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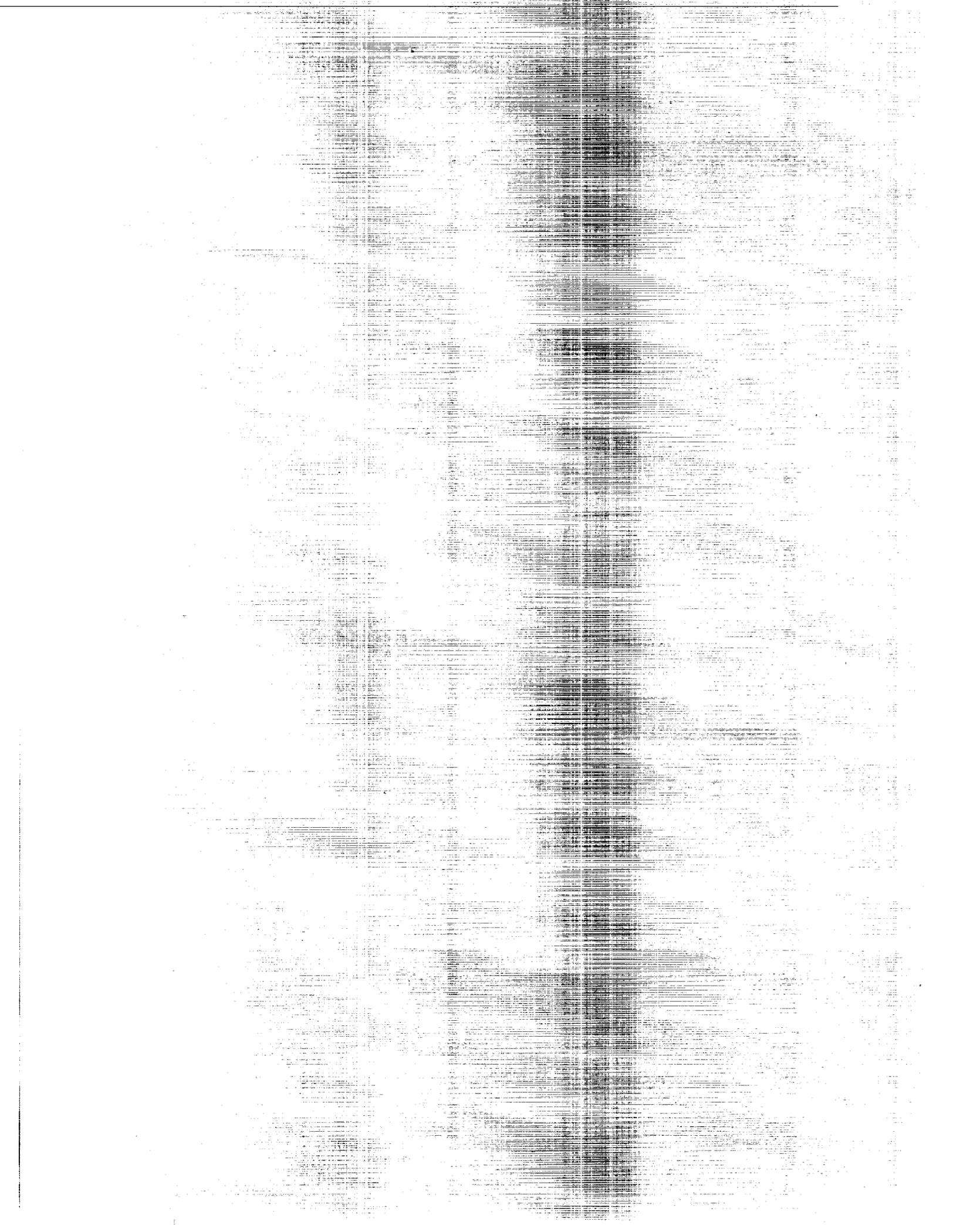
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1990–1991 NASA Space Biology Accomplishments

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1990–1991 NASA Space Biology Accomplishments

Edited by
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PREFACE

An individual technical summary of each research task within the NASA Space Biology Program for the period May 1990 through May 1991 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 activities of this related research are integrated in the document. Also included are reports on activities related to gravitational biology conducted under the auspices of the NASA Innovative Research Program, which seeks to encourage creative, unique approaches to research by funding innovative research projects. 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 Elizabeth L. Hess, Janet V. Powers, and Katherine J. Dickson 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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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 of one down to almost zero, effectively providing the full spectrum of gravity for prolonged periods of time 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.

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 microgravity of the space environment as a tool to advance fundamental scientific knowledge in the biological sciences to improve the quality of life on Earth and contribute to NASA's goal of manned exploration of space.

The current Space Biology Program is divided into the following five research areas:

Plant Biology. Gravity has profound influences on the growth and physiological processes of plants, yet the basic mechanisms underlying these effects are still not well understood. The goals of plant gravitational biology are to achieve a fundamental scientific understanding of the effects of gravity on plants, and to provide basic knowledge of plant development and physiology through experimentation on Earth and in the microgravity environment of spaceflight that can contribute to the utilization of plants in space and on Earth. Research is focused in three primary areas: understanding the basic mechanisms whereby plants perceive, transduce, and respond to a gravitational force; elucidating the role of gravity and microgravity on developmental and reproductive processes in plants; and determining the role of gravity and microgravity in metabolism, photosynthesis, and transport processes in plants.

