat-grown, actively lignifying plants is presently being investigated.

Significance of the Accomplishments

First, it was established that the uptake of exogenously supplied phenylalanine and its subsequent translocation and incorporation into lignified root and stem tissue did not adversely affect the lignification process; i.e., the lignin content and monomer ratios were essentially identical to "normally grown" plants. This was not the case when ferulic acid was supplied as a precursor.

Second, this methodology allowed us to probe the exact intermonomeric unit bonding environments of specific side chain carbons in the phenylpropanoid polymer; these represent most of the interunit linkages. The spectra following metabolism of [1-^{13}C], [2-^{13}C], and [2-^{13}C] Phe into lignin are shown in Figure 1. It can be seen that the most abundant bonding environment corresponds to the β-0-aryl linkage (Figure 1b). In addition, the first direct evidence for lignin-carbohydrate bonding was obtained (Figure 1c).

Third, a clinostat design for growing and maintaining plants hydroponically on aseptic media was developed. This now permits the determination of (1) any changes in lignin composition and structure with plants grown under "reduced" gravitational loads, and (2) any differences in the enzymatic activities of key enzymes involved in the lignification process.

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ANALYSIS OF COMPONENTS OF THE GRAVITROPIC RESPONSE IN PLANTS USING PURIFIED PLASMA MEMBRANE VESICLES

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Description of Research

The Committee on Space Biology and Medicine (National Research Council) and the Workshop on Plant Gravitational and Space Research (NASA) both have emphasized the need for subcellular investigations into the mode of action and regulation of the transport of the plant growth regulator auxin and of calcium. The goal of our research is to examine components of the plasma membrane (PM) which are involved in the gravitropic response of plants. The technical breakthrough which made this study possible was the development of a method to obtain large quantities of highly purified PM from plant tissues. The method involves separation of different kinds of membrane vesicles on the basis of both surface charge and density; it yields PM vesicles of extremely high purity (95-98%). We adapted this technique to membrane preparations from zucchini *(Cucurbita pepo)* hypocotyls which yield vesicles that will stay tightly sealed for many hours.

Accomplishments

In the past year, we have used the PM vesicle system to analyze: (1) the interaction of Ca^{2+} with auxin transport, (2) Ca^{2+}-stimulated protein kinases, and (3) auxin-binding proteins. We have also expanded the investigation of auxin-binding proteins to a gravitropic mutant of tomato.

(1) *Effects of Calcium on IAA Transport.* Calcium and movement of the auxin indole-3-acetic acid (IAA) are linked in the gravitropic response, but *in vivo* studies of the transport and localization of IAA and Ca^{2+} using either intact tissue or stem segments have been complicated by the intra- and extracellular compartmentalization of IAA and Ca^{2+}. This led us to examine the effect of Ca^{2+} on the transport of IAA across the PM. We have found that ***a wide range of Ca^{2+} concentrations applied either internally or externally had no significant effect upon IAA uptake into or efflux from isolated PM vesicles. This indicates that the influence of Ca^{2+} on IAA movement may be more indirect and speaks against the mechanism of coupled Ca^{2+}/IAA flux.*** We also attempted to elucidate the mechanisms of Ca^{2+} movement across the PM. Our results showed that the published method for measuring Ca^{2+} uptake into plant PM vesicles using the fluorescent chelator chlorotetracycline is invalid and based on a biophysical artifact. We established that another commonly used Ca^{2+} indicator compound, murexide, is also unreliable for *in vitro* measurements. Problems with murexide have also recently been observed by other workers. We are currently exploring the use of ^{45}Ca for such studies.

(2) *Calcium-Stimulated Protein Kinases.* Ca^{2+}-regulated protein kinases can be important regulators of physiological processes including several which are associated with gravitropism. It has been suggested that Ca^{2+} may exert its effect on IAA transport via phosphorylation of the carrier proteins by a Ca^{2+}-stimulated protein kinase. This

prompted us to identify and partially purify several PM protein kinases which are dependent upon Ca^{2+} for autophosphorylation activity. We have not yet been able to demonstrate any changes in the *in vitro* autophosphorylation of these serine/threonine kinases in response to gravistimulation. However, our assay is limited in that it can only detect those covalent modifications of the kinase which survive the isolation procedure. It remains possible that less permanent modulations of activity may take place. We are currently purifying the Ca^{2+}-stimulated protein kinases in order to generate specific antibodies which will allow us to study their *in vivo* involvement in the gravity response.

(3) *Auxin-Binding Proteins Involved in the Gravity Response.* Although the molecular mechanism of auxin action is still unknown, it is likely that auxin initiates cellular responses by binding to specific receptor proteins. One objective of our research is to understand the role of auxin-specific plasma membrane (PM) receptors and transport proteins in plant growth and development, especially with regard to the gravitropic response. This information will provide the cornerstone of future studies aimed at the molecular mechanism of both auxin action and gravitropism. We have identified auxin-binding proteins in highly-enriched PM vesicles from zucchini hypocotyls by photoaffinity labeling of polypeptides using $^{3}H\text{-}5N_3$-IAA (azido-IAA). Photolysis of azido-IAA in the presence of membrane proteins from auxin-responsive tissues of a variety of species results in the high specific-activity labeling of a low abundance polypeptide doublet of 40-42 apparent molecular weights (kD), which displays properties consistent with those expected for an auxin receptor or transport protein. The polypeptides: (1) are widespread in dicotyledonous, monocotyledonous, and coniferous species and in plant tissues that respond to IAA, but are not detected in auxin non-responsive tissues such as leaves; (2) are present at low abundance in the PM; (3) are saturated by increasing concentrations of IAA; and (4) are selective for binding only those auxin analogs that are physiologically active auxins or specific antagonists.

In an attempt to determine if the azido-IAA labeled polypeptides are indeed involved in auxin mediation of the gravitropic response, we have examined the auxin insensitive, agravitropic mutant of tomato (*Lycopersicon esculentum,* Mill.) known as *diageotropica (dgt).* We have shown that membranes from roots and hypocotyls of the wild-type progenitor of *dgt,* VFN8, contain the characteristic two polypeptides (40-42 kD) which are labeled with high specific activity by azido-IAA. These proteins are also azido-labeled at similar levels in roots of *dgt.* In contrast, membrane proteins from *dgt* hypocotyls are only slightly labeled relative to similar preparations from VFN8 hypocotyls.

Significance of the Accomplishments

The lack of specific azido-IAA binding in dgt is an exciting finding as it identifies an auxin-binding protein which is missing or altered solely in the mutant line of tomato. Since the *dgt* mutation appears to involve auxin perception, this provides not only confirmation of the probable receptor identity of the 40-42 kD polypeptides, but also associates that receptor with a specific set of morphological and physiological attributes which are known to be connected to auxin (i.e., altered gravity response and vascular system, reduced growth, lack of lateral roots, and failure to grow or produce ethylene in response to auxin). We believe that these results will be important in more precisely identifying the relationship of the 40-42 kD polypeptides to the *dgt* lesion.

Publications

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MECHANICAL STRESS REGULATION OF PLANT GROWTH AND DEVELOPMENT

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Description of Research

The objectives of this research are: (1) to characterize the effects of physical forces other than gravity on plant growth and development, and (2) to determine the physiological basis for these responses. Physical disturbances in the form of shaking, vibration, or contact rubbing affect straight growth as well as differential growth (e.g., tropistic curvature), and the responses can be modified by prevailing environmental conditions (e.g., light intensity, temperature). The usual response is reduced growth of unhardened herbaceous plants relative to that of undisturbed plants as assessed by measurements of both productivity (photosynthetic rate, dry weight gain) and dimensional parameters (e.g., leaf area, stem length). However, in some cases mild stimulation of plant growth has been detected in response to mild vibration.

The long-range objectives of this research are to: (1) elucidate which physiological mechanisms are common to and which are different between mechanically stressed and gravistimulated plants, and (2) determine the threshold of sensitivity of a sensitive test species to the frequencies and amplitudes of vibration found in orbiting spacecraft in the absence of a confounding gravity vector.

We have selected dark-grown legume seedlings as model test systems for both seismomorphic (shaking or vibrational effects) and thigmomorphic (contact rubbing effects) stimuli. Seismic forces are less damaging overall, can lead to net stimulation of growth, and are typical of the kinds of physical perturbations likely to be encountered on spacecraft (e.g., thruster firing and astronaut activity, such as treadmill exercises and acceleration sled). Thigmic forces are a good model for the abrasive action of soil particles encountered by germinating seedlings growing upward through soil. Since many first-generation Space Biology plant flight experiments will be done with tender seedlings growing in darkness or under low-light conditions (where plant sensitivity to seismic stress is greatest), dark-grown seedlings are appropriate test systems, and they are treated in our ground-based research program either with vibric or thigmic stress.

Elongating epicotyls and hypocotyls of pea and soybean, respectively, represent a model growth system that depends upon axial enlargement of cells in a discrete zone just below the plumule or hook of the dark-grown seedlings. Kinetic growth rate studies are being performed to determine the likelihood of biophysical triggers for more sustained growth rate changes following the initial. *In vitro* studies with cut sections, as well as intact seedlings with chemical probes, are being used to elucidate the role of changes in growth-promoting hormones and stress hormones in controlling the sustained growth changes caused by mechanical stress.

The principal investigator (CAM) has been collaborating with engineers and scientists at NASA Ames Research Center (ARC) in the Centrifuge Facility Project Office for Space Station regarding the conduct of a ground-based Plant Vibration Sensitivity Study with dark-grown seedlings of *Pisum sativum* L. cv. Alaska and *Zea mays* L. cv. Merritt.

During 1989 protocols were established for growing test subjects and exposing them to continuous vibration equivalent to either 10^{-3} or 10^{-2} g in the horizontal or vertical planes. Growth, development, and ethylene evolution protocols were established for plant response to these treatments, and will, of course, be overlain with a continuous, static, vertical 1 g force. The ground-based studies at ARC will be completed during the second half of 1990. The results could reinforce but will not replace the need for plant vibration studies under real spaceflight conditions.

Accomplishments

Growth of intact seedlings was monitored using an angular position-sensing transducer capable of measuring growth with a resolution of microns per minute. Dark-grown soybean (*Glycine max* L. Merr. cv. Century 84) and pea seedlings exhibited immediate growth reduction following a single mechanical exposure (20X symmetric contact to hook). Peas began growth recovery within 10 min of treatment and recovered to 70% of pre-stress rates by 30 min. However, preliminary results obtained with soybeans suggest that not all plants respond to mechanical stress similarly. In a recent experiment, 60% of test plants showed post-stress growth rates that were 36% of pre-stress rates, whereas remaining subjects showed no effect. In those plants that did show post-stress depression of growth rate, the effect was immediate and dramatic. Moreover, the effect was sustained for up to 7 hr post-stress.

Significance of the Accomplishments

The rapid reduction in growth rate caused by mechanical stress suggests that initial events are biophysical, possibly involving turgor reduction within the cell elongation zone. Following this immediate response is a sustained component resulting in long-term growth inhibition. Subsequent hormonal changes are responsible for the slower growth rate. Less auxin is transported through the elongation zone of thigmo-stressed plants. Production of stress ethylene during the longer-term growth reduction interferes with basipetal auxin transport and likely contributes to the reduction in axial growth.

Publications

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SIGNAL TRANSMISSION AND THE ROLE OF THE ROOT CAP DURING GRAVITROPISM

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Description of Research

The objective of our research is to understand signal transmission and the role of the root cap in root gravitropism. We studied this by examining (1) a newly discovered mutant of *Zea mays* having deformed root caps to determine how changes in this gravity-perceiving organ affect root graviresponsiveness, (2) the role of the apoplast and outer cell layers of roots as potential pathways for stimulus transmission during root gravitropism, (3) roots of the ageotropic (and mucilage-lacking) cultivar of *Zea mays* to determine how the absence of mucilage affects root gravitropism (this cultivar secretes negligible amounts of mucilage and is not responsive to gravity), and (4) the gravitropic responses of capless roots of *Tristerix aphyllus*.

Accomplishments

(1) In the ageotropic mutant of corn, gravitropic effectors such as calcium (Ca) move from the root cap to the root in mucilage (Figure 1). The gravitropic response can be turned on and off by applying and removing exogenous mucilage. Thus, the ***apoplast***, including the mucilage, ***is a primary route for movement of gravitropic effectors in roots.***

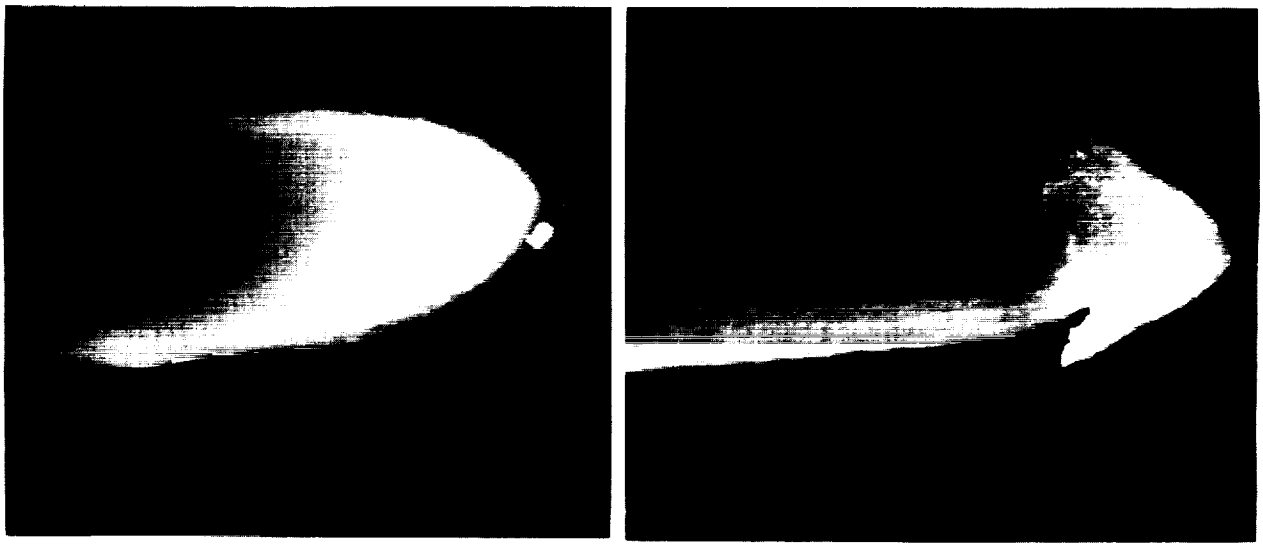

Figure 1. Root tips of a wild-type (left) and the 'Ageotropic' (right) cultivar of *Zea mays*. Tips of wild-type (graviresponsive) roots are covered by mucilage secreted by the root cap and epidermis. Root tips of the mutant lack mucilage. Applying mucilage to tips of these roots induces graviresponsiveness, indicating that in this cultivar, gravitropic effectors move in mucilage from the root cap to the root.

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(2) Symplastic connections occur between the root cap and root across only about 10% of the area of the cap junction in primary roots of *Zea mays*. These connections are unnecessary for root gravitropism.

(3) Primary roots of *Tristerix aphyllus* are graviresponsive despite lacking root caps. Thus, ***root caps are unnecessary for root gravitropism in this species.***

(4) ***Putative gravity-perceiving cells in primary roots of Tristerix aphyllus are located immediately adjacent to the elongating zone.*** To our knowledge, ***this is the only higher plant in which putative statocytes form in this position.***

(5) The outer cell layers, especially the epidermis, are important for signal transmission in primary roots of *Zea mays*.

(6) Plants grown on a rotating clinostat have significantly different cellular ultrastructures and metabolisms than those grown in microgravity. ***Plants grown in microgravity*** aboard the space shuttle ***produce different amounts and types of fatty acids than controls*** grown at 1 g or on a rotating clinostat.

Significance of the Accomplishments

Our finding that gravitropic effectors move through outer cell layers and mucilage is significant because (1) it provides important information about signal transmission during root gravitropism, and (2) it provides a relatively simple system for identifying the gravitropic signal(s).

Our finding that symplastic connections are unnecessary for root gravitropism is consistent with the observation that gravitropic effectors move apoplastically. This confirms the value of our mutant as a system for identifying the gravitropic effector(s).

Our studies of *Tristerix* indicate that (1) root caps are unnecessary for root gravitropism, and (2) putative statocytes may be located immediately adjacent to the elongating zone of roots. This system is unique in that putative statocytes in other higher plants (e.g., primary roots of *Zea*) are located in the root cap.

Our studies of flight-grown plants indicate that microgravity strongly affects lipid metabolism. These data are consistent with our previous observations indicating that microgravity affects cellular structure. Our comparisons of clinostat-grown plants with those grown in microgravity indicate that clinostats do not mimic the ultrastructural effects of microgravity.

Publications

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GRAVITROPISM IN *ARABIDOPSIS THALIANA:* A GENETIC APPROACH

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Description of Research

Our ultimate objective is to achieve an understanding at the molecular level of the mechanism whereby a plant measures and responds to a gravitational stimulus. Toward that end, this project is developing a collection of mutant strains of *Arabidopsis* with alterations in gravitropism. These lines will permit the identification of the elements in the transduction pathway, and will permit an analysis of the interrelations between the two sensory responses, gravitropism and phototropism.

The work is divided into several aspects. First, a family of mutants has been identified with alterations in gravitropism and phototropism. Second, individual mutants are characterized physiologically and genetically. Third, particularly interesting mutants will be mapped with respect to morphological markers and restriction fragment length polymorphisms in preparation for cloning the genes of interest.

Accomplishments

(1) Several mutants have been identified which exhibit no hypocotyl gravitropism, but which exhibit normal phototropism. These include a mutant which is starch-deficient and one which contains wild-type amounts of starch. Thus, ***a loss of starch is not necessary for a loss of graviperception.***

(2) A critical review of the older literature (particularly German) shows that the data on site of graviperception in roots indicates that perception is located in the apical 2-3 mm. This is not compatible with the assumption stated in the more recent literature without supporting data that perception is localized to the columella cells of the root cap.

(3) Roots of corn seedlings grow toward the warmer side in a thermal gradient. Thus, ***thermotropism must be added to the list of environmental stimuli to which roots respond.***

(4) A system has been developed with which gravitropism of seedlings can be monitored in physiological "darkness," using infrared radiation (wavelengths greater than 800 nm) and an infrared-sensitive CCD camera. This system permits the kinetics of a single seedling's response to gravity to be monitored in the absence of light as a confounding factor.

Significance of the Accomplishments

An evaluation of the role of starch in graviperception has proven to be an intractable problem. The identification of mutants in which graviperception is independent of starch synthesis demonstrates the utility of this genetic approach.

Publications

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CALCIUM MESSENGER SYSTEM IN GRAVITROPIC RESPONSE IN PLANTS

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Description of Research

Investigations during the last decade indicate that calcium and calmodulin play a central role in gravity signal transduction in plants. Recent results suggest that key enzymes involved in the calcium messenger system such as calcium- and calmodulin-dependent protein kinases can be modulated in two ways: (1) alteration in the calcium flux in response to the signal which activates calcium-calmodulin-dependent enzymes and (2) changes in the amount of calmodulin. Thus the calcium messenger system may involve changes in the concentrations of both the messenger (calcium) and receptor (calmodulin). It is likely that target cells regulate the levels of these components to evoke a specific response. In our laboratory we have taken the approach that one or both of these possibilities exist in gravity signal transduction.

Accomplishments

We have cloned a plant calmodulin cDNA and shown signal-induced changes in the expression of calmodulin (*Proceedings of the National Academy of Sciences, USA* 86: 3644-3648, 1989). To study the consequences of altered levels of calmodulin, we transformed tobacco protoplasts with calmodulin cDNA driven by cauliflower mosaic virus (CaMV) 35S promoter. ***We observed increased levels of calmodulin mRNA and calcium-dependent protein phosphorylation in protoplasts transformed with calmodulin.*** To further study the expression of calmodulin mRNA and the level of protein in transgenic plants, we regenerated several independent transgenic plants containing short (mostly the coding region) or long (the entire cDNA containing 5' and 3' untranslated regions) constructs in sense or antisense orientation driven by the CaMV 35S promoter (Figure 1).

Characterization of these plants revealed up to 50-fold higher levels of calmodulin mRNA in plants containing the sense constructs, whereas plants carrying the long construct in antisense orientation showed generally lesser amounts of the calmodulin message as compared to controls. In addition, the steady-state level of the calmodulin antisense RNA was much lower compared to the calmodulin mRNA driven by the CaMV 35S promoter. However, the amount of calmodulin protein was, at most, two-fold higher in sense plants, and there was a slight reduction in the level of calmodulin in plants carrying the long antisense construct as compared to control. These results suggest that the amount of calmodulin in the plant cell is regulated stringently at the translational and/or post-translational level.

To detect calmodulin-binding proteins, microsomal fractions were obtained from the tip and base of corn roots and analyzed for calmodulin-binding proteins using a [^{125}I] calmodulin gel overlay assay. A distinct difference with respect to number and abundance of calmodulin-binding proteins was observed between the microsomal membrane fractions

from the tip and the base of the root. The root tips were found to have more calmodulin and also more calmodulin-binding proteins. ***These results suggest the existence of coordinated regulation in the expression of calmodulin and its target peptides.***

Changing the orientation of corn roots from a vertical to a horizontal position increased the phosphorylation of specific polypeptides. These changes were observed within 3 min after changing the orientation. Likewise, depletion of Ca^{2+} with EGTA reduced this phosphorylation, and replenishment of Ca^{2+} restored it.

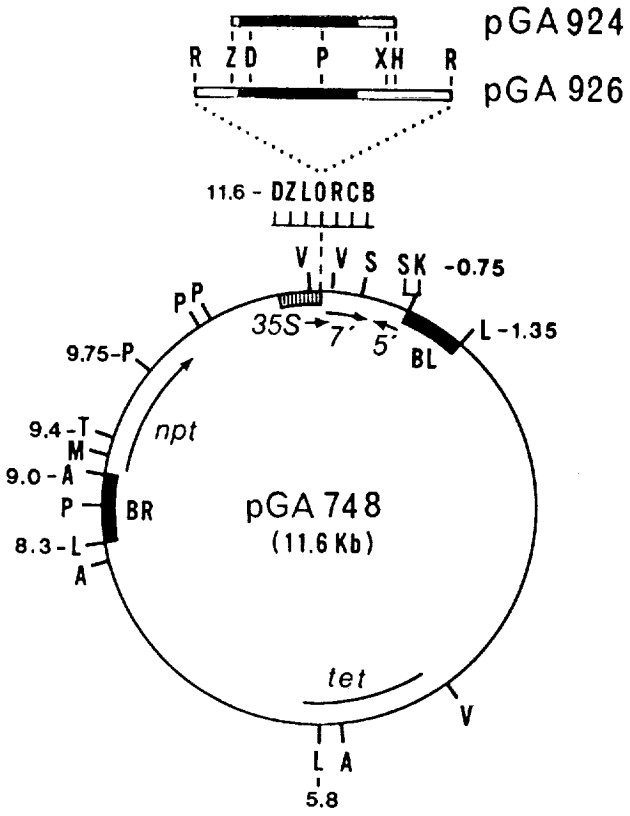

Figure 1. Illustration of the constructs of calmodulin that were used for stable transformation. Cloning of calmodulin cDNA from the pPCM-1 clone (Jena et al., *Proceedings of the National Academy of Sciences USA* 86: 3644-3648, 1989) into pGA748 in both sense and antisense orientations. pGA924-925 contains the short fragment (*Sca*I-*Hpa*I fragment) of calmodulin cDNA, mostly the coding region, in sense and antisense orientation, respectively (short constructs). pGA926-927 contains the intact *Eco*RI fragment of calmodulin cDNA including the 5', 3' untranslated regions and the coding region (long constructs). A, *Sac*II; B, *Bgl*I; C, *Cla*I; D, *Hind*III; H, *Hpa*I; I, *Mlu*I; K, *Kpn*I; L, *Sal*I; M, *Bam*HI; O, *Xho*I; P, *Sph*I; R, *Eco*RI; S, *Sac*I; T, *Pst*I; V, *Eco*RV; X, *Xba*I; Z, *Sca*I.

Significance of the Accomplishments

The seedlings of R1 and R2 generations of transgenic plants could be used to study the role of calmodulin in gravitropism. Furthermore, our observation that there is a coordinate

regulation of calmodulin and its binding proteins in roots suggests that the binding proteins might have a role in the calcium messenger system. Our studies also indicate that calcium-dependent changes in protein phosphorylation are involved in gravity signal transduction.

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EFFECTS OF HYPERGRAVITY ON SPORE GERMINATION IN THE SENSITIVE FERN *ONOCLEA SENSIBILIS*

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Description of Research

Previous studies have shown that germination of spores of the sensitive fern *Onoclea sensibilis* is delayed when they are subjected to hypergravitational forces of low to moderate intensity and that the delayed germination and recovery are accompanied by the synthesis of new proteins. The long-range goal of this research is to analyze the role of centrifugation-stress proteins during germination of spores of *O. sensibilis* and to use this as a model system to study the molecular biology of centrifugation stress in plants.

Shock proteins are known to be synthesized when cells are subjected to various treatments such as temperature or pressure. The possible role of these proteins in compensating for gravity-induced stress during germination of spores of *O. sensibilis* was investigated by pulsing spores at various times after centrifugation with ^{35}S-methionine, followed by SDS-PAGE and fluorography. Results of these experiments showed that at least six new proteins are synthesized after hypergravity stress, the new bands showing up stronger in spores stressed at 10,000 g than in spores stressed at 100 g. The bands were visible within the first two hours after centrifugation and persisted for the next 4-8 hours, but disappeared as spores recovered from stress. ***These results suggest that hypergravity stress may be tied to specific changes in gene expression as part of the spore's strategy to overcome the effects of the stress.*** From this perspective the general thrust of our research is to construct and screen a cDNA library of mRNA sequences from centrifuged spores and to select cDNA clones specific for centrifugation stress. By a multifaceted approach involving Northern blot, RNA dot blot, and Southern transfer, we intend to determine the centrifugation-stress specifically of the cDNA clones, the timing of their transcription, their relative abundances during germination, and their copy number. Preparatory to the proposed work on the isolation and characterization of centrifugation-stress mRNA sequences, we have already made a cDNA library to polyA+RNA of dormant spores and by differential screening have isolated several germination-specific clones.

Accomplishments

(1) ***Construction of cDNA library to polyA+RNA of dormant spores*** by the Gubler and Hoffman method using a commercial cDNA kit.

(2) Primary screening of transformed bacterial clones with random-prime labeled single-stranded cDNA prepared from polyA+RNA of dormant spores.

(3) Secondary screening of positive clones with single-stranded cDNA probes prepared from 20-day old gametophytes and leaves of *O. sensibilis* and identification of spore-specific recombinants.

(4) Establishment of spore-specificity of recombinants by Northern blot of RNA from spores, gametophytes, and leaves with labeled probes of recombinants.

(5) Selection of germination-specific mRNA clones by Northern blot of RNA from germinating spores and dark-imbibed non-germinating spores probed with labeled recombinants.

Significance of the Accomplishments

We have isolated from dormant spores of *O. sensibilis* a series of mRNA sequences which are used to code for proteins of germination. Since the hypergravity environment delays spore germination, with a concomitant appearance of new proteins, we are in a position to follow the effect of centrifugation stress on the expression of these mRNA sequences. This information, along with our proposed studies on the isolation of cDNA clones specific for centrifugation-shock, will provide great insight into the molecular biology of centrifugation effects on the germination of spores of *Onoclea sensibilis*.

Publications

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CELLULAR AND MOLECULAR PROCESSES OF THE GRAVITROPIC RESPONSE

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Description of Research

The mechanism by which plants transduce information about the direction of gravity into a predictable pattern of growth is an interesting problem in developmental biology and has important ramifications regarding our ability to grow and utilize plants in the microgravity environment of space. When a plant shoot is placed in a horizontal position, it begins to curve upward in a smooth arc within minutes, and it reestablishes its original vertical orientation within several hours. For the purpose of discussion and experimentation, this phenomenon, known as negative gravitropism, can be divided into three components: (1) gravity perception, (2) signal transduction, and (3) asymmetric cell elongation. The long-range goal of this research is to understand the cellular and molecular processes that occur between signal transduction and asymmetric growth.

Asymmetric shoot growth in most plant species involves a reduced rate of cell elongation on the upper side of a gravistimulated shoot and an increased rate of cell elongation on the lower side relative to vertical controls. It is quite likely that an asymmetric distribution of some growth factor must precede asymmetric cell elongation. There is a large volume of literature suggesting that, in shoots, the hormone auxin (IAA) is a growth factor that modulates asymmetric shoot elongation. This in turn makes it likely that auxin-specific receptors are an essential link between transduction events and the resulting response of gravistimulated tissue. Thus, a logical starting point toward a molecular understanding of the gravitropic response is the identification and characterization of auxin-specific receptors.

The analysis of mutants is a powerful approach to determining the physiological function of certain proteins. Several mutants of higher plants exist which are insensitive to the growth hormone auxin. The tomato (*Lycopersicon esculentum*, Mill.) mutant known as *diageotropica (dgt)* is a particularly promising candidate for an analysis of auxin-regulated growth and development. *Diageotropica* is a spontaneous, single gene recessive mutant of the parental variety VFN8. Mutant plants do not elongate in response to IAA. They also exhibit other morphological abnormalities which suggest that these plants may have a defect associated with a primary site of auxin perception or action. We have recently shown that the photoaffinity auxin analogue ^{3}H-5N_3-IAA (azido-IAA) labels a polypeptide doublet at 40-42 kD at high specific activity in membrane preparations from shoots of VFN8, but the identical procedure fails to label polypeptides from *dgt* shoots. These findings suggest that this 40-42 kD polypeptide doublet is a physiologically important auxin receptor which displays an altered pattern of expression in the mutant. Additional evidence supports the notion that these polypeptides are receptor proteins. The polypeptides are: (1) observed only in plant tissues that respond to IAA, (2) present at low abundance, (3) associated with the plasma membrane, (4) saturable with increasing concentrations of IAA, and (5) specific for binding to those auxin analogs which are also active auxins or specific antagonists.

The next step towards more precisely identifying the nature of the 40-42 kD polypeptides is the isolation of specific cDNA clones encoding these auxin-binding polypeptides. In

working toward this goal, an oligonucleotide probe corresponding to an internal region of an auxin binding protein was used to screen a tomato fruit cDNA library in λgt 11. Out of 200,000 recombinant plaques screened, 12 apparent positive clones were selected. Of these, four remained clearly positive upon rescreening. These clones are currently being characterized as to insert size. Verification of the identity of the cloned cDNAs will come from a variety of experimental approaches. These will include: direct sequence comparison with known protein and nucleic acid sequence information; expression of the proteins, either by direct expression of the fusion proteins or by translation of *in vitro* transcripts followed by azido-labeling, and/or the complementation of the *dgt* phenotype by the production of plants transgenic for wild type sequences.

Accomplishments

(1) Auxin-binding proteins were photoaffinity labeled by the addition of ^{3}H-$5N_3$-IAA to membrane vesicles prior to exposure to UV light (15 sec; 300 nm) and detected by subsequent PAGE and fluorography.

(2) At -196°C, ***high specific-activity labeling of a 40-kD and a 42-kD polypeptide was observed in a variety of plant species.***

(3) ***Shoots of the tomato mutant diageotropica (dgt) were found to have reduced amounts of the 42 kD-labeled polypeptides*** relative to wild-type controls.

(4) The above findings and physiological studies of *dgt* and wild-type tomato seedlings ***suggest the radiolabeled polypeptides are auxin receptors.***

(5) A tomato library was screened in an attempt to isolate specific cDNA clones encoding these auxin-binding polypeptides.

(6) Preliminary information suggests we have isolated clones encoding the 40-42 kD polypeptides.

Significance of the Accomplishments

It has been shown that the azido-labeled 40-42 kD membrane proteins are (1) ubiquitous in plant tissues that respond to IAA, (2) of low abundance, (3) saturable with increasing concentrations of IAA, and (4) capable of binding specific analogues that are also active auxins or specific antagonists. These data suggest that the two polypeptides are part of a physiologically important auxin receptor system. It is hoped that further characterization of these polypeptides will make it possible to dissect the mechanism of auxin action and thus provide us with a clearer understanding of plant gravitropism. Toward that goal, cDNA clones have been isolated which appear to encode the 40-42 kD polypeptides. Sequence information from clones representing both *dgt* and wild-type genes should help to distinguish both the nature of the mutation and the relatedness of these polypeptides to other recently characterized auxin binding proteins. Such knowledge should prove useful in efforts to manipulate the growth and yield of plants.

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ASSESSING POTENTIAL TARGETS OF CALCIUM ACTION IN GRAVITROPISM

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Description of Research

Light greatly accelerates the gravitropic response of roots, coleoptiles, and stems in a wide variety of plants. This indicates that some cellular response initiated by light affects at least one of the gravity-induced cellular responses necessary for gravitropism. Our research objective is to identify one or more of these responses, thus clarifying the cellular mechanisms regulating gravitropic growth.

Several lines of evidence indicate that calcium may play an important role in transducing the stimuli of both light and gravity into growth changes in plants. However, full confidence in the validity of this hypothesis will require that at least two additional pieces of evidence be obtained: (1) the identification of specific targets of calcium action that have a clear and significant impact on growth and (2) a demonstration that the gravity and/or the light stimulus alters the concentration of cytosolic free calcium ($[Ca^{2+}]_{cyt}$) in the responding cells. As yet there is little information on these two critical sets of evidence. This past year we carried out some experiments aimed at narrowing this information gap.

In most well-described cases, the immediate target of calcium action during the transduction of an environmental stimulus is a calcium-binding protein. Such proteins tend to be highly conserved evolutionarily, with very similar types occurring in both plants and animals. The best known target of calcium action in plants and animals is the calcium-binding protein calmodulin. More recently, calcium-binding kinases, including protein kinase C and phospholipid-independent kinases, have been described in both plants and animals. Still other calcium-binding proteins are well characterized thus far only in animals, including, prominently, the annexin family of proteins. One sub-set of this family was originally named the calcimedins, and two years ago we reported finding cross-reactivity between a pea antigen and an antibody to a mammalian calcimedin. This year we succeeded in partially purifying and characterizing an annexin-like protein from peas, and here we describe these results.

Wall isoperoxidases have been reported to be under the control of calcium, and last year we reported evidence that a 89 kD acidic isoperoxidase present in the cell walls of corn tissues may help mediate the growth responses of corn coleoptiles and mesocotyls to light. These results predicted that selectively inhibiting this isoperoxidase could alter growth rates in corn. This year we report the results of our initial efforts to inhibit this isoperoxidase *in situ* by reacting it with a monoclonal antibody that blocks its activity.

Detecting gravity-induced changes in $[Ca^{2+}]_{cyt}$ in complex multicellular tissues would be technically difficult. This measurement, however, would be easier in organisms with only a few cells. This past year we succeeded in measuring $[Ca^{2+}]_{cyt}$ in germinating spores of the fern *Onoclea* and in the rhizoid cell produced by the first cell division of the newly germinated spore, using incorporated fura-2 as a fluorescent indicator of $[Ca^{2+}]_{cyt}$ in these cells. The rhizoid cell of a newly germinated *Onoclea* spore shows normal growth and

gravitropism even with fura-2 present in its cytoplasm. This finding sets the stage for us to learn whether $[Ca^{2+}]_{cyt}$ changes in the rhizoid cell when it is gravistimulated, i.e., reoriented by 90°.

Accomplishments

(1) ***We have purified to near homogeneity a calcium-binding calpactin-like protein (CLP) from peas.*** Like animal calpactin, it has a mass of 35 kD and binds to phospholipids in a calcium-dependent fashion. It also cross-reacts with antibodies raised to animal calpactin, a protein thought to help regulate calcium-dependent secretion from cells.

(2) We have raised polyclonal antibodies to the pea CLP and have completed an initial study of its localization in pea stem and root cells. We found that the CLP appears to be most concentrated in the periphery of cells that are actively engaged in secretion, such as root cap cells and young developing tracheary elements.

(3) ***Perfusing coleoptile segments of etiolated corn seedlings with a monoclonal antibody (mAb) that binds to and blocks the activity of a 98 kD wall isoperoxidase inhibits the growth rate of these tissues.*** Perfusing coleoptiles with pre-immune serum or mAbs to phytochrome (a cytosolic protein) has no effect on their growth.

(4) ***The calcium-sensitive fluorescent dye, fura-2, can be successfully electroporated into intact fern spores and into intact newly germinated gametophytes,*** such that they continue to show both normal growth and development and normal responsiveness to light and gravity after the electroporation and incorporation of the dye. The dye appears to be restricted to the cytoplasm, where the $[Ca^{2+}]_{cyt}$ measured is 0.1-0.2 μM.

Significance of the Accomplishments

Findings #1 and #2: As yet there is only one other report of an annexin-like protein in plants, and it is preliminary and incomplete. Our results provide more convincing evidence both that calpactins are present in plants, and that they may play a role in secretion, a critical process which is likely to be a major control point in the regulation of growth.

Finding #3: All prior reports on the use of antibodies to inhibit wall enzyme activity also report growth inhibition by the antibodies. Because peroxidases are thought to inhibit growth by their cross-linking activities, inhibition of these wall enzymes would theoretically be expected to promote growth. The fact that growth is inhibited by mWP3 suggests either that peroxidase function may be needed for normal cell wall extension or that the reaction of any antibody with any antigen in the wall can create steric problems that nonspecifically inhibit growth. Further experiments will be needed to resolve which of these two alternatives is the more likely explanation of the results.

Finding #4: Successful incorporation of fura-2 into intact fern cells such that they continue to grow, develop, and respond to growth stimuli normally after the dye incorporation will allow us to measure even very rapid kinetics of any calcium changes that result from either gravitropic or light stimulation. Thus the germinating fern spore can serve as a valuable model system for directly testing whether phytochrome activation or gravitropic stimuli can induce changes in $[Ca^{2+}]_{cyt}$.

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CELLULAR POLARITY AND INTERACTIONS IN PLANT GRAVIPERCEPTION

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Description of Research

Our long term goal is to understand the mechanism(s) of gravitropic sensing in higher and lower plants. Although it had long been thought that the movement of amyloplasts — heavy, starch-filled plastids — probably triggers sensing, the isolation of several starch-deficient, but gravity-perceiving, mutants indicates that amyloplasts are not necessary for sensing. However, in the last few years, we have demonstrated that starchless roots of *Arabidopsis* and starch-deficient roots of tobacco are less sensitive to gravity than are wild-type roots with amyloplasts. We therefore concluded that starch (or presumably the weight it confers) is necessary for full sensitivity and that amyloplasts function as statoliths.

Last year we provided an initial description of gravitropism in the moss *Ceratodon*, which has filaments of cells (protonemata) that grow upward in the dark. We found that this cell has a distinct plastid zonation which includes a zone that is apparently specialized for amyloplast falling when the cell is horizontal, but not vertical. Also, using infrared videomicroscopy to study upward curvature in living cells reoriented to the horizontal, we found that before this cell curves up, there is a "wrong-way" (downward) curvature that takes place within minutes after reorientation.

Accomplishments

(1) ***A tobacco mutant with reduced starch has very disoriented, weakly gravitropic stems (hypocotyls).*** Electron microscopy confirmed that the tobacco mutant stems contain plastids with <10% of wild-type (WT) starch in comparable cells which have sedimented amyloplasts in the WT. Vertically grown (in the dark) mutant hypocotyls are severely disoriented (ratio of variabilities of mutant: WT = 5.3). In contrast, in vertically maintained tobacco roots, the corresponding ratios for light and dark-grown roots were 1.3 and 1.6, respectively. The tobacco mutant hypocotyls are somewhat gravitropic since (a) they are oriented a mean of 47° from the vertical (vs. 11° for the WT) and (b) upon a 90° reorientation, they curve upwards 10° in 72 hours (vs. 70° in the WT).

(2) ***The presumed gravity-sensing cells in roots have been visualized in the electron microscope after entire roots were rapidly frozen using high pressure freezing followed by freeze substitution.*** The cytoplasmic faces of all membranes displayed electron dense coats. Other new details of columella cell structure were seen such as vacuolar contents, turgid compartments, and intercisternal elements in the Golgi apparatus.