Cell and Developmental Biology. Cells, whether as single unicellular organisms or as the basic structural and functional unit of multicellular biological systems, have been shown to be sensitive to gravity. The scientific goal of cell gravitational biology is to understand how gravity influences biological function at the cellular level. To attain this goal, research is focused on identifying how single cells "sense" gravity, including both direct and indirect environmentally mediated effects, how this information is transduced into a biological response, and how cells respond to both acute and long-term variations in gravity.

Developmental biology research examines the influence of gravity and microgravity on reproduction, genetic integrity, differentiation, growth, development, life span, senescence, and subsequent generations of animals. The goals are to determine

whether specific animal species have evolved developmental mechanisms that depend on a gravity force or vector for normal function and, if so, to identify the mechanisms; and to determine if and how gravity affects the capacity of animal species to reproduce and develop over serial generations.

Gravity Sensing. Animals have developed gravity-sensing systems that facilitate orientation and locomotion within Earth's environment. The goals of this discipline are to understand how the brain processes information by understanding the organization and functioning of gravity-sensing organs; to test mechanisms of adaptation by exposure to variable gravity; to elucidate the stages by which the gravity-sensing system evolved, from invertebrates through vertebrates; and to begin to understand where in the developmental process gravity influences genetic transcription/translation to direct production of a specific functional architecture in gravity-sensing organs.

Structural Biosystems. In response to the force of gravity under which all biological species on Earth evolved, organisms have developed structures to withstand gravity loads. The goal of this discipline is to understand the role of gravity in modulating biological processes that regulate musculoskeletal systems. Research focuses on identifying how gravity level and direction influences the type, pattern, and amount of biomineralization and muscle in structural biosystems; understanding interactions between gravity and internal (e.g., metabolic regulatory substances) and external (e.g., environmental) factors in these systems; and specifying the time course of musculoskeletal adaptation to altered gravity and readaptation to normal Earth gravity.

Integrative Biology. Living systems have evolved in a gravitational field and are regulated to function within tightly controlled limits. Asymmetries in the environment (including presumably gravity) result in disturbances in these regulatory mechanisms to which living systems adapt and evolve. The goal of integrative biology is to understand how gravity affects mechanisms regulating homeostasis, adaptation, and the ability of living systems to respond to internal and external signals. Research focuses on understanding the role of gravity on animal regulatory mechanisms: the generation and/or entrainment of circadian rhythms, the internal synchronization of several circadian functions, and its role in one or more selected homeostatically controlled systems, e.g., the regulation of body temperature and the associated neuroendocrine regulation of energy and water.

The use of the unique microgravity environment of spaceflight is an integral part of these research efforts. Flight experiments in gravitational biology permit identification, confirmation, and understanding of effects due to microgravity. At the same time, ground-based research activities lay the groundwork for validation by flight experiments, identify gravity-sensitive biological systems, and utilize modern research techniques and instrumentation to target analyses at the most fundamental levels.

ACCOMPLISHMENT HIGHLIGHTS

SPACE BIOLOGY ACCOMPLISHMENTS HIGHLIGHTS

PLANT

Gravitropism: Sensing

- Nature of the Receptor
 - Amyloplasts (believed to be necessary for full gravitropic sensitivity) and nuclei sediment in the elongation zone as well as in the rootcap of *Equisetum* (horsetail) roots. This is the first modern report of sedimentation implying gravity sensing in the root apart from the rootcap, and is also the first report of nuclei sedimenting in roots. (Sack)
 - The tip cell of the moss *Ceratodon* appears to use sedimentation of amyloplasts followed by microtubule redistribution in gravitropic sensing. It was discovered that the zone of the lower wall of the tip cell where microtubules concentrate is rich in wall-building Golgi complex organelles, suggesting that microtubules may influence the position of Golgi. (Sack)
 - Hydrolytic enzymes, including cellulases and proteinases that can attack the outer part of the cell but that are impermeable to the cell itself, inhibit gravisensing in characean (algal) cells. This finding suggests that either the gravireceptor or elements of the signal transduction chain are localized in the extracellular matrix, which includes the outer part of the plasma membrane and the cell wall. (Wayne)
- Role of Membranes
 - It is proposed that gravitational pressure within a characean cell compresses the lower plasma membrane and produces tension on the upper plasma membrane, thereby releasing sufficient potential energy to open ion channels involved in signal transduction and graviresponse. In support of this model, it was shown that increasing the external density to a level greater than that inside the cell causes the direction of cytoplasmic streaming to reverse. (Wayne)
 - Applied hydrostatic pressure also can induce a polarity in cytoplasmic streaming in characean cells, with a response that varies with the external calcium concentration. Hydrostatic pressure can reverse, but not augment, the gravitational pressure-induced polarity of cytoplasmic streaming. These data support the gravitational pressure model of gravisensing. (Wayne)
 - Not only is the density of the extracellular medium important for determining the direction of the vector of gravity during gravisensing in characean cells: the magnitude of the response also depends upon the turgidity of the plasma membrane (PM). A turgid PM is a prerequisite for gravisensing in these cells. (Wayne)
 - Increasing the density of the external medium inhibits gravisensing in rice roots. Because this treatment affects movement of the protoplast but not of individual organelles, this result indicates that the plasma membrane may be the gravireceptor. (Wayne)

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- Preliminary characterization of two relatively non-voltage dependent potassium channels from wild type *Phycomyces* sporangiophores has been achieved. These channels pass both K⁺ and Cl⁻ and may represent two different conductance states. Ion channels are hypothesized to play a role in plant gravitropism. (Edwards)
- Measurements of conductance recorded between stele and cortex tissues of the corn seedling stem revealed that conductance changes as a function of stem orientation with respect to gravity. (Desrosiers/Bandurski)

Gravitropism: Transduction

- Role of Calcium
 - Sunflower seedlings grown with various levels of Ca²⁺ differ greatly in the amount of extracellular Ca²⁺ they contain, yet exhibit the same rate of gravitropic curvature. This discovery indicates that the amount of extracellular Ca²⁺ is unimportant in the gravitropic curvature mechanism. (Cleland)
 - Addition of exogenous Ca²⁺ to sunflower hypocotyl sections results in a rapid and nearly total inhibition of growth for 30-120 min, after which growth resumes at a reduced rate. This finding suggests that free calcium does not directly inhibit growth, but must inhibit growth in a secondary way. (Cleland)
- Role of Hormones
 - The Growth Hormone Concentration and Distribution (GHCD) experiment was flown on the shuttle Atlantis in October 1989. Free and total IAA and free and total ABA in shoots, kernels, and roots of *Zea mays* seedlings grown in microgravity and on the ground were compared. Both flight and control plants appeared to be of normal size and weight. The content of IAA, both free and total, clearly fell within the normal range. (Bandurski)
 - Antibodies against the plant hormones IAA and ABA fail to retard the movement of the gravitropic signal from the cap to the elongation zone. This tentative finding suggests that the gravitropic signal is not traveling as either of these hormones, contrary to popular models of root gravitropism. (Evans)
 - Maize roots exhibit "adaptation" to inhibitory levels of auxin in that they can resume rapid growth after an initial strong growth inhibition due to application of auxin. Roots adapted to elevated auxin levels also show higher than normal rates of elongation upon sudden withdrawal of the inhibitory hormone. This indicates that time-dependent adjustments in auxin sensitivity may play a role in the motor mechanism of root gravitropism. (Evans)
 - Asymmetry in free IAA levels in top and bottom halves of oat pulvini starts within 3 hr and is fully manifested by 6 hr following start of gravistimulation; thereafter, free and total IAA levels generally drop during the 24 hr after start of gravistimulation. The time-course of change in free IAA in the pulvini halves correlates closely with rate of upward bending of the pulvinus. (Kaufman)

- Role of Proteins
 - A calcium-binding, 35 kDa, annexin-like protein has been purified and localized in root, stem, and leaf cells of pea seedlings. The primary amino acid sequences of two peptides proteolytically derived from it have been obtained. This protein may play an important role in secretion, particularly in root caps where the secretion of mucilage has been proposed to be a critically important process in the control of root gravitropism. (Roux)
 - A protein kinase inhibitor, staurosporine, has been identified and shown to block phytochrome-induced spore germination in the fern *Dryopteris*, specifically during the calcium-dependent phase of the germination process. This supports the hypothesis that calcium-dependent protein kinases may be important targets of calcium action during light-induced germination. (Roux)
 - Studies investigating the structure and function of calmodulin genes indicate that plants contain multiple genes of calmodulin and that these genes are developmentally regulated. (Poovaiah)
 - Protein kinases that appear to be second-messenger-regulated are active in roots of maize. Kinases are elements of general signal transduction pathways that are already well characterized in microbial and mammalian systems with regard to their involvement in transduction of environmental stimuli. Gravity transduction in root caps may therefore involve elements found in other organisms. (Feldman)
 - A 56 kDa protein found in *Zea mays* roots may be a homolog of the mammalian multifunctional calcium/calmodulin-dependent protein kinase CaM KII. (Poovaiah)
 - The role of GTP-binding proteins (G-proteins) in auxin action and gravitropism is being examined. It was discovered that GTP-binding and hydrolysis in zucchini plasma membrane is similar to that in animals and yeast. The plant GTP-binding proteins can be stimulated by auxin, suggesting that these proteins may be involved in plant signal transduction. (Lomax)
- Use of Mutants
 - Comparison of sporangiophores of wild type and two mutant strains of the fungus *Phycomyces* has shown that wild type has a slow graviresponse with a 30 min delay to onset of positive gravicurvature, while the mutants show a faster graviresponse with little or no delay between sensing and response. (Edwards)
 - Four mutant strains of *Arabidopsis* exhibiting alterations in gravitropism have been genetically and physiologically characterized; these mutants carry single recessive nuclear mutations with respect to the gravitropism phenotype. The gravitropism alterations occur in darkness; the wild type phenotype is exhibited upon exposure to white light. (Poff)
 - Stem cells of the *dgt* gravitropic tomato mutant display greatly diminished binding of IAA to plasma membrane proteins; *lazy-2* gravitropic tomato mutants, however, appear to be altered at another gene, possibly within the signal transduction pathway between graviperception and graviresponse. (Lomax)