(3) Gravitropic curvature in corn roots does not start with an asymmetrical growth of the rootcap itself. Over the years, it has been reported that the very first growth response of a root to gravistimulation is a differential growth of the rootcap, a differential which later extends to the elongation zone. Growth markers were placed on the rootcap of

(4) ***Amyloplast sedimentation correlates with the recovery of gravitropism after basipetal centrifugation of Ceratodon protonemata.*** When amyloplasts were centrifuged out of the tip, growth resumed but gravitropism was absent until amyloplasts migrated back close to the tip and sedimented. Upward curvature sometimes took place without amyloplasts in the apical dome, but wrong-way curvature only occurred when amyloplasts were present in the very tip (apical dome).

Significance of the Accomplishments

Finding #1: The severely reduced gravitropism of the *Nicotiana* mutant hypocotyls demonstrates again that starch is necessary for full gravitropic sensitivity and that amyloplasts function in gravitropic sensing hypocotyls. While starch-deficiency depresses gravitropic functioning much more in hypocotyls than in roots, it does not completely eliminate all sensing. It is hypothesized that the starch-deficient plastids that remain in the mutant account for the residual sensing that exists in the hypocotyls. A comparison with *Arabidopsis* hypocotyls (starchless mutant and WT) is in progress.

Finding #2: Development of a successful protocol for freezing an entire plant organ without ice crystal damage means that one of the first freeze-substituted cells to be seen below the surface of an organ is of the cells that are presumed to sense gravity in roots. This new technique provides a means of preserving tissues that appears to be less artifactual than conventional chemical fixation techniques. This technique should not only provide a more accurate view of the structure of these cells, but it should also open up the possibility of doing high-resolution immunolabelling, e.g., of labile membrane antigens in cells that have been heretofore inaccessible.

Finding #3: This experiment shows that the rootcap is not the initial target of its own orientational signals in corn. This was one of the most sensitive assays used to study this question. Thus, rootcap asymmetry is not a universal feature of gravitropism, and this method provides a way of testing other genera to determine where, if anywhere, in the rootcap an asymmetry develops.

Finding #4: These and previous results suggest that the sedimenting amyloplasts function in gravitropic sensing in upward curvature and that the non-sedimenting amyloplasts in the apical dome are associated with an initial wrong-way curvature. This is the first modern description of a cell that both perceives and responds to gravity and that appears to employ amyloplasts as statoliths. These results reinforce the concept that the sedimentation of amyloplasts is generally associated with sensing. But the absence of dramatic sedimentation before the start of wrong-way curvature in *Ceratodon* protonemata indicates that sedimentation is not required for the earliest phase of protonematal gravitropism. An all or none interpretation regarding the importance of sedimentation appears inappropriate.

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GRAVITROPISM IN DICOT STEMS

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Description of Research

We continue to concentrate on the role of auxin (indoleacetic acid, IAA) in the gravitropic response of stems. The Cholodny-Went hypothesis explains gravitropic stem bending by suggesting that auxin moves to the lower side of a horizontal stem, causing those tissues to grow more than top tissues and thus causing the stem to bend up. We have emphasized an alternative mechanism; namely, that tissue *sensitivity* to auxin changes in response to gravistimulation whether auxin *concentration* changes or not. We obtain dose-response curves for upper and lower surfaces of stem segments immersed in auxin solutions over a wide concentration range (0, 10^{-8} to 10^{-2} M IAA). Sections are photographed at intervals (usually half an hour), and negatives are projected onto a digitizer. Suitable software is used to measure bending and growth in length of top and bottom surfaces. Maximum growth of *bottom* tissues occurs in zero or very low auxin concentrations; higher concentrations progressively inhibit growth. Growth of *top* tissues, on the other hand, is minimal in low auxin concentrations, increasing to a maximum at about 10^{-4} M IAA and then decreasing in the higher concentrations. Maximum growth of top tissues never quite equals maximum growth of bottom tissues. Because of these relationships, stem segments bend *up* in low auxin solutions and *down* in high auxin concentrations (because top surfaces are growing more than bottom surfaces). These data suggest that V_{max} sensitivity of top tissues is never as great as that of bottom tissues (because maximum growth is always less), and K_m sensitivity of bottom tissues *greatly* exceeds that of top tissues (because *much* lower auxin concentrations will cause one half of maximum growth).

During the past year, we began studies of the kinetics of changing sensitivity induced by gravistimulation to auxin in top and bottom tissues. We have investigated effects of temperature and the initial changes in stem growth immediately after stems are turned to the horizontal. We have also tested some species other than the sunflower and soybeans used previously.

Accomplishments

(1) For several months after moving to a new laboratory, we did not have temperature control in the two physiological darkrooms. Experiments were run at room temperature (18-22°C) instead of the higher temperature (33°C) we had used previously. Bending was reduced, and the downward bending at high auxin concentrations was often not apparent. Eventually, temperature control was installed in both darkrooms, so we could compare sunflower hypocotyl sections from the same batch at two different temperatures. No new phenomena were discovered. Although bending is slower at reduced temperatures, and sections at lower temperatures sometimes fail to bend as much as those at higher temperatures, in some experiments bending is essentially the same at both temperatures if one waits for segments at the lower temperature to catch up with those at the warmer temperature. Because sections are immersed in non-aerated solutions, it may not be surprising that bending is somewhat reduced when it is extended (i.e., by cool temperatures) over a long interval.

(2) We have observed the kinetics of bending and growth of top and bottom surfaces following gravistimulation. Changes were small after short times, so there is some uncertainty in the results. Nevertheless, there was clearly a lag of about 30 to 60 minutes after gravistimulation before curvature began; that is, before growth of top and bottom surfaces differed. Typically, sections bent down slightly during that initial lag period, even though, at all concentrations, there was a slight burst of growth that lasted about 15 minutes immediately following gravistimulation (i.e., top grew slightly more than bottom). ***Then, after about 2.5 hr in buffer with no auxin or in 10^{-8} M IAA, there was a sharp increase in growth rate of the bottom surface; growth rate of top surfaces stayed fairly constant. As auxin concentration increased, the lag time became shorter until, at about 10^{-5} M IAA and higher, there was no detectable lag but a large burst of rapid growth of both surfaces that slowed after about 15 minutes.***

(3) We tested the following species in our system, using hypocotyl, stem, or coleoptile sections from seedlings: cabbage, pea, tomato, maize, and oats. Responses were similar to those of sunflower and soybean, but there were some interesting differences. In several experiments, pea stem sections failed to bend (although we did observe the usual response once). Although we grew the plants at different temperatures as well as in light or darkness, we have not resolved the discrepancies. Variability was always high. We were interested in maize because most evidence for the Cholodny-Went hypothesis has been obtained with monocots, including maize. In three experiments completed so far, ***maize coleoptiles exhibited bending much like sunflower and soybean hypocotyls: rapid upward bending in zero and low auxin concentrations, virtually no bending or some downward bending (one experiment) at the highest concentrations.*** A single analysis of top and bottom growth of maize coleoptiles, however, produced the unexpected result that growth of *bottom* surfaces continued to increase with increasing auxin, reaching a maximum at 10^{-3} M IAA, the *same* auxin concentration that produced maximum growth of the *top* surface, which was a little higher, accounting for the downward bending. Growth of the top surface was essentially zero in buffer, increasing to its maximum at 10^{-3} M IAA, but increasing more steeply than did bottom growth.

Significance of the Accomplishments

Finding #1: Temperature experiments were somewhat disappointing. We had expected to find qualitative differences at cool and warm temperatures (based on earlier observations), but the differences proved to be only quantitative.

Finding #2: Studies of the lag in bending are more interesting. We wonder if this lag time is required for sensitivity to auxin to change in response to gravistimulation. Disappearance of the lag at high auxin concentrations is especially interesting. It is as if auxin penetration and response to auxin were virtually instantaneous at those concentrations. If the initial lag represents the development of auxin sensitivity, its shortening by higher auxin concentrations suggests (as others have also suggested) that sensitivity to auxin can develop in response to auxin itself and that higher concentrations lead to more rapid increases in auxin sensitivity. In bottom tissues, however, sensitivity is already so great at zero or very low external auxin levels that endogenous auxin leads to maximum response, and increases in solution auxin push the response system over the top of the curve to the descending or inhibitory part. The striking thing remains the marked difference in response to auxin of top and bottom tissues.

Finding #3: Results so far suggest that the generalization gained from sunflower and soybean can probably be extended to other species, but the observed differences in response are also interesting. It is especially important to extend the observations with maize to see if the pattern observed so far appears in future experiments.

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AMIDE OXIDASE AND PEROXIDASE REGULATION OF DIFFERENTIAL GROWTH RESPONSES IN GRAVISTIMULATED PLANTS

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Description of Research

Our long-term goal is to understand the biochemical and physiological mechanisms by which plants respond to a perceived gravity stimulus. An elucidation of such mechanisms will facilitate our understanding and prediction of plant growth responses in altered gravity environments, such as the hypogravity environment of space.

Our specific goal is to determine the extent to which peroxidase-mediated coupling mechanisms play a role in the regulation of plant growth by modulating cell wall extensibility. In particular, we want to characterize specific peroxidase isozymes (PODs) that are responsive to short-term gravistimulation and, therefore, may regulate differential growth (i.e., tropistic bending). We expect that cross-linking reactions catalyzed by these inducible PODs should be largely reversible, while other "housekeeping" PODs may carry out lignification and other types of cross-linking reactions which are irreversible, providing long-term structural support for the plant.

We have been examining a new model for plant growth regulation, the key components of which are cell wall-localized: (1) amine oxidases generating H_2O_2 as a product of polyamine oxidation, and (2) PODs utilizing the H_2O_2 to carry out cross-linking of phenolic moieties of wall constituents. This cross-linking would decrease wall extensibility and growth potential of the tissue. Differential growth accompanying tropistic curvature of the gravistimulated organ could result from gradients in the activities of amine oxidases, their polyamine substrates, and/or PODs.

We have shown previously, using both cytochemical and biochemical methods, that lateral gradients in amine oxidase activities are not seen in either gravistimulated pea epicotyls or corn coleoptiles. However, these organs do exhibit a lateral asymmetry in concentrations of polyamines (higher on the upper, slower growing sides, consistent with the model), which could be sufficient to produce H_2O_2 gradients and differential growth responses. In addition, corn coleoptiles exhibit lateral asymmetries in the expression of certain POD isozymes, some of which are induced after only 30 min gravistimulation. Expression of a few of these PODs also appears to be induced by Ca^{2+} and auxin, which are thought to play important, often antagonistic, roles in regulating plant growth. We are presently characterizing these PODs in relation to the gravitropic bending response in the coleoptile.

Accomplishments

(1) We have carried out kinetic analyses of changes in the activities of soluble and ionically and covalently bound PODs in gravistimulated corn coleoptiles.

(2) We have shown that ***the activities of two cationic PODs are increased on the upper, slower growing sides of gravistimulated corn coleoptiles; induction of these isozymes occurs as early as 30 min after presentation of the gravity stimulus.***

(3) We have characterized changes in the expression of various POD isozymes in response to exogenous applications of Ca^{2+}, the Ca^{2+} ionophore A23187, and H_2O_2 in non-gravistimulated corn coleoptiles.

(4) Ca^{2+} inhibition of PODs *in vitro* has been demonstrated.

Significance of the Accomplishments

Finding #1: While the expression of specific isozymes has frequently been correlated with growth and development in plants, expression is generally examined over a period of days or weeks. To my knowledge, ***this is the first time that rapid changes in the expression of specific POD isozymes has been demonstrated for any growth response. Our findings suggest that it is largely ionically bound PODs that respond to the gravity stimulus;*** thus they are the most likely candidates for further studies involving the role of PODs in growth regulation. Isozyme staining profiles for soluble and covalently bound PODs are essentially unchanged during gravistimulation.

Finding #2: After only 30 min, the activities of two cationic POD isozymes are induced in tissues on the upper, slower growing side of the gravistimulated coleoptile (Figure 1). Induction of these PODs precedes visible bending in the coleoptile and these enzymes may initiate cross-linking of the cell wall, setting up the differential growth response. In addition, a weakly anionic POD from the soluble fraction is expressed in tissues on the *lower* side. Interestingly, this anionic POD is responsive to Ca^{2+}; in non-gravistimulated coleoptiles, its activity is increased in tissues on the side opposite that side to which exogenous Ca^{2+} is applied. All three PODs are found in cell wall exudates. Ca^{2+}-dependent secretion of extracellular POD has been reported by other workers and may function in POD expression in the gravistimulated coleoptile.

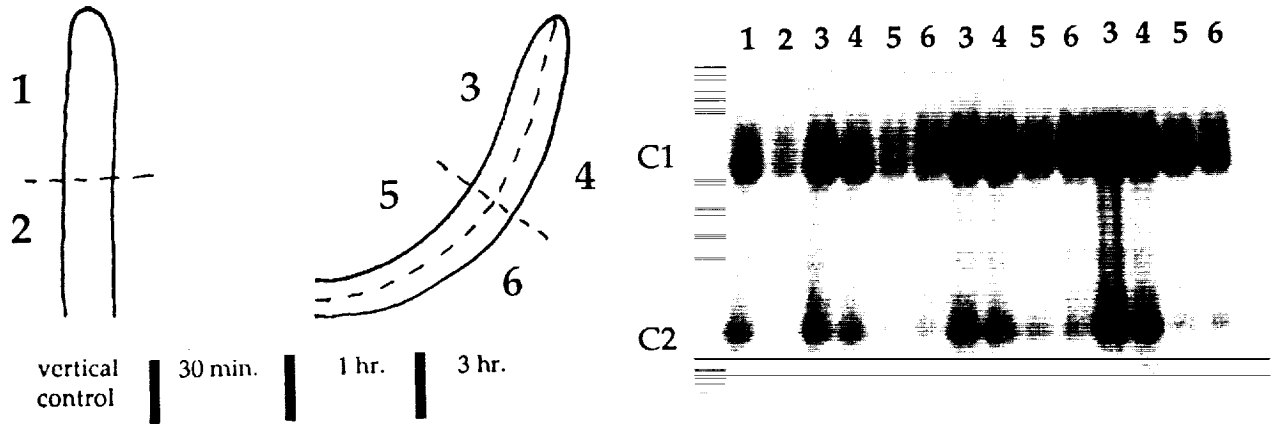

Figure 1. Kinetic analysis of cationic peroxidase isozyme (POD) expression in gravistimulated corn coleoptile. Lane numbers on gel correspond to tissue sections from which the PODs were extracted, as shown in diagram at left. Tissue segments #3-6 were harvested at 30 min, 1 hr, or 3 hr after the onset of gravistimulation. Each lane represents 5 µg of total protein. Two major isozymes are seen; both are located predominantly in apical coleoptile tissues. POD activity (isozymes C1 and C2) is higher on the upper, slower-growing section #3 side than on lower side (section #4). In more basally located tissues, weak POD activity in isozyme C1 is initially higher on lower side (section #6) than on upper side (section #5), but becomes uniform after 3 hr gravistimulation.

Finding #3: Unilateral application of H_2O_2, Ca^{2+}, and A23187$\pm$ Ca^{2+} all produce marked changes in the activities of several weakly anionic/cationic PODs from the soluble and ionically bound POD fractions. We have observed that application of H_2O_2 to vertical coleoptiles stimulates bending toward the side of application and it now seems likely that this growth response is due to the induction of specific PODs, several of which are responsive to gravistimulation (see above) or Ca^{2+} treatment. The most exciting discovery to date is that an alternating lateral asymmetry in the activity of one cationic POD is consistent with an "autotropic straightening" mechanism by which plant growth is guided relative to the gravity vector.

Finding #4: We wanted to know whether Ca^{2+} availability could regulate the *activities* of POD, apart from its possible involvement in POD secretion into the cell wall, indirectly influencing growth by regulating POD-mediated cross-linking reactions. Ca^{2+} inhibits the activity of all classes of PODs in enzyme assays, but only at high concentrations (5-10 mM). Gel staining assays suggest that it is the weakly anionic/cationic PODs that are inhibited to the greatest extent. Ca^{2+}, however, is an integral part of the POD molecule and does stimulate POD activity in preparations in which Ca^{2+} has been depleted using a chelator such as EDTA. In summary, it seems likely that Ca^{2+} does not regulate POD activity directly, but may control POD secretion into the wall.

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ANIMAL PROJECTS

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EFFECT OF SKELETAL UNLOADING ON BONE FORMATION

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Description of Research

The long range goal of this research program is to understand the effects of gravity on skeletal development and bone metabolism. Present objectives are to define the effects of gravity or mechanical stress on bone formation and resorption, and to determine the mechanisms (hormonal or paracrine) by which mechanical stress is coupled to bone cell activity.

Bone is a dynamic, living tissue. It is continually undergoing change, or remodeling, which involves a delicate balance between bone formation and bone resorption. This balance is influenced by systemic hormones such as parathyroid hormone, glucocorticoid hormones, growth hormone, and the vitamin D metabolites, as well as local factors such as blood flow, neuromuscular activity, and mechanical stress. Recently, a number of cytokines have been observed to stimulate or inhibit bone formation and resorption. Some of these cytokines, such as insulin-like growth factor-1 (IGF-1), transforming growth factors, and fibroblast growth factors, have been identified in bone. IGF-1 production by bone appears to be regulated by growth hormone. Such cytokines may participate in coupling mechanical stress to bone cell activity, or the activity of one type of cell to the activity of another type of cell, for example, osteoblast activity to osteoclast activity.

The major focus of work conducted during the past year concerned the role of IGF-1 in bone formation. IGF-1 is produced by bone and stimulates bone formation in an autocrine or paracrine fashion. Growth hormone stimulates IGF-1 production in a number of tissues including bone. Since IGF-1 levels may be reduced in bone from the hindlimb-unloaded rats (the model of skeletal unweighting used in this research), it was determined whether either growth hormone, IGF-1, or IGF-2 (an insulin-like growth factor similar to IGF-1 produced primarily in fetal tissues which may also stimulate bone formation infusion) could reverse the bone loss seen in the tibiae of the unloaded rats. In addition we developed the cDNA probes to permit the examination of whether unloading leads to a reduction in the message for IGF-1 and IGF-2.

Accomplishments

(1) Demonstrated that ***growth hormone sustains growth of bone in hypophysectomized rats, but that skeletal unweighting blocks this response.***

(2) Demonstrated that ***IGF-1 had a greater effect on bone mass than IGF-2, but that neither IGF-1 nor IGF-2 prevented the decrement in bone mass due to unweighting.***

(3) Demonstrated that the growth plate from rat bone contains a comparable complement of IGF-1 transcripts to the liver, and that hypophysectomy reduces the amount of IGF-1 transcript in both tissues to comparable degree.

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(4) Demonstrated that fetal bone and liver contain IGF-2 transcripts in much higher concentrations than adult tissues.

Significance of the Accomplishments

Current efforts are directed toward determining whether a systemic hormone, $1,25(OH)_2D$ (the active metabolite of vitamin D), interacting with a local growth factor, IGF-1, regulates bone formation, and if so whether it is this regulation which is disrupted by skeletal unweighting. To date it has not been possible to reverse the inhibition of bone formation caused by unweighting with infusions of $1,25(OH)_2D$, IGF-1, or growth hormone which should increase the local production of IGF-1. Nevertheless, it is known that serum $1,25(OH)_2D$ levels in the blood fall with skeletal unloading, and preliminary evidence suggests that local levels of IGF-1 do likewise. We are now in a position to test, in a more sensitive fashion because of the availability of cDNA probes for the IGF-1 message in bone whether skeletal unloading suppresses IGF-1 production by skeletal tissue. This will permit the reexamination of whether IGF-1 production falls with skeletal unloading, and the reassessment of whether growth hormone and $1,25(OH)_2D$ fail to prevent the loss of bone with unloading because of their failure to raise IGF-1 production.

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PHYSIOLOGY OF DEVELOPING GRAVITY RECEPTORS AND OTOLITH-OCULAR REFLEXES IN RAT

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Description of Research

Our long-term objective is to examine the effects of microgravity on the physiology of the developing and adult mammalian gravity receptors. This proposal examines: (1) the physiological responses of otolith afferents in the adult rat and during postnatal development, and (2) the otolith organ contribution to the vertical vestibulo-ocular reflexes (VOR).

These experiments will provide important new information on the physiology of the rat gravity receptors during development, interaction of otolith-canal information in trochlear motoneurons and at the motor output (vertical vestibulo-ocular reflex), and will examine the adaptive behavior of the vestibular system subsequent to otoconial demineralization, i.e., reduced otoconial mass, as a model for examining microgravity effects on the developing and mature otolith system. Such information will be required to interpret the results from future experiments with orbited rats to assess the effects of microgravity on the developing and mature labyrinth.

Accomplishments

We have made progress in three areas:

(1) Physiology of adult otolith afferents. Studies have been conducted to characterize the physiologic response of otolith afferents in the adult rat. In response to ramp changes in head position, the vast majority of otolith units (57/68) show predominantly "tonic" responses in which the discharge rate is proportional to head position and is independent of the velocity of transition. The few remaining otolith units (11/68) have "phasic-tonic" responses characterized by an overshoot (or undershoot) during transition followed by a return, within 4-12 sec, to a new steady state rate. Within the range of stimulus parameters used, tonic and phasic-tonic afferents respond in an essentially linear manner. These data from first-order otolith afferents will permit a quantitative comparison between peripheral and central parts of the otolith-ocular pathways and will allow us to chart the postnatal stages in the physiological development of the peripheral otolith system.

(2) Physiology of otolith afferents in early postnatal development. Studies have been conducted to examine the discharge patterns of otolith afferents in neonatal rats. ***Otolith afferents of postnatal day 14 rats already have a high level of spontaneous discharge and show sensitivities to sinusoidal and velocity steps similar to the adult.*** Additional experiments are underway to characterize the other postnatal periods.

(3) Postnatal development of gravity-related postural reflexes; studies using labyrinthectomized animals and animals with demineralized otoconia as a result of their

rearing on manganese-deficient diets. Considerable progress has been made this year in characterizing the postnatal development of gravity-related postural reflexes, including air-righting, surface-righting, swimming, negative geotaxis, and head elevation. There is some evidence for otoconial demineralization with even short-duration spaceflight (6 days). The ***manganese-deficient paradigm produces some variability in the completeness of otoconial defect from animal to animal.*** Behavioral testing revealed that over half of the animals showed asymmetries in three or more tests, and there was consistent laterality of the symptoms within one animal. The most sensitive indicators of vestibular disorders in the Mn-deficient animals were swimming, surface- and air-righting. Studies are now underway to analyze the changes in the otoconia of the animals undergoing the behavioral trials.

Significance of the Accomplishments

Research analysis results on the characterization of the otolith afferents in the adult rat are now being compared to the same afferents during postnatal development in an attempt to identify critical milestones for development of the otolith system. Detailed studies in other sensory systems (visual, auditory) show that it is during development that neural systems are most susceptible to alterations in the stimulus environment. A knowledge of these developmental milestones will be helpful in assessing possible effects of microgravity on animals born and reared in space. The study of animals with demineralized otoconia will be important for evaluating the ability of the vestibular system to adapt to changes in otoconial demineralization possibly occurring with microgravity. Our results have shown that the neonatal animals compensate for demineralization, and this compensation proceeds with a time course similar to recovery from hemilabyrinthectomy in young animals. A significant factor in compensation is the animals' ability to see, as evidenced by a correction in postural asymmetry at a developmental time when the eyes are open.

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STUDIES OF INTERCELLULAR COMMUNICATION AND INTRACELLULAR RESPONSES BY BONE CELLS TO SIMULATED WEIGHTLESSNESS

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Description of Research

The hypogravity environment encountered both in space and in models of weightlessness here on Earth results in a reduction of new bone formation. The mechanism(s) that cause this reduction in formation are unknown. These researchers have previously shown that a change in blood flow or vascular injury to the blood vessels in bone may be one explanation. However, the bone forming process is complex. The bone forming cells (osteoblasts) are dependent on a normal blood supply, but they are also sensitive to hormonal and mechanical influences which regulate the normal physiology of the skeleton. For example, significant changes have not been found in non-weight-bearing bones such as the ribs or calvaria compared with the changes found in the long bones which are weight-bearing and subject to high levels of mechanical stress.

Because of the variability in anatomy and physiology of different bones of the skeleton the investigators have chosen to use histological techniques to study bone cell function. Enzyme activity is localized with histochemical methods, cell structure is studied with electron microscopy, and cellular components are described by cytochemical localization of monoclonal antibodies for specific proteins and carbohydrates. Studies to date have concentrated on the bone forming cells (osteoblasts) and the cells embedded within the bone matrix (osteocytes), as well as the vascular association with these cells.

Accomplishments

(1) It has been established that ***vascular changes occur within bone as a result of spaceflight and that these alterations are anatomically confined to the subperiosteal region of the long bones.*** Results from spaceflight onboard Cosmos 1887 have helped establish this finding.

(2) In areas of bone where severe vascular changes are seen, ***osteocytic death and significant histochemical changes in the perivascular cells also occur.***

(3) Techniques have been developed to embed fresh unfixed bone in plastic resin in such a way as to preserve enzyme and structural information in the osteocyte population. Using this technique it is now possible to visualize cytoskeletal components of the osteocyte population and study cell-cell relationships within the bone matrix.

Significance of the Accomplishments

The vascular changes in bone are unusual in that they are confined to only subperiosteal regions of the weight-bearing bones (Finding #1). This suggests that the cardiovascular changes which occur during spaceflight may have a skeletal component that significantly alters bone formation. This effect has not been found in simulated weightlessness, although a slow down in blood flow through bone has been observed. Thus the model and actual spaceflight are not entirely comparable with regard to the intensity of physiological

changes that occur during non-weight-bearing. It appears that the vascular changes also initiate cellular necrosis in the osteocyte and perivascular cell population (Finding #2). It was interesting that histochemical studies demonstrated which cells were undergoing cell death even when morphological studies indicated no significant change.

The new techniques of working with fresh unfixed embedded bone will permit cytochemical studies not previously possible. For example it is now possible to localize specific proteins and cellular components with antibodies (Finding #3) as well as enzyme activities which previously were denatured by the tissue preparation. Other studies indicate that the osteocyte population contains some enzymes that are very responsive to mechanical stimuli. This new methodology will permit the study of these cells and relate their activity to mechanical and hormonal changes that take place during studies of weightlessness.

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EFFECT OF ALTERED G ON CHONDROCYTE DIFFERENTIATION

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Description of Research

The response of the skeletal system to microgravity is well-documented, with all components thus far examined (bone, tendon, cartilage) showing significantly altered differentiation, including altered matrix organization, after spaceflight.

The projects in our laboratory are directed toward the question of the effect of gravitational changes on pre- and postnatal skeletal development, with emphasis on endochondral ossification. Several approaches to this question are used: *in vivo* and *in vitro* centrifugation, whole animal unloading, and spaceflight. In the last case, we are analyzing bones from recent Cosmos spaceflight missions and are developing hardware for what will be the first study of cultured skeletal cells in space.

Because of the complexities involved in whole animal systems, and the lack of inflight animal sacrifice, cell culture systems to address mechanisms of gravitational responses in the skeletal system are of importance. One such system, using differentiating cartilage cells in high density micromass culture, will be flown in space on the Spacelab mission International Microgravity Laboratory-1 (IML-1). Besides being the first culture of skeletal cells in space, this experiment also has the most extensive medium changes and fixations ever carried out in space.

Accomplishments

Centrifugation, in vitro

(1) ***Centrifuged (2.9 g) micromass cultures developed cartilage 24 hrs prior to appearance of cartilage in control cultures.***

Centrifugation, in vivo

(2) Analyses of skeletal muscle of mice centrifuged at 3 g for 14 weeks were carried out in Berne, Switzerland, and demonstrated an increase in mitochondrial and lipid total volumes. This finding is similar to changes seen in endurance training. However, there was also an increase in muscle mass which is seen in strength rather than endurance training.

(3) Dr. Cogoli of Zurich found ***a four-fold higher incorporation of ^{3}H-thymidine in lymphocytes from the spleens of animals centrifuged for 12 months and stimulated with the mitogen Concanavalin A*** when compared to controls.

Spaceflight

(4) ***Increased height and cell number in the proliferative zone of growth plates of flight rats*** were found after exposure to microgravity for 14 days aboard the Soviet biosatellite Cosmos 2044.

(5) ***The hypertrophy/calcification zone of rat growth plates was significantly reduced in height and cell number after spaceflight*** aboard Cosmos 2044.

(6) ***No significant changes were found in growth plates of the unloaded control group,*** in contrast to previous studies which had shown this to be a good simulation model for spaceflight.

(7) ***Response of the growth plate to microgravity depends on age.*** Cosmos 2044 results provided the first indication that growth plate may respond differently to microgravity at different ages of the animal.

Hindlimb unloading

(8) Hypergravity was used as a countermeasure to the effects of hindlimb unloading. Unweighted animals were allowed to bear weight for 1 or 2 hrs/day, at 1 g (ground), or by centrifugation at 1.5 or 2.6 g. Height and cell number per zone of the growth plate were determined. In all experimental groups, total plate height and total cell number/column were significantly less than in controls. However, the results indicate some preventive or reparative effect of reloading at 1 g for 2 hrs or 1.5 g for 1 hr/day. In 1.5 g (1 hr) animals, the reduction in proliferative and hypertrophic/calcification zones was less than in other centrifuged groups and not significantly different from controls. Longer or higher g exposures produced significant reductions in cell number and height.

Significance of the Accomplishments

(1) Previous studies in this laboratory showed that precocious chondrogenesis occurs in intact embryonic limb buds exposed to hypergravity. The current study shows that the same result occurs when the cells of the limb are dissociated and exposed to hypergravity in culture. With the micromass culture system, the response of precartilage cells to a complete range of g forces, including simulated microgravity using a clinostat and true microgravity on our shuttle flight, can be determined. With cartilage cells and quantitative measurements, it should be possible to come to some scientific conclusions regarding the relationship of centrifugation to spaceflight and the validity of clinostating.

(2) This study demonstrates the benefits of long range tissue sharing, and the value placed upon operation of our animal centrifuge by other laboratories. It also adds to our knowledge of centrifugation effects, and with the advent of centrifugation on shuttle and space stations and the current lack of centrifuge scientists, such studies have become extremely important.

(3) The increased activation seen in hyper-g exposed animals is in contrast to the lower activation seen in spaceflown animals.

(4&5) These animals were exposed to earth gravity for about 10 hrs prior to sacrifice, so these results represent both effects of microgravity exposure and initial stages of recovery from that exposure. The results also confirm previous observations of altered chondrocyte proliferation and differentiation in growth plates exposed to microgravity.

(6&7) The lack of response to unloading seen in these animals is likely due to age, because these were the oldest unloaded group we have studied. However, we have not studied plates from the particular model used by the Soviets, which does not allow movement in 360^{o}. Whichever the case, the results demonstrate the need for caution in interpretation of unloading studies.

(8) This pioneering study, carried out with Dr. Frank Booth of University of Texas Medical School, demonstrates the advantages and disadvantages of using g forces higher than 1 to prevent effects seen in unloading or spaceflight. The advantage is that with 1.5 g, shorter loading times are needed than with 1 g; the disadvantages are that too much g force compresses the plate and stops the growth, and that the animals are somewhat stressed by the centrifugation. Additional studies are being carried out to determine what combination of g force and time is best to maintain bone growth. It is also possible that different systems, e.g., muscle and bone, require different thresholds for health maintenance.

Publications

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INFLUENCES OF SYNAPTIC GENESIS ON THE ARCHITECTURE OF THE VESTIBULAR EPITHELIA

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Description of Research

The objective of this project is to study, with the electron microscope and modern biotechnological applications, the vestibular epithelia of the utricle and saccule in order to: (1) delineate modifications of cells known to be influenced by synaptic genesis in other sensory organs and (2) investigate the relationship that might exist between refinements of the epithelia and changes in the formation of the statoconial membrane that rests above the maculae.

This study is needed because the maculae utriculi and sacculi are gravity detectors and thus their development should be influenced by the microgravity environment of spaceflight. The modifications caused by changes in the gravity vector and force in the developing gravity sensors could occur at the organ level, at the tissue level, and/or at the cellular level. At each level, morphological and biochemical manifestations of the changes can be followed during development and critical periods can be determined based on the significance of the changes. Normal values from Earth-based experiments are needed in order to identify changes that may occur in spaceflight.

Accomplishments

In order to establish normative Earth-based data, the saccule and utricle of chick embryos and newly hatched birds were carefully studied with the light and electron microscopes and with computerized video microscopy. In this report analysis of the bipolar neurons in the vestibular nerve is described. ***The bipolar neurons are cable-like structures that connect the inner ear with the brain. These neurons change in size as the distance between the inner ear and the brain becomes larger due to embryonic growth.*** It is a well known fact that an increase in the length of nerve fibers is accompanied by an increase in the axoplasmic cross sectional area and wrapping thickness of myelin sheaths. The ear from 11-day old embryos (E11) processed in parallel with newly hatched chicks (P2) was analyzed.

Statistical manipulations of the data obtained from computerized video-morphometric measurements showed a ***significant increase ($p<0.00003$) in the surface area of the bipolar neurons of P2 over E11.*** No significant difference was found in the shape of the neurons from these groups. At E11, the nerve fibers of the vestibular neurons have almost no myelin sheaths, and the perikarya of the neurons still display the characteristic appearance of actively growing structures, i.e., heterochromatic nuclei and large quantities of polysomes. At P2, the utricle and saccule resemble the adult, and their appearance corroborates the precocious development of chicks, a characteristic essential for evaluating the processes of embryonic development and its relationship to maturation.

Significance of the Accomplishments

The neurons of the vestibular nerve that connect the inner ear to the brain undergo a significant change in size of perikarya but not in shape (Figures 1 and 2). This probably

means that the shape factor (circle = 1.0) of perikarya, through a given sectional plane, tends to remain nearly the same. On the other hand, the perikaryal size increases probably to accommodate the larger size of its axons. A constant change of perikaryal size would occur as transition between temporary and permanent synapses takes place. Such has been the case for the auditory portion of the inner ear of chicks.

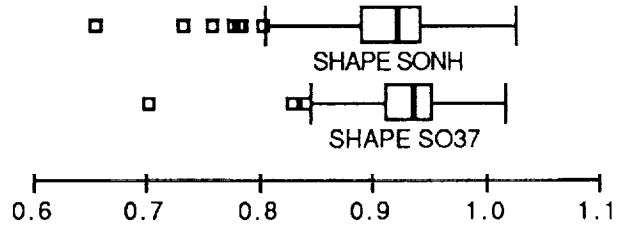

Figure 1. Comparison of neuronal shape between E11 (bottom) and P2 (top) vestibular nerves. A value of one represents a perfect circle. The main body of the neurons (perikarya) does not significantly change between the onset of myelination and time of hatching.

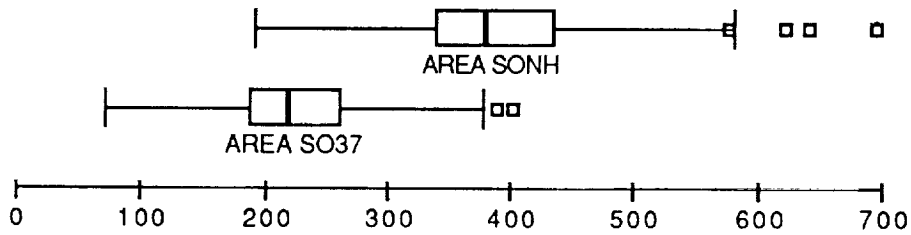

Figure 2. Contrary to the shape of the perikarya, the cross sectional area (μm^2 on the x axis) changed significantly between E11 (bottom) and P2 (top).

Since studies in chicks by other investigators show a progressive maturation of vestibular responses, ***a gradual change and correspondence between morphology and physiology is reasonable. In fact, in many other sensory systems, behavioral responses do have an underlying morphological basis.*** Future aims of this project are to examine other aspects of the inner ear and vestibular nerve in order to provide morphological data needed by other investigators in this field. A corresponding alteration of the perikarya and axons of neurons should also exist, which should be evident in statistical analysis of data obtained from various developmental groups. In the utricle, hair cells proximal to the nerve entrance (habenula perforata) are innervated first while those away from the entrance of the nerve are innervated last. This pattern of innervation constitutes a gradient which has also been shown in the auditory lagena by this laboratory.

Image processing is being used in this laboratory in conjunction with true color/real time analysis for examining the association of structural components in the developing inner ear. The computerized video system allows us to emphasize subtle differences barely visible in

unprocessed raw images. The real advantage of this technology is its capacity for true objective quantification of differences between structures, since this system can recognize millions of color variations and then match the color threshold with those presented for analysis. For instance, gray levels of intensity cannot distinguish between the density of a blue nucleus counterstained with hematoxylin and the density of a nucleus stained brown with peroxidase precipitate for a given antibody. True color image processing, however, can make this distinction, and thus it is a dream come true for researchers wishing for objective quantification of immunological reactions.

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NEURAL MECHANISMS BY WHICH GRAVITATIONAL STIMULI AND STRESS AFFECT THE SECRETION OF RENIN AND OTHER HORMONES

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Description of Research

The long-term goal of this research is delineation of the brain pathways and neurotransmitters that mediate gravitational and stress-induced changes in the secretion of renin and other salt and water-regulating hormones. Evidence from this laboratory indicates that stimulation of certain serotonin-secreting neurons in the dorsal raphe nucleus of the midbrain increases renin secretion, and that these neurons project to the hypothalamus. One goal of this research has been determination of the specific parts of the hypothalamus that affect circulating renin and angiotensin. Another goal has been analysis of the importance of this region in the physiologic control of renin secretion. A third goal has been determination of the pathways from the nervous system to the renin-secreting cells in the kidney. To study the role of the hypothalamus and other parts of the brain in the regulation of renin secretion, stimuli have been used which increase renin secretion in diverse ways. Emphasis has been placed on the postural stress of 45^{o} head-up tilt. Other standard tests include: (1) administration of the serotonin-releasing drug p-chloro-amphetamine (PCA); (2) the psychological stress of immobilization; (3) the chronic volume depletion stress of a low sodium diet; and (4) the acute volume depletion stress of nonhypotensive hemorrhage. The role of the vasopressin-secreting neurons that connect the hypothalamus to the brainstem and spinal cord in the regulation of renin secretion is also being studied.

Accomplishments

(1) ***Demonstration that the sympathetic nervous system plays a major role in the increase in renin secretion produced by a low sodium diet.*** The drug propranolol blocks the receptors that mediate the effects of sympathetic nerves on renin secretion, and its effect on various responses have been studied. We previously showed that propranolol blocks the renin responses to immobilization, head-up tilt, and PCA. The effect of propranolol on the response to a low sodium diet has been studied and it has been found that renin is reduced compared to vehicle-injected controls on the same diet. However, injection itself is a stress, and in the animals that have been handled and injected, it is not possible to say whether propranolol is reducing the overall response to sodium depletion or simply blocking the response to injection stress. Therefore, chronic cannulas were placed in rats so that we could inject the animals without handling them. In the animals on a normal sodium diet, propranolol did not reduce renin secretion when administered in this fashion.

(2) Demonstration that vasopressin probably acts within the brain to inhibit renin secretion. In addition to regulation of angiotensinogen secretion, the paraventricular nuclei of the hypothalamus are involved in the regulation of the secretion of renin from the kidneys in some situations. Most of the paraventricular neurons secrete vasopressin into the circulation from the posterior pituitary, but others project to the cardiovascular regulatory areas in the brainstem and spinal cord. The vasopressin secreted by the neurons

Brattleboro rats that (due to a genetic defect) have no vasopressin in their brains or circulating blood, we found that β-adrenergic blockade produced by propranolol lowered circulating renin to essentially normal levels. This indicates that the Brattleboro rats have chronically increased sympathetic discharge. In another set of experiments, we used osmotic minipumps to infuse vasopressin subcutaneously in Brattleboro rats in a dose which restored circulating vasopressin to normal. The increase in renin secretion was unaffected even though urine volume and water intake were markedly reduced and the concentration of the urine was increased. This seems to rule out a peripheral stimulus to sympathetic discharge. However, since very little vasopressin penetrates the blood-brain barrier, the increased sympathetic discharge that produces the renin hypersecretion could be central in origin. If our working hypothesis is correct, then injection of vasopressin directly into the brain should inhibit renin secretion. In experiments carried out during the past year, this appears to be the case. Additional experiments are underway to make sure that this central inhibition is reproducible, clear cut, and dose related.

(3) ***Confirmation of the hypothesis that paraventricular regulation of angiotensinogen secretion is mediated via neuroendocrine control of the thyroid gland.*** Several years ago, we demonstrated that lesions of the paraventricular nuclei decreased circulating angiotensinogen. We postulated that this effect was neuroendocrine, since the paraventricular nuclei regulate the secretion of thyroid and adrenocortical hormones via the anterior pituitary gland, and these hormones increase circulating angiotensinogen. We found that removal of the anterior pituitary duplicated the effect of paraventricular lesions. This year, we completed experiments in animals with paraventricular lesions in which thyroid hormones or adrenocorticotropic hormone (ACTH) were replaced by injection. ACTH did not restore angiotensinogen to normal, but replacement of thyroid hormones did. Therefore, it seems clear that ***paraventricular lesions decrease circulating angiotensinogen because they decrease secretion of the brain hormone that stimulates the pituitary to secrete the hormone that in turn stimulates thyroid hormone secretion.***

(4) Discovery of a unique effect of anesthetics on circulating angiotensinogen. In the course of experiments described in the preceding paragraph, we made the chance observation that ***24 hours after surgical stress and some anesthetics, plasma angiotensinogen was elevated with little if any change in plasma renin activity***. This year, we have demonstrated that this increase cannot be explained by an increase in ACTH, thyroid hormones, luteinizing hormone, or renin. However, it is blocked by hypothosectomy. Therefore, we are actively investigating the role of the remaining pituitary hormones in mediating the response.

Significance of the Accomplishments

The experiments described above and experiments conducted in previous years have done much to elucidate the role of the brain in the regulation of renin secretion. This has appreciable significance for NASA because both postural changes and stressful stimuli increase renin secretion, and renin not only stimulates the secretion of the salt-retaining hormone aldosterone, but affects water balance and maintains blood pressure. In addition, our demonstration that brain lesions lower circulating angiotensinogen is important because it demonstrates for the first time that there is a neuroendocrine mechanism regulating the circulating level of this important component of the renin-angiotensin system.

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VECTOR-FREE GRAVITY INTERFERES WITH SYNAPSE FORMATION

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Description of Research

Prolonged spaceflights and plans for the colonization of space have opened two new areas of research in biology. The first concerns the well-being of developing biological systems in flights of even short durations, and the second concerns the possibility of investigating, from an evolutionary perspective, the contribution of gravity to development.

The continued objective of this research is to examine, in-depth, specific aspects of nervous system development, under simulated microgravity, to gain a better understanding of how gravity influences ontogenetic development of the brain while at the same time exploring the possibility that the constancy of gravity on Earth has contributed significantly to cellular evolution.

The investigators have already shown that exposure of neurons and myocytes to vector-free gravity profoundly affects synapse development including abnormalities in neuronal morphology. Over the past 12 months, attention has been focused on the distribution of cytoskeletal proteins which may be the underpinnings of the alterations in myocyte acetylcholine receptor distribution and the alterations in neuron morphology (appearance of swellings along neuritic shafts). Using immunocytochemistry to label cytoskeletal proteins, we are probing changes in the structure of the cellular scaffolding responsible for the anchoring of proteins to specific locations in the cell subsequent to growth of cells in vector-free gravity.

Accomplishments

Our results show that synapse development in a slow clinostat (1-10 rpm) results in dramatic reductions of the appearance of nerve-induced acetylcholine receptors (AChR) accumulation at the point of contact between nerve and muscle cells in culture. Figure 1 shows typical results from cells which were rotated and then stained for 43kD protein and for vinculin, both of which are cytoskeletal protein markers. Our results show that ***43kD protein always co-distributes with AChRs.*** This is not surprising since 43kD is now known to be directly, and very tightly, linked to the cholinergic receptor. Therefore, in the absence of AChR (after rotation), 43kD protein is also expected to be absent. The story for vinculin is more complicated mostly because the exact location of vinculin, in reference to AChR, is still unknown. Our data show that ***in rotated cells the ratio of vinculin to AChR is larger than in control cells. This implies that cytoskeletal organization in response to nerve contact takes place at least up to the level of vinculin*** (vinculin is present in rotated cells even when AChR numbers are diminished, hence the increase in vinculin/AChR ratio). Thus, ***the loss of anchoring of AChR may be due to cytoskeletal elements which are located between vinculin and the receptors.*** We are now pursuing this lead. Possible candidates for these elements include: talin, actin oligomers, spectrin, and/or extracellular matrix proteins (see Figure 2 for a model of the system).

DISTRIBUTION OF CYTOSKELETAL MARKERS IN MYOCYTES

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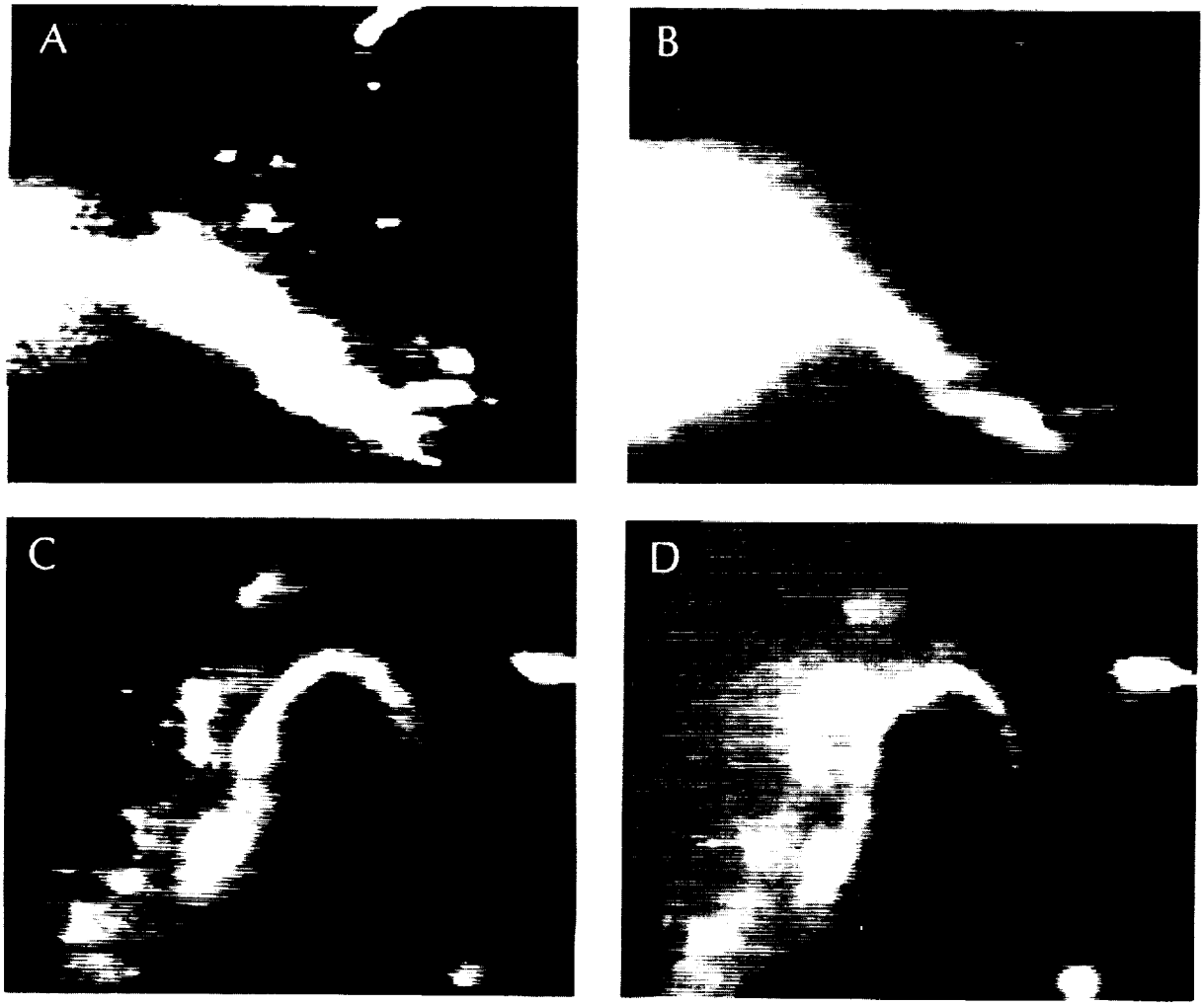

Figure 1, Panels A-H. Preliminary data showing distribution of cytoskeletal markers in control and rotated myocytes.

Figure 1A-D: Coincidence of acetylcholine receptors (AChRs) with 43kD protein. Panel A shows the distribution of AChRs in a control myocyte. Panel B shows that 43kD protein codistributes with the AChRs indicating the close relationship between the receptor and cytoskeletal proteins. Panel C shows AChR distribution from a rotated myocyte (most myocytes do not in fact have AChR at the point of innervation after rotation; see text). Panel D shows the corresponding distribution for 43kD, the cytoskeletal marker. Note that although 43kD is present, its distribution appears to be reduced when compared to the control (A, B). In cases where AChRs are absent from the nerve-muscle contact zone, after rotation, 43kD is also absent. This indicates that the tight binding between the two proteins is not affected by rotation. Absence of receptors, after rotation, also results in absence of 43kD. The two proteins behave as a unit.

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DISTRIBUTION OF CYTOSKELETAL MARKERS IN MYOCYTES

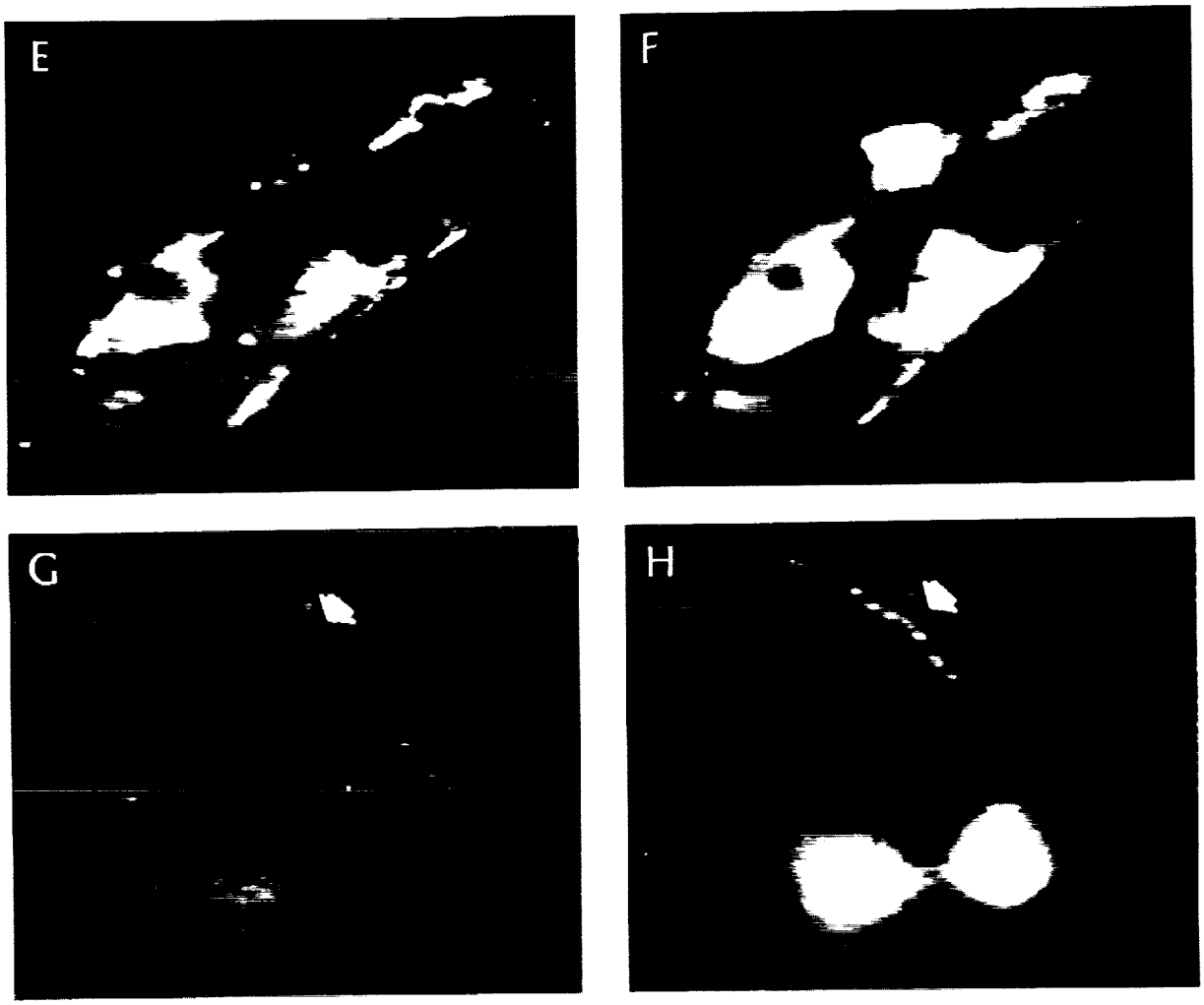

Figure 1E-H: Coincidence of AChR binding sites with vinculin binding sites. Panel E shows AChR distribution in a control myocyte, and panel F reveals that vinculin, a cytoskeletal linker protein, codistributes with the AChRs. In contrast, panels G and H, from a rotated cell, show that vinculin is present at the nerve-muscle contact zone despite the absence of AChR subsequent to rotation. This implies that nerve-muscle interactions, during rotation, take place normally as far as assembly of the cytoskeleton is concerned up to the level of vinculin, but that despite this partial assembly, the AChRs cannot be retained at the nerve-muscle contact zone.

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PROPOSED MODEL OF RECEPTOR ACCUMULATION/CYTO-SKELETAL ASSEMBLY AT THE NEUROMUSCULAR SYNAPSE

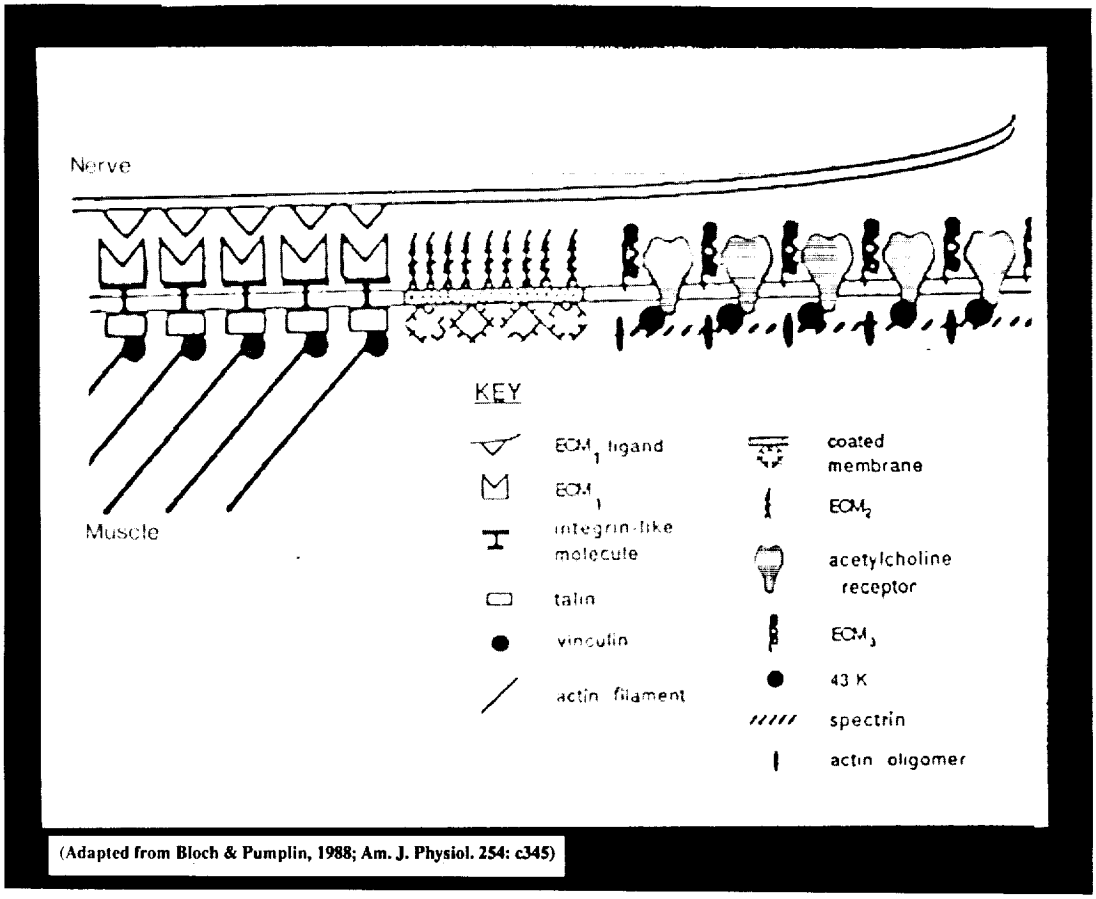

Figure 2: Cartoon model of the mechanism of receptor patching and its association with the cytoskeleton at the neuromuscular junction. This model, adapted from Bloch & Pumplin (1988), provides a hypothetical framework through which we examine how simulated μg may affect the receptor accumulation process and the assembly of the associated cytoskeleton. For example, the initiating signal is derived from the nerve (or polystyrene beads as a substitute) which contains extracellular matrix ligands (ECM_1) that bind to their hypothetical receptor on the ECM of the myocyte. This initiates the assembly of the underlying cytoskeleton (e.g., vinculin). By an as yet unknown process this signal is carried to non-patched, randomly distributed AChR in the myocyte. This signal results in receptor accumulation at the nerve-muscle zone. Shortly after the arrival of AChR, at the forming neuromuscular junction, the receptors are capped by anchorage to the underlying cytoskeleton (via 43kD protein, spectrin, actin, etc.). By probing for distribution of specific elements, we expect to gain information concerning the specific mechanism(s) affected by clinostat rotation which may be associated with the failure of AChR to accumulate at the nerve-muscle contact zones (see text for further details).

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THE EFFECTS OF GRAVITATIONAL FIELDS ON NEURAL SIGNALING IN THE HIPPOCAMPUS

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Description of Research

Our research has as an ongoing, long-term objective the elucidation of the effects of altered gravitational fields on modulatory and regulatory neural networks. Recently our experiments have dealt with the effects of serotonin, a neuromodulator, on neurons in the hippocampus. We studied the hippocampus because rats flown on Spacelab-3 showed an increased number of serotonergic receptors in this region of the central nervous system after seven days in space. In addition, a more recent study has shown that rats exposed to a hypergravic field of 2.0 g for seven days show a decreased number of receptors. Serotonin is a well-studied neuromodulator involved in many types of neural activity, including sleep and temperature regulation. In addition, in the hippocampus serotonin may modulate long-term potentiation, a proposed cellular mechanism for memory and learning. Our immediate goal is to determine how serotonergic effects on hippocampal cells are altered by gravitational fields.

An additional motive for studying the effect of serotonergic activity in the hippocampus is that other types of neural activity are known to be modified by exposure of animals to hypergravic fields. Temperature regulation of animals in a microgravity environment has been reported to be impaired. This year we also completed studies on thermoregulation in hypergravic-acclimated rats (briefly described below), and found that when raised from birth in a 2.1 g field rats were better able to cope with cold exposure in hypergravic environments than were 1 g controls. While there are many studies by several investigators showing that thermoregulatory systems are altered by changes in gravitational fields, there are relatively few studies on the effects of gravitational fields on serotonergic mechanisms, and more studies are needed to more fully delineate altered serotonergic activity.

Accomplishments

(1) One set of experiments focused on selected aspects of serotonergic modulation of neural activity in rats exposed for seven days to 2 g fields. Experiments showed that ***serotonin was effective in modulating the amplitude of one neural response (the population spike) in both the rats exposed to 2 g for seven days and the 1 g controls.*** More specifically, even though there was a 26.9% decrease in receptor number in rats exposed for seven days to a 2 g field, this was not sufficient to markedly alter the action of serotonin at 5-HT_{1A} receptors. (Additional experiments are in progress to determine if there is a subtle change in serotonin's effectiveness in decreasing population spike amplitude.)

(2) In extending the experiments to the action of serotonin acting at other receptor types on hippocampal cells, experiments were performed on rats and hamsters. In experiments at 1 g, population spikes had a clear biphasic response, with a depression in neural activity averaging 52.4 ± 10.1% less than control levels followed by a rebound of neural excitability which averaged 33.7 ± 9.3% greater than control levels. In rats at 1 g

the depression was also clearly present, averaging 76.4 ± 5.5% less than control levels; however, the rebound was often small or masked in background activity, averaging only 7.4 ± 4.8% of controls. The rebound effect was not blocked by 5-HT_{1A} blockers, and thus was the result of the activation of cellular mechanisms by serotonin acting on a second type of hippocampal serotonergic receptor.

(3) When rats were exposed to cold (9°C), concurrently with a hypergravic field of 2.1 g, the core temperature of rats raised at 1 g fell markedly (by 6°C), while that of rats born and raised at 2.1 g remained relatively constant (falling only 1°C). Measurements of O_2 consumption showed that the rats reared at 1 g and then cold exposed at 2.1 g failed to activate thermogenic mechanisms.

Significance of the Accomplishments

Finding #1: Depression of the amplitude of population spikes during serotonin perfusion in rats exposed to hypergravic fields showed that one modulatory effect of serotonin is not abolished in animals exposed to hypergravic fields. Thus the decreased number of receptors observed under the same experimental conditions (exposure to a hypergravic field using the same small animal centrifuge) is insufficient to abolish a primary modulatory action of serotonin.

Finding #2: The rebound following serotonin administration was larger in the hamster than in the rat. Thus to study the characteristics of rebound (an enhancement elicited by a different serotonergic receptor than the 5-HT_{1A} receptor type) under hypergravic conditions, the hamster is the preferred model.

Finding #3: While a hypergravic field of 2.1 g is a novel environment for animals raised at earth gravity (1 g), it is not novel for the animals reared from birth in a 2.1 g field. The rats acclimated to 2.1 g were able to maintain their core temperature when cold exposed in the 2.1 g environment whereas the 1 g acclimated rats could not. These experiments show that ***an animal acclimated to hypergravic fields has an altered neural thermocontrol system and can more effectively regulate core temperature in a hypergravity field than can an animal acclimated to 1 g.***

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MECHANISM OF CONTROL OF BONE GROWTH BY PROSTAGLANDINS

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Description of Research

Biomedical studies of manned spaceflight have consistently shown a loss of weight-bearing bone. Various lines of evidence from human and animal studies suggest that this loss is due to the lack of bone formation in the absence of gravity. The mechanism by which bone growth is regulated is not known; however, clinical observations have demonstrated that increases of endogenous cortisol as seen in Cushings syndrome are associated with bone loss and osteoporosis. Treatment of patients with asthma and rheumatoid arthritis has also demonstrated the deleterious effects of glucocorticoids on bone formation. The bone loss associated with glucocorticoids involves trabecular bone and examination of patients treated with synthetic glucocorticoid prednisone shows a reduction in bone formation which is probably due to a direct inhibition of osteoblastic function.

These data suggest that the glucocorticoids could also play a role in the loss of bone that occurs in spaceflight. In the Skylab missions urinary cortisols of the nine crew members increased almost two fold. The direct cause of glucocorticoid-induced bone loss is not known and an understanding of the relationship between glucocorticoids and prostaglandins in bone cell growth and differentiation may increase our ability to understand the underlying mechanism of bone formation in microgravity. The glucocorticoids and prostaglandins may play a role in the physiological control of bone cell growth and mineralization at the cellular and molecular level. We are using an *in vitro* osteoblast model treated with comparable amounts of glucocorticoid as seen in spaceflight to determine if: (a) glucocorticoids affect prostaglandin synthesis and cell growth; (b) if glucocorticoids affect bone mineralization; and (c) if these glucocorticoid-induced changes are related to changes in protein synthesis or assembly.

Accomplishments

Our bone model is the MC3T3-E1 cell, a cloned osteoblast line that retains synthetic functions of normal bone tissue, including production of alkaline phosphatase, prostaglandin E_2 and mineralized matrix containing hydroxyapatite. In these studies, we have used 100 μM dexamethasone treatment on the osteoblast MC3T3-E1 cells to simulate the effect of increased plasma cortisols on bone cells seen in spaceflight over a 13-day period of growth and differentiation.

(1) The levels of collagen synthesis in the growing osteoblast are barely detectable. However once the cells are confluent and differentiated, collagen is the major protein synthesized by the osteoblast. Using western blot analysis and affinity purified collagen antibody, we have found that ***collagen type I accounts for approximately 97% of the total collagen synthesis in this cell line.***

(2) Dexamethasone treatment of the mineralized osteoblast causes a decrease in ^{14}C proline incorporation into new protein synthesis. Using western blot analysis with radioautography and video densitrometry, this observation has been expanded to

demonstrate that collagen type I synthesis is specifically inhibited by glucocorticoids. Procollagen a_1(I) and a_2(I) synthesis is decreased by approximately 50% while collagen a_1(I) and a_2(I) is decreased up to 70% when compared to control after 13 days of growth and mineralization.

(3) The glucocorticoid treated cells were examined by microscopy for changes in cell structure. Using phase microscopy, the shape of the treated cells was found to be irregular when compared to controls. Using specific probes for f-actin cytoskeleton we found that the ***irregularity seen in overall cell shape is reflected by the actin cytoskeleton.***

(4) The distribution of the collagen matrix in the treated cells differed from the control osteoblasts. In general, the collagen matrix formation in the treated cell was not as uniform as the control and appeared to be discontinuous in portions of the matrix.

Significance of the Accomplishments

The importance of these findings is that the temporal regulation of collagen synthesis in this cultured osteoblast corresponds to published observations made *in vivo*. Collagen synthesis is of minor importance during the first stages of osteoblast growth, but becomes increasingly important during mineralization. Finding #2 suggests that collagen synthesis is inhibited by the glucocorticoids at concentrations approximating the levels of cortisol seen in man during spaceflight. In addition to decreased collagen synthesis, there are changes in the f-actin cytoskeleton assembly and collagen matrix formation in the mineralizing cultures. These findings suggest that not only do the glucocorticoids decrease the ability of the bone cell to synthesize collagen matrix needed for mineralization, but also interfere with the cytoskeleton formation and assembly of the collagen matrix during mineralization. This, along with other findings, suggests that the glucocorticoids may inhibit bone formation, both by interfering with the growth of the osteocyte and also by inhibiting synthesis and uniform assembly of the collagen matrix during mineralization.

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MECHANOCHEMICAL TRANSDUCTION ACROSS EXTRACELLULAR MATRIX

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Description of Research

The general goal of this project is to characterize the molecular mechanism by which mechanical signals, such as those that result from the action of gravitational forces, are transduced into intracellular metabolic alterations. The more specific aim is to analyze the process by which mechanical forces are conveyed by extracellular matrix molecules, transmitted across the cell surface, and transduced into changes of actin polymerization inside the cell. This approach is based on the concept that cell shape and, thus, the form of the cytoskeleton, depends on a dynamic equilibrium between tensile forces that are generated within contractile actin-containing microfilaments and resisted both by internal structural elements and by extracellular matrix attachment sites on the surface of the cell. If this type of tensional integrity or "tensegrity" mechanism is used by cells, then externally applied mechanical loads may affect complementary force interactions, change local thermodynamic parameters, and thereby alter cytoskeletal filament assembly. Changes of cytoskeletal organization, in turn, can alter the distribution and hence function of much of the cell metabolic machinery. Characterization of this process could have much greater implications for understanding the effects of gravity on a wide variety of metabolic functions.

We have focused on the biomechanical mechanism by which actin filament assembly is regulated inside the cell. Cells normally generate tension within their contractile microfilaments and apply these forces on their points of attachment on extracellular matrix molecules. Thus, one specific objective of this research is to study the process by which transmembrane matrix receptors resist cell-generated mechanical forces and transduce them into alterations of microfilament assembly in the cell. Our long term goal is to determine whether externally applied forces utilize a similar matrix-based mechanism for transmembrane mechanochemical coupling.

Accomplishments

(1) ***Demonstration of the Importance of Cell Shape in Growth Control:*** We have developed a simplified *in vitro* system for regulation of capillary endothelial cell growth responsiveness to soluble growth factors, such as basic fibroblast growth factor. In this system, cell shape and DNA synthesis are controlled by varying the coating density of a single type of extracellular matrix molecule, such as fibronectin. Using this system in combination with chemically defined medium, we have been able to clearly demonstrate that ***fibronectin (FN) controls cell growth based on its ability to support changes of cell and nuclear shape.*** We have also demonstrated that the ***effects of fibronectin on cell form and function are mediated by specific binding interactions with cell surface matrix receptors,*** specifically the alpha 5, beta 1 integrin receptor.

(2) ***Microfilament Assembly Mediates the Effects of Fibronectin on Growth***: Immunofluorescence studies using antibodies directed against G-actin or using rhodaminated-phalloidin demonstrate an increase in the number and length of actin bundles as fibronectin coating densities are raised. However, no increase in actin filament staining is seen in suspended cells cultured in the presence of soluble fibronectin or fibronectin-coated microbeads which do not support cell spreading. Furthermore, use of cytochalasin-D, an inhibitor of microfilament assembly, revealed that actin polymerization is required for both cell spreading and entry in S-phase. Importantly, cytochalasin had no effect when added at later times after cells have entered the synthetic phase of the cell cycle. These results suggest that ***actin assembly is necessary for progression through the G-1 phase of the cell cycle.***

(3) ***Quantitation of Actin Polymerization within Cells Spreading on Fibronectin***: We have used quantitative immunoblotting techniques in combination with anti-actin antibodies and a NBD-phallicidin binding assay to measure actin polymerization during the initial phases of cell attachment and spreading on fibronectin-coated dishes. Both of these techniques showed similar results and demonstrated that monomeric actin shifts into a polymerized F-actin form and becomes associated with the cytoskeleton beginning approximately 10 minutes following cell attachment to fibronectin-coated dishes. The effects of FN on actin assembly nicely parallel its early effects on cell spreading.

(4) ***Application of Mechanical Loads to Fibronectin Receptors:*** We have just begun to develop a system in which cells are plated on silastic membranes to which controlled mechanical loads can be applied. These membranes are non-adhesive for endothelial cells under the culture conditions we utilize. We can therefore vary the number of structural interconnections between the cell and the substratum (and thus the amount of force experience by the cell) by varying the density of fibronectin coated on these elastic substrata. Experiments measuring the effect of externally applied mechanical loads on actin polymerization are just beginning to get underway.

Significance of the Accomplishments

Finding #1: The finding that cell proliferation can be controlled by varying the density of fibronectin attachment sites on the surface of the cell suggests that extracellular matrix may serve as a basic regulator of cell growth responsiveness in the local tissue microenvironment. These studies also suggest that ***matrix molecules regulate cell proliferation based on their ability to provide a physical anchoring substrata that can resist cell-generated tensile forces and thereby promote cell extension.*** The finding that fibronectin regulates cell growth by modulating cell shape also suggests ***that cells may utilize a biomechanical signal transduction system during growth control.***

Finding #2: The demonstration of the effects of fibronectin on actin assembly as well as inhibition of cell growth by the addition of inhibitors of actin polymerization strongly suggests that ***the effects of fibronectin on growth are mediated via its effects on the cytoskeleton.***

Finding #3: The finding that fibronectin promotes cell spreading and actin polymerization in parallel, whereas soluble FN does neither, is consistent with our hypothesis that ***fibronectin regulates actin filament assembly both by binding to cell surface receptors and by physically resisting cell-generated loads that are applied to those receptors.***

Finding #4: We now have an *in vitro* system which can be used to analyze the molecular path of force transmission across the cell surface as well as the mechanism of mechanochemical transduction.

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VESTIBULAR ELECTROPHYSIOLOGICAL RESPONSE THRESHOLDS: EFFECTS OF SPACEFLIGHT AND LINEAR SINE WAVE MOTION

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Description of Research

The principal aim of this research is to examine the role played by gravity in controlling or influencing the ontogeny of peripheral and central vestibular function. Ultimately an in-depth comparison will be made of how vestibular function develops and matures under the influence of gravitational fields having strengths less than (< 1.0 g, hypodynamic) equal to (1.0 g) and more than (>1.0 g, hyperdynamic) the natural gravitational field strength of the Earth. Studies have been undertaken to examine how the gravitational vector strength and direction may modulate or influence normal response to transient stimuli. Efforts have been directed to investigate how vestibular function may adapt to changes in gravitational fields and to evaluate the vestibular system's ability to adapt as a function of the organism's age. Through these kinds of studies we may begin to appreciate the limits to and determinants of physiological adaptation in the vestibular system under a variety of gravitational environments.

This research program has led to the recording of short latency vestibular responses to pulsed linear cranial acceleration for the first time. Salient features of these responses include short latencies (4 to 7 peaks occurring within 8 msec following the stimulus) and resistance to intense auditory masking (96 to 100 dB SPL). Moreover, the responses are abolished upon complete bilateral destruction of the labyrinth but are little affected by selective bilateral destruction of the cochlea and lagena. This research has demonstrated that responses to pulsed linear acceleration in anesthetized birds are vestibular in origin and likely represent compound action potential of the vestibular nerve and central relays. Although the precise generators are not yet known, our results suggest that the lagena contributes little to response components.

Concurrent studies in rats demonstrated that similar responses could be obtained using pulsed linear acceleration stimuli. These responses occurred within 8 msec following the stimulus, had amplitudes less than 10 and were resistant to high intensity auditory masking (up to 105 dB SPL). As in birds, response latencies and amplitude were linearly dependent upon stimulus intensity. Moreover, response thresholds decreased as a function of the rate of change in acceleration (i.e., da/dt) in both species. The effects of selective bilateral destruction of the cochlea and labyrinth in rats are currently being studied.

New research described in the current report sought to: (a) determine the effects of spaceflight on vestibular ontogeny by measuring vestibular response thresholds in birds flown as embryos on the shuttle Discovery (March 1989) and (b) complete pilot research evaluating the effects of linear sine wave motion on vestibular responses to pulsed linear acceleration.

Accomplishments

(1) **Does linear sine wave motion affect vestibular responses?** Hypothetically, vestibular responses to pulsed linear acceleration are generated, at least in part, by neurons innervating otolith structures of the labyrinth. It is well known that linear sine wave motion can be used to dynamically stimulate otolith sensory structures while producing little or no effect on canal sensors. Evidence suggesting that linear motion can modify vestibular responses to pulsed acceleration would support the hypothesis that these responses depend on activity in otolith organs.

Pilot research was undertaken in the Vestibular Research Facility at the NASA-Ames Research Center, Moffett Field, CA. In a series of experiments a linear sled was used to examine the effects of sine wave linear motion on vestibular responses. ***Linear motion was found to produce a small phase-dependent modulation of vestibular response thresholds, latencies and amplitudes.*** These preliminary findings provide some support for the 'otolith' hypothesis. They demonstrate that such measurements can be made and serve as a preface to indepth studies exploring the precise vestibular origins of the responses.

(2) **Vestibular responses in unanesthetized animals:** An innocuous method of recording vestibular responses was developed to enable measurements of vestibular function in unanesthetized birds. ***Responses to pulsed linear acceleration in unanesthetized animals were highly resistant to intense auditory masking (104 dB SPL), were little affected by the removal of both cochleae and lagenae, but were virtually eliminated with bilateral destruction of the labyrinth.*** Therefore, responses to pulsed linear acceleration in unanesthetized animals, like those in anesthetized animals, are of vestibular origin and represent compound action potentials of the vestibular nerve and central relays.

(3) **A role for gravity in shaping embryonic vestibular development?** ***Eight birds (n=8) incubated for 7 days in space as embryos*** (approximately embryonic days E9-E16 during the March 1989 flight of the space shuttle Discovery) ***had elevated vestibular thresholds when compared to 8 sibling Earth controls raised at 1.0 g.*** Indeed, to elicit vestibular responses, the animals exposed to weightlessness required stimulus intensities nearly twice those of their Earth counterparts (mean threshold ± sd: space birds = 0.053 gm ± 0.018, Earth = 0.033 g ± 0.026; Mann/Whitney U:$p < 0.028$). Moreover, the effect of spaceflight was not transient since the measurements of threshold were made approximately four weeks after the animals returned to Earth. These results are provocative and support the hypothesis that gravity may play a role in determining vestibular sensitivity.

Significance of the Accomplishments

The results support the hypothesis that, during ontogeny, gravity may help shape the ultimate functional attributes of the vestibular system. The research leads directly to studies designed to evaluate the effects of hyper- and hypodynamic environments on the ontogeny of vestibular function. Responses to pulsed linear acceleration appear to depend on neurons that are more sensitive to the rate of change in acceleration than acceleration itself. Hypothetically, the responses therefore may reflect the activity of the so-called 'irregular' type of vestibular primarily afferents that innervate the utricles and saccules. Moreover, the sensitivity of these neurons may be modified during ontogeny as a result of exposure to weightlessness. These findings may serve to improve our understanding of how organisms develop normally on Earth and be of value in forming the strategies used in planning for space exploration.

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MOLECULAR BASIS OF TENSION DEVELOPMENT IN MUSCULAR ATROPHY

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Description of Research

The long-range goal of our research has been to assess the effects of immobility and hypokinesia on skeletal muscle function — in particular, the effect of exercise on skeletal muscle following prolonged atrophy. It is the aim of this project to describe the mechanism by which calcium ions and calcium-mediated physiologic mechanisms act in conjunction with gravity during hypokinesia.

Our work has focused on the atrophy-associated cellular changes which might predispose skeletal muscle to injury during re-use and recovery. Preliminary studies in this laboratory have shown that exercising atrophied skeletal muscle following hypokinesia produces myofibrillar damage and degeneration. A major finding was the appearance of Type IIC or transitional fibers and extensive fiber damage coincident with training during exercised recovery from hypodynamia. The damage appeared in the form of necrotic fibers, central nuclei, phagocytosis, and fiber debris in the intrafasicular and intrafibrillar spaces of the soleus.

Initial studies have sought to: (1) characterize the occurrence and distribution patterns of control, transitional, and damaged fibers which occur in atrophied soleus during the course of recovery; (2) determine the contribution of the cytoskeletal element Titin in the production of skeletal muscle damage during the recovery of skeletal muscle from atrophy; and (3) determine the role nuclear control domains play in skeletal muscle.

Accomplishments

(1) ***Spatial Analysis of Control and Atrophied Skeletal Muscle:*** It is well known that fiber type grouping can occur in response to denervation; indeed, "type grouping" is often taken to be the pathognomonic sign of neurogenic atrophies. Therefore, it is of interest to determine whether fiber types are distributed randomly in the normal muscle and also to determine whether they are distributed randomly in non-neurogenic pathologies, for example, in disuse atrophy.

We sought to determine: (1) whether there were any nonrandom spatial patterns in soleus muscle of control adult rats, and (2) the spatial patterns of fiber types of unloaded soleus muscle relative to controls. Specifically, our purpose was to determine whether fibers of any type showed a preference to be adjacent to fibers of the same or any other type, and whether any fiber type showed a preference for being non-randomly found in association with fascicle boundaries. The presence of spatial patterns in control groups has to be determined in order to serve as a standard for all other groups. In addition, the presence of nonrandom patterns in hypokinetic groups may offer clues to the nature of the processes that produce atrophy in a manner analogous to the fact that fiber type grouping is generally held to be a pathognomonic sign of neurogenic atrophy.

Our principal finding is that the ***normal muscle fiber type pattern of actively interspersed fibers persists throughout the hypokinetic paradigm.*** In contrast

to the fiber type grouping that is commonly seen in neurogenic atrophies, our finding is that ***both normal and hypokinetic atrophic soleus muscle show "anti-grouping";*** that is, a tendency for fiber types to avoid themselves and to seek adjacencies with other fiber types.