- In wild type tomato, a transcript of size identical to that of a small auxin up-regulated gene (SAUR 15A) is strongly induced within 1 hr of auxin application. In the auxin-insensitive *dgt* mutant, no SAUR 15A homolog is expressed. Characterization of SAUR genes such as 15A should aid in establishing a causal relationship between molecular induction (using auxin) of specific mRNAs and a physiological system such as gravitropism. (Rayle)
- Role of Phytochrome
 - In a variety of corn that requires light for complete gravisensitivity, phytochrome was found to be most active in the central-most portion of the root cap, which is also the region containing amyloplasts. In addition, the kinetics of phytochrome processes in the root cap closely parallel the actual gravity-induced curvature of the root, further suggesting that phytochrome plays a role in the transduction of gravity. (Feldman)

Gravitropism: Growth Response

- Proteins isolated from cucumber cell walls are able to induce inactivated cell walls to extend. This provides the first direct evidence that walls contain enzymes that can induce extension of isolated cell walls. (Cosgrove)
- In studying the biochemical basis for cell wall relaxation, isolated cucumber cell walls have been found to extend at much faster rates when wall thiols are reduced. Thiol reduction alone is insufficient for stimulation of cell expansion, but in the presence of auxin, it prolongs cell expansion. These results indicate that a reducing environment in the cell wall may be important (perhaps regulatory) for wall loosening reactions. (Cosgrove)
- Phloem unloading into the growing region of the cucumber hypocotyl becomes asymmetrical during graviresponse, indicating that sugar unloading from the phloem is coordinated with cell expansion rate changes. (Cosgrove)
- Both epidermal and cortical tissues of pea epicotyls show the same ability to respond to auxin. This finding is evidence against the epidermis as the primary site of auxin action in growth. (Rayle)
- Outer cell layers are not required for nor do they control either the growth rate or the gravitropic curvature of stems or coleoptiles of several plant species, suggesting that other cells are capable of responding to auxin and controlling growth rate. (Cleland)
- Gravistimulation induces rapid cell elongation in maize within the postmitotic isodiametric growth zone, a region of the root near the tip in which cell division is absent and cell elongation is normally minimal. This finding draws attention to the possible recruitment of new cells in the adjustment of growth rates during gravitropism. (Evans)
- Several antibodies against higher plant pectic polysaccharides crossreact with *Chara* cell walls. The antibodies will serve as probes to study the cell wall and apical vesicles in *Chara* rhizoids during gravitropic curvature. (Kiss)

- Cryofixation, a method in which samples are fixed very rapidly, preserves structural features and antigenic determinants in *Chara* better than conventional fixation techniques. This method is being used to investigate the role of the Golgi apparatus in differential cell wall growth in response to gravity. (Kiss)
- An anionic peroxidase isozyme (A3) is induced by exogenous Ca^{2+} or IAA application to upright corn coleoptiles in a manner that is consistent with growth inhibition. A3 activity also increases dramatically in upper side tissues of gravistimulated coleoptiles within 30 min. A3 appears to be a cell wall-localized enzyme and increased activity may promote localized wall cross-linking and "stiffening," contributing to the gravitropic bending response. IAA and Ca^{2+} effects may be mediated, in part, through their effects on peroxidase isozyme expression. (Slocum)
- Role of pH
 - The outer cell layers of maize roots show the strongest growth promotion in response to low pH, indicating that these cells are most likely to be involved in establishing the differential growth pattern that leads to gravitropic curvature. (Evans)
 - The pH optimum for growth of oat coleoptiles is lower in the first 2 hrs after the hormone auxin is added than during prolonged growth. This finding suggests that while rapid growth rate changes such as occur in gravitropism are mediated by an acid-growth mechanism, the steady-state, prolonged growth response is caused by a different mechanism. (Cleland)
 - Changes in pH of oat pulvinus tissue during the gravitropic response were measured for the first time. The bottom half of the pulvinus showed a transient alkalization followed by an enhanced rate of acidification, whereas the top half showed virtually no difference in pH, compared to controls. This result may be related to the asymmetric distribution of IAA in pulvinus halves during graviresponse. (Kaufman)
 - When stem segments containing whole oat pulvini were gravistimulated at various buffered pHs, the graviresponse of pulvini at high pHs was less than that of non-buffered control pulvini and of pulvini at lower pHs. When pulvinus halves were gravistimulated at various pHs, the bottom halves showed 63% and the upper halves only 24% of the full graviresponse that is manifested in non-buffered control pulvini. This and the previous finding demonstrate the importance of pH changes in the gravitropic response of cereal pulvinus tissue. (Kaufman)

Plant Metabolism

- The growth rate of *Neurospora* cultures flown on STS-32 in January 1990 increased during flight, independent of a temperature effect. This response may suggest an altered metabolic rate in space. (Ferraro)
- Amounts of lignin and monomer compositions of radish plants grown for 54 days in microgravity on the Mir space station and on Earth as ground controls were analyzed. Flight-grown plants produced more pectinaceous/non-cellulose polysaccharide polymers, and their cell walls had a lower α -cellulose content and a lignin of greatly reduced sinapyl alcohol moieties. These results have implications

for reduced lignin formation and therefore for plant structural integrity in microgravity. (Lewis)