(2) ***Titin Banding Patterns in Skeletal Muscle:*** Using single fibers from soleus and plantaris muscles dissected in a relaxing solution (pCa 10.0; EGTA 0.05), we have been able to stain fibers with monoclonal anti-Titin 9D10 IgG (M. Greaser, Univ. Wisc.) which were incubated with 1:20 diluted FITC conjugated goat anti-mouse IgG. We measured the stained Titin doublets and sarcomere lengths. ***A ratio of Titin spacing/sarcomere length revealed that there was a significant difference ($p \leq 0.001$) between slow soleus and fast plantaris muscles*** (Figure 1). Soleus muscle was 0.380 ± .004 SEM, while plantaris ratios were 0.290 ± .004 SEM. Furthermore, when compared by fiber type, irrespective of muscle of origin, there was a significant difference ($p \leq 0.001$) between fast and slow fibers. Results are consistent with morphometric data from previous ultrastructural studies that have shown that the width of Z bands vary between fiber type with slow (type I) fibers the widest at 1445 Å and fast (type II) fibers narrower at 611-881 Å. These findings are consistent with others that show ultrastructural differences between fibers of different types.

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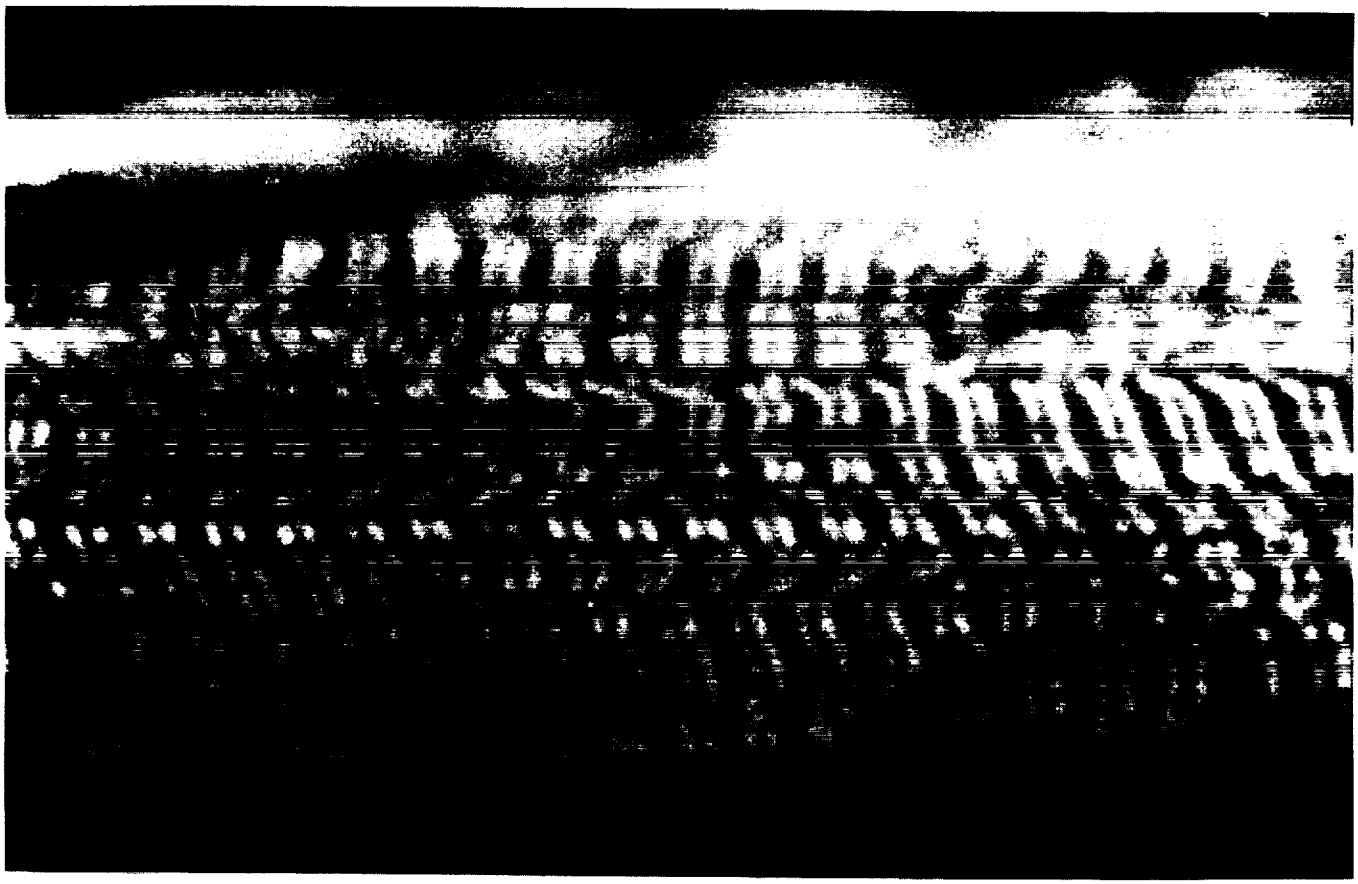

Figure 1. Myofibrils from a single skeletal muscle fiber labeled with monoclonal anti-Titin 9D10 IgG which were incubated with 1:20 diluted FITC conjugated goat anti-mouse IgG (magnification X 1000).

(3) ***Nuclear Control Domains in Skeletal Muscle:*** Using the intact single skeletal muscle fiber technique, fiber segments from soleus and plantaris muscles of adult female rats were mechanically dissected and analyzed for nuclear control domains by fiber type. Pieces from the same cell were classified according to fiber type and the third fiber piece was stained with Hematoxilyn and Eosin for determination of the number of nuclei, estimated fiber volume, and sarcomere spacing. In order to count only the sub-sarcolemmal nuclei, fiber segments with extraneous fiber debris or disrupted sarcolemma were excluded from this study. Cytoplasmic volume (μm^3)/nucleus differences were found to be significant ($p \leq 0.01$) between the fast and slow fibers (139,116 ± 13,541 SEM vs. 71,843 ± 5776 SEM, respectively). Furthermore, significant ($p \leq 0.01$) fiber type differences were found in the number of sarcomere lengths per nucleus (5.98 ± 0.38 SEM in fast versus 3.40 ± 0.21 SEM in slow fibers.) Results of volume/nucleus and sarcomere lengths/nucleus may be interpreted to be theoretical nuclear control domains and are consistent with data from previous biochemical studies that have shown that slow muscles have higher DNA concentrations than fast muscles. Since lower threshold motor units with slow skeletal muscle fibers are recruited tonically, we interpret our results to reflect a higher protein turnover rate and smaller nuclear control domain necessary for cell maintenance in the slow versus the fast fibers.

Significance of the Accomplishments

Finding #1: ***Spatial Analysis:*** Our findings indicate that the atrophy produced by the unloading of skeletal muscle does not appear to be neurologically mediated but seems solely due to myogenic variables.

Finding #2: ***Titin:*** Titin (Mr, 3×10^6) is a long, flexible filamentous muscle that links thick myosin filaments to Z lines in the sarcomere and is responsible for the maintenance of the structural and mechanical stability of skeletal muscle. It has been proposed that Titin is a major component of the "third" filament system of the myofibril, and has been immunologically detected to be among "gap" filaments that are ultrastructurally visible in overstretched muscle. Direct evidence of this elastic role was described by using ionizing radiation to ablate preferentially Titin and nebulin from isolated myofilaments. Removal of these filaments causes the myofibril to become much less elastic and become axially misaligned. The significant difference in Titin doublet spacing between fiber types may relate to the differential ability of slow and fast muscle types to withstand increased loading following atrophy.

Finding #3: ***Nuclear Control Domains:*** A Nuclear Control Domain is, or a DNA unit is defined as, the volume of cytoplasm controlled by a single nucleus. In skeletal muscle, the cumulative nuclear output gives rise to the genetic products that comprise all metabolic, membrane, and structural muscle proteins. Accurate enumeration of the numbers and appearance of nuclei between different fiber types has not previously been accomplished. The unique linear array of nuclei along the length of slow fibers differs greatly from that of fast (type II) fibers which appear to be randomly distributed. The reason for this distinct nuclear arrangement is not readily apparent.

If the area that each nucleus controls is so significantly different between fiber types, then one may speculate that transitions between fiber types may be the result of alterations in the domain of the nucleus. It is known that cardiac nuclei change shape during contraction. Perhaps the nuclei is able to sense changes in the use of a muscle, as in the case of decreased activity during weightlessness and hypokinesia, and consequently decrease its rate of protein synthesis. Further study of this area may give rise to the initial activity sensing mechanism that produces skeletal muscle atrophy. Further studies are planned in

experimental animals that have undergone functional adaptation, e.g., hypertrophy, atrophy, denervation, and exercise training.

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MECHANISMS OF VESTIBULAR ION TRANSPORT

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Description of Research

The two classes of vestibular sensory organs, the maculae and ampullae, differ by virtue of the accessory structures which mediate their respective mechanical sensitivities to linear or angular acceleration. In contrast, the mechanoreceptive hair cells associated with each type of sensory organ show many similarities of organization and function within the sensory epithelia. The hairs of the receptor cells, in each type of structure, extend toward the lumen of a fluid compartment filled with endolymph. This specialized extracellular fluid is distinguished by its unique low-sodium/high potassium ionic composition. Vestibular transduction is thought to commence when mechanical displacement of the sensory hairs results in the opening of "transduction channels" at the apical cell surface, with augmented flow of ionic current from endolymph into the hair cell. This "transduction current" — which is carried, in mammalian hair cells, almost entirely by endolymphatic potassium ion — exits the hair cell by way of ion channels situated in the basolateral membrane.

Given the importance of potassium movements for vestibular transduction, and the documented losses of body fluid and electrolytes which occur upon exposure to microgravity, the long-term objective of this project is the characterization of molecular mechanisms mediating active and passive ion flux in vestibular endolymph and sensory epithelia. As an additional goal, we seek to determine whether these mechanisms participate in possible adaptive responses to changes in fluid and electrolyte balance, and to altered patterns of vestibular stimulation experienced in microgravity.

The only mechanism yet demonstrated for active transepithelial transport of K^+ into the endolymphatic compartment is Na^+, K^+-ATPase, a transmembrane enzyme which "pumps" Na^+ and K^+ in opposite directions across cell membranes and across epithelia. Much physiological evidence implicating Na^+, K^+-ATPase in endolymphatic K^+ transport has been obtained by local application of ouabain, a specific inhibitor which binds to an extracellular site on the enzyme. Exposure of inner-ear tissues to ouabain *in vivo* is associated with a decrease of endolymphatic [K^+], and an increase of [Na^+].

Virtually nothing is known of possible mechanisms which may modulate levels of inner-ear ATPase. However, this enzyme also plays a major role in the regulation of whole-body fluid and electrolyte balance, primarily in ion-transporting epithelia of exocrine organs such as the kidney. In many of these tissues, cation transport is subject to regulation by the steroid hormone aldosterone ("aldo"), which promotes resorption of Na^+ and secretion of K^+. One known mechanism by which the hormone exerts these effects involves an increased population of Na^+, K^+-ATPase enzyme sites in the target tissues. Since aldo levels are significantly altered in human subjects upon exposure to microgravity, demonstration of an aldo effect on inner-ear ATPase levels would raise the possibility that alterations in endolymphatic fluid and electrolyte balance occur during vestibular adaptation to the microgravity environment.

Studies completed in the past year were intended to evaluate the hypothesis that aldosterone may modulate the population of inner-ear ATPase sites. To estimate enzyme site densities, guinea pig tissues were isolated by microdissection and incubated with ^{3}H-ouabain *in vitro*; specific binding was normalized to tissue dry weight. Parallel studies of blood chemistry were conducted.

Accomplishments

(1) Scatchard analysis of cochlear lateral wall from normal guinea pigs produced a linear function, with kD of 2.82 μM. This value represents the ouabain concentration required for half-maximal saturation of binding sites. Cochlear lateral wall incorporates the ion-transporting epithelium responsible for maintenance of cationic concentrations in cochlear endolymph, and is more readily isolated than the corresponding vestibular ion-transporting epithelia.

(2) Pairs of male guinea pigs, weighing 200-250 gm, were maintained on a high Na^+/low K^+ diet for 4-5 days (to suppress endogenous secretion of aldosterone). Twenty-two hours prior to sacrifice, the experimental animal received an injection of aldosterone (10 μgm/100 gm body weight), while the paired control was sham-injected. Tissues were incubated with ^{3}H-ouabain at a concentration of 5 μM; in each experiment, ouabain binding was assayed in lateral wall of the basal cochlear turn and in the three ampullae of either ear. The duplicate determinations of ouabain-binding in lateral wall and ampullae from each animal were then averaged. In every experiment, ouabain binding in the aldo-injected animal was higher than that in the control, and these differences attained statistical significance, both in the lateral wall and ampullae (Table 1).

Tissue	n(1)	Δ, (2) (Aldo-Control)	p(3)
Lateral wall (basal cochlear turn)	(4)	+ 2.35 ± 1.64	<0.05
Ampulla	(5)	+ 2.33 ± 1.70	<0.02

(1) Means of duplicate determinations

(2) Mean difference, aldo-control: picomoles ^{3}H-ouabain bound per mg dry tissue, ± standard deviation; the concentration of ^{3}H-ouabain in the incubation medium was 5μM

(3) p-values determined by one-tailed t-test for paired scores

Table 1. Changes in ^{3}H-ouabain binding: Aldosterone injection vs. sham-injected control. Pairs of male guinea pigs were maintained on a high Na^+/low K^+ diet, as described in Finding #2. Twenty-two hours prior to sacrifice, one animal received an injection of aldosterone (10 μgm/100 gm body weight), while the paired control was sham-injected. Inner-ear tissues were isolated by microdissection, and incubated with ^{3}H-ouabain. Tissue-bound ouabain was assayed by liquid scintillation spectrometry.

(3) Recognizing that differences in ouabain binding might arise from indirect effects of the experimental treatment upon whole-body fluid/electrolyte balance, we analyzed blood sera obtained by exsanguination at the time of sacrifice. In both the

aldosterone and control animals maintained on a high Na^+/low K^+ diet, [Na^+] and [Cl^-] were elevated, and [K^+] was reduced, relative to normal values.

(4) Maintenance of animals on a high Na^+/low K^+ diet resulted in a statistically significant decrease of aldosterone levels relative to normal values. Twenty-two hours following injection, aldo levels in aldo-injected animals were significantly higher than those in sham-injected animals, but still did not reach normal values.

(5) There were no significant differences in serum electrolyte values or serum osmolarity between aldo-injected and control animals maintained on the altered diet.

Significance of the Accomplishments

Finding #1: Linearity of the Scatchard plot is indicative of a single class of saturable binding sites, presumably associated with Na^+, K^+-ATPase. Non-specific binding, in contrast, is not saturable. Determination of the kD value permits the selection of an ouabain concentration which should produce nearly complete saturation of specific ouabain-binding sites in the experiments described under Finding #2.

Finding #2: A statistically significant increase in ouabain binding was observed in both cochlear and vestibular tissues of aldo-injected animals. This result is consistent with the hypothesis that ***aldosterone promotes an increase in the population of inner-ear Na^+, K^+-ATPase sites.***

Findings #3 and #4: These results confirm the anticipated effects of the experimental manipulations. ***Maintenance of subjects on a high Na^+/low K^+ diet resulted in an increase of serum [Na^+], decrease of [K^+], and decrease of aldosterone. Administration of exogenous aldosterone resulted in a significant elevation of serum aldo, but not beyond physiologic levels.***

Finding #5: The absence of significant differences, in serum electrolyte values and osmolarity, between aldo-injected and sham-injected subjects maintained on a high Na^+/low K^+ diet argues against the possibility that observed differences in ouabain binding resulted from an indirect effect on inner-ear ATPase. Such an effect could be mediated, for example, by changes in extracellular electrolyte concentrations. The present observations, however, are consistent with the hypothesis that ***inner-ear ion-transporting epithelia represent target organs subject to a direct influence of aldosterone, and that the hormone's action includes modulation of inner-ear ATPase levels.***

Findings #1-#5: Taken together, these results provide first ***evidence of a treatment capable of increasing levels of inner-ear ATPase, and suggest that endolymphatic fluid/electrolyte balance may be altered, at least transiently, by alterations of circulating aldosterone induced by exposure to microgravity.***

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COLLAGEN SYNTHESIS, ASSEMBLY, AND MINERALIZATION IN CHICKEN OSTEOBLAST CELL CULTURES

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Description of Research

The purpose of this research is to describe the basic molecular biology and the biochemistry, morphology, and ultrastructure of a bone cell culture system to be utilized at a later date for spaceflight experiments under microgravity conditions. At the current time, we are characterizing the culture system in the laboratory under normal gravity. We are interested in understanding the growth and development of cultured bone cells derived in our case from osteoblasts of chicken calvaria and in studying the onset and progression of mineralization in the culture extracellular matrix. We are concerned with elaborating (1) the mechanisms of collagen gene expression, (2) the temporal relationship between collagen cross-links and collagen ultrastructure, (3) the assembly of collagen fibrils into orthogonal arrays, (4) the precise interaction between collagen fibrils and mineral deposition in the cultures, and (5) the effects of non-collagenous proteins on mineralization in the system.

In our previous experiments, we have found that the cultured chick osteoblasts reproducibly mineralize in a temporal sequence of differentiation following the osteoblast phenotype. Over a 30-day culture period, parameters of both cell and extracellular matrix development have been examined, including determinations by biochemical and electron microscopic means of collagen gene expression; collagen synthesis, processing, and accumulation; cellular growth; presence and function of non-collagenous proteins; and extracellular matrix composition, assembly, and mineralization. Of major importance, our earlier work with our bone cell cultures has shown that they closely approximate chemical, physical, and biological events described for bone *in vivo*; that extracellular matrix formation is dependent on post-translational modification affecting collagen accumulation and not collagen synthesis; that collagen accumulation is not dependent on the rate of synthesis but may also be dependent on the efficiency of collagen fibril formation; and that mineralization occurs only if a preliminary matrix is present in the system.

Accomplishments

Continuing studies with the culture system have now established the following results:

(1) In cooperative research with Dr. David Eyre (University of Washington, Seattle), we have found that ***HP and LP forms of non-reducible pyridinium crosslinks, specific to collagen, increase over the 30-day time course of bone culture development, the HP:LP ratios and molar values are identical to those of bone collagen controls, and the kinetics of such crosslink formation are found to correlate directly with increases in collagen fibril diameters.***

(2) By assaying crosslink formation and collagen turnover, through collaboration with Drs. Lila Graham and Paul Gallop (Children's Hospital and Harvard School of Dental Medicine, Boston), ***it was found that the presence of remodeling or***

collagenase activity appears to occur in culture, affecting newly synthesized collagen molecules.

(3) ***Treatment of bone cell cultures with α-aminopropionitrile, a potent crosslink inhibitor, results in a 50% decrease of collagen accumulation over the 30 day culture period, the appearance of collagen fibrils unusual in their diameter sizes and size distribution as a function of culture time, the absence of extensive orthogonal arrangements of collagen fibril layers, and the loss of bulk mineral.*** Alkaline phosphatase and osteocalcin levels remain unchanged in the presence of the inhibitor.

(4) A 66 kD phosphoprotein, which we previously found by biochemical and immunocytochemical methods to be expressed concurrently in culture with alkaline phosphatase and osteocalcin and localized in association with initial extracellular mineral foci in the system, contains an arginine-glycine-asparitic acid sequence among its amino acids. ***This result is suggestive of a role in cell attachment for the phosphoprotein, but its accumulation at mineralization sites distant from cell surfaces implies alternative function(s)*** which are also being investigated.

(5) ***Mineralization of the culture system occurs in association with collagen,*** and the interaction, size, shape, and distribution of mineral crystals within fibrils were demonstrated with topographic imaging, a microscopic technique we have developed and applied to both tissue *in situ* and the bone culture system. The method provides a unique and direct three-dimensional view of ultrastructure. The ***mineral crystals are thin platelets, rather than rodshaped crystals, apparently arranged in stacks within collagen hole zones*** (the putative nucleation sites for mineral within the fibrils). ***Platelet lengths and widths exceed the size of the hole zones,*** a result with significant implications into the basic pattern of collagen fibril aggregation and mineral growth.

(6) Initial experiments in collaboration with Dr. Marc Grynpas (Mt. Sinai Hospital and the University of Toronto, Ontario, Canada) utilizing inductively coupled plasma ashing measurements show that ***strontium in low concentrations substitutes in a proportional manner for calcium in the extracellular matrix of the bone cell cultures.***

Significance of the Accomplishments

The findings are very important in terms of describing some of the events of biological mineralization in an *in vitro* system closely resembling the mineral deposition of normal calcified vertebrate tissues. The results are providing insights into details of calcification still incompletely understood and otherwise unattainable with other models. The results cited in findings # 1, 2, and 3 above suggest that chemical crosslinks unique to collagen are responsible for the aggregation of smaller fibrils to larger ones and for the assembly of the extracellular collagen matrix as a whole. Changes in the characteristics of collagen fibrils accompanying crosslink inhibition noted in finding #2 indicate the culture osteoblasts may produce collagenase as a means for remodeling the system. The observation in finding #4 that the 66 kD phosphoprotein is localized at culture sites distant from the osteoblast implies that the cell binding capacity of the molecule may be only transient in nature and that this culture constituent has additional functions. From finding #5, collagen-mineral interaction seen by microscopic topographic imaging, a newly applied technique in its own right for direct three-dimensional viewing of ultrastructure, shows the manner by which the crystals are normally accommodated by the fibrils, perhaps as oriented stacks of thin platelets which

follow gaps or channels within specific regions of large collagen assemblies. Finding #6 suggests that strontium may be used as a reliable marker for determining the rate of mineral accumulation in collagen in the culture matrices.

In total, these data characterize more extensively the development and growth of bone cells, the assembly of their extracellular matrix, and the deposition of mineral in culture. The information from the bone cell cultures is extremely valuable for comparison with that obtained from calcifying tissues themselves and for its contributions toward explaining the mechanism(s) of vertebrate mineralization. Such ground-based cultures also provide the control model against which mineralization events may be analyzed from the same cultures subjected to the microgravity conditions of later inflight experiments. From those results, it may be possible to understand the basis of one of the most serious problems of spaceflight suffered by humans and experimental animals, the mass loss of the skeleton and its related effects.

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MICROMOTIONAL STUDIES OF UTRICULAR AND CANAL AFFERENTS

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Description of Research

The long-range goal of this research is to refine our understanding of the sensitivity of the vestibular components of the ear to very-low-amplitude motion, and especially of the role of gravity in this sensitivity.

We focused on the American bullfrog, a common animal subject for vestibular sensory research. Our principal experimental method was to apply precise, sinusoidal microrotational stimuli to an anesthetized American bullfrog, to record the resulting responses in an individual vestibular nerve fiber from the intact ear, and to use intracellular dye to trace the fiber and thus identify the vestibular sensor that gave rise to it. In this way, we were able to identify specific micromotional sensitivities and to associate those sensitivities definitively with specific sensors. Furthermore, by recording from nerve fibers after they leave the intact inner-ear cavity, we were able to achieve these identifications without interrupting the delicate micromechanics of the inner ear. We were concerned especially with the relative roles of the utricle and the anterior semicircular canal in the sensing of microrotational motion of the head about horizontal axes, and with the role of gravity in mediating that sensing process in the utricle. The functional characterization of individual nerve fibers was accomplished with a conventional analytical tool, the cycle histogram, in which the nerve impulse rate was plotted against the phase of the sinusoidal stimulus.

Accomplishments

Using intracellular dye to trace functionally characterized vestibular axons to their peripheral origins, we separated anterior vertical canal axons from utricular axons and demonstrated conclusively that there is a sizable population of utricular striolar axons that mimic the rotational sensitivity usually attributed to anterior vertical canal axons. For small-amplitude, low-frequency rotations about horizontal axes, the firing rate in a subset of utricular axons follows rotational velocity more faithfully (in both phase and amplitude) than does that in any of the vertical canal axons we studied. We have demonstrated definitively that the ***stimulus for this faithful representation of rotational velocity is the rate of change of the projection of the gravity vector onto the utricular surface and not centripetal acceleration of the otoconial mass of the utricle***. This rotational velocity response spans a very large part of the normal motional range of the head, from approximately 0.1 Hz to more than 4.0 Hz.

Scanning electron microscopy studies have shown that the sensory surface (crista) of the anterior semicircular canal is divided into five regions, characterized by different distributions of sensory hair bundles (the transduction apparatus). The hair bundles have been classified as types A (those with the longest sterocilia equal in length to the kinocilium) and B (those with the longest stereocilia being less than 2/3 as long as the kinocilium). The two peripheral (planar) regions have only type B bundles, which are densely distributed. Between the two planar regions is the ridge. The central region of the

ridge has only type A bundles, also densely distributed. The regions of the ridge lying between this central region and the planar regions have sparsely distributed types A and B bundles.

Individual axons tend to innervate either the ridge regions or a planar region, but not both. Axons from the planar regions consistently have conspicuously lower gain and more periodic (less random) spontaneous firing than those from the ridge regions. On the other hand, no differences in gain or regularity of firing was found between the axons innervating the central region of the ridge and those innervating the other two ridge regions. Thus, ***gain and regularity of firing do not appear to be determined by hair bundle type.***

In principle, if the spontaneous firing of a nerve fiber were perfectly regular, then even the smallest stimulus-induced variation in firing pattern would be detectable. Irregularity of firing makes detection more difficult, and can be interpreted as being equivalent to noise in the channel. We found that the stimulus amplitude required to exceed the nerve-fiber's noise level was independent of where the fiber arose. Those from the ridge exhibited higher gain, but also higher noise; those from the planar regions exhibited lower gain, but also lower noise. In terms of signal detectability, both types of fibers were approximately equivalent.

Significance of the Accomplishments

It is well known that the human vestibulo-ocular reflex operates very well for minute head rotations. Traditionally, the sensors responsible for this reflex have been assumed to be the semicircular canals. The otoconial organs of mammals have been considered sensors of lineal motion and of head orientation relative to gravity. Our observations of gravity-mediated rotational velocity sensitivity in the frog casts serious doubt on this traditional view. Furthermore, our results suggest that serious deficits in rotational motion sensitivity could occur in microgravity environments.

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GRAVITY AS A PROBE FOR UNDERSTANDING EMBRYONIC PATTERN SPECIFICATION IN THE AMPHIBIAN MODEL SYSTEM

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Description of Research

The fertile amphibian egg shows a clear gravitational response. Fertile eggs naturally orient with respect to the gravity vector, and the polarity of the future embryo can be influenced by manipulation of the force environment ranging from hypergravity to microgravity. The large size of the egg, the polar distribution of its cytoplasmic components, and the ease of experimental manipulation make the amphibian egg a unique model system to study how a single cell senses gravity and how this information is transduced into changes in pattern specification.

We are using gravity as an experimental tool to investigate the developmental biology of primary pattern specification. Our focus has been directed along two main lines: (a) the biological explanation for the considerable variation in the response of individual amphibian eggs to given gravitational perturbation and (b) the extent to which the reorganization of the egg cytoplasm in response to gravity drives embryonic pattern specification.

Accomplishments

(1) ***Individual spawnings of eggs differ in cytoplasmic rigidity.*** The apparent cytoplasmic viscosity of individual inverted eggs can be quantified. Previous reports from our laboratory utilized cytoplasmic mobility (CM) as the scoring system. We have changed the designation to cytoplasmic immobility (CIM) to more accurately reflect what is being measured. Individual spawnings of eggs show a wide range in mean CIM values (52 μm-192 μm/20 spawnings), and each individual spawning shows a unique spread of individual CIM values around its mean.

(2) ***Eggs with a more rigid cytoplasm display a higher survival rate in response to centrifugation.*** The survival of fertile eggs subjected to a centrifugation regimen that induced twinning was monitored. Eggs with high CIM values have a higher survival rate.

(3) Individual spawnings of eggs show considerable variation in twinning. Each spawning had a distinctive percentage of eggs that showed a double axis (% twinning=%T) (Figure 1a) and a distinctive severity of twinning (twinning index=TI) in response to centrifugation. In agreement with data reported by others, the variation in %T was substantial (the range at 15 g was 0.0 to 100%; at 30 g it was 0.0 to 87%).

(4) ***Variation in cytoplasm rigidity (CIM) can account for variation in gravity sensitivity measured by twinning frequency.*** The relationship between CIM and twinning was analyzed. There was a significant negative correlation between CIM and TI (e.g., r=0.80 at 15 g) and CIM and %T (Figure 1b). Low CIM eggs produce more twins.

TWINNING AND CYTOPLASMIC IMMOBILITY IN TADPOLES

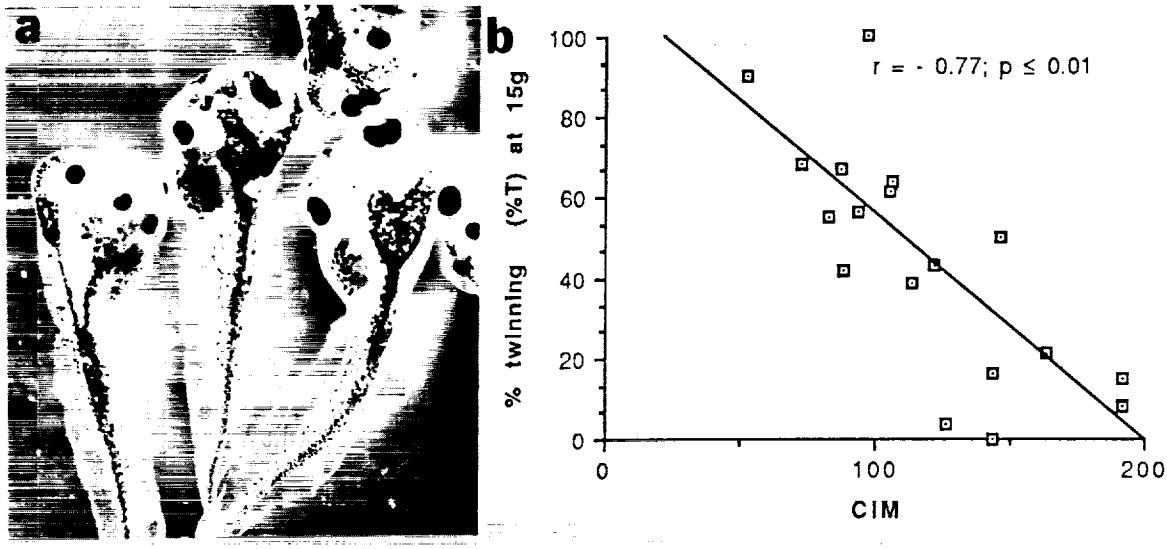

Figure 1. Relationship between twinning and cytoplasmic immobility (CIM). (a) Representative surviving tadpoles from a spawning with high % twinning (%T) and a low CIM. (b) Correlation between % twinning (%T) and CIM. Simple linear regression line correlation coefficient (r), and p value for two-tailed significance test is shown.

MICROTUBULES IN AN INVERTED FERTILE FROG EGG

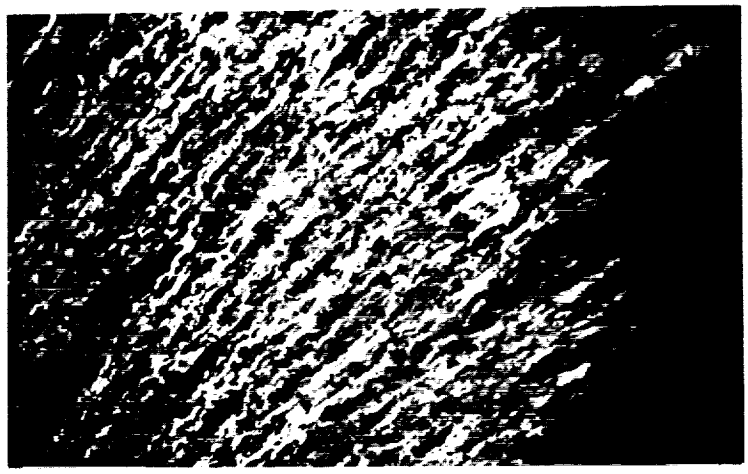

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Figure 2. Confocal scanning microscope image of the parallel microtubule array in the original vegetal hemisphere of an inverted fertile egg at 75% of the time between fertilization and first cleavage. Microtubules are visualized by immunofluorescence. Control normal orientation eggs showed identically oriented arrays, and no arrays were detectable in the animal hemispheres of either control or inverted eggs.

(5) Subcortical parallel microtubule arrays remain in the original vegetal hemisphere in eggs inverted with respect to the gravity vector. An extensive array of parallel microtubules appears transiently in the subcortical vegetal hemisphere in normally oriented eggs during the time the egg cortex rotates in normal fertile eggs. Inverted eggs show considerable reversal of pattern formation such as a cleavage furrow initiation site and blastomere size. The location of the parallel microtubule array in inverted eggs was investigated using antibodies to β tubulin and immunocytology (Figure 2). The parallel microtubule arrays did not shift in inverted eggs.

Significance of the Accomplishments

Eggs from individual spawnings show considerable variation in cytoplasmic rigidity. This variation can be quantified (CIM) and used to uniquely classify each spawning of eggs (Finding #1). Amphibian eggs vary considerably in their response to a given gravitational challenge such as directed centrifugation (Finding #3). This degree of variation, which can range from 0 to 100%, has made gravitational experiments difficult to interpret. The current study has provided an important new discovery. For the first time, a clear correlation between a property of the cytoplasm (CIM) and the response to an amphibian egg to a directed gravitational force (survival and twinning) has been documented (Findings #2 and #4). Future interpretation of gravitational studies on amphibian eggs will require an understanding of the physical and/or biochemical basis for CIM.

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STRUCTURAL DEVELOPMENT AND GRAVITY

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Description of Research

The goal of this research is to understand the role of gravity in skeletal growth and development. To achieve this goal, we must first learn if gravity turns bone cells on or off, if/how these cells communicate with each other and with their environment, if/how secretory products are altered by different gravity levels, how alterations in organic matrix affect mineralization and material properties, and the role of local and systemic factors (including endocrine, blood flow, and fluid shifts) in gravitational responses. To accomplish these studies, both ground-based and spaceflight experiments are essential.

Gravity is a major factor determining the amount of structural support required by Earth organisms. The hypotheses of this research effort are: (1) skeletal support structures will change during spaceflight and/or unloading, (2) the magnitude of change will be dependent upon the modeling or remodeling activity in each bone and the length of exposure, (3) changes will be manifested primarily through altered matrix formation and/or mineralization, (4) changes in both quantity and quality of bone will occur, and (5) systemic and local factors will be involved in the changes. Most ground-based research involves growing rats exposed to simulated spaceflight; three flight experiments have been approved and will allow gathering of more information to support or negate the hypotheses. During this report period, ground-based studies focused on innervation and the response to skeletal unloading, correlation between bone mechanical properties and bone biochemistry during one to four weeks of unloading, response of adult female rats to skeletal unloading, and further definition of a bone cell culture system. Spaceflight experiments included analysis of bones from rats flown on Cosmos 2044, and preparation for experiments on the first and second dedicated Spacelab Life Sciences missions and the Japanese Spacelab mission.

Accomplishments

(1) Analysis of ***Cosmos 2044 rat tissues showed that minimal bone growth occurred during the mission even though the body mass gain was similar to the rats flown on Cosmos 1887.***

(2) Neonatal guanethidine-induced sympathectomy or neonatal capsaicin-induced denervation ***(destruction of unmyelinated and fine myelinated sensory nerves) did not dramatically alter the hindlimb response to unloading. However, mechanical and chemical parameters were different in the weight-bearing humerus of the guanethidine-treated unloaded animals as compared with the humerus of the guanethidine-treated controls*** (collaborative study with Dr. Esther Hill).

(3) Preliminary observations of rat bone cell cultures suggest that ***bone cells grown on collagen-coated beads appear morphologically more like authentic osteoblasts than control growth without beads and mineralization without addition of organic phosphate*** (collaborative study with Charlotte Cone and Dr. Steve Doty).

(4) ***Minimal bone changes occurred in adult female rats after two or four weeks of unloading while muscle changes were similar to those reported in growing rats.***

Significance of the Accomplishments

Finding #1 suggests Cosmos 2044 rats may not be directly comparable with Cosmos 1887 animals. Cosmos 1887 rats were euthanized about two days after recovery of the spacecraft while Cosmos 2044 rats were euthanized within hours of recovery, but Cosmos 2044 rats were approximately three weeks older at the beginning of the experiment due to launch delays. Although weight gain was similar in both experiments, Cosmos 2044 rats, if anything, had less bone than basal controls while Cosmos 1887 rats displayed evidence of bone growth.

Finding #2 indicates that the skeletal response to unloading is not altered by the selective destruction of unmyelinated and fine myelinated sensory nerves. However, sympathetic innervation may be important in a systemic and/or hormonal response to unloading.

Finding #3 describes a unique bone cell culture system using primary rat osteoblast-like cells grown on collagen coated beads; these cells have an eccentric nucleus surrounded by abundant cytoplasm containing well developed endoplasmic reticulum and Golgi systems, produce alkaline phosphatase receptors, and appear to mineralize without the addition of organic phosphate. These cells may develop a more comprehensive, osteoblast-like phenotype due to mechanical forces or pressures not present in monolayer cultures. This cell culture system will be flown in the Japanese Spacelab mission.

Finding #4 suggests that detection of bone changes in the unloaded long bones of adult rats might require more sensitive measurement techniques, longer unloading periods, or different sampling sites than were used in this experiment whereas muscle changes appear to be age-independent.

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SKELETAL COLLAGEN TURNOVER BY THE OSTEOBLAST

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Description of Research

Among the most overt negative changes experienced by man and experimental animals under conditions of weightlessness are the loss of skeletal mass and attendant hypercalciuria. These clearly result from some disruption in the balance between bone formation and bone resorption (i.e., remodelling) which appears to be due to a decrease in the formative functions of the osteoblast. In the present study, the clonal osteoblastic cell line, UMR 106-01, and sections of bone have been used to investigate the regulation of several proteins whose expression is central to both bone formation and bone resorption; these are Type I collagen (the major organic constituent of bone), collagenase and tissue inhibitors of metalloproteases. Expression has been monitored at the protein level using a combination of techniques including the radiolabelling of the proteins, gel electrophoresis, ELISA assays and immunohistochemical staining, and subsequently at the level of RNA, by Northern blot analysis and nuclear run-on assays. This project will shed some light on the comprehensive role of the osteoblast in the remodelling process, and, in so doing, provide some insight into how the process might be disrupted under conditions of microgravity.

Regulation of Collagenase Synthesis and Turnover. We have shown that collagenase is secreted by UMR cells and then rapidly removed from the medium by a cell-mediated process. Using radioiodinated collagenase, we have now been able to demonstrate the presence of specific receptors for collagenase on UMR cells. Binding, conducted at 4°C, was shown to be maximal at 2 hr. Scatchard analysis revealed 1800 receptors/cell, with a single class of high affinity sites (kD~10^{-11}M). Specificity of the receptor was further demonstrated by the lack of competition for binding by either human or bacterial collagenases. This has led us to formulate the hypothesis that the collagenase receptor on osteoblastic cells is involved in regulation of extracellular bone collagenase, and may be responsible for either activation and/or endocytosis of the secreted enzyme.

UMR 106-01 Collagenase Clones and Regulation of Collagenase mRNA. We have isolated two clones for rat collagenases from a UMR 106-01 cDNA library. These clones are identical except one (UMRCase54) is 245 nucleotides longer at the 5' end. These clones contain the entire coding sequence of the proenzyme; missing are approximately 2 amino acids of the signal sequence and 100-200 nucleotides of the untranslated 5' region of the transcript (estimates are based on comparison to the human collagenase cDNA).

Nuclear run-on studies, together with Northern blot analyses, indicated a 4-8 fold increase in the rate of synthesis of collagenase mRNA resulting in a 100-fold elevation in accumulated mRNA, 4 hr after treatment with parathyroid hormone (PTH). These studies demonstrate that the regulation of collagenase gene expression in UMR 106-01 cells is, in part, transcriptional.

Our next goals are to isolate the regulatory regions of the rat collagenase gene. To do this, we have constructed a UMR genomic library in lambda DASH II. Using a sequence at the

5'-most end of UMRCase54 we have isolated a rat collagenase genomic clone. We are currently characterizing this clone which will be used to decipher the mechanism involved in the transcriptional regulation of the UMR collagenase gene.

Immunohistochemical Detection of Collagenase in Sections of Bone from Cosmos 2044 Flight Animals. Frozen sections near the sagittal suture (an area of high turnover) of 64 day old rat calvariae were stained for collagenase by immunohistochemistry. The results indicated the presence of collagenase in the same restricted endocranial matrix-associated region that was seen in developing 14 day old rats although amounts of staining were lower in the older age group. The results also demonstrated that the enzyme could be detected in fixed, decalcified tissue. [Note: Only small amounts of collagenase have been detected in weightbearing bones (tibiae) and mostly associated with the primary spongiosa. These observations have led us to hypothesize that collagenase is more important in non-weightbearing skeletal tissues and that weightlessness and the related changes in blood distribution may affect the amount and location of collagenase in calvariae.]

Prior to receiving the tissues from the Cosmos flight, we conducted control experiments with similar pieces of calvarial tissue from 90 day old rats to ensure the validity of the flight experiments. This included testing the time of fixation and type of fixative since the time between dissection of the rats and arrival in Moscow was greater than usual. We found that fixation in paraformaldehyde for 72 hr did not affect the immunohistochemistry of collagenase staining.

We received the Cosmos samples in very good condition and clearly labelled. We have had frozen sections cut from animals from the basal, flight and vivarium groups and have carried out experiments to assess our techniques with these materials. We have stained sections from these groups both with Hematoxylin and Eosin and have used immunohistochemistry for collagenase. The vivarium sections are excellent, but when using the other groups we need to cut deeper into the blocks or at a different orientation to obtain better materials. With the latter, there have been problems with the tissue adhering to the slides. Alternative techniques to attach the sections were tested and we finally found that the use of chrome-gel helped. We are now ready to conduct a complete comparative assessment of the treatment groups from the flight experiment.

Accomplishments

(1) Demonstrated that the neutral metalloprotease, collagenase, is not only secreted by osteoblastic cells but is also removed from the media by a specific receptor.

(2) Showed that ***PTH regulates collagenase gene expression, in part, by a transcriptional mechanism.***

(3) ***Isolated a genomic clone for rat collagenase from a UMR 106-01 genomic library.***

(4) Observed collagenase staining in adult rat calvariae in a restricted endocranial matrix-associated region.

Significance of the Accomplishments

Finding #1: Treatment with PTH appears to change the phenotype of the osteoblast from a matrix-synthesizing cell to one actively involved in the resorption process. Collagen synthesis declines while production of enzymes associated with matrix removal increases. This situation may resemble that seen in situations of weightlessness where bone formation

is perturbed. Nevertheless, the cell appears to exert tight control over the amount of collagenase in the extracellular medium by rapidly re-extracting it *via* a specific cell-surface receptor.

Finding #2: The demonstration that rat bone collagenase is regulated at the transcriptional level and the availability of the cDNA clone will lead us into a new realm of experimentation which will help us to dissect the signal transduction pathways involved in PTH action on the osteoblast. We will also use the cDNA to assess the effects of weightlessness on transcript abundance in bones from animals subjected to microgravity.

Finding #3: Isolation of the gene for rat collagenase and its regulatory elements will enable us to construct materials which can be used to monitor PTH action as well as the effects of other mediators. One use of these reagents will be to transfect osteoblasts and send them into space to determine the effects of microgravity on hormonal responsiveness of bone cells.

Finding #4: The detection of collagenase in adult calvariae forms the basis of studies which are currently underway on calvarial tissues from rats flown on Cosmos 2044.

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GRAVITATIONAL EFFECTS ON EARLY DEVELOPMENT IN AMPHIBIAN EMBRYOS

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Description of Research

The long range goals of this project fall into two parts: (1) to understand how the neural axis is determined and patterned in amphibians, and (2) to study how this pattern is altered when embryos are fertilized and grown in an extraterrestrial environment. The more immediate goals are to understand how the neural axis is patterned on Earth. We have made extensive studies of the tissue interactions involved in establishing the dorsal axis and more specifically the neural tissue. In order to understand how morphogenetic signals pattern the neural system at the molecular level, we needed to construct molecular probes. Therefore, another of our immediate goals has been to construct the appropriate molecular probes so that we can study the signals which program the neural patterns and how the presumptive neural cells respond to these signals at the molecular level.

Accomplishments

We have made considerable progress in two areas of the problem of neural pattern formation. In the first area, we have shown that the old paradigm of neural induction and patterning resulting from interactions from the underlying dorsal mesoderm is not entirely correct. We have shown that ***signals which induce the ectoderm to become neural are traveling from the blastopore lip area (the cells which initiate the cell rearrangements at gastrulation) through the plane of the ectoderm*** as well as possible signals from the underlying dorsal mesoderm. This finding has allowed us to re-examine several classical questions and provide more meaningful assays to understand the mechanisms involved in neural induction.

The second accomplishment during 1990 is that ***we have constructed a recombinant DNA library of all the active genes (RNA) in the cells which we have shown previously to be sending the signals for neural induction.*** We have screened this recombinant library and have isolated clones which represent RNAs which reside in only the cells sending the signals or those responding to the signals and becoming neural tissue. These clones fall into two categories. One class of clones represent RNAs which were made during oogenesis (when the egg was made). These RNAs must become physically localized to the dorsal side of the embryo where the signaling cells will eventually reside. The localization process of this class of RNA will be sensitive to any gravitational perturbations which lead to an abnormal dorsal axis or a repositioned dorsal axis. Therefore, these clones will provide excellent probes for looking at how changes in the gravitational environment affect the distribution of specific molecules that are implemented in the dorsal patterning process. The second class of clones represent the earliest biosynthetic response of presumptive neural cells to the signals from the organizing region.

Significance of the Accomplishments

Understanding the tissue interactions which lead to neural induction and pattern formation of the brain region are extremely important if we are to understand the mechanisms which

control this process. There has been a major quest for finding the signals and understanding the mechanisms controlling neural induction since 1924. In 1933 scientists became convinced that all of the signals come from the underlying dorsal mesoderm and have approached the problem accordingly. However, we now know that there are several signaling events occurring prior to the time the ectoderm has contact with the dorsal mesoderm and that some of these events appear to be necessary before the dorsal mesoderm can have an effect within the amount of time before neural tissue is determined. Therefore, these findings should enable us to reapproach this very old problem with completely different assays and expectations.

The clones which we have isolated from the recombinant cDNA library will enable us to study the mechanisms for neural induction at the molecular level. Several studies have been done which show that the dorsal axis can be repositioned by altering the embryo's position relative to Earth's gravity. Recent studies indicate that amphibian embryos fertilized in space will experience developmental abnormalities. A very likely candidate for the developmental abnormalities is that the cytoplasmic rearrangements which normally occur between fertilization and the first cleavage division do not occur correctly. We have clones for RNAs which are normally distributed on only the dorsal side of the embryo, precisely where the organizer or signaling cells will reside. Therefore, we now have the capability to study the mechanisms that localize a specific molecule within the single celled embryo and to study this localization process in embryos that have been fertilized and/or raised in a space environment. The second class of clones corresponds to RNAs synthesized very early in the process of establishing a dorsal axis and neural tissue. These clones will be important in helping to identify the signals responsible for the dorsalization and neural induction processes. We can use these clones in assays to isolate the signals and begin to understand how the first class of clones which have been physically localized to the dorsal side of the embryo during the first cell cycle are initiating the process of pattern formation.

It appears that at least some of the signals involved in the induction of dorsal structures and the resultant pattern formation are highly conserved between species. Therefore, it is highly likely that many of the cloned probes which we have isolated will also be useful in the study of how other organisms might respond to a space environment.

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CELL KINETIC AND HISTOMORPHOMETRIC ANALYSIS OF MICROGRAVITATIONAL OSTEOPENIA

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Description of Research

Previous studies of cell differentiation supporting bone formation have shown that osteoblast (bone forming cell) histogenesis is inhibited by decreased skeletal loading and microgravity. The present goals and objectives for research in this area are: (1) to define the cellular regulation of osteoblast production, (2) to determine how this process is suppressed in microgravity, and (3) to develop *in situ* hybridization methodology enabling further characterization and verification of previously described osteoblast precursors. Presently, DNA labeling (^{3}H-thymidine uptake), mitotic activity and nuclear size are used as indices of the proliferation and differentiation aspects of osteoblast production.

Using our established techniques, kinetically and/or morphometrically distinguishable cell compartments in the osteoblast histogenesis sequence have been described: (1) self-perpetuating, less differentiated precursor cells (**A** type), (2) committed osteoprogenitor cells (**A'** type), (3) nonosteogenic **B** cells, and (4) preosteoblasts (**C+D** cells). The osteoblast (**Ob**) histogenesis sequence is **A** $\rightarrow$ **A'** $\rightarrow$ **C** $\rightarrow$ **D** $\rightarrow$**Ob** (Figure 1). An increasing nuclear volume (**A** $\rightarrow$ **C**) is a morphological manifestation of change in genomic expression (differentiation) and is necessary for the production of the immediate precursor cells to osteoblasts. The nuclear volume assay is applicable to all skeletal sites tested, i.e. periodontal ligament (PDL), tibial metaphysis, mandibular condyle and mandibular periosteum. The rate-limiting osteogenic induction step (**A'** $\rightarrow$ **C**) is stimulated by mechanical (orthodontic) force and inhibited following exposure to microgravity. The mechanical sensitivity of the **A'**$\rightarrow$ **C** shift appears to involve the action of prostaglandins since administration of the prostaglandin synthesis inhibitor, indomethacin, results in a block of osteoblast histogenesis downstream from the microgravity block.

Research conducted in the past year focused on (1) further definition of osteoblast production in rats exposed to a ground-based simulated weightlessness model, (2) preflight Experiment Verification Test (EVT) specimen processing and evaluation for the upcoming SLS-1 (Spacelab Life Sciences-1) shuttle mission, and (3) development of *in situ* hybridization as an advanced method for studying specific messenger RNA production within osteoblast precursor cells.

OSTEOGENESIS HISTOGENESIS PATHWAY

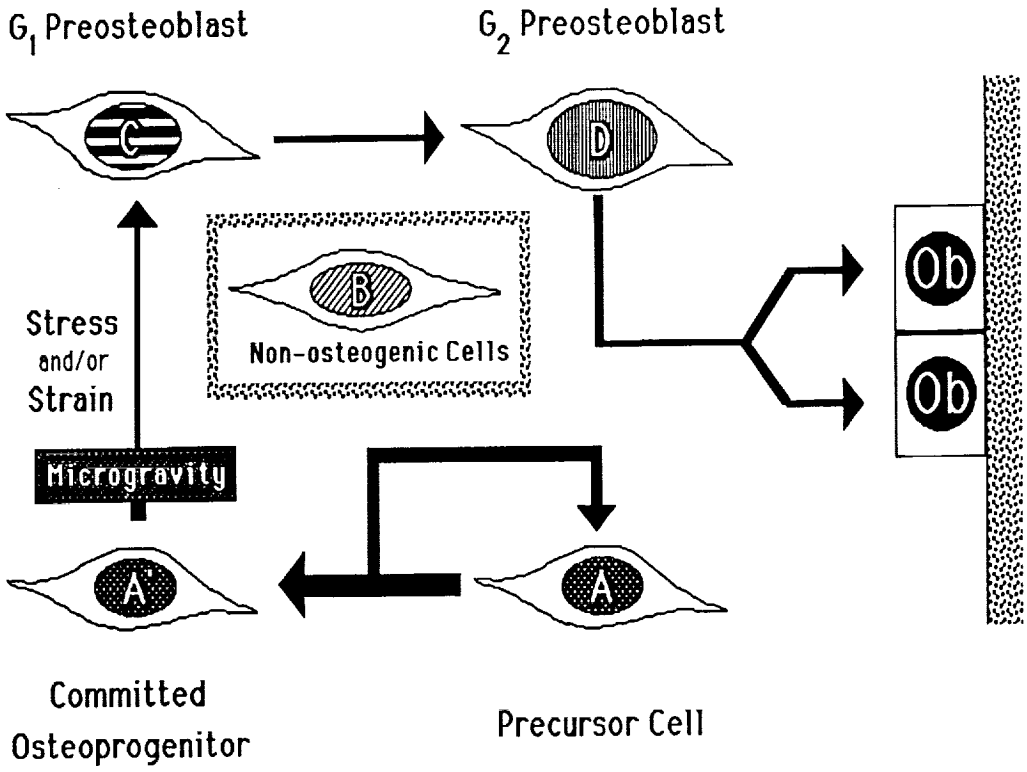

Figure 1. Schematic of cell kinetic model describing the proliferation and differentiation of cells associated with osteoblast histogenesis. Cellular compartments are classified as **A+A'**, **B**, **C** or **D** according to the following nuclear volume categories: 40-79, 80-119, 120-169 and >170 μm^3, respectively. Microgravity blocks the induction of osteogenesis (i.e., **A'→ C** shift).

Accomplishments

• Flight Preparation

(1) Mandibular condyles, maxillae, lumbar vertebrae and tibiae from EVT animals showed acceptable ^{3}H-proline labeling of bone matrix formation (Figures 2 and 3). This test was very important since the animals also received other radioisotopes that might have interfered with specific detection of the ^{3}H-proline bone matrix label.

• Simulated Weightlessness (SW) Data - Unloaded Rat Model

(2) ***Unloading (3 days) resulted in a 52% increase ($p < 0.05$) in osteoblast progenitor (A+A') cells and a 47% decrease ($p < 0.05$) in preosteoblast (C+D) cells in the tibial metaphysis*** of animals euthanized in the evening (9 PM). AM-euthanized animals showed a similar pattern, but only the decrease (39%) in the **C+D** cell compartment was significantly different from control.

(3) No significant differences between the AM and PM time groups were noted in the fractional distribution of tibial metaphyseal cell types.

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Figure 2. Autoradiography ^{3}H-proline labeling of bone and PDL adjacent to maxillary first molar from EVT rat. Concentrated band of autoradiographic grains depicts bone matrix formation adjacent to PDL. More diffuse labeling within PDL is indicative of ongoing collagen synthesis.

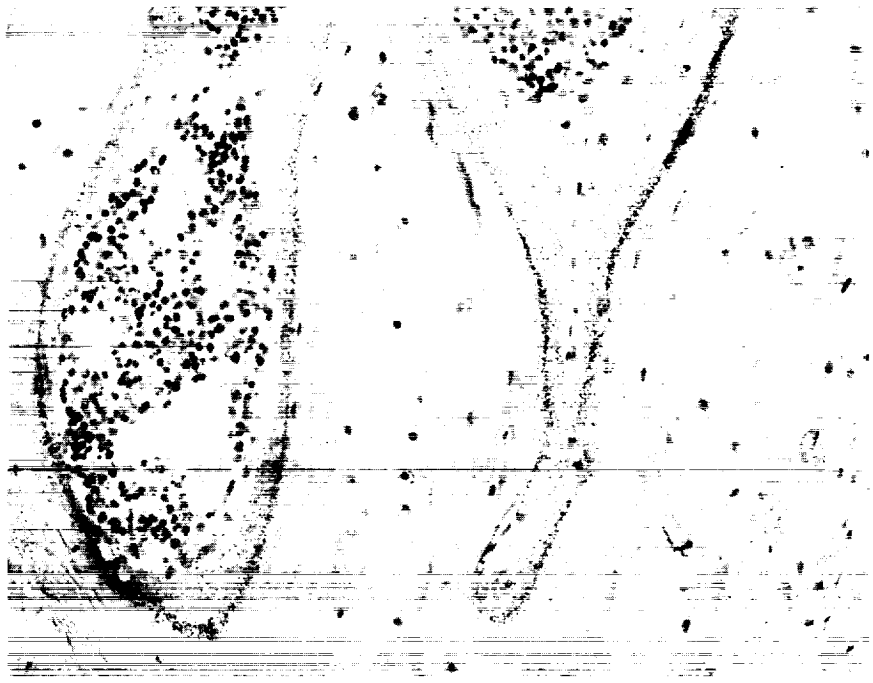

Figure 3. Autoradiographic ^{3}H-proline labeling of trabecular bone within a lumbar vertebra from EVT rat. The concentrated band of autoradiographic grains depicts bone matrix formation adjacent to marrow space.

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(4) ^{3}H-thymidine labeling of cells undergoing DNA synthesis tended to follow shifts in the fractional distribution of cell types.

(5) Unloading (3 days) did not result in significant differences in mandibular condyles with regard to fractional distribution of osteogenic cells, incidence of cells undergoing DNA synthesis, width of condylar cartilage zones, or volume percent ratio of cartilage to bone.

- ***In Situ* Hybridization Methods Development**

(6) Initial studies have focused on the methodological problems involved in performing this advanced technique in paraffin and methylmethacrylate embedded tissues as well as in fresh frozen specimens. We prefer to use methylmethacrylate-embedded tissue for the *in situ* hybridization because this embedding media is most amenable to the nuclear volume cell kinetic assay. The use of methylmethacrylate-embedded tissues alone will allow adjacent serial sections to be processed for nuclear volume cell kinetic assays or *in situ* hybridization, greatly improving correlations between the two techniques.

Significance of the Accomplishments

Finding #1: Evaluation of EVT specimens indicates that the ^{3}H-proline labeling methods to be used in SLS-1 rats will adequately label bone matrix formation following spaceflight, despite concomitant dosing of the animals with other radioactive substances (used for non-bone SLS-1 experiments).

Finding #2: These data indicate that the block of tibial metaphyseal osteoblast histogenesis in hindlimb unloaded rats is similar to that seen in the periodontal ligament (PDL) of animals exposed to both actual and simulated weightlessness.

Finding #3: However, in contrast to PDL cells, these data indicate that the ***transient suppression of preosteoblast formation within 72 hours after initiation of SW is not associated with disturbance of circadian rhythm of tibial metaphyseal osteogenic cells.*** Alternatively, the tibial metaphyseal osteogenic cells may have a different rhythm period.

Finding #4: Despite the size of the precursor cell (**A+A'**) and preosteoblast (**C+D**) compartments, the number of proliferating cells remained relatively constant. This indicates a ***relatively constant control of cell turnover which is independent of the total number of a particular cell type.*** These data are similar to that seen in PDL.

Finding #5: The unloading model for simulated weightlessness does not mimic the effects of spaceflight on the mandibular condyle.

Finding #6: Development of an *in situ* hybridization technique will allow visualization of specific messenger RNAs as a means of further defining the state of differentiation of osteoblast precursor cells previously described by nuclear volume assay. The maintenance of section adhesion to the slides has proven to be a major problem that must be overcome if methacrylate-embedded tissues are to be utilized.

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STRUCTURE AND FUNCTION OF MAMMALIAN GRAVITY RECEPTORS

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Description of Research

The long-term goal of this research is to understand how information is processed in gravity receptors on Earth and in space, with special reference to understanding adaptive responses to altered gravitational environments. To help achieve this goal, more than 25 three-dimensional (3-D) reconstructions of terminal/receptive fields and of small portions of the macular neural network of the Sprague-Dawley rat have been made, and the connectivities of about 400 cells have been mapped. This ground-based work will serve as a basis for interpretation of any observed changes in microstructure that occur in maculas of rats to be flown in April 1991 on shuttle mission SLS-1 (Spacelab Life Sciences-1) and later, on SLS-2.

For our most recent reconstructions we have cut a new series of 175 serial sections at 20 nm. This is twice as thick as is usual for transmission electron microscopy. However, this thickness is easily handled by the Zeiss 902 transmission electron microscope (TEM) available to us, and has many benefits in our work. Doubling the thickness has meant that fewer sections need to be handled to cover the same distance in the macula, and has permitted better visualization of small-diameter collaterals from calyces and nerve fibers. Currently, we photograph alternate sections in their entirety but have also focused on a small area for more intense study through the use of every section.

The method of reconstruction remains the same as reported previously. Following photography at 3000X in the TEM, the micrographs are printed and assembled into montages of the sections. Objects of interest are traced onto acetate from the sections and are digitized into a PC where the slices are placed in register and portrayed as filled slices. These data are then transferred to a Silicon Graphics IRIS high performance workstation where the objects are reconstructed as shaded solid images. Animated films of the objects turning around, or being cut into and reassembled, are made using equipment at the Numerical Aerodynamic Simulation Facility.

Accomplishments

The accomplishments fall naturally under two headings: Results, and Theoretical Interpretations.

Results: This study continues to provide evidence that maculas are organized as weighted neural networks for parallel distributed processing of information. We have mapped the distribution of over 300 ribbon and spherule synapses in type I and type II hair cells as part of the study of weighting of information flow in the macular neural network. No two macular terminal/receptive fields are identical in details, so that macular wiring appears to be accomplished through constrained randomness. Moreover, various macular areas appear to be morphologically organized for differing analyses of the incoming acceleratory signal. This is called segregation, and means that the various parts of the macula are likely analyzing the input for different components and, possibly, for centrally different purposes. Thus far, we have ***identified four network organizations that differ from one another in kinds of nerve fibers supplying the area, in***

terminal fields, and degree of collateralization. With respect to collaterals, our findings continue to indicate that the intrinsic collaterals are significant in information processing. We still have not identified an extrinsic source for efferent terminals on macular neural elements, but this finding must be interpreted cautiously because of the difficulty in tracing efferents over distance within the macula. The nerve fibers are often only 0.2 μm in diameter between boutons.

In related work, we carried out cupric ion-ferricyanide labelling of impulse initiation zones in rat vestibular nerve fibers. The zones had not been labelled previously except in fish. Our results showed that, whether branched or not, ***each vestibular nerve has but one impulse initiation zone.*** It is ***located just distal to the heminode.*** Coincidentally, it was learned that a substance in subsurface cisterns of type I hair cells is reactive to the procedure employed, but type II hair cell cisterns react little or not at all. This indicates that there is a fundamental difference in cisternal material in the two kinds of hair cells.

Theoretical Interpretations: The observation that no two macular terminal/receptive fields are identical prompted an attempt to reproduce the fields by computer, using the Monte Carlo method of simulation. Data concerning branch lengths, number of branches, and numbers of hair cells communicating with individual calyces were obtained from the reconstructions and montages. These provided constraints, so that nerves could terminate, branch or grow within specific distances and could have hair cells within a certain range. The constraints were coupled with a random number series in a software program to generate symbolic representations of the terminal/receptive fields. The outcome of this study is that the fields actually observed were reproduced as were others, within the range of possibility but not seen. The simulations lend support to the idea that an element of randomness is introduced developmentally when the connectivities of the maculas are being established.

A second interpretation concerns the fundamental organization of maculas, and whether it corresponds to that in other neural tissue such as retina and other parts of the brain. The results of the 3-D studies are being interpreted to indicate that there are channeled and distributed modifier circuits in the maculas, as in all other neural tissue. In the mammalian macula, type I hair cells provide channeled input to the calyx (Figure 1, left) but type II hair cells and the intrinsic collateral system comprise the distributed circuitry that modifies calyceal and vestibular nerve output (Figure 1, right). This interpretation brings maculas into line organizationally with other, more complex neural tissue.

A corollary concept is that the distributed modifier microcircuits provide the system with its greatest potential for adaptability to new environments, and have the most influence on mechanisms underlying memory and learning. This concept will be tested in the microgravity of space.

In collaborative work with a mathematician trained in signal processing, we have shown that the stereocilia of the hair cell tufts have nearly optimal hexagonal organization for detection of signals. The stereociliary tufts are compared to phased array detectors.

Significance of the Accomplishments

Our research has identified the site and number of impulse initiation zones in mammalian vestibular nerve fibers. This information is vital to any interpretation of macular functioning. Likewise, the research on stereocilia should provide new insights into their functioning. While the hexagonal arrangement of the stereocilia has been known since they were first studied using electron microscopy, the functional significance of this

organization has eluded discovery. These and other of our findings have been placed into a global interpretation of macular functioning that will be helpful when flight results are obtained. That is, we shall be looking not only for changes in number and size of ribbon and spherule junctions, but also for alterations in subsurface and subsynaptic cisterns in the two kinds of hair cells. The total effect is that we have a solid base of knowledge on which to build our future research, and a solid basis for comparison of results obtained in microgravity with those of Earth-bound studies. Our theoretical interpretation of the fundamental organization of macular and other neural tissue will be sharpened by experiments but, if proven to be basically correct, it provides a new conceptual framework for studying adaptation to altered gravitational environments and for investigating the possible causes of macular disorders wherever they occur.

INPUT OF TYPE I AND TYPE II HAIR CELLS IN THE MACULAR CIRCUITRY

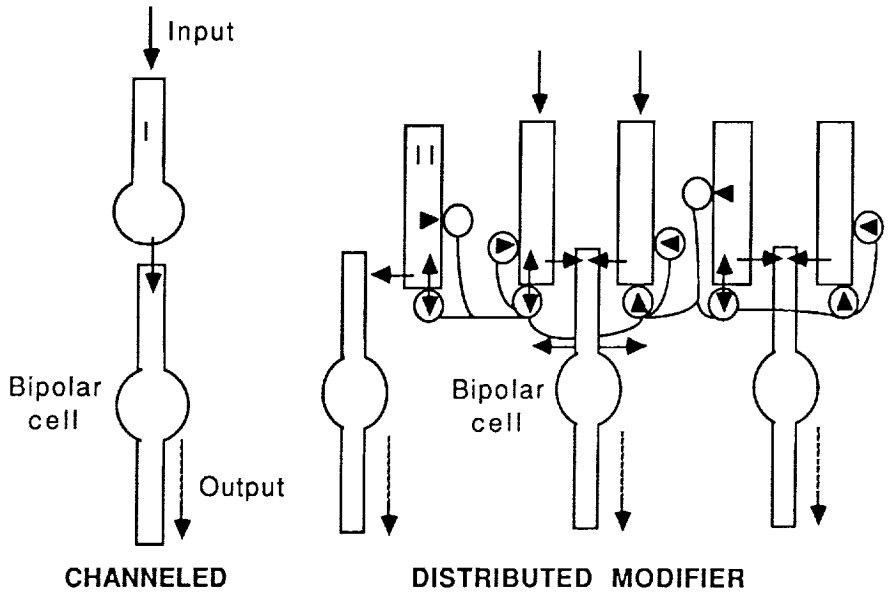

Figure 1. The type I (I) cell channels its output to its calyx (left). Type II cells (II) and the collateral system comprise the distributed modifier circuit (right) that shapes calyceal output. Solid arrows indicate input and output of hair cells; arrowheads show direction of synapses on type II cells; double headed arrows are reciprocal synapses; dashed arrows represent modified output of bipolar cells.

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DEVELOPMENT, MATURATION AND PHYSIOLOGY OF THE BRAIN-PITUITARY-GONADAL AXIS OF FISH IN THE CEBAS/AQUARACK SYSTEM

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Description of Research

The long-range goal of this project is to study the effects of near zero gravity on the development and function of the brain-pituitary-gonadal (BPG) axis in the swordtail fish, *Xiphophorus helleri*. The first phase of the work is to collect fundamental, relevant data in the Closed Equilibrated Biological Aquatic System (CEBAS)/Aquarack (C/A), a project developed by the German Space Agency (DARA), at ground level conditions in order to establish a database that will serve as a comparison for results obtained from future space experiments. The specific aims of this project are to: (1) Determine the effects of the C/A system on the structure and function of the brain, pituitary gland and gonads in sexually mature animals. This includes an analysis of the effects of the C/A system on fertilization and gestation; and (2) Determine the effects of the C/A system on the development (birth to maturity) of the neuroendocrine axis concerned with reproduction.

The biology of the reproductive system will be analyzed by evaluating the changes in the synthesis, storage and release of neurotransmitters, neurohormones and pituitary and gonadal hormones in mature and neonatal animals placed into the C/A system, and in young immature fish born in, and permitted to reach puberty in, the system. Immunocytochemical and morphometric methods will be the principal tools of analysis.

Accomplishments

In the initial 17 months of this program, we have processed for histological study and immunocytochemical analysis brains, pituitary glands and gonads from 39 mature and immature, male and female swordtails ranging in weight from 0.23 to 1.85 grams. Each animal was photographed in Bochum before they were forwarded to Brooklyn College for study. We have tested in pilot studies antisera to five different gonadotropins, gonadotropin releasing hormone (GnRH), and four neurotransmitters (serotonin, dopamine, neuropeptide Y [NPY], and dynorphin [DYN]).

Of special significance is our recent ***localization by immunocytochemical methods of NPY and DYN in the brain and pituitary gland of immature and mature Xiphophorus maculatus*** (the platyfish). Immunoreactive (ir-) -NPY was found in perikarya and nerve tracts of the nucleus olfactoretinalis, telencephalon, ventral tegmentum, the neurohypophysis and in specific cells of the adenohypophysis. Ir-DYN was found in nerve tracts in the olfactory bulb and in association with the cells of the pars intermedia and caudal pars distalis of the pituitary gland. The ***association of NPY and DYN with brain structures and pituitary gland regions previously found to be involved with sexual maturation, growth and differentiation, suggests that these neuropeptides play a role in these events.***

Significance of the Accomplishments

The data that we have already gathered on the distribution of brain neuropeptides and neurotransmitters and pituitary hormones in *Xiphophorus* are enabling us to construct a "map" that will permit us to assess brain-pituitary-gonadal axis activity under experimental conditions. These data will enable us to study the effects of space travel on reproductive system structure, development, and function under the conditions of the CEBAS/Aquarack system.

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EFFECTS OF WEIGHTLESSNESS ON *AURELIA* EPHYRAE DIFFERENTIATION AND STATOLITH SYNTHESIS

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Description of Research

The long-range goals of this research are: (1) to discover the role(s) of gravity on the behavior and development of *Aurelia* ephyrae (tiny jellyfish) and on their graviceptor structures (rhopalia) and (2) to discover the effects of microgravity on ephyrae and graviceptor development after short-term (9-day space shuttle flight) and long-term (space station and biosatellite) exposure to a microgravity environment.

Specific objectives are: (1) to determine whether the microgravity of space will modify the development of ephyrae from polyps, the development of the graviceptors of ephyrae, the mineralization and/or demineralization of statoliths of rhopalia, or the swimming/pulsing behavior of ephyrae; (2) to discover, by comparing the features listed above in ephyrae that develop in space with those of ephyrae that develop on Earth, the roles that gravity plays in the development of ephyrae, their graviceptors, and their behavior on Earth; (3) to develop a method for maintaining rhopalia in nutrient media for 1-3 months in order to study development of more mature rhopalia (than those found in ephyrae) on Earth and in space; and (4) to compare behavior of ephyrae with and without rhopalia.

Accomplishments

(1) **Touch-plate Development in Graviceptors:** For the first time, touch-plate development was studied in the graviceptors (rhopalia) of living ephyrae using the light microscope. The touch-plate is a part of the rhopalium which is involved in graviception in medusae. A series of rhopalia were examined at 24 hr intervals following induction of ephyra formation with iodine. At 96 hr, ephyrae which were attached to the strobila were excised from the strobila. Rhopalia from these ephyrae and from free-swimming ephyrae at later time periods were excised and put into a wet film for microscopic examination. The results are seen in Figure 1. Touch-plates are not found until 144 hr and contain only a small ocellus and a few straight vibrating cilia (these may represent the formation of a new type of mechanosensory cell not found earlier in the rhopalia). Another observation made on these jellyfish was the formation of the first ocelli by 96 hr while the ephyrae are still attached to the strobila. These ephyrae pulse slowly and weakly at this time. Statolith numbers increase from the 96 hr to the 144 hr time period.

Earlier studies (see *1988-89 NASA Space/Gravitational Biology Accomplishments* report) revealed that rhopalia of ephyrae given nutrient media after release from the strobila continued growing in size and had increased numbers of statoliths. The vibrating cilia, thus far, have been found only in the touch-plate area of rhopalia, and they increase in number as the touch-plate grows and the ephyrae mature into medusae.

TOUCH-PLATE DEVELOPMENT IN EPHYRAE GRAVICEPTORS

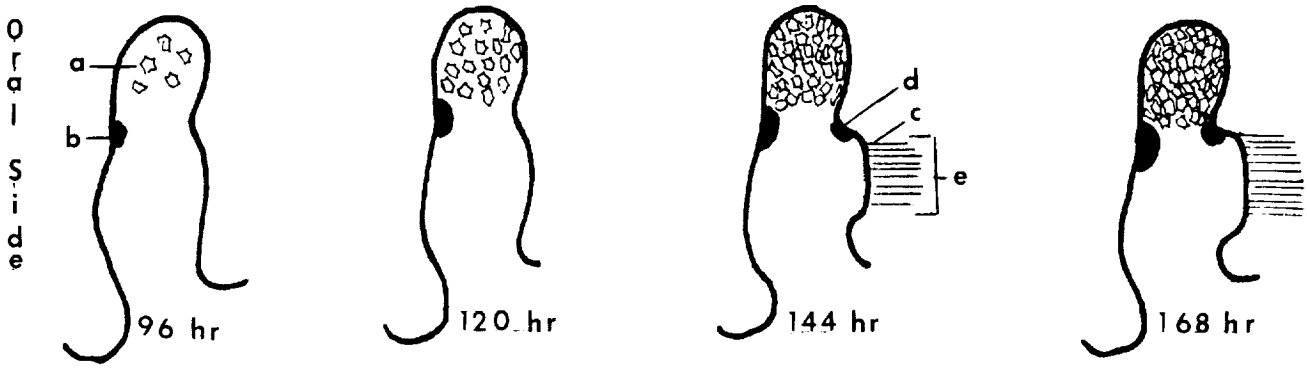

Figure 1. Touch-plate formation in graviceptors of ephyrae developing after metamorphosis induction with iodine. Side view of rhopalia: a. statoliths; b. 1st ocellus; c. vibrating cilia; d. 2nd ocellus; e. touch plate.

(2) **FMRF-amide-like Immunoreactivity in Graviceptor Nerves** (in collaboration with Dr. Millie Hughes-Fulford, University of California and the VA Medical Center, San Francisco, CA): The neurons of graviceptors of *Aurelia* ephyrae were examined for the presence of FMRF-amide-like (FMRF-a-l) neuropeptide using both the immunofluorescence and peroxidase-labelled antibody methods. Microscopic examination of the ephyrae treated with antibody against FMRF-amide revealed immunoreactive neurons and neurites throughout the ephyrae, especially in the diffuse nerve net. Graviceptors were immunoreactive using both methods but the peroxidase-labelled antibody revealed individual neurons in the graviceptors. A cluster of neurons was seen at the bases and tips of the rhopalia. Immunoreactive neurites were very closely associated with the statoliths and the 1st ocellus. Longer neurites extended along the sides of the rhopalia connecting the two concentrations of nerves and extending to the radial muscles.

Significance of the Accomplishments

Our research is directed toward learning as much about *Aurelia* development and behavior as possible in the time interval before the Jellyfish Experiment is flown on the SLS-1 (Spacelab Life Sciences-1) shuttle flight (currently scheduled for August 1991). In addition, efforts are being made to culture and study the jellyfish in nutrient media so that they can be flown on a long-term unmanned flight in the future.

Finding #1: ***The sequential development of graviceptor structures was determined using normal jellyfish.*** It was most exciting to find that ***touch-plate formation proceeds in unfed newly released ephyrae and is visible within 24 hr after release from the strobila.*** The recent change of the SLS-1 flight from 7 to 9 days will permit a study of the formation of one group of ephyrae to the stage when the touch-plates would form on Earth. This means, therefore, that we will have the opportunity to learn whether microgravity interferes with the initiation of touch-plate development, including the formation of the 2nd ocellus. In addition, of course, the determination of this sequence of development is important from the standpoint of understanding more about the developmental processes in differentiating and growing ephyrae. This information will be incorporated into studies in progress, including those in which developing ephyrae are given nutrient media.

Finding #2: It is very important that nerves in the ephyrae can be positively identified using the FMRF-a-l immunoreactivity method. The presence of a natural neuropeptide which is related to FMRF-amide in *Aurelia* graviceptors and in the diffuse nerve net which has sensory function suggests that this substance plays an important role in the regulation of pulsing and may also be involved in the orientation of the organisms while swimming. We will be able to determine whether this neuropeptide is present in ephyrae which had developed in space, especially in organisms which may return to Earth with swimming/pulsing abnormalities after a spaceflight.

Publications

Spangenberg, D.B. and Hughes-Fulford, M. 1989. FMRF-amide-like immunoreactivity in neurons of *Aurelia* ephyra graviceptors (Abstract). *ASGSB Bulletin* 3: 27.

SKELETAL MUSCLE METABOLISM IN HYPOKINETIC RATS

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Description of Research

This work concerns the mechanisms of atrophy and metabolic alterations associated with the lack of load-bearing (unweighting), which may occur with prolonged bedrest or weightlessness. To further understand this muscle wasting, comparisons are made with measurements in muscles whose nerve supply is interrupted. Our work this year has dealt primarily with carbohydrate and protein metabolism.

For carbohydrate metabolism we analyzed further the effects of isoproterenol (beta-adrenergic agonist) on muscle. Measurements included glucose phosphorylation, glycogen synthesis and production of cyclic AMP, the hormone second messenger. In relation to our interest in the sparing of membrane proteins, we assayed several enzyme activities in plasma membranes of unweighted muscle and compared with denervated muscles. In terms of protein metabolism, the focus was on the mechanisms of accelerated protein breakdown in unweighted and denervated muscle. Of particular interest were the roles of lysosomal versus cytosolic protein breakdown.

Accomplishments

(1) Carbohydrate Metabolism

(a) ***Unweighting results in an increased isoproterenol (beta-adrenergic agonist) response of glycogen synthesis and cyclic AMP production.*** Previous studies of glycogen synthesis suggested no difference in response but the more recent work showed that elevated glycogen in unweighted muscle can mask isoproterenol responses. For cyclic AMP production, non-receptor-mediated effects do not differ.

(b) Greater insulin sensitivity antagonizes the effects of isoproterenol in unweighted muscle.

(c) Insulin control of 3-0-methylglucose uptake shows increased sensitivity in unweighted muscle just as for 2-deoxyglucose.

(d) In preparation for our spaceflight study concerning insulin response of glucose transport, we have found that a flight as short as 2.5 days with animal availability by 4 hours or longer flight (5 days) with as much as a 12-hour delay in receipt of animals will likely yield satisfactory data.

(2) Membrane Proteins

While membrane receptor proteins likely are spared during unweighting atrophy, membrane enzyme activities show differential responses. Likely this difference reflects varying metabolic effects on enzymes which are not evident for receptors.

(3) Protein Metabolism

(a) ***Protein breakdown is accelerated more with denervation than unweighting atrophy.***

(b) ***Accelerated protein breakdown with unweighting is primarily a cytosolic event.***

(c) ***Proteolysis in unweighted soleus is more sensitive to alterations of intracellular calcium.***

(d) In vivo proteolysis in unweighted soleus muscle can be lowered by intramuscular injection of mersalyl, a thiol protease inhibitor, leading to termination of atrophy for 24 hours.

(e) Accelerated protein breakdown with denervation atrophy is associated with a role for the lysosome in accord with greater lysosomal permeability (fragility).

(f) Intramuscular injection of chloroquine, a lysosomotropic agent reduces proteolysis and terminates atrophy of denervated muscle but not unweighted muscle.

Significance of the Accomplishments

Finding #1: Parts 1a, b, c support further our observations of the sparing of membrane receptor proteins in general (i.e., insulin and beta-adrenergic receptor). Part d results have helped to define the potential limitations of our spaceflight experiment on shuttle flight STS-43 in 1991.

Finding #2: Membrane enzymes do not seem to behave identically as membrane receptor proteins in terms of protein sparing. Likely these results indicate some metabolic alteration instead of these activities since denervated muscle behaves in a similar manner. In contrast, the response of receptors is different between unweighted and denervated soleus muscle.

Finding #3: The most significant aspect of these results is the finding that accelerated protein breakdown in unweighted muscle occurs mostly in the cytoplasm. It seems to be linked to increases in calcium-dependent proteolysis (3c) and may in particular involve the calcium-activated protease, which is mersalyl-sensitive (3d). In contrast, denervation may lead to increased lysosomal proteolysis (3e). If so, then an intact nerve supply may tend to attenuate this process while with denervation the absence of some factor(s) may lead to fragility of this organelle (3d). Of particular note was our success in differentially terminating atrophy for 24 hours of unweighted or denervated soleus muscles.