- The first proof of lignification in the cell walls of cultured plant cells was achieved. (Lewis)
- Changes in the concentration of starch and sucrose were noted in soybean plants grown in altered gravity conditions. Because starch is a form of storage carbohydrate and sucrose is the primary form of translocated carbon in most plants, alterations in starch and sucrose metabolism due to changes in gravity could have profound effects on plant growth and yield. (Brown)
- Photosynthetic CO₂ uptake increased in pea plants and respiratory CO₂ evolution decreased in maize plants grown on a horizontal clinostat, compared to vertically rotated or stationary control plants. These findings suggest that clinorotation has an effect on the carbon exchange rate of plants. (Brown)

Plant Cell & Developmental Biology

- Competent cell cultures of carrot and daylily (*Hemerocallis*) can be derived from a hormone-free medium and the process of embryogenesis can be controlled by changes in pH. The ability to generate embryogenic cultures predictably without a long lead time provides systems amenable for the first time to reliable study of mechanisms involved in the generation and manipulation of free cells, and to their study at altered *g*. (Krikorian)
- Fixation procedures for use in studying embryo sac development in *Arabidopsis thaliana* were successfully determined. This will allow for investigation of the potential consequences of cell ultrastructural changes on embryo sac development in an upcoming flight experiment. (Musgrave)
- A prototype fluid mechanical shear apparatus was designed and built for measuring algal swimming and orientation in the presence of gravity, illumination, and fluid shear. It was shown that algal swimming trajectories are oriented in a manner expected by the combination of gravity and applied shear forces. (Kessler)

Environmental Factors

- Although it temporarily inhibits growth rates in soybean seedlings, mechanical stress also preconditions these plants to withstand even higher levels of physical stress, in part because the support tissues of mechanically stressed plants become stronger and more fibrous due to altered cell wall composition and deposition. (Mitchell)
- Wheat grown under cool-white fluorescent light (rich in blue light) was significantly taller than plants grown under greenhouse conditions or using high-pressure sodium light (rich in far-red light). Plants grown at low light levels were considerably delayed in wheat head emergence. Because current and near-term spaceflight opportunities will require short plants with short life cycles, these phenomena may influence the selection of spaceflight light sources. (Salisbury)

ANIMAL

Gravity Receptors and Neurophysiology

- The eyes of the larval frog exhibit a counter-rolling response when exposed to static tilt, an example of a gravitational reflex. Experiments have shown that the inner ear is the source of the tilt sensitivity of the nerves supplying the eye muscles. The CNS apparently passes this gravitational sensing information via the vestibular nuclei onto the extraocular motoneurons. (Cochran)
- Hair cells from different macular zones of the bullfrog differ in their passive and active membrane conductance and in their sensitivity and response dynamics, particularly in the rate and extent of their adaptation to low-frequency mechanical stimulation. In addition, the macular origin of isolated utricular hair cells can be inferred from their hair bundle morphology and lectin binding patterns. (Baird)
- The earliest vestibular response peaks of chicks to pulsed linear acceleration are generated by vestibular primary afferents and are not of central origin. The peaks represent compound action potentials of the vestibular nerve. (Jones)
- It was demonstrated for the first time in rats that short latency responses to pulsed linear acceleration depend exclusively on VIIIth nerve neurons. (Jones)
- In the chick, microtubule-associated proteins and neurofilament proteins, essential for the survival and functioning of neurons, change in amount in developing VIIIth neurons before, during, and after synaptogenesis. These proteins may be involved in the establishment of connections between hair cells and the VIIIth neurons, which convey vestibular information from the inner ear to the brain. (Fermin)
- Studies of maculas in centrifuged rats indicate that synapses in type I hair cells are comparable in size and number to those of control rats, but that synapses in type II hair cells are reduced in number by one-third in centrifuged rats. This finding suggests that it is the distributed modifying circuitry that would be most subject to change in response to an altered g environment. (Ross)
- Reconstructions and electron micrographs of synapses in the rat demonstrate that there are synapses between type II hair cells and the nerve terminals that supply type I cells with opposing directional polarizations. This observation indicates that the neural network is continuous across the macula. (Ross)
- The discharge patterns, synaptic gains, and synaptic organization of mammalian vestibular afferent units depend upon the epithelial region of the end organ they innervate. (Goldberg/Lysakowski)
- Type I and II hair cells occur in equal numbers in chinchilla cristae. In the monkey, however, type I hair cells outnumber type II cells 3:1. The latter finding is due to relatively more calyx units, fewer bouton units, and central dimorphic units with fewer bouton endings. Functionally this represents a larger proportion of low-gain, irregularly discharging afferents. (Goldberg/Lysakowski)
- Dimorphic units have been considered the rarest type of afferent. They are, however, the most numerous type found going to the cristae and utricular macula. (Goldberg)

- Results indicate that gravity receptors and vestibulospinal output in rats are active at birth and exert powerful control over the antigravity postural reflexes. Disrupted gravity receptor function, by either early labyrinthectomy or Mn-deficiency, produces minimal effect on the time of onset of antigravity reflexes but has major effects on response symmetry. Adaptive mechanisms successfully develop during the first postnatal days. (Blanks)
- Embryos of a marine mollusc raised at 2 g appear to have smaller statoliths than those reared at 1 g. (Wiederhold)
- When sufficient artificial lift was added to make killifish neutrally buoyant without swimbladder lift, thereby simulating weightlessness, approximately 7-10 days were required for the complete resorption of gases from the swimbladder. Fish exposed to artificial lift display a slower rate of gas secretion into the swimbladder, suggesting that atrophy of the gas generating tissue occurs after just one week of disuse. (Goolish)
- The mechanism for regulating gas secretion and resorption in the killifish's swimbladder is not simply dependent on maintaining a net hydrostatic lift force of zero. Rather, the stimulus for regulation appears to be the rotational (pitching) forces experienced by the fish. (Goolish)