Publications

Fagan, J.M. and Tischler, M.E. 1989. Effects of oxygen deprivation on incubated rat soleus muscle. *Life Sciences* 44: 677-681.

Henriksen, E.J., Kirby, C.R., and Tischler, M.E. 1989. Glycogen supercompensation in rat soleus muscle during recovery from nonweightbearing. *Journal of Applied Physiology* 66: 2782-2787.

Jaspers, S.R., Henriksen, E.J., Satarug, S., and Tischler, M.E. 1989. Effects of stretching and disuse on amino acids in muscles of rat hind limbs. *Metabolism* 38: 303-310.

Kirby, C.R. and Tischler, M.E. 1989. Evidence for sparing of membrane proteins during atrophy of unweighted rat soleus (Abstract). *ASGSB Bulletin* 3: 87.

Tischler, M.E., Cook, P., Hodsden, S., McCready, S., and Wu, M. 1989. *In vivo* protein metabolism of developing flight muscle of tobacco hornworm *Manduca sexta* (Abstract). *FASEB Journal* 3(3): A264.

Tischler, M.E., Henriksen, E.J., Jaspers, S.R., Jacob, S., and Kirby, C. 1989. Changes in muscle accompanying non-weight-bearing and weightlessness. In: *Advances in Myochemistry Volume 2* (ed. by G. Benzi). London: John Libbey Eurotext, p. 325-338.

Tischler, M.E., Kirby, C.R., Rosenberg, S.B., and Chase, P. 1989. In vivo evidence for different sites of accelerated proteolysis in unweighted soleus (US) or denervated soleus (DS) (Abstract). *ASGSB Bulletin* 3: 72.

Tischler, M.E., Satarug, S., and Henriksen, E.J. 1989. Comparison of atrophy mechanisms in unloaded and denervated soleus muscle. In: *Intracellular Proteolysis. Mechanisms and Regulation* (ed. by N. Katunuma and E. Komanami). Tokyo: Japan Scientific Societies Press, p. 483-484.

Toth, A., Tischler, M.E., and Johnson, P.C. 1989. *In vivo* quantitative determination of NADH level (Abstract). *FASEB Journal* 3(4): A1403.

THE ROLE OF CALCITE SKELETAL MATRIX PROTEINS IN BIOMINERALIZATION

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Description of Research

The ultimate goal of our work is to learn how calcified tissue matrix proteins preside over the process of biomineralization resulting in the formation of hard tissues. Disturbances in bone and calcium metabolism occur in humans and in other vertebrates during extended spaceflight. The basic molecular and biochemical basis of how mineralized skeletal elements are formed is poorly understood. Therefore, basic information about biomineralization will be required to better understand the complex events that occur in microgravity environments. We believe that the analysis of simpler model systems to study basic aspects of biomineralization will be of substantial benefit to the space biology program.

With this in mind, our studies have focused on the formation of the calcite spicules of the developing larva of the California purple sea urchin, *Stronglycentrotus purpuratus*. The calcite spicules of the *S. purpuratus* pluteus larva have an integral organic matrix consisting of at least ten glycoproteins. Our studies have centered on learning more about these matrix proteins. These studies have entailed isolating cDNA clones encoding spicule matrix proteins from expression libraries by using antibodies generated against the total spicule matrix proteins. After we isolate cDNA clones that react with the anti-total spicule matrix antibody, we then further characterize the cDNA clone to be sure that it encodes a genuine spicule matrix protein. Once we have isolated the appropriate cDNAs, we will not only know the derived amino acid sequence of the various spicule matrix proteins, but we will then be able to generate immunological and molecular probes as well as recombinant spicule matrix proteins to use as tools to address the structure and function of these proteins. We already have detailed knowledge of one of the spicule proteins, an approximately 50 kilodalton protein called SM50. So, for the past year, we have been attempting to isolate cDNA clones that encode the several other spicule matrix proteins.

Accomplishments

(1) We have isolated a cDNA clone, pNG3/7, that encodes another of the spicule matrix proteins.

(2) We have begun to characterize pNG3/7 and its expression at a molecular level. This analysis entails sequencing the cDNA clone as well as determining when and where this gene is expressed.

(3) We have begun to characterize pNG3/7 expression at a cellular level. It is apparent from our studies that a ***cue from the extracellular matrix in the sea urchin's blastocoel instructs the primary mesenchyme cells to express this gene.***

(4) We have isolated a number of other cDNA clones that may encode other spicule matrix proteins but which have not yet been well characterized.

(5) ***We have isolated and sequenced a cDNA clone from the sea urchin Lytechinus pictus that encodes a protein homologous to the SM50 protein*** of the *Strongylocentrotus purpuratus* embryo.

Significance of the Accomplishments

The isolation and characterization of the pNG3/7 cDNA clone as well as the isolation of a number of other possible spicule matrix cDNA clones are significant first steps towards approaching the problem of how spicule matrix proteins preside over biomineralization.

After several screens of several cDNA libraries we have isolated a number of expression cDNA clones that reacted with the anti-total spicule matrix antiserum. One of these clones, pNG3/7, encodes a 30 kilodalton spicule matrix protein whose amino acid composition is similar to that of other invertebrate matrix proteins. The derived amino acid sequence of this clone and that of SM50 provides us with invaluable information about the structure of these matrix proteins. While both proteins are acidic, the pNG3/7 sequence is very different from SM50. Most notable among other differences between the two proteins is that pNG3/7 does not have a repeating amino acid motif as does the SM50 protein. While we still do not know the structure and function relationship of these proteins, these protein sequences will be informative in comparing them to the other spicule matrix proteins.

The molecular and cellular characterizations of when, where, and how the pNG3/7 gene is expressed are invaluable pieces of information for determining if in fact the pNG3/7 clone encodes a spicule matrix protein. In addition, this information provides indirect clues to how the sea urchin embryo might use this gene product to construct a spicule. For example, experiments looking at when the RNA transcripts encoded by pNG3/7 and SM50 appear in the sea urchin embryo show that they are expressed at slightly different times. SM50 mRNA is first expressed early by the primary mesenchyme cells (the cells that construct the spicules), many hours in advance of the first appearance of spicules. Alternatively, pNG3/7 transcript is first expressed relatively later by the primary mesenchyme cells, just prior to the initial formation of spicules. These findings suggest that there may be different functions for these proteins.

Our finding that a cue from the extracellular matrix is needed for expression of pNG3/7 is significant because it is different from that of SM50. SM50 RNA transcripts will begin to accumulate in the absence of any cues from the extracellular matrix or from other cells. Here again these differences may be reflecting different functions for these proteins. It will be interesting to compare the expression of other spicule matrix protein genes to these two genes.

Our isolating and sequencing of a cDNA clone homologous to SM50 from another species of sea urchin (*Lytechinus pictus*) provides us with valuable information about important structural features of the SM50 protein that has been conserved over the 60 million years since the two species of sea urchins diverged. It is apparent that the repeating amino acid motif as well as a proline rich region of the SM50 protein are highly conserved in the *L. pictus* homologue. We are currently trying to isolate a cDNA clone from *L. pictus* that is homologous to pNG3/7.

While we have a number of other possible clones that encode spicule matrix proteins, these clones have not yet been well characterized. However, we are hopeful we have isolated cDNAs that encode other spicule matrix proteins.

Publications

Killian, C.E., George, N.C., and Wilt, F.H. 1989. The isolation of cDNA clones encoding the integral acidic matrix proteins of the sea urchin embryo's calcareous spicules (Abstract). *ASGSB Bulletin* 3: 71.

Livingston, B.T., Shaw, R., and Wilt, F.H. 1989. Cloning and characterization of a cDNA encoding a *Lytechinus pictus* spicule matrix protein (Abstract). *Journal of Cell Biology* 109(4, Part 2): 155a.

EFFECTS OF MICROGRAVITY ON MAMMALIAN DEVELOPMENT AND DIFFERENTIATION

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Description of Research

The primary objective of our research is to evaluate the affects of altered gravitational environments on mammalian germ cell development and early embryogenesis at the molecular level.

We previously showed that mouse female germ cells rotated on a clinostat exhibit anomalies of the meiotic maturation process. No marked alterations in the efficiency of fertilization or gross abnormalities in very early embryo development were noted. We have extended these studies to include examination of the effects of altered gravity environments on the male reproductive system. Our studies include examination of the effects of exposure to simulated hypogravity (using a hindlimb unweighting model) and to actual microgravity (from spaceflight) on testicular development and spermatogenesis in rats. We are using sensitive molecular markers of normal development in order to examine the response of reproductive tissues to alterations in the gravity environment and to identify stages of germ cell and embryonic development that might be most susceptible to altered environments such as microgravity. The particular parameter that we are evaluating in flight and unloaded testicular tissue is the expression of genes known to exhibit specific patterns of activation during normal testicular development. Our study has focused initially on characterization of the expression of cellular stress protein genes, also known as heat shock protein (*hsp*) genes, in the rodent testis and embryo. Special attention is also focused on examining the pattern of expression of various genes which are known to be normally expressed in developing bone and muscle, such as certain homeobox-containing genes. The experimental methods involve molecular cloning of these assorted genes and both hybridization analysis of the mRNAs isolated from tissues exposed to altered gravity and their normal counterparts and immunohistochemical and electrophoretic analysis of their proteins.

Accomplishments

(1) Characterization of normal heat shock protein gene expression is essential in order to evaluate alterations at the molecular level in tissues from animals in ground-based and flight studies. To determine if the pattern of expression of heat shock genes are modified by altered gravitational environments, we have demonstrated that the ***normal patterns of expression of subsets of the hsp90 and hsp70 members of the heat shock protein gene family have unique cellular and temporal patterns of expression in the developing mouse testis and the midgestation embryo.*** Certain members of both the HSP 70 and HSP 90 families were shown to exhibit spermatogenic stage-specific patterns of expression in the absence of exogenous stress. Two different *hsp*90 transcripts were detected in the mouse testis. The testicular transcripts exhibited cellular and developmental stage specificity of expression. The larger and more abundant transcript was expressed at high levels in the germinal compartment of the testis, particularly in germ cells in meiotic prophase. The smaller *hsp*90 transcript was expressed

predominantly in the somatic compartment of the testis. Expression of the two *hsp*90 transcripts was seen in the testis of other species. In addition, expression of both *hsp*90 transcripts was detected in the embryonic and extra-embryonic compartment of midgestation embryos. A stress-inducible member of the HSP 70 family was shown to be induced by elevated temperatures in certain stages of spermatogenesis, namely the stem cell stages, and/or in the somatic compartment of the testis, but not in later stages of germ cell development.

(2) In collaboration with Dr. Emily Morey-Holton at NASA Ames Research Center, we received testicular tissue from rodents used in ground-based studies on the effects of altered gravity. We are investigating the effects of hypogravity on testicular development and spermatogenesis at the molecular level in animals subjected to the hindlimb unloading model system. The model was designed to simulate and test the effects of microgravity by removing the weight-bearing function of the muscle and bone of the hindlimbs of an animal. The mechanism used allows the animals to move freely and enables an *ad libitum* access to food and water. Dramatic decreases in testis weight have been observed in unweighted animals, but virtually no information exists as to the nature of this pathology. We have isolated RNA from testicular tissue of unloaded rats for 2, 5, 7, and 11 days, respectively, plus their normally weighted controls. RNA isolated from testis of unloaded rats is being examined with probes for cellular stress protein genes.

(3) An important aspect of our experiments is comparison of the observations obtained in our studies using simulated altered gravity environments with changes observed during spaceflight. We have received testicular tissues from rodents flown on a Soviet Cosmos biosatellite. RNA was isolated from testes of spaceflight exposed rats and is being examined with probes for cellular stress protein genes. RNA was isolated from the testicular tissues of 5 rats that were flown for two weeks on Cosmos 2044, 5 rats that were Soviet unloaded ground controls, 5 rats that were synchronous simulated-flight ground controls, and 5 rats that were vivarium normal controls. Total testicular RNA from each of the Cosmos-flown rats plus their controls and the unloaded rats at Ames plus their controls was fractionated by electrophoresis and transferred to Nytran membranes. DNA probes to *Hox*-1.4 (a homeobox containing gene), and *hsp*90 and *hsp*70 (subsets of the heat shock protein gene family) were labeled with radioactive markers. Transfer and immobilization of the RNA to the Nytran membranes facilitated retention of the RNA, thus allowing the samples to be hybridized sequentially with multiple probes. We are currently using densitometric scanning to semiquantitatively compare the relative sizes and levels of transcripts from different genes within each testicular sample. Immunohistochemical analysis will be used to extend this evaluation of gene expression by examining variation at the level of the actual protein products.

Significance of the Accomplishments

Finding #1: *Hsp* genes are expressed in distinctive patterns during both normal development and cellular stress; they are therefore useful molecular markers of cellular perturbation. The unique cellular and temporal patterns of expression of members of the *hsp*90 and *hsp*70 families lend support to the possibility of an important role for these cellular stress protein genes in mammalian testicular function. The particular pattern of expression of the stress-inducible member of the HSP 70 family raises the possibility that cells in early stages of spermatogenesis may be especially vulnerable to changes in their environment. This information will be valuable in predicting stages of development likely to be sensitive to spaceflight and will be useful in designing the best experiments for actual flight.

Finding #2: Northern blot hybridization permits detection of very subtle relative differences in the expression of different genes in tissue samples from space flown animals and their ground-control counterparts. Such molecular analyses enable the use of small samples of the very precious flight tissues to examine the possible effects of altered gravity or other biological stresses.

Publications

Tennyson, V.M., Sherman, D.L., Behringer, R.R., Brinster, R.L., Palmiter, R.D., Tasch, J., Crotty, D., Wolgemuth, D.J., and Gershon, M.D. 1989. Abnormalities of enteric neuronal development in transgenic mice overexpressing the homeobox-containing gene Hox-1.4 (Abstract). *Society for Neuroscience Abstracts* 15: 1121.

Wolgemuth, D.J., Gruppi, C.M., Zakeri, Z.F., and Grills, G.S. 1989. Cellular stress protein gene expression in normal gametogenesis and embryogenesis and in tissues subjected to altered environments (Abstract). *ASGSB Bulletin* 3: 107.

SPECIAL ACTIVITIES

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SPACE BIOLOGY RESEARCH ASSOCIATES PROGRAM

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The Space Biology Research Associates Program provides a unique opportunity to train individuals to conduct biological research in areas relevant to NASA's interest. To maximize the potential for Space Biology as an emerging discipline, there is a need to develop a cadre of scientists interested in working in this area. This grant was developed to train biologists by offering Research Associate Awards to young scientists. These awards provide opportunities for individuals to work on projects directly related to Space Biology and in laboratories that provide the necessary facilities and a relevant research environment. It is anticipated that these scientists will develop research careers in the evolving discipline of gravitational biology, a focused area of Space Biology. The field of gravitational biology is rapidly growing and its future will reflect the quality and training of its scientific personnel.

The program began on June 1, 1980 with funding to support several Research Associates each year. As of April 30, 1990, 76 annual awards had been made to 46 awardees, of whom 30 have received a second year of funding. Table I illustrates the variety of projects, laboratories and institutions to which the 46 Research Associates have been assigned. These scientists represent different disciplines including: zoology, developmental biology, botany, and physiology (animal and plant). In June 1980 there were 19 laboratories participating. Presently there are 61 laboratories in the program. Table II illustrates the number of awards given out each year to new recipients and to renewal candidates.

Many of the Research Associates have been asked to participate in NASA panels, national workshops and national meetings. There have been 112 publications in refereed journals and as many abstracts of papers presented at national and international meetings. Each year in the fall, Research Associates attend the annual meeting of the American Society for Gravitational and Space Biology (ASGSB). The Research Associates are active participants in these meetings, presenting papers and posters along with their senior colleagues. All of the current Research Associates and many of the former Research Associates are members of ASGSB. Research Associates are encouraged to participate in other national meetings in their own disciplines.

The scientists who have completed this program have accepted positions in colleges and universities, with research laboratories and with NASA. An individual listing of the 46 Research Associates is provided in Table I. A more detailed description of the awardees follows Tables I and II.

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Table I. SPACE BIOLOGY RESEARCH ASSOCIATES

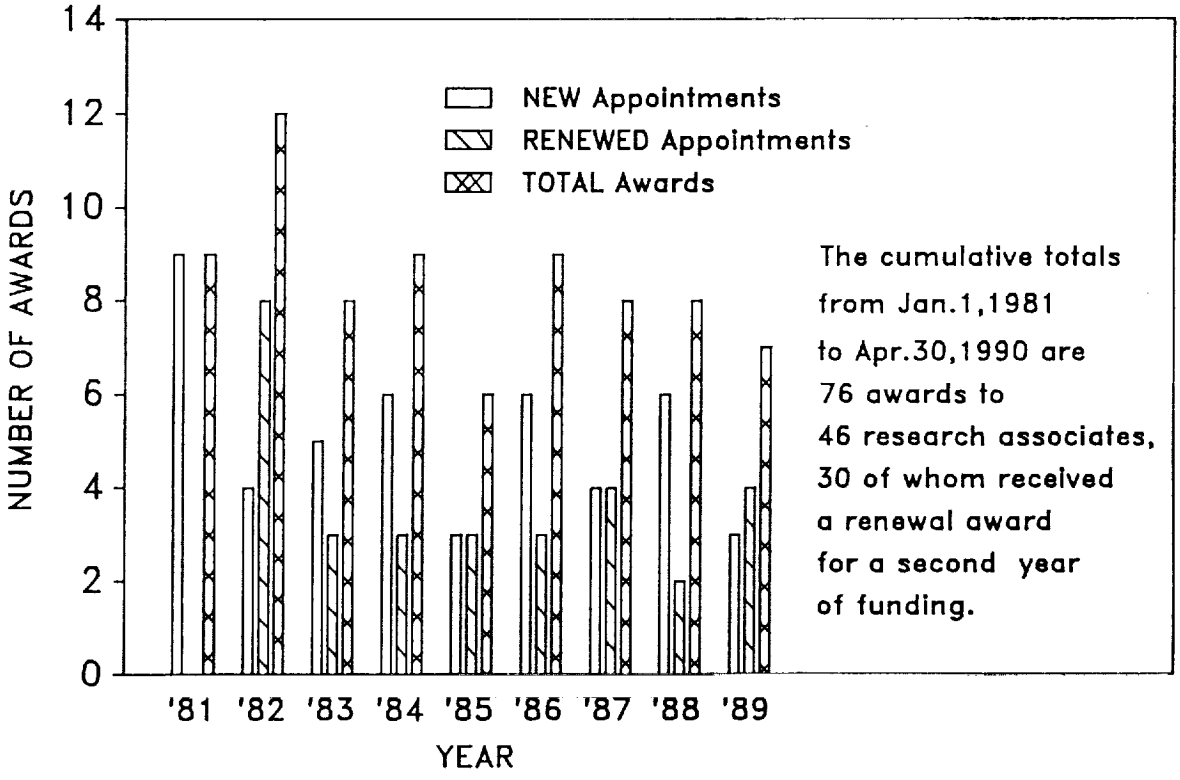
ANIMAL PROJECTS

S. BAIN	SUNY, Stony Brook	SKELETAL BONE REMODELING
W. BERRY	U. Louisville	ROLE OF VITAMIN D/IMMUNE FUNCTIONS
M. BINDER	Dartmouth Col.	CARDIAC PATHOLOGY
S. BLACK	U. Cal., Berkeley	AMPHIBIAN DEVELOPMENTAL ORIENTATION
H. BLAIR	Washington U.	CELLULAR/BONE ATROPHY
J. BUCKEY	U. Texas, Dallas	CARDIOVASCULAR RESPONSES
G. BURROWS	N.I.H.	SYNAPTOGENESIS/NEUROPATHOLOGY
D. CLOHISY	Washington U.	OSTEOCLASTOGENESIS/BONE ATROPHY
M. COOPER	U. Cal., Berkeley	OSTEOPOROSIS/BONE ATROPHY
D. DICKMAN	U. Texas, Galveston	SEMICIRCULAR MUSCLE CANALS
S. GLOTZBACH	Stanford U.	NEUROPHYSIOLOGY/CIRCADIAN RHYTHM
C. GOULD	U. Louisville	IMMUNOLOGY/INTERFERON FUNCTIONS
M. GRAY	Tufts U.	MECHANICAL ENVIRONMENT IN BONE ARCHITECTURE
E. GREENFIELD	Washington U.	OSTEOBLAST ROLE/OSTEOCLAST ACTIVITY
T. JONES	U. Cal., Davis	NEUROPHYSIOLOGY/BRAINSTEM POTENTIALS
T. KERR	Wayne State U.	MAMMALIAN VESTIBULAR SYSTEM
D. KLIGMAN	N.I.M.H.	NEURITE EXTENSION FACTOR RESPONSES
A. LYSAKOWSKI	U. Chicago	VESTIBULAR HAIR CELLS/SYNAPTIC RELATIONS
K. MCLEOD	SUNY, Stony Brook	ELECTRICAL FIELDS IN BONE REMODELING
D. MEYERS	U. Pennsylvania	GRAVITY PERCEPTION/MICROCRUSTACEAN (FW)
L. MINOR	U. Chicago	RESPONSES OF SECONDARY VESTIBULAR NEURONS
D. MURAKAMI	U. Cal., Davis	HYPERDYNAMIA/VISUAL SYSTEMS
S. PERKINS	Washington U.	VITAMIN D/OSTEOCLAST DIFFERENTIATION
K. POTE	U. Virginia	OTOCONIA Ca BINDING PROTEIN
G. RADICE	Indiana U.	GRAVITY-SENSORS/AMPHIBIAN EMBRYOLOGY
F. ROBINSON	U. Pittsburgh	SENSORY MOTOR PROPERTIES IN UVULA
J. STEFFEN	U. Louisville	GLUCOCORTICOID RECEPTORS & MUSCLE RESPONSES
J. SZILAGYI	Cleveland Clinic	HYPODYNAMIC RESPONSES/ANIMAL MODEL
D. THOMASON	U. Texas, Houston	DECREASED ACTIN SYNTHESIS/MUSCLE ATROPHY
Y. TORIGOE	U. Cal., Irvine	NEUROPHYSIOLOGY OF GUT

PLANT PROJECTS

S. BARSEL	Michigan St. U.	PLANT CELL PHYSIOLOGY
T. BJÖRKMAN	U. Washington	ELECTRICAL RESPONSES/GRAVITATIONAL SENSITIVITY
T. BROCK	U. Michigan	AUXIN & PROTEIN SYNTHESIS IN GRAVITROPISM
M. DESROSIERS	Michigan St. U.	ELECTRICAL POTENTIAL IN HORMONE TRANSPORT
J. GARAVELLI	Texas A&M U.	PLANT/ALGAE CELL CHEMISTRY
J. GAYNOR	Yale U.	AMYLOPLAST/GRAVITATIONAL SENSITIVITY
M. HARRISON	Washington U.	ENVIRONMENTAL ETHYLENE/GRAVITROPISM
G. JAHNS	U. Houston	LIGNIN BIOSYNTHESIS IN PLANT DEVELOPMENT
K. KUZMANOFF	Stanford U.	ENZYME REGULATORS IN PLANT CELL WALL
M. MATILSKY	Princeton U.	GRAVITY PERCEPTION/COENOCYTE
M. MUSGRAVE	Duke U.	PLANT RESPIRATORY METABOLISM/SPACEFLIGHT
D. REINECKE	Michigan St. U.	IAA DISTRIBUTION/PLANT GEOSENSING
B. SERLIN	U. Texas, Austin	CELL WALL GROWTH/CORN ROOTS
R. SLOCUM	Yale U.	ROLE OF CALCIUM/GRAVISTIMULATION
J. SLONE	Washington U.	AUXIN TRANSPORT/GRAVITROPISM
L. TALBOTT	Washington U.	STEM GRAVICURVATURE/SPECIFIC POLYMERS

Table II. **NASA Space Biology Research Associate Awards**



RESEARCH ASSOCIATE AWARDEES

The 46 awardees are listed alphabetically, including their award term in parentheses, their host laboratory, and current location.

DR. STEVEN BAIN (6/1/88 - 5/30/90) is working on "The Interaction of Skeletal Remodeling With Systemic Disorders: An Obstacle to Extended Space Flight?" in Dr. Clinton Rubin's laboratory at SUNY, Stony Brook, New York.

DR. SARA-ELLEN BARSEL (6/1/87 - 5/30/88) worked on "Molecular and Genetic Phototropism in *Arabidopsis thaliana*" in Dr. Kenneth Poff's laboratory at Michigan State University, East Lansing, Michigan. She is now working for Chemical Abstracts in Columbus, Ohio.

DR. WALLACE BERRY (7/1/88 - 6/30/90) is working on "Lymphokine Producing Capacity of Antiorthostatically Suspended Rats: Relationship to 1, 25-dihydroxyvitamin D_3" in Dr. Gerald Sonnenfeld's laboratory at the University of Louisville, Louisville, Kentucky.

DR. MICHAEL BINDER (1/1/83 - 12/30/83) worked on "Congenital Heart Malformations and Situs Inversus" in Dr. W.M. Layton, Jr.'s laboratory at Dartmouth Medical School. He is now on a research fellowship in the Pathology Department at Brown University, Providence, Rhode Island.

DR. THOMAS BJÖRKMAN (10/1/86 - 9/30/88) worked on "The Mechanism of Gravity Sensing in Plants" in Dr. Robert Cleland's laboratory at The University of Washington, Seattle, Washington. He is continuing to work in Dr. Cleland's laboratory at the University of Washington, Seattle, Washington.

DR. STEVEN BLACK (7/1/82 - 6/30/84) worked on "Determination by Gravitational and Centrifugal Force of the Amphibian Dorsal-ventral Axis" in Dr. Raymond Keller's laboratory at the University of California, Berkeley. He is continuing research with Dr. Keller and is also working with Dr. Kenneth Souza at NASA-Ames Research Center, Moffett Field, California.

DR. HARRY BLAIR (7/1/84 - 6/30/86) worked on "Cellular Mechanisms of Bone Degradation" in Dr. Steven Teitelbaum's laboratory at The Jewish Hospital/Washington University Medical Center, St. Louis, Missouri. He is continuing to work in Dr. Teitelbaum's laboratory funded by a NIH Physician Scientist Training Grant.

DR. THOMAS BROCK (8/1/86 - 7/30/88) worked on "Comparison of Changes in Protein Synthesis Induced by Gravity and Auxin Treatment in Pulvini and Coleoptiles of Oat (*Avena sativa* L.)" in Dr. Peter Kaufman's laboratory at The University of Michigan, Ann Arbor, Michigan. He is continuing to work with Dr. Kaufman at the University of Michigan.

DR. JAY BUCKEY, JR. (7/1/82 - 6/30/84) worked on "2-D Echocardiography as an Accurate Mean for Measuring Left Ventricular Volume and Central Venous Pressure During Zero-gravity" in Dr. C. Gunnar Blomqvist's laboratory at the University of Texas Health Sciences Center, Dallas. At the present time he is the project manager for the cardiovascular experiment scheduled on Spacelab Life Sciences-1 and a Research Assistant Professor/Instructor in Clinical Medicine at the University of Texas Health Sciences Center, Dallas, Texas.

DR. GEORGE H. BURROWS (7/1/81 - 6/30/83) worked on "Studies of Synaptogenesis" in Dr. Marshall Nirenberg's laboratory at NIH, Bethesda, Maryland. He is now on the staff of the National Heart, Lung, and Blood Institute, Bethesda, Maryland.

DR. DENIS CLOHISY (7/1/86 - 6/30/87) worked on "Mechanisms of Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at The Jewish Hospital/Washington University Medical Center, St. Louis, Missouri. He is now completing his clinical training in Orthopaedic Surgery at the University of Minnesota, St. Paul, Minnesota.

DR. MARK COOPER (1/1/85 - 12/30/86) worked on "Osteoporosis of Weightlessness and the Electrophysiology of Bone" in Dr. John Miller's laboratory at The University of California at Berkeley, California. He is now a Research Associate in the Department of Molecular Neurobiology at Yale Medical School, New Haven, Connecticut.

DR. MARK DESROSIERS (7/1/86 - 6/30/88) worked on "A Search for Voltage-gating of Plant Hormone Transport Channels" in Dr. Robert Bandurski's laboratory at Michigan State University, East Lansing, Michigan. He is continuing to work in Dr. Bandurski's laboratory at Michigan State University.

DR. J. DAVID DICKMAN (6/1/87 - 5/30/90) worked on "High Frequency Response Properties of Semicircular Canal Fibers" in Dr. Manning Correia's laboratory at the University of Texas, Galveston, Texas. He is contining to work with Dr. Correia at the University of Texas.

DR. JOHN S. GARAVELLI (1/1/82 - 4/30/82) worked on "Chemical Characterization of Volatile Products of Algal Cell Cultures" in Dr. Franklin Fong's laboratory at Texas A&M University. He is now working for the Extraterrestrial Research Division at NASA-Ames Research Center, Moffett Field, California.

DR. JOHN GAYNOR (1/1/81 - 12/30/82) worked on "Purification and Characterization of Amyloplasts from *Pisum sativum*" in Dr. Arthur Galston's laboratory at Yale University. He is now an Assistant Professor and Henry Rutgers Scholar in The Botany Department at Rutgers University, Newark, New Jersey.

DR. STEVEN GLOTZBACH (1/1/84 - 12/30/84) worked on "Neurophysiological Studies of Circadian Rhythm Control Mechanisms" with Dr. H. Craig Heller at Stanford University and Dr. Charles A. Fuller at the University of California, Riverside. He is continuing to work in Dr. Heller's laboratory funded by a NIH-NIRA grant, Palo Alto, California.

DR. CHERYL GOULD (7/1/84 - 8/30/85) worked on "Effect of Weightlessness on Various Immunological Functions Using a Murine Simulated Space Flight Model" in Dr. Gerald Sonnenfeld's laboratory at The University of Louisville, Louisville, Kentucky. She is now an Assistant Professor at the University of Kentucky, Lexington, Kentucky.

DR. MARTHA GRAY (7/1/86 - 6/30/87) worked on "The Correlation of Applied Strain Distributions to the Location of New Bone Formation: A Rigorous Mechanical Analysis of an *in vivo* Bone Preparation" in Dr. Clinton Rubin's laboratory at Tufts University School of Veterinary Medicine, North Grafton, Massachusetts. She is now an Assistant Professor at the Massachusetts Institute of Technology, Boston, Massachusetts.

DR. EDWARD GREENFIELD (7/1/88 - 6/30/90) is working on "Regulations of Osteoclastic Bone Resorption by Osteoblasts" in Dr. Steven Teitelbaum's laboratory at Washington University, St. Louis, Missouri.

DR. MARCIA HARRISON (7/1/83 - 8/30/85) worked on "Participation of Ethylene in Two Modes of Gravitropism of Shoots" with Dr. Barbara Pickard at Washington University, St. Louis, Missouri. She is now an Assistant Professor in the Biology Department at Marshall University in Huntington, West Virginia.

DR. GARY JAHNS (1/1/83 - 4/30/84) worked on "Interactions of Light and Gravity on the Growth, Orientation, and Lignin Biosynthesis in Mung Beans" in Dr. Joe Cowles' laboratory at the University of Houston. He is now working at NASA-Ames Research Center, Moffett Field, California.

DR. TIMOTHY JONES (1/1/81 - 12/30/82) worked on "The Effects of Hypergravic Fields on Brainstem Auditory-evoked Potentials" in Dr. John Horowitz' laboratory at the University of California, Davis. He is now an Associate Professor at the University of Nebraska, Lincoln, Nebraska.

DR. THOMAS KERR (1/1/83 - 12/30/84) worked on "Cellular Localization of Na^+, K^+-ATPase in the Mammalian Vestibular System"; the first year in Dr. Muriel Ross's laboratory at the University of Michigan and the second year in Dr. Dennis Drescher's laboratory at Wayne State University. He is now an Assistant Professor at Wayne State University, Detroit, Michigan.

DR. DOUGLAS KLIGMAN (7/1/82 - 6/30/84) worked on "The Role of Neurite Extension Factor Nerve and Muscle Tissue Response to Stress or Injury" in Dr. David Jacobowitz' laboratory at the National Institute of Mental Health, Bethesda, Maryland. He is now on the staff at NIMH, Bethesda, Maryland.

DR. KONRAD KUZMANOFF (7/1/83 - 7/30/85) worked on "Isolation and Identification of B-glucan Synthetase: A Potential Biochemical Regulator of Gravistimulated Differential Cell Wall Loosening" in Dr. Peter Ray's laboratory at Stanford University. He is now a Research Associate working with Dr. Craig Beattie at the University of Illinois at Chicago, Illinois.

DR. ANNA LYSAKOWSKI (7/1/89 - 6/30/90) is working on "Synaptic Relations of Type I and Type II Vestibular Hair Cells" in Dr. Jay Gould's laboratory at the University of Chicago, Chicago, Illinois.

DR. MICHAEL MATILSKY (1/1/81 - 12/30/82) worked on "Gravity Perception in the Algal Coenocyte *Caulerpa prolifera* " in Dr. William Jacobs' laboratory at Princeton University. He is now a Senior Research Scientist with Plant Biotech Industries in Ashrat, Israel.

DR. KENNETH MCLEOD (11/1/87 - 10/30/89) worked on "*In-vivo* Measurement of Strain Generated Potentials in Bone During Controlled Mechanical Loading" in Dr. Clinton Rubin's laboratory at the State University of New York, Stony Brook, New York. He is continuing to work in Dr. Rubin's laboratory at SUNY.

DR. DEWEY MEYERS (7/1/81 - 6/30/83) worked on "Response, Adaptation and Gravitational Perception in a Parthenogenic Freshwater Microcrustacean, *Daphnia galeata mendotae*" in Dr. Allan Brown's laboratory at the University of Pennsylvania. He was the Science and Curriculum Coordinator in the Space Life Sciences Training Program at Kennedy Space Center, Florida. Recently he became an Adjunct Associate Professor at West Virginia School of Osteopathic Medicine, Lewisburg, West Virginia.

DR. LLOYD MINOR (7/1/87 - 6/30/88) worked on "Primary Vestibular Afferent Inputs to Central Pathways Mediating the Vestibulo-ocular Reflex" in Dr. Jay Goldberg's laboratory at the University of Chicago, Chicago, Illinois. He is now finishing his clinical training at the University of Chicago Medical Center, Chicago, Illinois.

DR. DEAN MURAKAMI (1/1/85 - 12/30/86) worked on "Influences of the Hyperdynamic Environment on the Development of the Visual System in the Rat" in Dr. Charles Fuller's laboratory at the University of California at Davis. He is continuing to work with Dr. Fuller at the University of California, Davis.

DR. MARY MUSGRAVE (6/1/86 - 10/30/88) worked on "Studies of Respiratory Metabolism" in Dr. Boyd Strain's laboratory at Duke University, Durham, North Carolina. She is now an Assistant Professor at Louisiana State University, Baton Rouge, Louisiana.

DR. SHERRIE LYNN PERKINS (7/1/88 - 6/30/89) worked on "Vitamin D Effect on Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at Washington University, St. Louis, Missouri. She is now an instructor in the Department of Anatomic Pathology at Jewish Hospital, Washington University.

DR. KENNETH POTE (6/1/88 - 5/30/90) is working on "An Otoconial Calcium Binding Protein; Its Temporal Expression and Tissue Distribution" in Dr. Robert Kretsinger's laboratory at the University of Virginia, Charlottesville, Virginia.

DR. GARY RADICE (7/1/81 - 6/30/83) worked on "Control of Gravity-sensing Mechanism in Amphibian Eggs" in Dr. George Malacinski's laboratory at Indiana University. He is continuing to work with Dr. Malacinski, Bloomington, Indiana.

DR. DENNIS REINECKE (11/1/88 - 9/30/89) worked on "Does Indole-3-Acetic Acid Turnover Correlate with Topically-induced Asymetric Growth?" in Dr. Robert Bandurski's laboratory at Michigan State University, East Lansing, Michigan. He is now an Assistant Professor in the Department of Horticulture at the University of Minnesota, St. Paul, Minnesota.

DR. FARREL R. ROBINSON, JR. (7/1/84 - 6/30/86) worked on "Sensory Motor Properties of the Uvula and Nodulus" in Dr. David Tomko's laboratory at the University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. He is now working as a Research Associate with Dr. Albert Fuchs in the Physiology Department of the University of Washington School of Medicine in Seattle, Washington.

DR. BRUCE SERLIN (7/1/84 - 6/30/85) worked on "Differential Wall Growth in Gravistimulated Corn Roots: Its Timing and Regulation" in Dr. Stanley Roux's laboratory at the University of Texas at Austin. He is now an Assistant Professor at DePauw University, Greencastle, Indiana.

DR. ROBERT SLOCUM (1/1/81 - 12/30/83) worked on "Studies on the Localization and Functional Role of Calcium in Gravistimulated Plant Organs;" the first year in Dr. Stanley Roux's laboratory at the University of Texas at Austin and the second year in Dr. Arthur Galston's laboratory at Yale University. He is now an Assistant Professor at Williams College, Williamstown, Massachusetts.

DR. J. HENRY SLONE (7/1/85 - 6/30/87) worked on "Characterization of the Protein Responsible for the Lateral Transport of Auxin During Gravitropism of Pea Shoots and Determination Whether Phosphorylation Participates in Gravitropic Activation" in Dr. Barbara Pickard's laboratory at Washington University in St. Louis, Missouri. He is now working in the Plant Photobiology Laboratory at USDA, ARS, Beltsville, Maryland.

DR. JOSEPH STEFFEN (7/1/81 - 6/30/83) worked on "Glucocorticoid Receptor Levels in Hindlimb Skeletal Muscles and Diaphragm During Prolonged (2 Week) Antiorthostatic Hypokinesia and Recovery" in Dr. X.J. Musacchia's laboratory at the University of Louisville. He is now an Assistant Professor at the University of Louisville, Louisville, Kentucky.

DR. JULIANNA SZILAGYI (7/1/81 - 12/30/81) worked on "Progressive Hemodynamic Changes in Simulated Weightlessness" in Dr. Carlos Ferrario's laboratory at the Cleveland Clinic. She is now an Assistant Professor at the University of Houston, Houston, Texas.

DR. LAWRENCE TALBOTT (7/1/89 - 12/30/89) worked on "Actuation of Gravicurvatur in Pea Stems by Alteration of Specific Wall Polymers" in Dr. Barbara Pickard's laboratory at Washington University, St. Louis, Missouri. He is now in a Postdoctoral Fellowship at UCLA, Los Angeles, California.

DR. DONALD THOMASON (11/1/89 - 10/30/90) is working on "Mechanisms of Decreased Actin Synthesis During Rodent Hindlimb Unweighting" in Dr. Frank Booth's laboratory at The University of Texas Medical School, Houston, Texas.

DR. YASUHIRO TORIGOE (1/1/84 - 12/30/85) worked on "Anatomical Correlated Underlying Vestibulo-autonomic Outflow to the Gut" with Dr. Robert H.I. Blanks at the University of California, Irvine. He is continuing to work with Dr. Blanks at the University of California.

THE INTERACTION OF SKELETAL REMODELING WITH SYSTEMIC DISORDERS: AN OBSTACLE TO EXTENDED SPACEFLIGHT?

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Description of Research

The objectives and long-term goals of this research are intended to determine how an organism's metabolic status interacts with bone tissue to modulate the skeleton's ability to adaptively remodel in response to changes in its mechanical environment. As participation in space exploration expands to include individuals of increasingly diverse metabolic status, the question of how microgravity will interact with each individual's physiological milieu becomes critical. For example, considering the differences in endocrine-driven remodeling between gender, the skeletal changes in female astronauts may differ significantly, and be potentially far more deleterious, than those stimulated in their male counterparts. Therefore, we have proposed that the systemic state of an organism will not simply influence, but will in fact control, the nature and extent of skeletal remodeling in response to microgravity conditions. Ultimately, a means to predict the potential skeletal risk of each astronaut candidate could play a pivotal role in the future selection of potential space travelers by identifying those in greatest danger of skeletal distress.