Development

- The location of the third (horizontal) cleavage furrow in *Xenopus laevis* eggs varies directly with the applied gravitational field. This finding represents a natural gravity response during amphibian development. (Malacinski)
- *Xenopus laevis* embryos exposed to various gravitational fields up to the midblastula stage show specific changes in morphology associated with a gravitational field. However, these induced alterations in blastula morphogenesis become normal as development proceeds, and exposed and control feeding tadpoles are grossly indistinguishable. (Malacinski)
- By using the monoclonal antibody Epi 1 to study the establishment of pattern in the nervous system during early amphibian embryonic development, it was determined that neural induction occurs by at least three mechanisms. This indicates that the process is much more complex than originally believed. (Phillips)
- Gonadotropin, gonadotropin releasing hormone, and approximately twelve other neuropeptides and neurotransmitters have been localized in the brain and pituitary glands of two species of the fish *Xiphophorus*. The localization of these substances indicates that they are involved in the neuroendocrine regulation of reproduction in *Xiphophorus*. (Schreibman)
- Significantly less Epidermal Growth Factor (EGF) was found in the salivary glands of centrifuged male and female mice, compared with the amount found in control mice. (Duke)
- When beads were substituted for neurons in samples rotated on a clinostat in studies of synapse development, acetylcholine receptor aggregation on muscle cells at the point of contact was significantly reduced. Moreover, where receptors did accumulate after clinostat rotation, the structure of the synapse assembly was less well defined and was spread over a smaller area. These data are consistent with the

idea that the response of the muscle to the nerve signal in simulated microgravity is the root cause of its failure to accumulate receptors and therefore to form a functional synapse. (Gruener)

- In hindlimb unloaded rats, testicular weight loss correlates with a dramatic change in the population of spermatogenic cells. Also, the developmental stages of spermatogenic cells affected by hindlimb unloading correlate with changes in the expression of the *hsp 70* and *hsp 90* genes. (Wolgemuth)
- Rats flown on Cosmos 2044 did not demonstrate an obvious induction of *hsp 70* or *hsp 90* genes at the mRNA level in the somatic compartment of the testis. (Wolgemuth)

Bone and Muscle

- Compact bone of the tibia of rats flown on Cosmos 2044 for 14 days contained bone-forming cells with reduced Golgi synthetic activity. (Doty)
- Following flight on Cosmos 2044 and an approximate 10-hr recovery period, no significant differences were observed in early osteoblast progenitor cells, nonosteogenic cells, or preosteoblasts in rat periodontal ligament. These data fit the predicted recovery pattern derived from previous spaceflight experiments, thus confirming the time course of recovery. (Roberts)
- The morphology of calvaria (top of skull) from rats flown on Cosmos 2044 was altered, suggesting that the shift in blood flow and the change in loading that occur during spaceflight lead to thickening of the matrix of the calvaria. (Partridge)
- Increased vascularization (i.e., more open blood columns and more capillary sprouts) was found in cartilaginous growth plates of rats flown aboard the 7-day flight of Spacelab 3. (Duke)
- Cells associated with blood vessels in weight-bearing bone and the adjacent osteocytes showed damage following spaceflight on Cosmos 2044 and Cosmos 1887. (Doty)
- Lesions observed in the longitudinal sections of adductor longus muscle from rats flown on Cosmos 2044 were not randomly distributed, but rather were laterally grouped. Elevated tension rather than nerve damage seems a more likely cause of these grouped lesions. (Thompson)
- A muscle is under higher tension for a longer period during eccentric contraction than during isometric or concentric contraction. The lesions observed in Cosmos 2044 flight rats were structurally similar to those found following eccentric contraction or other activities producing high tension, suggesting that antigravity muscles exposed to microgravity are more susceptible to high tension lengthening damage. (Thompson)
- Hindlimb unloading of immature, growing rats results in a rapid initial reduction and eventual complete shutdown of satellite cell proliferative activity in soleus muscle. Initiation of the response precedes any measurable changes in the soleus muscle, and the initial reduction in proliferative activity appears to be unrelated to physical changes in the muscle. (Schultz)

- The satellite cell response during hindlimb unloading is associated with both a reduction in the number of cells undergoing mitosis and a reduction in the total number of satellite cells. Satellite cells play an important role in skeletal muscle growth as a source of myonuclei for growing myofibers and in the regeneration response following injury as a source of myoblasts. (Schultz)
- Insulin and IGF-1, but not IGF-2, induce rapid and pronounced hypertrophy in avian skeletal myofibers incubated in a new serum-free medium. Mechanical stimulation of the muscle fibers increases cell sensitivity to insulin and IGF-1. The glucocorticoid dexamethasone induces atrophy of the skeletal muscle fibers; mechanical stimulation of these cells significantly reduces the atrophic response. These findings demonstrate the interaction of mechanical forces and growth factors in regulating muscle size. (Vandenburgh)
- A comparison study of hindlimb unloaded and control rats suggests that alterations in bone metabolism occur before alterations in bone biomechanics, that unloaded bones appear to be 1-2 wks younger than control bones, that both quality and quantity of bone are altered by unloading, and that cortical and cancellous bone respond differently to unloading. (Morey-Holton)
- The ability of osteoblast-conditioned media to maintain viability of osteoclast precursors does not involve prevention of programmed cell death, unlike a variety of hematopoietic factors that utilize the prevention of programmed cell death as the mechanism whereby their target cells remain viable. (Greenfield)
- Mineral from bone (osteoblast) cell cultures is similar in size, shape, surface texture, crystallographic properties, and relation with collagen to that from *in vivo* bone. A chondrocyte culture system assembles and mineralizes as does normal growth plate cartilage. These similarities permit the use of the cell cultures in the study of bone mineralization. (Landis)
- When indomethacin (an inhibitor of prostaglandin synthesis) was added to growing osteoblasts, both DNA and protein synthesis levels decreased. Addition of exogenous prostaglandin to these inhibited cells returned these levels to normal. These data support the view that prostaglandins play a role in the regulation of bone growth. (Hughes-Fulford)
- Studies on hormonal regulation of bone collagen synthesis indicate that protein kinase A activation is the major route for parathyroid hormone (PTH) inhibition of collagen synthesis. It was also shown that PTH induces collagenase transcription primarily through the cAMP-dependent pathway in rat osteosarcoma cells, and that it requires the expression of other genes. (Partridge)
- Insulin injections reduce cyclic AMP in unloaded diabetic rat muscle, more so in unloaded than in weight-bearing muscle. This effect and results from other experiments indicate that this hormone may limit the rate of muscle atrophy caused by unweighting relative to the rate of atrophy seen with denervation. (Tischler)
- Hindlimb unloading results in an increased isoproterenol (a β -adrenergic agonist) response of glycogen degeneration and lactate production. (Tischler)
- Intramuscular injections of isoproterenol *in vivo* show a more dramatic effect in unloaded animals. (Tischler)

- Changes in both the production of and metabolic clearance of the hormone $1,25(\text{OH})_2\text{D}$ are responsible for the fall in bone serum level of this hormone during skeletal unloading. (Bikle)
- Although levels of IGF-1 (insulin-like growth factor 1) are reduced during skeletal unloading in rats, there is an increase in mRNA for IGF-1 in epiphyseal growth plates during the first 5 days of unloading. A demonstrated decrease in mRNA for collagen is consistent with the reduction of bone formation that occurs during unweighting. (Bikle)
- Levels of insulin growth factor 1 increase in rat muscle 2-5 days following a single bout of eccentric exercise. (Kirby)
- Examination of the distribution patterns of like muscle fibers of rats indicated that, unlike with denervation, exercise of skeletal muscle during recovery following hindlimb unloading does not cause any change in the grouping behavior among like fiber types. (Kasper)
- The shape and spatial distribution of myonuclei in rat skeletal muscle fibers are interdependent (that is, related to each other), suggesting that there is some functional significance to their morphological features and transcriptional activities. (Kasper)
- An increase in polysome size observed in rat skeletal muscle as a result of simulated microgravity also occurs in rat cardiac muscle under the same conditions. This suggests that atrophy of cardiac muscle has a biochemical basis. (Thomason)
- Analysis has revealed that the proteinaceous matrix of sea urchin calcite spicules is twice as complex as previously believed, involving at least 20 proteins. The majority of proteins are acidic and glycosylated. Characterization of the previously isolated SM50 spicule matrix protein from *S. purpuratus* has revealed that it is basic and non-glycosylated, however, and that it shows good homology with a similar protein isolated from a distantly related sea urchin, *L. pictus*. (Wilt)
- A gene encoding a second spicule matrix protein (SM30) in the sea urchin has been isolated and characterized. Comparative analysis of the *SM30* and *SM50* genes implies that there are different functions for their encoded proteins. (Wilt)

Regulatory Biology

- In the dentate gyrus region of the hippocampus in rats exposed to 2 g for 7 days, the decrease in population spike amplitude during serotonin perfusion was significantly less than in rats exposed to 1 g. This finding demonstrates that serotonergic effects on hippocampal cells are altered by gravitational fields, and is consistent with the hypothesis that hypergravity causes a down regulation of serotonergic receptors in the rat hippocampus. The findings are also significant in that the gravitational effect was associated with a specific cell type, granule cells in the dentate gyrus. (Horowitz)
- Xylazine, an anesthetic shown to elevate plasma angiotensinogen levels in rats, causes an increase in plasma oxytocin and vasopressin. This finding is part of an effort to determine the specific parts of the hypothalamus and related areas that affect components of the circulating renin-angiotensin system. (Ganong)

- Electrical stimulation of the ventromedial nuclei increases renin secretion in rats. Data from preliminary experiments indicate that the ventromedial nuclei are not integrating centers per se, but are part of a descending pathway from the hypothalamus to the kidneys. (Ganong)
- The body temperature of rats exposed to long-term 2 g centrifugation and constant lighting conditions exhibits a decreased daily mean and a loss of circadian rhythmicity. Neither the mean body temperature nor the freerunning period of body temperature circadian rhythms recovers to baseline levels. (Fuller)
- The body temperature of rats exposed to 1-hr pulses of 2 g once a day exhibits a significant decline during the pulses, but never adapts to them. The response to the daily 2 g exposure appears to be an entrained circadian rhythm: when the rats are no longer exposed to the pulses, the body temperature appears to freerun from the point of 2 g release. (Fuller)