Determining the potential impact of a "distressed" metabolic state on the skeleton's response to disuse requires the capacity to enhance or exclude certain components of the bone's mechanical environment, as well as the ability to control specific aspects of the animal's systemic milieu. These experimental criteria can be satisfied by utilizing an animal model of disuse osteoporosis, the functionally isolated turkey ulna. In this model, while the bone is completely deprived of any mechanical stimuli, its vasculature and innervation are preserved. In addition, through changes in diet (calcium deficiency), hormonal balance (endocrinopathy), or age, the skeletal response to the superimposed effects of systemic factors can be quantified.

In our research, we are using the ulna model to address how the bone responds to the absence of mechanical loading in each of the following systemic populations:

(1) Adult normal (healthy males, 1 yr. old, fused physes);
(2) Nutritionally deficient (calcium poor diet);
(3) Hormonally imbalanced (castrated adult males);
(4) Growing normal males (5 months of age); and
(5) Old normal males (3 years of age).

By compiling a detailed morphologic, cellular, and physical profile of the skeleton's response to disuse in healthy adult, endocrinopathized, nutritionally deficient, growing, and aged populations we are developing a framework with which to evaluate the impact of metabolic status on the bone's ability to adapt to an altered mechanical environment.

Accomplishments

To date, we have obtained preliminary results from 4 of the 5 systemic populations; adult normal, hormonally imbalanced, growing normal, and old animals. Results are summarized as follows:

(1) Eight weeks of functional isolation of the ulna in normal adult males results in a 15% decrease of bone cross-sectional area. This bone loss is a result of cortical thinning by the expansion of the marrow cavity with minimal intracortical porosity (Figure 1a). In castrated males, the same 8 week period of functional isolation generates a decrease in cross-sectional area equivalent to that observed in the disuse ulnae of normal males. However, the bone is removed intracortically, *not* by expansion of the marrow cavity (Figure 1b). Thus, the ability of the bone cells to perceive and/or respond to changes in the mechanical environment are modulated by the animal's systemic state.

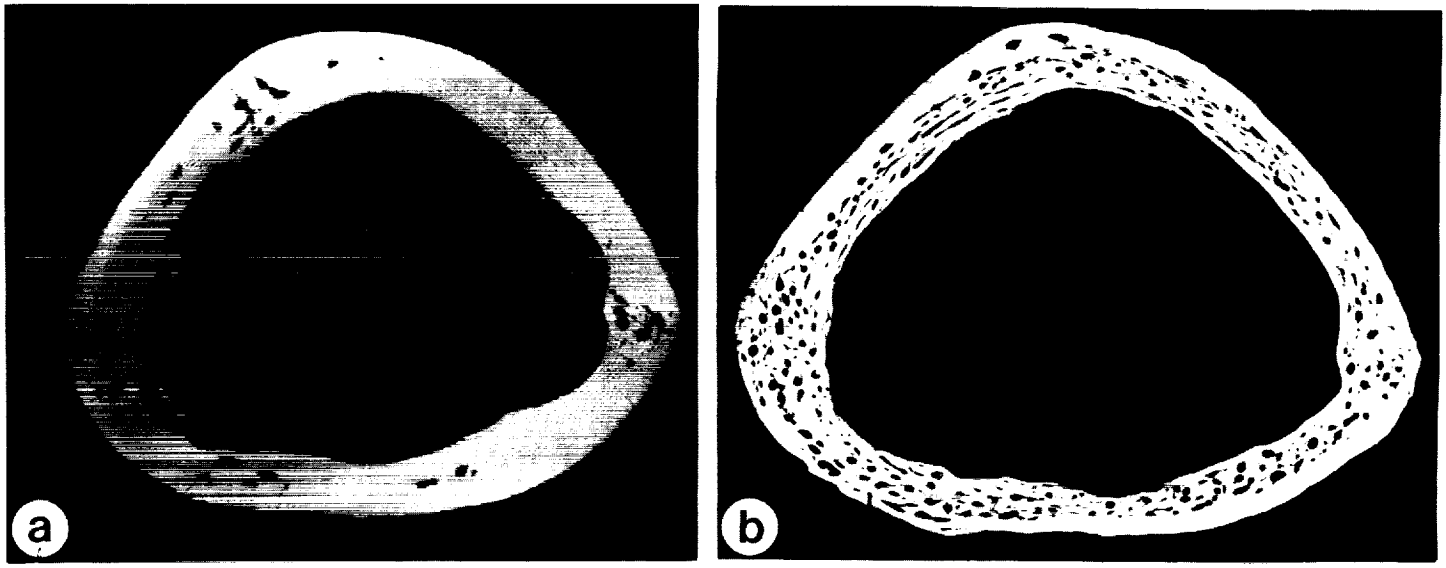

Figure 1. Microradiographs of 100 μm thick midshaft sections from the functionally isolated ulnae from normal (a) and castrated (b) male turkeys. Compared to their respective, intact control ulnae, disuse in the normal male is dominated by cortico-endosteal resorption. This contrasts markedly with the intracortical remodeling triggered by disuse in the ulnae of castrated males.

(2) Comparisons of the *intact* ulnae of castrated and normal males shows an increase in porotic area with no real increase in remodeling events. This indicates the presence of a remodeling imbalance in the castrated animals prior to functional isolation. Therefore, when disuse is superimposed on this imbalance, increases in the number of cortical remodeling events exacerbates this imbalance, leading to the development of intracortical osteopenia.

(3) In animals systemically primed for growth (i.e., 5 month old males), exposure of the ulnae to disuse does not trigger a decrease in bone mass. In fact, functional isolation actually appears to accelerate bone formation rates. Compared to intact control bones, periosteal bone apposition was increased 64% in the disuse ulnae of growing animals. These results suggest that in the young, rapidly growing skeleton, functional stimuli exert a strong controlling influence on bone forming and resorbing activities. Once this control is

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removed, the active cells escape the regulatory influence of function, and in this case, increase their bone forming and resorbing actions.

(4) In normal adult males, total bone cross-sectional area increased 30.2% in response to an artificial, externally applied load (3,000 microstrain, 300 cycles/day). In the three year old males, total cross-sectional area was unaffected by the same loading protocol. The reluctance of the bone to produce new tissue suggests that the cells in the aged animals are "looking" for a different, perhaps more intense, signal.

Significance of the Accomplishments

It is apparent from our preliminary results that the morphologic and cellular aspects of remodeling are dominated by the animal's systemic state. It is clear, from the endocrine dependent site-specific resorption in the castrated animals, accelerated bone formation in the growing animals, and the inability of the aging skeleton to respond to osteogenic stimuli, that the systemic state must be considered in evaluating the skeletal response to disuse. By evaluating the impact of hormonal imbalance, growth, aging and nutritional deficiency, as regulatory factors in the skeleton's response to disuse, a profile of the remodeling interaction potentiated by each metabolic condition can be assembled. Indeed, if there are distinct mechanisms by which each systemic state interacts with the normal adaptive process, then we should expect a unique morphologic response for each metabolic population.

Spaceflight is becoming accessible to an increasingly diverse population. This will no doubt include scientists chosen not for their physical fortitude, but for their expertise across a wide spectra of disciplines. Certainly, the metabolic profiles of these payload specialists will be equally diverse. To minimize the potential trauma to the skeletal system, investigations must be designed to evaluate the influence of age, nutrition, and endocrine related factors on the bone's response to microgravity.

Publications

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Bain, S.D., Impeduglia, T.M., and Rubin, C.T. 1990. Cement line staining in undecalcified thin sections of cortical bone. *Stain Technology* 65(3): 1-5.

Rubin, C.T., Bain, S.D., and McLeod, K.J. 1990. The inability of the aging skeleton to respond to osteogenic stimuli: The origins of Type II osteopenia? (Abstract). *Transactions of the Annual Meeting, Orthopaedic Research Society* 15: 75.

EFFECTS OF THE UNLOADING MODEL ON DIHYDROXYVITAMIN D AND CELLULAR IMMUNITY

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Description of Research

The long range objective of this research is to define the mechanisms responsible for immune system changes during exposure to the microgravity environment of spaceflight. Our present goal is to determine how changes in the concentration of circulating 1,25-dihydroxyvitamin D affect components of cellular immunity using the unloading model to simulate the physical effects of microgravity.

Functional changes in components of cellular immunity accompany exposure to the spaceflight environment. Lymphocytes from humans and animals that have flown in space show reduced proliferative responses to mitogenic stimulation and may have reduced ability to produce cytokines. It is presumed that these phenomena are at least partly due to the lack of inertial loading referred to as "weightlessness" or "microgravity". The mechanisms responsible for the immunological effects of spaceflight are unknown. However, it is probable that physiological changes brought about by unloading interact with the immune system during spaceflight (Figure 1).

It has been discovered that the mineral homeostatic hormone, 1,25-dihydroxyvitamin D, is also an immunoregulatory hormone. This suggests that bone metabolism and mineral homeostasis, which are altered during spaceflight, may interact with the immune system through dihydroxyvitamin D. The main points of our hypothesis are: (1) altered mineral homeostasis during spaceflight causes changes in the plasma concentration of dihydroxyvitamin D and (2) changes in plasma concentrations of dihydroxyvitamin D affect immune cell activity and production of cytokines.

We have employed rat whole body unloading, with and without head down tilt to model the musculoskeletal unloading and headward fluid shift experienced by humans during spaceflight. Adult rats were unloaded with or without head down tilt for periods of one to fourteen days. Rats were also placed in the unloading apparatus with no head down tilt and full loadbearing on all four limbs to assess the effects of restraint. Persistency of unloading effects were assessed by allowing unloaded rats to recover in normal caging for two or seven days. Plasma levels of dihydroxyvitamin D were measured following each unloading period.

Parameters of cellular immunity were quantified in the unloaded and recovered rats. Lung macrophages were tested for changes in their ability to phagocytize foreign particles (sheep erythrocytes). Spleen cells (monocytes and lymphocytes) were assayed for their ability to produce the immune cytokines interleukin-1 (IL-1), interleukin-2 (IL-2), interferon-gamma (IFN-gamma), and interferon-alpha/beta (IFN-alpha/beta).

Additional experiments have been conducted to determine the role of dihydroxyvitamin D in the immunological effects of unloading. To prevent unloading-induced changes in dihydroxyvitamin D, the hormone was administered to unloaded rats through implanted osmotic pumps.

MODEL OF PHYSIOLOGICAL AND IMMUNE SYSTEM INTERACTIONS DURING SPACEFLIGHT

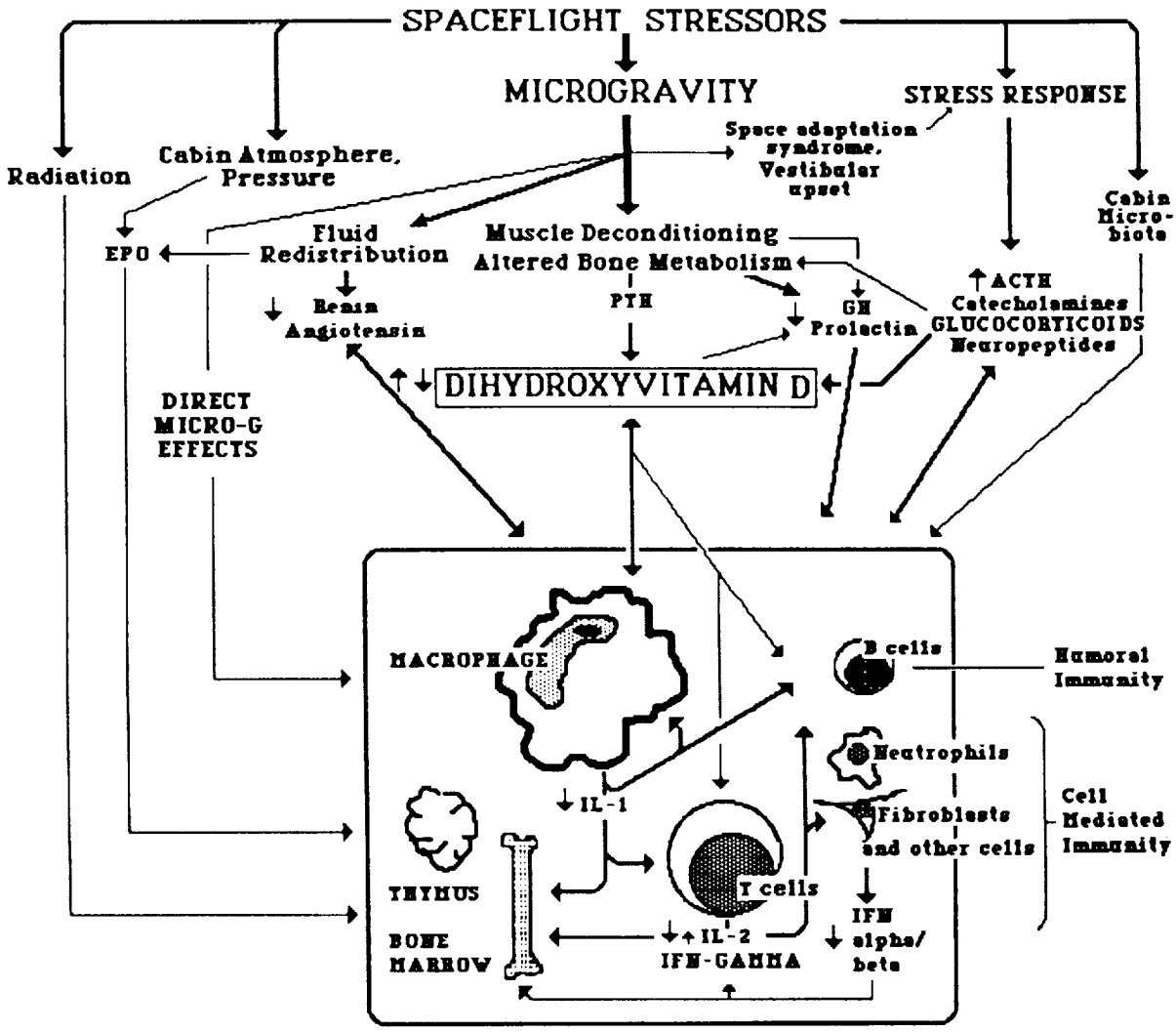

Figure 1. Possible interactions between physiological systems and the immune system during spaceflight. Abbreviations: PTH=parathyroid hormone, GH=growth hormone, ACTH=adrenocorticotrophic hormone, IL-1=interleukin-1, IL-2=interleukin-2, IFN-gamma=interferon-gamma, IFN-alpha/beta=interferon-alpha/beta, EPO=erythropoietin.

Accomplishments

(1) Plasma dihydroxyvitamin D levels were significantly reduced in unloaded rats regardless of the type of unloading used. Circulating 1,25(OH)2 D was reduced by more than 60% following seven days of head down unloading and by more than 70% following fourteen days of head down unloading as compared to pair fed controls. Unloading without head down tilt or with full loadbearing produced results essentially identical to those for head down unloading. Plasma dihydroxyvitamin D levels returned to normal in all unloaded rats following seven days of recovery in normal caging. Administration of exogenous dihydroxyvitamin D prevented the decrease in plasma levels of the hormone during unloading.

(2) Unloading with or without head down tilt for seven or fourteen days or restraint in the harness system with load bearing tended to reduce macrophage phagocytosis. Seven days of recovery were sufficient to normalize macrophage phagocytosis in unloaded rats. Phagocytic function was normal or enhanced in unloaded animals given exogenous dihydroxyvitamin D.

(3) Spleen cell production of IL-1 was reduced by seven or fourteen days of unloading with or without head down tilt and also by harness restraint. Production of IL-1 was further suppressed following two days of recovery but had returned to normal after seven days of recovery. Unloaded animals receiving exogenous dihydroxyvitamin D had significantly increased IL-1 production compared to unloaded and unloaded controls.

(4) Spleen cell production of IL-2 was either unaffected or enhanced following seven or fourteen days of unloading with head down tilt. Unloading without head down tilt or with loadbearing tended to suppress IL-2 production. Dihydroxyvitamin D administration either had no effect or suppressed IL-2 production in unloaded and control animals.

(5) Early reports that unloading for seven or fourteen days suppressed the IFN-gamma response of spleen cells have not been confirmed. In further experiments, unloading with or without head down tilt actually appeared to enhance IFN-gamma production. In contrast, unloading restraint with loadbearing tended to suppress IFN-gamma production as did two days of recovery following unloading. Exogenous dihydroxyvitamin D suppressed the IFN-gamma response in unloaded and control animals.

(6) The unloading model had variable effects on production of IFN-alpha/beta. Some experiments have shown as much as 70% suppression of IFN-alpha/beta production following unloading while other experiments have shown enhancement of production. Production of IFN-alpha/beta was lower in rats receiving exogenous dihydroxyvitamin D.

Significance of the Accomplishments

Finding #1. Experimental and clinical evidence indicates that dihydroxyvitamin D participates in the normal function of the immune and erythropoietic systems, brain, vestibular system, pancreas, and bone. Therefore, changes in vitamin D metabolism would be expected to affect these systems. These studies confirm and expand on previous findings that the unloading model does affect vitamin D metabolism. The direct causes and mechanisms for this phenomenon are unknown. However, because weightbearing restraint in the unloading apparatus decreased plasma dihydroxyvitamin D levels to the same extent as other forms of unloading, it appears that musculoskeletal unloading and/or head-down tilt are not major contributors to the decrease in dihydroxyvitamin D observed during unloading. Glucocorticoid hormones, which are released as part of the

physiological stress response, are known to inhibit the key enzyme in dihydroxyvitamin D synthesis. Glucocorticoids elicited in response to the stress of unloading restraint may have inhibited production of dihydroxyvitamin D during unloading.

Findings #2-6 indicate that the ***unloading model had selective effects on the immune system rather than an overall suppression.*** Differences in the immunological effects of different unloading types indicate that unloading, head down tilt, and restraint stress each contributed to the immunological effects of unloading modeling. The recovery studies indicate that the observed immunological effects of unloading were reversible within one week.

Findings #2-3. Phagocytosis of pathogens and IL-1 production by macrophages are functions central to the initiation and regulation of the immune response. Impairment of these functions during unloading or spaceflight would be expected to result in a suppressed overall immune response. Enhancement of the IL-1 response in unloaded rats receiving the exogenous dihydroxyvitamin D suggests that changes in vitamin D metabolism may be immunologically relevant to spaceflight.

Finding #4. Changes in IL-2 production could impair lymphocyte differentiation and function. As with IL-1, this could lead to suppressed or inappropriate immune responses. Enhanced IL-2 production during unloading when dihydroxyvitamin D levels were low was consistent with the known effects of dihydroxyvitamin D on IL-2. However, because these effects were not consistent with respect to unloading without head down tilt or with loadbearing, other factors such as stress may have been responsible for changes in IL-2.

Findings #5-6. Interferons are important antiinfection, antitumor, and immune regulatory compounds produced by lymphocytes and other cells. Interferon-gamma regulates the expression of proteins that allow coordinated interaction with other immune cells. Interferon-gamma also stimulates macrophage activity and IL-1 production. Interferon-gamma is synergistic with dihydroxyvitamin D in promoting macrophage phagocytosis and killing of microorganisms. Interferon-alpha/beta functions significantly in immune cell differentiation and as an antiviral agent. Abnormally increased or reduced interferon responses could seriously disrupt immune function. As was observed for IL-2, enhanced IFN-gamma production during unloading was consistent with reduced plasma dihydroxyvitamin D. Suppression of IFN-gamma production in unloaded rats given exogenous dihydroxyvitamin D was also consistent with known dihydroxyvitamin D effects on lymphocytes. Interestingly, cell cultures flown in space have shown an increased interferon response that could not be due to vitamin D effects.

In summary, our studies indicate that the unloading model reduces plasma dihydroxyvitamin D levels by a mechanism not dependent on unloading or head down tilt. The glucocorticoid mediated stress response to restraint may be responsible. Changes in macrophage phagocytosis and IL-1 production showed the strongest correlation with plasma dihydroxyvitamin D levels indicating that changes in vitamin D metabolism may be immunologically relevant in unloading and spaceflight. The results obtained for the other cytokines suggest that other factors such as stress are contributing to the immunological effects of unloading. The recovery studies indicate that both the dihydroxyvitamin D changes and the immunological effects are reversible.

Publications

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Mandel, A.D., Sonnenfeld, G., Berry, W.D., Taylor, G.R., Wellhausen, S.R., Konstantinova, I.V., Lesnyak, A.T., and Fuchs, B.B. 1990. Experiment K-6-23: Effect of spaceflight on levels and function of immune cells. In: *Final Reports of the U.S. Experiments Flown on the Soviet Biosatellite Cosmos 1887* (ed. by J.P. Connolly, R.E. Grindeland, and R.W. Ballard). Moffett Field, CA: NASA Ames Research Center, p. 471-480. (NASA TM-102254)

C-3

REGULATION OF OSTEOCLASTIC BONE RESORPTION BY OSTEOBLASTS

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Description of Research

The long-term objective of this research is to understand the regulation of bone resorption during periods of net bone loss, such as those experienced in spaceflight. Conditions that increase bone resorption by osteoclasts, including weightlessness, are thought to function indirectly, that is, by inducing osteoblasts, the cells that form bone, to produce factors that stimulate osteoclast numbers or activity. Accordingly, this project has focused on osteoblast derived factor(s) that stimulate bone resorption by osteoclast-like cells that form in culture from isolated osteoclast precursors.

Accomplishments

(1) ***Osteoblast conditioned media stimulate resorption primarily by increasing osteoclast formation rather than by activating mature osteoclast-like cells.***

(2) ***Osteoblast conditioned media increase osteoclast formation by maintaining the viability of osteoclast precursors.***

Significance of the Accomplishments

Osteoblast derived factor(s) stimulate bone resorption by maintaining the viability of osteoclast precursors rather than by activating mature osteoclasts. This finding is reminiscent of the action of a variety of hematopoietic factors, including colony stimulating factor-1, which acts on cells of the monocyte lineage. Since osteoclasts derive from this lineage, similar factors might be expected to maintain the viability of osteoclast precursors. Prevention of apoptosis, programmed cell death, has recently been shown to be the mechanism whereby a number of these factors maintain the viability of their target cells. The possibility that the osteoblast derived factor(s) also inhibit the specific pattern of DNA fragmentation that is characteristic of apoptosis is currently being investigated.

These studies have begun to describe the mechanisms that regulate bone resorption. More complete understanding of this process may allow prevention of bone loss during conditions such as weightlessness.

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SYNAPTIC RELATIONS OF TYPE I AND TYPE II HAIR CELLS IN THE CRISTA AMPULLARIS OF THE CHINCHILLA

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Description of Research

The long-range goal of this research is to understand the structural and functional organization of the sensory epithelium in the vestibular end-organs. These end-organs include the crista ampullaris, which detects three-dimensional motion, and the utriculus, which detects gravity. Our study to date has concentrated primarily on the crista ampullaris, but preliminary data from the utriculus indicate that its regional synaptic organization is similar to that of the crista ampullaris.

Previous work on the light microscopic level has characterized three types of afferent nerve fibers and the two types of receptor cells (type I and type II hair cells) they innervate. The three types of nerve fibers are: (1) calyx (or chalice) fibers, which terminate in a cup-like ending around type I hair cells; (2) bouton fibers, which terminate as synaptic boutons at the base of type II hair cells; and (3) dimorphic fibers, which terminate in both calyx and bouton endings. In the adult pigmented chinchilla, type I and type II hair cells are present in equal proportion throughout the sensory epithelium, while the three types of nerve fibers show an unequal distribution. Calyx fibers (10% of the total) are found solely in the central zone. Bouton fibers (20% of the total) are found solely in the peripheral zone. Dimorphic fibers (70% of the total) are found throughout the epithelium. These three types of fibers also have different physiological properties. Simply stated, calyx fibers have irregular firing patterns, bouton fibers have regular firing patterns, and dimorphic fibers have a broad range of firing patterns, which depends on their location in the sensory epithelium. They are irregular in the central zone and regular in the peripheral zone. In addition to these differences in discharge regularity, there are differences among the three fiber types in galvanic sensitivity and in response dynamics. From these previous studies, it was concluded that the physiology of an afferent is more closely related to its location in the sensory epithelium than to its branching pattern or to the types and number of hair cells it contacts.

The present study was undertaken to determine whether the physiological differences described above could be related to regional differences in synaptic innervation. We have examined, using the electron microscope, serially sectioned material from several chinchilla end-organs. Our initial data were gathered from a series of 300 sections taken through the entire cross-section of one superior canal crista so that all three regions (central, intermediate and peripheral) were represented in each section. We have since switched to an approach utilizing stereological (quantitative sampling) methods to be able to examine tissue from several animals and to verify the general applicability of our results. Both approaches have yielded similar results. In each case, low-power photomontages were made of the entire sensory epithelium, using every fourth or fifth section in the series. High power examination of every section in the series was used to determine the number and location of all synaptic elements. These data were notated on the photomontages and compiled using a computer spreadsheet program. In the past year, over 25,000 hair cell profiles have been examined. In addition, we are reconstructing individual hair cells from

each region, using higher power serial photomicrographs and a PC-based 3-D reconstruction program.

Accomplishments

(1) ***Peripheral type II hair cells are innervated by 30 or more boutons, each making a single synaptic contact*** (Figure 1A). ***Central type II hair cells have few (2-6) afferent boutons, each of which receives several synaptic contacts*** (Figure 1B).

(2) ***Many type II hair cells in the central zone make multiple synaptic contacts with the outer face of one or more calyx endings. In contrast, outer-face synapses are rare in the peripheral zone.***

(3) ***Ribbon synapses between type I hair cells and calyx endings are plentiful in both central and peripheral zones*** (Figures 1C and 1D).

(4) ***The invaginations made by calyx endings into type I hair cells are also plentiful, especially in the central zone.***

(5) The efferent innervation of type II hair cells and of calyx endings is similar throughout the cristae.

Significance of the Accomplishments

Finding #1: There are differences in the afferent bouton innervation of type II hair cells in the central and peripheral zones of the crista. A higher convergence ratio of hair cell synapses to afferent boutons in the central zone of the crista implies that, anatomically, the central zone appears to be specialized for increased sensitivity. In thirty-five years of electron microscopic study of the peripheral vestibular apparatus, these regional differences have never been reported.

Finding #2: The importance of this observation is that most so-called calyx units can be functionally dimorphic.

Finding #3: This observation, along with the presence of outer-face calyx synapses, should lay to rest the notion that calyx endings are not important sites of chemical transmission.

Finding #4: Invaginating junctions are, as far as we know, not seen in other hair cell-afferent synapses and so may provide a key to the peculiar function of the calyx ending.

Finding #5: This suggests that the larger efferent responses seen in central (irregular) afferents may reflect differences in the neurotransmitter or in the density and type of postsynaptic receptors present on calyx endings and on type II hair cells.

An understanding of the synaptic relations of hair cells with their afferent and efferent endings is fundamental to understanding alterations during spaceflight, as well as during development of the vertebrate vestibular apparatus in extended spaceflights. These changes will probably be subtle, at best, and so quantitative baseline data from normal adult tissue, such as that shown in the present study, are needed for comparison to spaceflight experiments.

TYPE II HAIR CELLS OF THE SUPERIOR CANAL CRISTA OF ADULT CHINCHILLA

ORIGINAL PAGE
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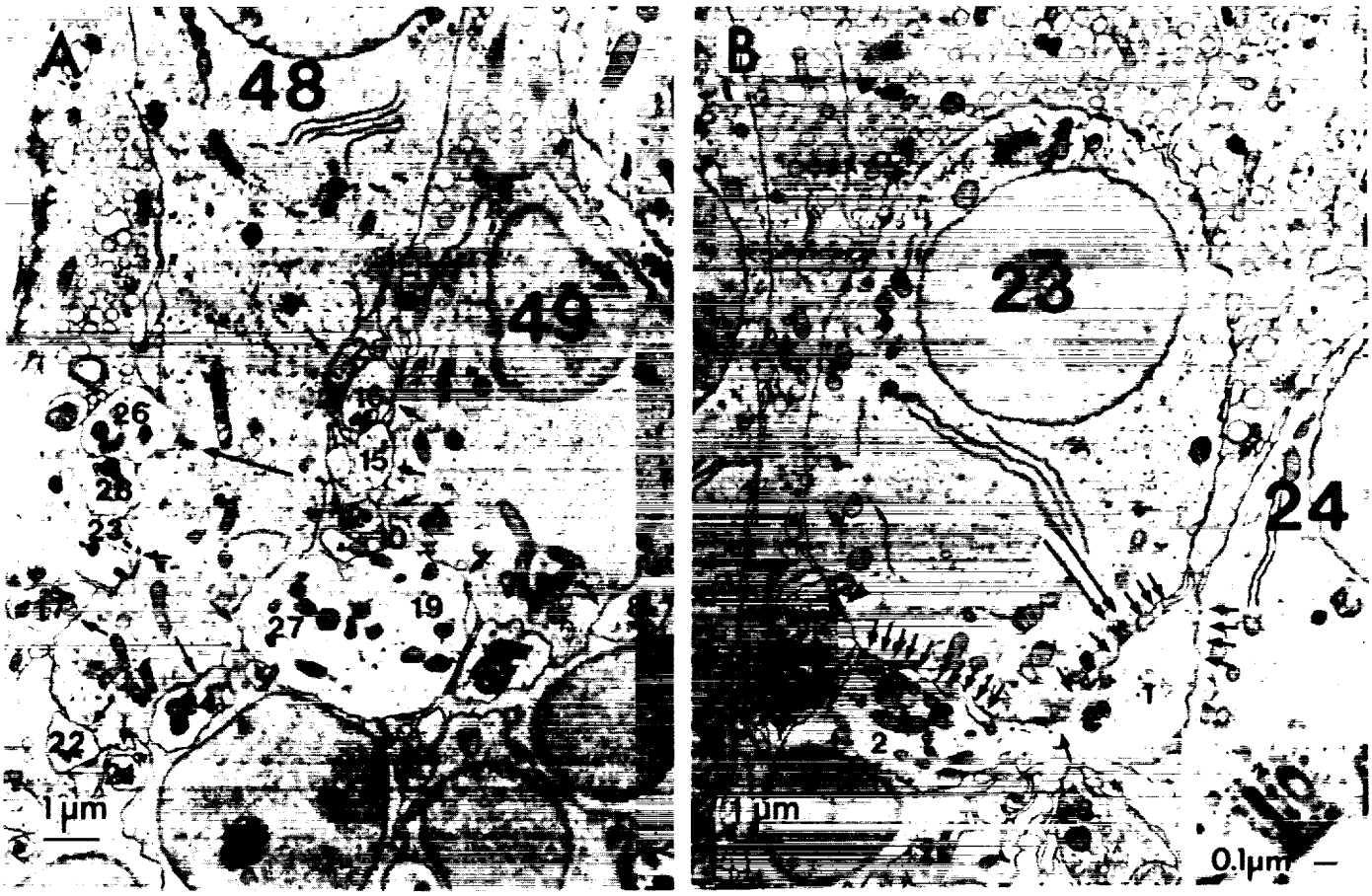
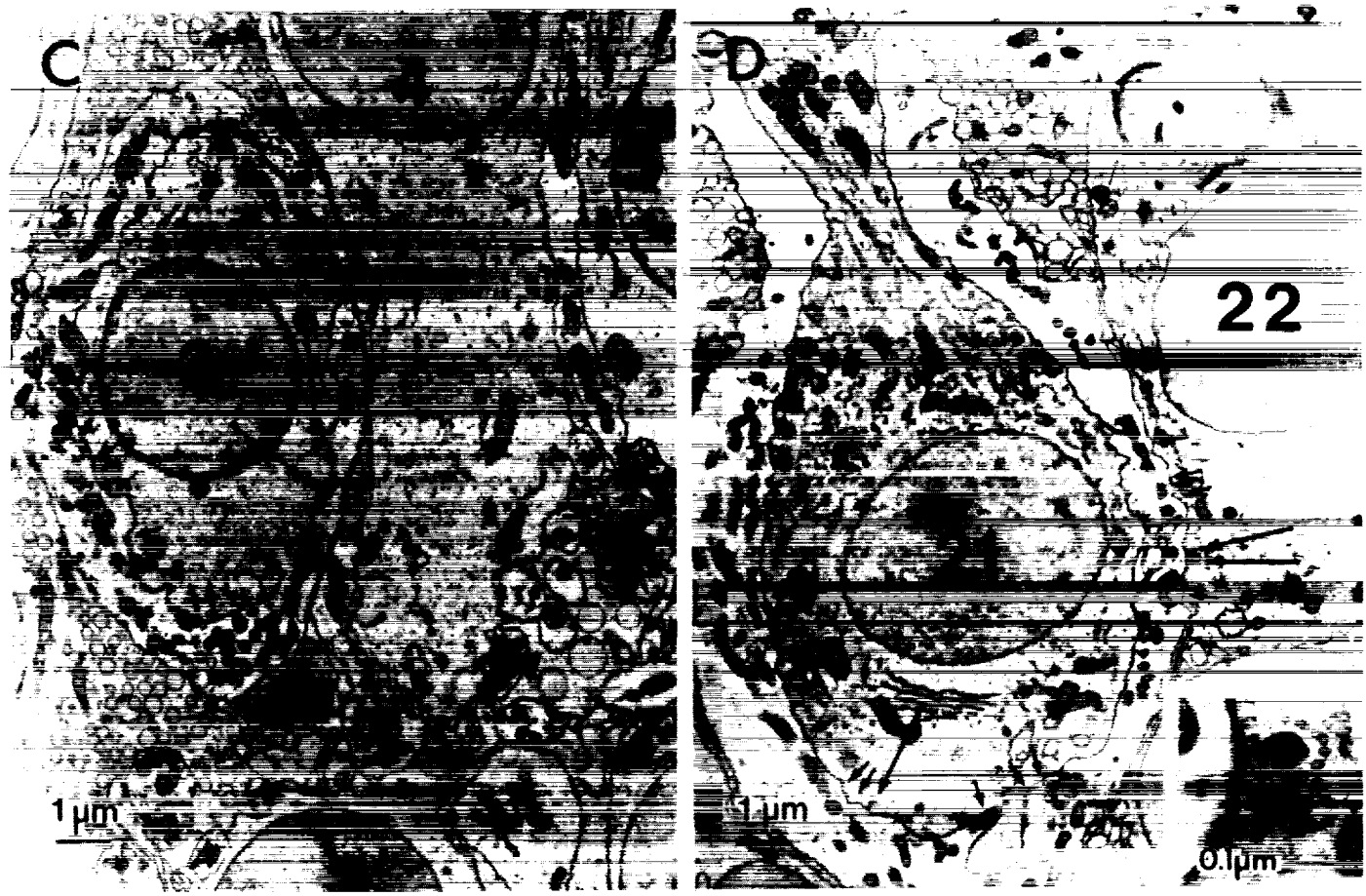


Figure 1, Panels A-D (pages 199 and 200). Electron micrographs taken from a superior canal crista in a normal adult pigmented chinchilla.

Panel A: Two type II hair cells (48, 49) in the peripheral zone that were serially reconstructed. The several boutons contacting each of them were numbered consecutively based upon their first appearance in the set of sections. Here and in the other panels, long arrows point to synaptic ribbons seen in the section, short arrows point to synaptic ribbons seen in nearby sections. Most peripheral boutons make only single ribbon synapses with their type II hair cells. Panel B: A serially reconstructed type II hair cell in the central zone. Bouton 1 makes 11 ribbon synapses, seven with cell 23 and four with cell 24, while bouton 2 receives 10 synapses from cell 23. Most of the ribbons appear in adjacent sections; the two seen in this section (long arrows) are shown at higher magnification in the inset. An efferent bouton (E2) synapses on cell 23 and is associated with a subsynaptic cistern (arrowhead).

TYPE I HAIR CELLS OF THE SUPERIOR CANAL CRISTA OF ADULT CHINCHILLA

ORIGINAL PAGE
BLACK AND WHITE PHOTOGRAPH



Panel C: A serially reconstructed type I hair cell (3) in the peripheral zone has eight synaptic ribbons. Most of these ribbons appear to be associated with calyceal invaginations; the asterisk marks a case where both structures are seen in the same section. An efferent bouton (E1) contacts the calyx ending at its base. Close appositions between type II hair cells and the outer surface of calyces are not common, but when they do occur, there are usually thin processes of supporting cells (arrowheads) interdigitating between them. Panel D: A type I hair cell in the central zone (21) has 14 synaptic ribbons. The calyx ending is also contacted by a type II hair cell (22) which contains six ribbons forming synapses with the outer face of the ending (arrows); the two synapses seen in this section of cell 22 (long arrows) are shown at higher power in the inset.

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REGULATION OF BONE REMODELING ACTIVITY THROUGH THE CONTROL OF STRESS GENERATED POTENTIALS

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Description of Research

The long-term objective of this research is to improve our understanding of the manner in which bone is able to sense the demands of its functional environment. The basis for this work lies in the electrophysiological properties of bone tissue. During normal functional activity, electrical currents are endogenously induced within bone. It is believed that skeletal tissue cells are sensitive to these currents, because connective tissue cells, of which bone cells are a derivative, are known to respond to electric currents similar in magnitude and frequency to those induced within bone. Moreover, electric fields exogenously induced into bone have been shown to modulate bone remodeling activity in the absence of normal functional loading. These observations have led us to suggest that an efficient mechanical loading protocol for the minimization or prevention of microgravity-induced bone loss might be developed by optimizing the endogenously induced electric currents.

The specific objective of this investigation was to define a loading paradigm for the maintenance of bone mass in the absence of normal functional loading. Our hypothesis was that a loading paradigm which ensures the endogenous generation of osteogenic electric fields will also maintain bone mass with a minimal mechanical work input. The investigation therefore required the isolation of the most osteogenic electric field component of functional strain, the establishment of the relationship between strain magnitude and induced field intensity, and the subsequent testing of the predicted mechanical loading paradigm.

The experimental work was based on the isolated avian ulna model of disuse osteopenia. In this model, the ulna of adult male turkeys is isolated by proximal and distal osteotomies such that the animal cannot apply functional loading to its ulna. Through the use of external magnetic coils, electrical fields mimicking those produced endogenously can be induced in the absence of functional loading. This permits the isolation of the fields which are most osteogenic. In addition, this preparation permits the recording of induced field intensities during controlled mechanical loading. Finally, the field characteristics and mechano-electric transduction measurements can be used to predict an appropriate mechanical loading protocol which can be subsequently tested in the isolated ulna preparation.

Accomplishments

(1) Initial work in this investigation was directed toward isolating the maximally osteogenic electric field characteristics. Through the application of broad spectrum electromagnetic fields, with subsequent theoretical analysis, we have isolated the regime of maximum electric field efficacy to the frequency range below 75 Hz. Using sinusoidal stimulation, we have shown that within this low frequency range, electric fields near 15 Hz demonstrate the greatest osteogenic effect. Calculations of the induced field intensity within the bone preparation show that fields as low as 10 microvolts/cm or less are sufficient to maintain bone mass at 15 Hz when applied for at least one hour per day.

(2) In order to associate these electric field parameters with mechanical strain, in vitro stress generated potential recordings were made in the ulna preparation under conditions of controlled mechanical loading. These recordings indicate that average field intensities on the order of 10 microvolts/cm can be induced in cortical bone with peak bone strains on the order of 10-100 microstrain.

(3) The prediction that 15 Hz mechanical strains, applied to bone for one hour per day, should be sufficient to maintain bone mass in the absence of any functional loading was tested in the isolated ulna preparation. As our mechanical loading apparatus cannot be regulated at strain levels below approximately 100 microstrain, we elected to test the predicted loading paradigm with a 250 microstrain load at 15 Hz for 5 minutes per day.

(4) Preliminary tests results suggest that very low level loading can indeed prevent the loss of bone mass due to disuse, if the loading is at an appropriately high frequency. ***While 1 Hz loading at 250 microstrain can be shown to result in up to a 11% loss of bone mass in eight weeks in the turkey, 15 Hz loading both prevented this loss, and initiated new bone formation.***

Significance of the Accomplishments

This work appears to indicate that bone senses and adapts to the relatively low amplitude but high frequency strains presented within its environment rather that the much larger amplitude but lower frequency components of bone strain. As the high frequency components arise predominantly from the dynamics of muscle activity, and not through the reaction loads associated with locomotion, these results suggest that microgravity-induced bone loss may be preventable without strenuous activity. However, to fully understand the normal maintenance of bone mass it will be necessary to specifically identify the origin of high frequency mechanical strains, the specific cell population which responds to this mechanical stimulus, and the effect of very long term controlled mechanical loading.

In the future, we will be pursuing each of these questions. Through *in vivo* strain gage, accelerometer, and electromyographic recordings we will be attempting to isolate the component within the muscular control system which gives rise to the high frequency strain within bone during normal functional activity. In *in vitro* studies we will be addressing the issue of whether the osteocyte population of bone is capable of responding directly to either the mechanical or electrical environment. We will also be continuing with the isolated ulna preparation in an effort to identify the minimum levels of strain necessary to maintain bone mass when loading durations are extended into the range of several hours per day. Finally, we will continue our use of theoretical models to address the more complex questions of the manner in which a mechanical strain signal is utilized in the adaptation of the morphology of bones.

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TEMPORAL EXPRESSION AND SPATIAL DISTRIBUTION OF THE MAJOR PROTEINS IN THE OTOCONIA OF TWO SPECIES OF VERTEBRATE

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Description of Research

This research is aimed at increasing our understanding of the function of the peripheral portion of the vestibular system found in the inner ear of vertebrates. Specifically, this research is interested in the evolution, chemistry, and function of a biomineralized structure found within the inner ear. The vestibular system has evolved to detect linear acceleration and gravity through sensory end organs, known as the maculae, through the interaction of extracellular mineralized structures with the underlying neuroepithelium. These biominerals, known as otoconia (literally "ear dust"), are embedded in a gelatinous mass known as the otoconial membrane. The otoconia are thought to increase the mass of the otoconial membrane thereby increasing the deflection of the apical projections on the underlying sensory cell, the hair cell, during accelerations.

Otoconia are composites of protein and mineral phases. The mineral phase varies within the vertebrates in a phylogenetic trend. Otoconia from some primitive fishes are mineralized by calcium carbonate deposited as vaterite. Reptiles and amphibians predominantly have otoconia mineralized by calcium carbonate in the crystal form of aragonite. The otoconia from birds and mammals contains calcium carbonate in the form of calcite. Each mineral type has a protein unique to that type of otoconia. This research has focused on the proteins contained within the otoconia of the rat (calcitic) and the African clawed frog, *Xenopus laevis* (aragonitic). Little is known about the nature of these proteins, save their molecular weights. The calcitic rat otoconial protein is M_r=90k and the aragonitic is M_r=22k. The cells of origin, developmental expression, or turnover of the proteins are all unknown. In addition nothing is known about interactions of these proteins with their mineral component. Understanding the structure of these proteins will enhance our knowledge of the development and evolution of the mammalian vestibular system, and will serve as a model system for the field of biomineralization.

I have begun to investigate the otoconial proteins using both immunological/histochemical and molecular biological approaches. My plan is to isolate the proteins from the otoconia of the rat and the clawed frog, raise antibodies against them, and sequence the proteins. The antibodies will be used for immunohistochemistry and to screen cDNA libraries constructed from inner ear tissues. In addition, the amino acid sequence will be used to synthesize oligonucleotide probes for screening the libraries. The clones coding for the proteins will be used to generate in situ hybridization probes and to construct expression clones for the production of the proteins in large amounts. Both the cDNA probes and the antibodies are to be used to study the development and evolution of otoconia. These approaches will enable me to answer the basic questions of the temporal and spatial expression of these proteins. In addition, they will allow the further characterization of these proteins and their interactions with the mineral phase using in vitro approaches.

Accomplishments

We have ***determined essentially the entire amino acid sequence of the 22 kD otoconial protein from Xenopus laevis*** using a combination of mass spectrometry and classical Edman degradation to analyze purified peptide fragments of the protein (see Figure 1). In addition, a homology search against the NBRF protein data bank has shown the N-terminal fragment of the otoconial protein has great sequence homology with the phospholipase A2. This has given us insight into the manner in which the protein should be treated prior to proteolytic digestion for sequence determination. Since the phospholipases have seven disulfide crosslinks, we have increased the amount of dithiothreitol used for reduction. This greatly enhanced the trypsin, CNBr, Asp-N, and Lys-C cleavages and allowed for determination of nearly the entire amino acid sequence (Figure 1). The entire sequence aligns with the phospholipase A2 (for example see Figure 2). We are currently mapping the disulfide linkages in this protein, to see if the linkages are similar to those of the phospholipase A2.

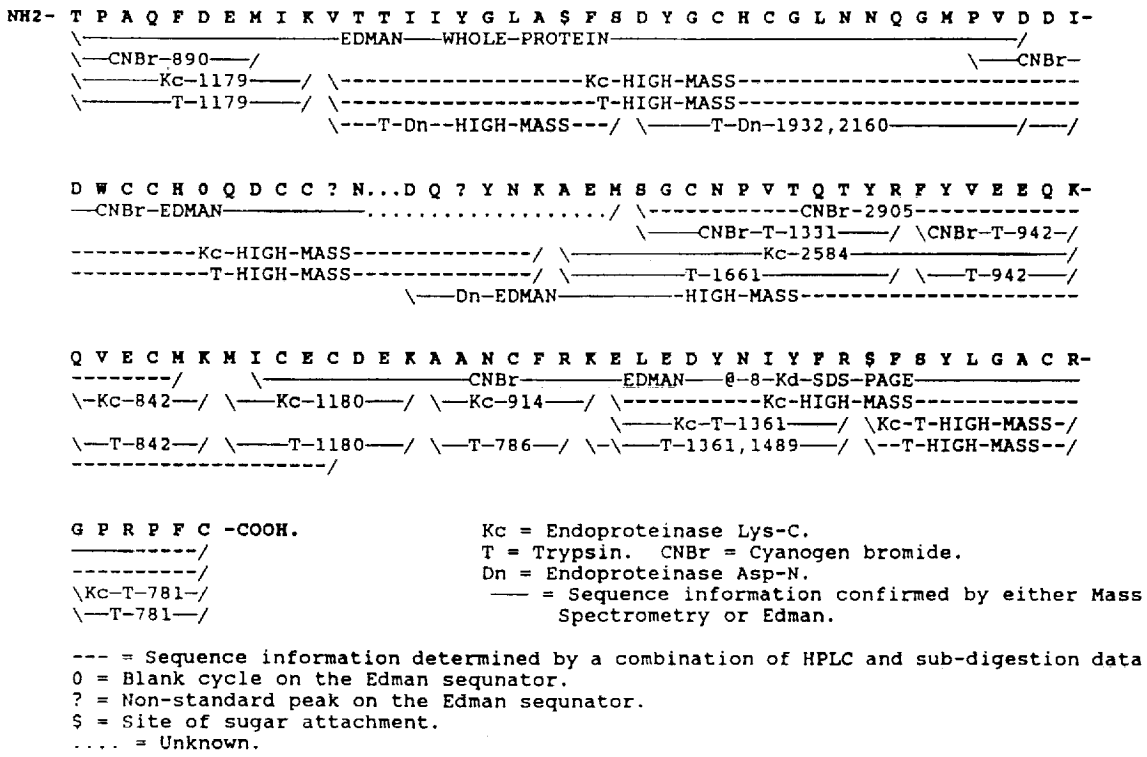

Figure 1. The amino acid sequence of the 22 kD aragonitic otoconial protein from *Xenopus laevis* aligned with the sequence of a phospholipase A2 (ammodytoxin A). The single letter code for the amino acids is used. Identical residues are identified by a colon, while conservative replacements are marked by periods. The asterisks are residues which coordinate calcium ions within the proteins of the phospholipase A2 family. These residues are within the calcium binding loop which have the invarient residues marked by I. The question marks have been identified as sites of glycosylation. There is 38% identity between these sequences.

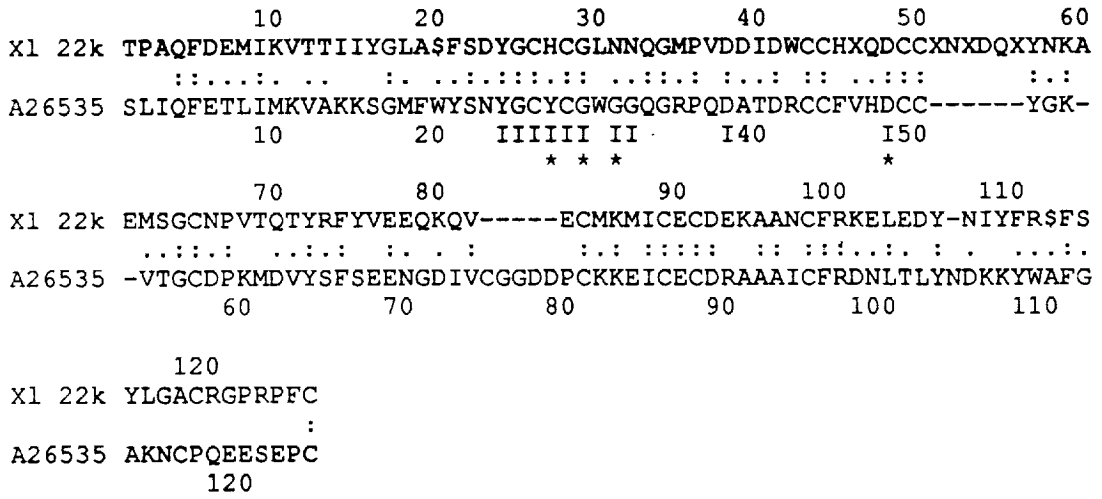

Figure 2. An alignment of the major protein from *Xenopus laevis* aragonitic otoconia with phospholipase A2 from viper toxin. The single letter amino acid abbreviations are used for the sequences. The colons indicate identical residues, while the periods are conservative replacements. The I's indicate invariant amino acids in the calcium binding domain of the phospholipases A2; Y27, G29, G31, and D48 (indicated by asterisks) are involved in the calcium coordination. Loss of D48 results in loss of activity of phospholipase A2. The dollar signs are glycosylation sites in X1 22 kD. The phospholipases A2 are not glycosylated. Dashed lines are insertions made by the alignment program. The sequences have 38% sequence identity.

The crystal structures for several phospholipases A2 have been solved. A model of the *X. laevis* otoconial protein was built using the published coordinates for the carbon backbone of the western diamondback rattlesnake (*Crotalus atrox)* venom phospholipase A2 by altering the amino acid side chains to those of the otoconial protein using the University of California, San Diego, Molecular Modeling System software. The resulting structure was put through 160 cycles of minimization using the X-plor program. The resulting structure was then compared to the otoconial protein before minimization and compared to the *C. atrox* phospholipase A2. All of the changes in amino acid composition could be accommodated by the backbone structure of the phospholipase A2.

We have started the carbohydrate analyses of the *Xenopus laevis* 22 kD otoconial protein. We have HPLC purified the peptides containing the glycosidic modifications to analyze the carbohydrates present in each side chain. The amount of sugar present in the protein and each glycopeptide is shown in Figure 3. We estimate that this protein may contain as much as 7000 molecular weight, or 34% carbohydrate.

Whole Protein Sugar Analysis

Sugar	Raw Data	#	Incremental M.W.	Weight in Protein
Fuc	1.9	2	147	294
GalNc	2.6	3	204	612
GlcNc	13.1	13	204	2652
Gal	8.7	9	163	1467
Man	6.8	7	163	1141
NeuAc	2.8	3	291	873
				7039

Percentage of sugar in protein = 7,039/21,000 = 34%

GLYCOPEPTIDE ANALYSIS

	COOH end #1	COOH end #2	NH2 end	Total
Fucose	1 / 1 / 1	1 / 1 / 1	1 / 1 / 1	2
GalNac	1?	1?	1?	2?
GlcNac	3.1/4.6/ 4	3.8/5.6/ 4	4 / 8 / 6	10
Galactose	2.4/2.6/ 3	2.6/2.9/ 3	3.1/5.2/ 4	7
Mannose	2.2/ 3 / 3	2.7/ 3 / 3	4.5/7.9/ 6	9

Figure 3. Summary of carbohydrate analyses of the intact *Xenopus laevis* otoconial protein (above) and the HPLC purified glycopeptides. The glycopeptide near the carboxy-terminus was purified twice for analysis. The glycopeptide near the nitroxy-terminus was purified once. All samples were run in duplicates. The three numbers for the glycopeptide analyses are the first run, the second run and their whole number average. N-acetyl-neuraminic acid (NeuAc) was not determined for the glycopeptides. Abbreviations are Fuc=fucose, GalNc=N-acetyl-galactosamine, GlcNc=N-acetyl-glucose, gal=galactose, man=mannose. There is general agreement in the total sugar present and that present in each glycopeptide.

Significance of the Accomplishments

Having determined that the entire amino acid sequence is significant because it allows use of the polymerase chain reaction for amplification of the cDNA coding for the protein, the homology search to the phospholipase A2, based on this sequence, has given insight into the methods required to solve the problems of limited hydrolysis by the proteolytic methods. This homology may also give insight into the evolutionary origins of the otoconial proteins. Evidence that this otoconial protein is not a phospholipase A2 per se, are the glycosylation (the phospholipases A2 are not glycosylated) and the modification of the calcium binding loop. The residues invariant in the calcium binding loop of the phospholipase A2 are shown in Figure 2. Four amino acids, tyrosine 27, glycines 29 and 31, and aspartic acid 48 coordinate the calcium ion. The first three residues are different in the otoconial protein. This suggests that the interaction of this protein with the calcium carbonate mineral phase in intact otoconia is not through calcium binding, per se. The model construction is a first step in determination of the interactions between the mineral and organic phases of otoconia.

This is the first demonstration of the type of glycosylation of an otoconial protein. It is often assumed that proteins of the otoconial matrices are glycosaminoglycans. The

carbohydrate analyses show that this is not true. ***The carbohydrates present show this protein is a glycoprotein.*** The sequence within the glycopeptide, X-Phe Ser, indicates it is likely a N-linked oligosaccharide. More important for our proposed use of the expressed protein for determination of the structure and interaction with the mineral phases, this shows we must use an approach which will glycosylate the expressed protein.

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PLANT HORMONE CATABOLISM AND TROPICALLY INDUCED ASYMMETRIC GROWTH

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Description of Research

Plant hormones play important roles in terrestrial plant growth and development including roles in cell division, cell elongation, and differentiation of cell tissues. The plant hormone indole-3-acetic acid (IAA) is rapidly redistributed to the lower faster-growing side of a shoot following a gravity stimulus. The long-range goal of this research is to understand how gravity induces a hormone/IAA asymmetry, and how microgravity may alter hormone metabolism and action and thus affect plant growth and development.

This research has examined IAA catabolism, an important regulatory control point in IAA metabolism which may contribute to the rapid hormone asymmetry. In corn, *Zea mays* L., I have discovered a new pathway for IAA catabolism with the oxidation of IAA to oxindole-3-acetic acid. This pathway is an irreversible oxidation of IAA resulting in loss of IAA/hormone activity. The regulation of this pathway may help maintain IAA growth-limiting in actively growing tissues by destroying the hormone following growth induction. The current research has examined the mechanism and developmental occurrence of the oxindole-3-acetic acid pathway.

Accomplishments

Accomplishments include the further characterization of the IAA to oxindole-3-acetic pathway: (1) indole-3-acetic acid oxygenase can be separated from peroxidase/IAA oxidase and lipoxygenase, (2) this pathway occurs in all corn tissues and developmental stages examined. The *in vitro* oxygenase assay depends on a lipid soluble cofactor which can be substituted by fatty acids and corn oil. The present study provides evidence that ***IAA oxidation to oxindole-3-acetic acid is assisted by a novel enzyme present in etiolated and light-grown tissues.***

Significance of the Accomplishments

This is the first *in vitro* study of this pathway. The enzyme indole-3-acetic acid oxygenase has been shown to be a novel enzyme which can be separated from peroxidase and lipoxygenase enzymes by ion exchange chromatography and gel filtration chromatography. The enzyme is stimulated by a heat-stable lipid-soluble cofactor which can be replaced by unsaturated fatty acids in the oxidation of IAA. In the regulation of the IAA oxygenase during gravitropism (as well as during vertical growth), the activity of the enzyme may be regulated by the availability of the cofactor. The natural cofactor needs to be unambiguously identified and quantified to determine how it affects IAA oxygenase activity. Unrefined corn oil has been shown to stimulate IAA oxidation and may be used in the isolation and characterization of the natural lipid-soluble factor. The mechanism of the oxidation reaction can be examined with the purified enzyme and the following questions asked: Is the cofactor co-oxidized during IAA oxidation? If so, does the oxidized lipid cofactor have biological activity? Measuring the turnover rate of IAA to oxindole-3-acetic acid, measuring IAA oxygenase activity, and measuring cofactor availability will clarify how catabolism down-regulates IAA activity during growth. The separation of interfering

enzymes from the IAA oxygenase and identification of the natural cofactor makes this research feasible.

Until recently, IAA levels were thought to be regulated by decarboxylation by peroxidase (IAA oxidase). This work with corn (and work by others with broad bean, scots pine, and poplar) has demonstrated that IAA can be catabolized without decarboxylation. This research has confirmed that IAA oxidation to oxindole-3-acetic acid occurs without the involvement of peroxidase, a nonspecific oxidase. Furthermore, the enzyme has been shown to occur in young green seedlings as well as in etiolated seedlings. The pathway occurs at two distinct developmental stages, supporting our hypothesis that catabolism is important in plant hormone regulation.

The basic understanding of how gravity induces a hormone asymmetry and how microgravity affects normal plant development by altering hormone metabolism and action will likely have practical benefits in our attempts to optimize plant growth in microgravity.

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CHANGES IN CELL WALL POLYMER SIZE AS INDICATORS OF GRAVITROPIC RESPONSE MECHANISMS

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Description of Research

We intend to use structural changes in the cell walls of gravitropically active tissue to understand the sensory and signal transduction mechanisms by which plants respond to gravity. The structural properties of the plant cell wall are the ultimate determinant of the rate of elongation. The length and/or degree of association of the carbohydrate polymers composing the plant cell wall are likely to be a critical factor determining these properties. Substantial progress has been made in understanding the changes in wall polymers during growth responses, although the system is quite complex and we are far from a complete understanding of the complexities and interactions of the system.

Our approach is to determine the extent and location of growth changes along a gravitropically responding stem. By then isolating and analyzing wall components from this stem at various times during curvature, we can correlate molecular size or compositional changes in cell wall structure with the growth response. By analogy to known effects and through experimentation, we will determine the likely biochemical mechanisms controlling these structural changes. Knowledge of the final biochemical action(s) resulting in growth control will provide a tremendous advantage for investigating the signal transduction chain of gravitropism, to say the least. Ultimately it may be possible to trace the chain of events from final biochemical action all the way back to the initial sensory event.

Accomplishments

(1) We developed an HPLC system for the molecular size analysis of soluble cell wall polymers and coupled it to an extremely sensitive electrochemical carbohydrate detector.

(2) In pea stem, the maximum gravitropic curvature takes place between 20 and 60 minutes after horizontal placement and involves reduction of elongation on the lower side and stimulation of elongation on the upper side. ***Polymers of the hemicellulose wall fraction undergo a reduction in size on the lower side and an increase in size on the upper side of the stem correlated with changes in elongation rates.***

(3) Monosaccharide composition analysis indicates that xyloglucan polymers account for most of the size changes seen in the hemicellulose fraction.

(4) ***Polysaccharide size changes occur primarily in the epidermal layer of the stem.***

(5) ***Electron microscopy of fractionated polymers indicates that size increases of xyloglucan are due to formation of a crosslinked network. Preliminary studies indicate that formation of this network may be pH and ion dependent.***

Significance of the Accomplishments

Finding #1. Until now, polymer size analysis has been done on low pressure chromatography columns requiring as long as 24 hr per separation. Recent advances in HPLC column technology now permit similar size separations in 1 hr with better resolution. When coupled with new electrochemical carbohydrate detectors, this system permits practical analysis of polymer size distributions in small amounts of tissue, such as individual stem segments or collections of epidermal peels.

Finding #2. Previously, decreases in hemicellulose polymer size have been correlated with hormonal (IAA) stimulation of growth and with pH reductions such as those stimulated by IAA. Increases in hemicellulose size are found in situations where cell wall extensibility should decrease and occur as a response to sudden turgor pressure increases. Both types of changes occur during gravitropic bending and may indicate the operation of at least two regulatory mechanisms in the gravity response.

Finding #3. Metabolic and turnover studies indicate that xyloglucan is the most active of the dicot hemicellulose polymers during growth. The auxin and turgor responses mentioned above affect primarily the size of xyloglucan polymers. We now confirm that polymer size changes in gravitropic growth regulation again involve primarily xyloglucan polymers.

Finding #4. Much indirect evidence has focused attention on the extensibility of the epidermis as the controlling factor governing overall extensibility of the plant stem. Our work confirms that the majority of observed wall size changes occur in the outer few cell layers of the gravitropically responding stem. These results focus the search for gravitropic mechanisms to actions involving primarily the epidermal tissue.

Finding #5. The existence of pH and ion dependent molecular size changes in wall polymers tends to support the emerging picture of the cell wall as a non-covalently linked network as opposed to a covalent structure requiring enzymatic action for alteration. Visualization of this network should permit direct *in vitro* studies of the biochemical actions responsible for changes in wall structure. Although enzymatic action is not excluded, our results encourage investigation of the role of ion movements and auxin-induced pH effects in gravitropism (Figure 1).

Publications

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POSSIBLE ROLE OF ION MOVEMENTS DURING GRAVITROPIC BENDING

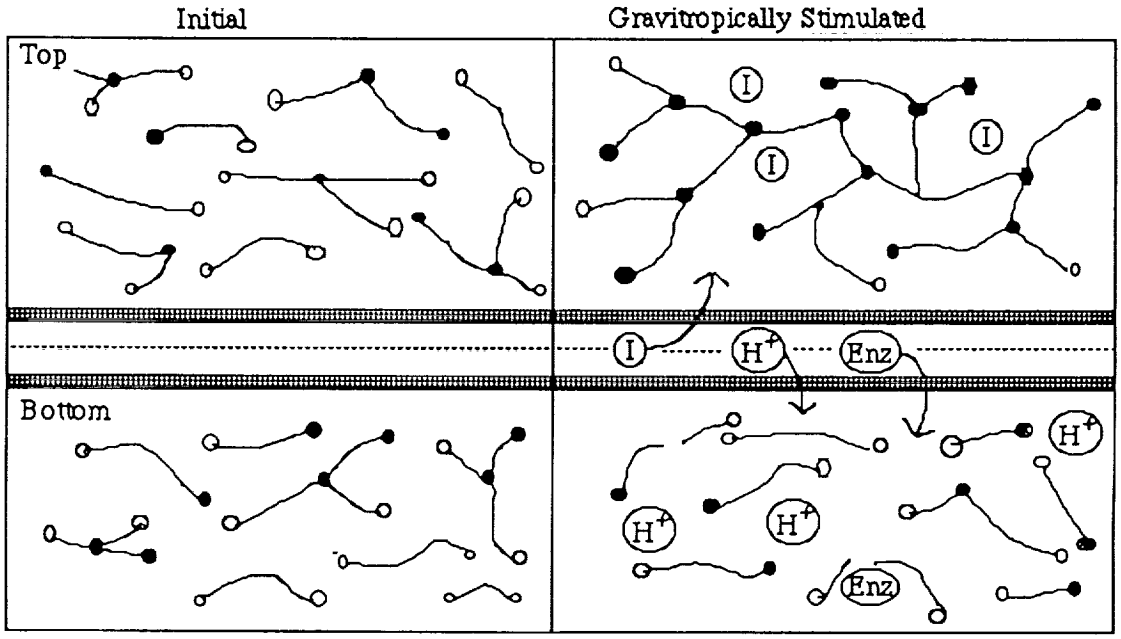

Figure 1. Possible mechanisms for controlling wall extensibility during gravitropic bending. Wall polymers in the initial state form an intermediately crosslinked network with filled • and unfilled o ionic binding sites. Gravitropic stimulation causes release of protons and/or enzymes on the bottom side which reduce the extent of crosslinking. Ions may be transferred to the upper side, causing increased crosslinking and slowing growth.

MECHANISMS OF DECREASED ACTIN SYNTHESIS DURING RODENT NON-WEIGHTBEARING

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Description of Research

The purpose of this research is to define the mechanisms of skeletal muscle atrophy in a non-weightbearing environment. The ground-based rodent hindlimb non-weightbearing protocol has been used as a model for the atrophy of human skeletal muscle that occurs during spaceflight. This protocol has a profound effect on the weightbearing muscles of the hindlimb, causing rapid atrophy of the soleus muscle. Previous work by me has shown that one of the most rapid responses of the soleus muscle is a large decrease in contractile protein synthesis within a few hours of the onset of non-weightbearing; in these few hours there is no change in the availability of components with which the muscle can synthesize protein.

The hypothesis of this work is that soleus muscle actin protein synthesis rate rapidly decreases during hindlimb non-weightbearing as a result of alterations in nascent protein elongation and/or initiation of protein synthesis. To test this hypothesis, I proposed experiments to measure the relative concentration of α-actin mRNA in the polysome pool, and the electrophoretic size profile of the polysomes containing the α-actin mRNA.

Accomplishments

(1) A novel analytical technique was developed to evaluate skeletal muscle polysome profiles. This technique allows simultaneous evaluation of nascent polypeptide elongation and protein synthesis initiation.

(2) ***Using this technique, the rapid downregulation of protein synthesis that occurs at the onset of non-weightbearing is accompanied by a shift of polysome size toward larger polysomes*** (more ribosomal subunits per mRNA).

(3) ***Using cDNA probes for α-actin mRNA, the mRNA for this protein is associated with the larger polysomes.*** The overall amount of the mRNA associated with the polysomes does not change significantly.

Significance of the Accomplishments

(1) Development of this analytical technique provides a sensitive means for evaluating the earliest changes that occur during muscle atrophy. This will be essential for a timely and cost-effective evaluation of countermeasures to the effects of weightlessness.

(2) The observation of larger polysomes associated with the rapid downregulation of protein synthesis in non-weightbearing skeletal muscle was surprising. The significance of this finding is that ***there is a direct coupling of mechanotransduction to the control of gene expression in muscle at the level of translation.***

(3) The association of α-actin mRNA with the larger polysomes indicates that this mechanism is active (and may even be unique) for the contractile proteins. The increased size of the polysomes is consistent with a slowing of nascent polypeptide elongation rate through some, as yet, undefined mechanism. The lack of a significant change in content of α-actin mRNA on the polysomes indicates initiation of protein synthesis is not a site of regulation in the rapid decrease in soleus muscle protein synthesis.

Publication

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NASA GRADUATE STUDENT RESEARCHERS PROGRAM

In 1980, NASA initiated the Graduate Student Researchers Program (GSRP) in order to cultivate additional research ties to the academic community and to support promising students pursuing advanced degrees in science and engineering. Since then, approximately 800 students have completed the program's requirements while making significant contributions to the nation's aerospace efforts. Universities have also benefitted through strengthening their research capabilities.

Each year, NASA selects approximately 80 new students for the opportunity to receive stipends and to work at the unique national laboratories of the NASA facilities or at their home universities. Awardees are selected based on competitive evaluation of their academic qualifications, their proposed research plan, and their planned use of NASA research facilities. Fellowships are awarded for one year and are renewable, based on satisfactory progress, for up to three years.

The Graduate Student Researchers Program is managed at NASA Headquarters by the University Programs Branch, Educational Affairs Division. Forty of the 80 new awards each year are sponsored by the NASA Headquarters Office of Space Science and Applications (OSSA) in the fields of life sciences, astrophysics, earth sciences, solar system exploration, and microgravity science and applications. OSSA fellows carry out their research or a plan of study at their home universities. Each year they attend a two- or three-day annual symposium at NASA Headquarters in Washington, D.C. The symposium provides an opportunity for GSRP fellows to exchange ideas, discuss progress, and learn more about space science and applications at NASA.

The remaining 40 new awards are distributed throughout the NASA field centers. Fellows selected by centers must spend some time in residence at the center, taking advantage of the unique research facilities of the installation and working with center personnel.

Of the awards currently sponsored by the Headquarters Office of Space Science and Applications, thirteen are in the space biology and biomedical research areas. These awardees' abstracts, which were compiled from the May 1990 annual symposium in Washington, D.C., are included in the following pages.

Further information about the OSSA Graduate Student Researchers Program may be obtained from: Mr. Joseph K. Alexander, Assistant Associate Administrator for Space Science and Applications, Code E, NASA Headquarters, Washington, D.C. 20546. Mr. Gary Gans or Mr. John Lynch, University Programs, Educational Affairs Division, Code XEU, NASA Headquarters, Washington, D.C. 20546, may be contacted for further information about the 40 GSRP fellowships to conduct research at NASA facilities and centers.

THE DEVELOPMENT OF A GENETIC SYSTEM FOR THE DISSECTION OF THE GRAVITROPISM PATHWAY IN *ARABIDOPSIS THALIANA*

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Gravitropism was described by Charles Darwin over 100 years ago; yet, the mechanisms of the response in higher plants remain unknown. In an effort to elucidate some of these mechanisms, a direct screening procedure for the isolation of gravitropism mutants in *Arabidopsis thaliana* was developed. Sixty mutant lines in four phenotype categories were isolated. Some of these mutant lines exhibited different gravitropism phenotypes in the root and the hypocotyl, supporting the hypothesis that some elements of the gravitropism pathway are organ-specific. Additional screening of the gravitropism mutants permits the examination of specific models for gravitropism, and the examination of the interaction of gravitropism with other sensory pathways. Screening with iodine-potassium iodide (IKI) showed that three of the 60 lines were also altered in starch content, allowing examination of the role of starch in gravitropism. Tests for phototropism revealed that some of the mutant lines were altered in their phototropic response, supporting the hypothesis that phototropism and gravitropism share some elements of signal transduction. Since the direct screening procedure avoids the bias inherent in procedures based on particular models for gravitropism, it is expected that these 60 mutants represent lesions at various steps throughout the entire gravitropism pathway.

EXPRESSION OF CELLULAR STRESS PROTEINS IN TESTIS AND EMBRYO

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The long-term goal of this project is to assess the effects of spaceflight on mammalian germ cell development and early embryogenesis at the molecular level. We are examining the expression of cellular stress protein genes, also known as heat shock protein genes (hsp). These genes serve as molecular markers of both normal mammalian cellular differentiation and the cellular response to stress. Our study has focused on the characterization of the hsp90 gene family expression in the murine testis and embryo. Two different hsp90 transcripts were detected in the mouse testis using two human hsp90 cDNA probes. The testicular transcripts were approximately 3.2. kb and 2.9 kb in size and exhibited cellular and developmental stage specificity of expression. The larger, more abundant transcript was expressed at high levels in the germinal compartment of the testis, particularly in germ cells in meiotic prophase. The smaller hsp90 transcript is expressed predominantly in the somatic compartment of the testis. Expression of two hsp90 transcripts was seen in the testis of other species, further supporting an important role for hsp90 in mammalian testicular function. In addition, expression of both hsp90 transcripts was detected in the embryonic and extra-embryonic compartment of midgestation embryos. These observations indicate that the hsp90 gene family is under developmental regulation in the mouse testis and midgestation embryo and that it will be useful as a sensitive molecular indicator of cellular stress.

EFFECTS OF LOADED, ISOTONIC MUSCLE CONTRACTIONS ON SKELETAL MUSCLE MASS AND BLOOD FLOW IN THE CONSCIOUS, UNLOADED RAT

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Cardiovascular and musculoskeletal systems are adversely affected when the body is removed from gravitational forces, as during spaceflight. In order to prevent muscle atrophy and cardiovascular deconditioning, both of which would hinder long-term spaceflight, adequate animal models need to be investigated and used to evaluate a variety of countermeasures to these adverse effects of spaceflight. In comparing unloaded isotonic, isometric, and loaded isotonic muscle contractions, a series of studies has determined high resistance, isotonic muscle exercise provides the greatest benefit in preventing muscle atrophy induced by hindlimb unloading. The purpose of current investigations is to study the effects of muscle substrate delivery and metabolic waste removal on atrophy and atrophy prevention on individual skeletal muscles. Initially, these studies have evaluated muscle blood flow (MBF) in the conscious animal. Untrained (UT), hindlimb unloaded control soleus (SOL) muscles significantly atrophied 33%, while plantaris (PLT) and gastrocnemius (GAST) muscles atrophied 9% and 8%, respectively. Loaded, isotonic training (T) resulted in full prevention of PLT and GAST muscle atrophy but only reduced SOL muscle atrophy by 10%. Absolute blood flow (ml/min) in atrophied muscles declines to a greater degree than would be predicted by the degree of muscle atrophy. SOL blood flow declined 68% following disuse while PLT and GAST flows decreased by 24% and 53%, respectively. Prevention of muscle mass appears to reduce alterations in MBF to these individual muscles. PLT and GAST blood flow declines in T hindlimbs were reduced by 88% and 67% from UT values, respectively. Relative muscle blood flow (RBF) (ml/min/100g tissue) demonstrated that not only muscle fiber type but muscle function determines the distribution of blood flow in the hindlimb. Muscles are composed of three fiber types: (1) slow contracting, oxidative, and highly fatigue resistant (SO); (2) fast twitch oxidative glycolytic (FOG) fibers which are fatigue resistant; and (3) fast glycolytic (FG) fibers with low fatigue resistance. Rodent SOL muscles, composed mostly of SO fibers, had significantly lowered RBF in both UT (-43%) and T (-44%) animals. PLT muscles composed of FG (41%) and FOG (59%) fibers, had increased RBF in UT (22%) and T (29%) hindlimbs. GAST muscles, composed of both FG (38%) and FOG (58%) twitch fibers, had decreased RBF in UT (-31%) while increasing 8% in T animal hindlimbs. These two muscles (PLT and GAST) are very similar in fiber composition, but dramatically different in blood flow distribution in both T and UT muscles. In summary, this study demonstrated the beneficial effects of highly resistant isotonic muscle exercise on atrophy prevention, and provided insight into differences in blood flow distribution between unloaded and loaded hindlimb muscles. This altered distribution may be a mechanism by which substrate delivery or metabolic waste removal may influence muscle mass during prolonged periods of disuse. The time course of distribution changes is important and would combine a potential mechanism if blood flow decreases prior to mass changes. These studies are presently underway.

EFFECT OF PHOTOPERIOD AND LIGHT INTENSITY ON BODY TEMPERATURE AND ACTIVITY RHYTHMS IN RHESUS MONKEYS

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Circadian rhythms of diverse physiological, behavioral and biochemical parameters are synchronized with environmental cues. Temporal changes in the environment often result in physiological and behavioral alterations, but these effects are not fully understood. This study examined the effects of light on the circadian rhythms of body temperature and physical activity. Four adult male Rhesus monkeys were studied at two light intensities, 200 and 1500 lux, in three different 24 hour light/dark cycles: LD12:12, LD14:10, LD16:08. Deep body temperature and activity were recorded via telemetry. At 1500 lux the mean body temperature was higher, the rhythm amplitude was larger, and the phase delayed compared with 200 lux. At both light intensities in longer photoperiods, mean temperature was elevated, the temperature rhythm amplitude increased and the phase delayed. Responses of physical activity were more variable than body temperature. Mean activity and rhythm amplitude were lower, and the phase delayed with increasing light intensity and photoperiod. We conclude that the circadian timekeeping system of the Rhesus monkey is sensitive to changes in light intensity and photoperiod.

A METHOD FOR STUDYING TRACHEARY ELEMENT DIFFERENTIATION AT SINGLE CELL RESOLUTION

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The differentiation of the unique plant cell type, the tracheary element, has long been considered a model system for the study of cellular differentiation in plants. In order to study cytoplasmic events occurring in the course of tracheary element differentiation, culture techniques were developed which permit continuous and high resolution observations of differentiating cells. Isolated mesophyll cells of *Zinnia elegans,* induced to differentiate as tracheary elements, were attached to glass cover slips and subsequently cultured in a Sykes-Moore chamber. Differentiating cells were observed with an inverted microscope equipped for video enhanced and differential interference contrast microscopy. Cells treated in this way completed differentiation within 96 hours.

EXERCISE INDUCED GLYCOGEN DEPLETION IN SELECT RAT HINDLIMB MUSCLES AFTER TWO WEEKS OF HINDLIMB UNLOADING

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This study was designed to measure the degree of glycogen depletion of different rat hindlimb muscles of various fiber type compositions with intense exercise after exposure to hindlimb unloading simulated weightlessness (SW). Adult male Sprague-Dawley rats (250-320g), familiarized with treadmill running were assigned to SW, hindlimb unloading with exercise (SW-E), cage control (CC) or cage control with exercise (CC-E) groups. The Overton-Tipton head-down unloading (45^{o}) model in which hindlimbs are non-weight bearing was utilized. After 14 days of SW or control conditions, the SW-E and CC-E groups were exposed to five 10-minute bouts of treadmill exercise at 85-90% of maximal oxygen consumption with 2-3 minute rest periods between bouts. Results for muscle glycogen concentration (mg/g) immediately after exercise or without exercise were as follows (means ± SE; n = 9-10; *indicates a value significantly different from the no exercise condition, †indicates a value significantly different from the corresponding control condition, $p < 0.05$):

	CC	CC-E	SW	SW-E
Soleus (SOL)	3.73 ± 0.42	1.81 ± 0.42*	6.15 ± 0.66†	2.75 ± 0.35*
Plantaris (PL)	4.91 ± 0.48	2.30 ± 0.43*	7.67 ± 1.01†	1.43 ± 0.31*
White Gastrocnemius (GW)	5.07 ± 0.28	3.32 ± 0.50*	5.44 ± 0.49	1.09 ± 0.32*†
Extensor Digitorum Longus (EDLP)	5.21 ± 0.53	2.44 ± 0.47*	6.58 ± 0.69	1.75 ± 0.30*

Hindlimb unloading resulted in the atrophy of the SOL (14%) and PL (12%) muscles, but not the EDL. Glucose-6-phosphate (G-6-P) concentration was measured for the SOL and GW; but, the only significant difference observed was an increase in SOL G-6-P in SW-E rats 30 minutes after exercise when compared to SW. These results indicate that resting glycogen concentrations were significantly elevated in hindlimb muscles that exhibited atrophy during SW. Furthermore, exercise induced glycogen depletion per mg of muscle is significantly greater after SW in the PL which consists predominantly of fast oxidative-glycolytic and fast glycolytic fibers, and the fast glycolytic GW and EDL muscles, but not in the slow oxidative SOL.

Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
NASA TM-4258		

4. Title and Subtitle
1989-90 NASA Space Biology Accomplishments

5. Report Date
February 1991

6. Performing Organization Code
SBR

7. Author(s)
Thora W. Halstead, Editor

8. Performing Organization Report No.

10. Work Unit No.

9. Performing Organization Name and Address
Space Biology Program, Life Sciences Division, NASA Office of Space Science and Applications and The George Washington University, Science Communication Studies, Washington, DC 20006

11. Contract or Grant No.
NASW-4324

13. Type of Report and Period Covered
Technical Memorandum

12. Sponsoring Agency Name and Address
National Aeronautics and Space Administration
Washington, DC 20546

14. Sponsoring Agency Code

15. Supplementary Notes
For previous edition, see NASA TM-4160

16. Abstract

This report consists of individual technical summaries of research projects of NASA's Space Biology Program, for research conducted during the period May 1989 to April 1990. This program is concerned with using the unique characteristics of the space environment, particularly microgravity, as a tool to advance knowledge in the biological sciences; understanding how gravity has shaped and affected life on Earth; and understanding how the space environment affects both plant and animal species. The summaries for each project include a description of the research, a list of the accomplishments, an explanation of the significance of the accomplishments, and a list of publications.

17. Key Words (Suggested by Author(s))
gravity
weightlessness
space biology
animal
plant
simulated weightlessness
life sciences
hypogravity
microgravity
hypergravity

18. Distribution Statement
Unclassified - Unlimited

Subject Category 51

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of pages	22. Price
Unclassified	Unclassified	236	A11

NASA FORM 1626 OCT 86 Available from the National Technical Information Service, Springfield, VA 22161

*U.S. GOVERNMENT PRINTING OFFICE: 1991-527-064/26012