Cell Biology

- It was demonstrated that cells use "tensegrity" (tensional integrity) architecture for their cytoskeletal organization. This mechanism suggests that cells sense changes in a balance of pre-existing forces rather than recognize the presence or absence of a force such as gravity. (Ingber)
- Actin bundles and microtubules act as internal support struts in living cells, serving to translate inwardly-directed tension into outward extension during cell spreading. While actin bundles are essential for spreading, microtubules appear to be redundant support elements. (Ingber)

PLANT PROJECTS

2023年12月27日

GROWTH HORMONE CONCENTRATION AND DISTRIBUTION: RESULTS OF THE MID-DECK SHUTTLE EXPERIMENT

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Description of Research

The Growth Hormone Concentration and Distribution experiment (GHCD) was flown on Atlantis on October 18, 1989. Four canisters were flown, each containing two compartments and each compartment containing thirteen seeds. Water was added to the compartments just 17 hrs prior to launch so that imbibition occurred on Earth but germination and subsequent growth was in microgravity. Two of the four canisters were frozen 121 hrs into the mission and the remaining two frozen immediately after landing at about 134 hrs after launch. Identical simultaneous ground controls were grown at KSC and ground controls with a temperature simulation of conditions during flight were grown at Michigan State.

The objective of the experiment was to compare free and total indole-3-acetic acid (IAA) and free and total abscisic acid (ABA) in shoots, roots, and kernels of *Zea mays* seedlings grown at 1 g and in μ g. The growth promoting hormone, IAA, appears to be the major growth controlling hormone in young seedling plants. The growth inhibiting hormone, ABA, appears to be the major regulating inhibitor of seed germination and is also a hormone that responds to stress conditions during plant growth. It is believed that chemical analysis of the hormonal status of the seedlings will be a sensitive indicator of metabolic stress as might be occasioned by growth under 1 g as compared to plants grown in μ g.

This report presents results only for free and total IAA in Table I, with results for ABA to be presented later. "EARTH" refers to the grouped results of material grown at KSC as a simultaneous ground control and material grown at MSU as a temperature control. Material frozen in flight is grouped with material frozen after landing as "SPACE" material. The detailed analysis of variance required to separate these variables is not complete. Results are presented as picomoles per plant plus or minus the standard error of the mean. The number of samples is indicated as (n).

Accomplishments

- (1) *The plants appear to be of normal size and weight grown both at 1 g and in μ g.*
- (2) *The content of IAA, both free and total, clearly falls within the normal range.*
- (3) To the extent that a growth hormone may be viewed as an indicator of metabolic status it appears that plants grown in μ g will not be appreciably chemically different from plants grown at 1 g.

Table I. Results of free and total IAA content in corn seedlings grown at 1 g and μ g.

	Pmole/Plant	
	EARTH	SPACE
Free IAA*		
Shoot	35 \pm 5 (11)	30 \pm 3 (8)
Root	26 \pm 2 (11)	18 \pm 2 (5)
Kernel	644 \pm 82 (11)	391 \pm 66 (6)
Total IAA		
Shoot	393 \pm 31 (15)	308 \pm 15 (7)
Root	144 \pm 9 (12)	167 \pm 34 (6)
Kernel	64,361 \pm 2791 (13)	70,465 \pm 1538 (8)

* Free IAA means IAA occurring in the plant as the free acid, as this is regarded as the active growth promoting form. Total IAA includes free IAA and esterified IAA liberated by mild alkaline hydrolysis that is free plus stored IAA.

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PHOTOSYNTHESIS AND CARBOHYDRATE METABOLISM IN HIGHER PLANTS IN ALTERED GRAVITY CONDITIONS

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Description of Research

Photosynthetic uptake of atmospheric CO₂ and its subsequent conversion into carbohydrates for transport, storage or further metabolism are characteristic features of higher plants. At present there is preliminary evidence that altered gravitational conditions, including microgravity, affect these physiological processes.

The specific objectives of this research are to determine the effect of altered gravity conditions on: (1) photosynthetic and respiratory carbon exchange rates in higher plants, and (2) the concentration and metabolism of carbohydrates, particularly starch, in the same species. An additional objective is to develop a water and nutrient delivery system which could be used for the culture of plants in microgravity.

To examine how photosynthesis and respiration may be altered under different gravity conditions, seedlings of maize (*Zea mays* cv. Silver Queen) and pea (*Pisum sativum* cv. Burpee Bush Snapper) were grown on horizontally or vertically rotating clinostats (1 rpm) or in the stationary mode. Measurements of whole plant carbon exchange rates were taken in both the light and the dark. To investigate the possible role that gravity plays in starch metabolism, soybean seeds were germinated and grown in darkness while on a low-speed centrifuge (150 rpm at a radius of 20 cm = 5 g), on a horizontally rotating clinostat or on a vertically rotating clinostat. Measurements were made of the concentration of starch and soluble sugars in the cotyledons after six days.

Concomitant to this research was the continuing refinement of the porous tube nutrient delivery system. This system utilizes porous ceramic tubes through which water and nutrients are supplied to the surrounding plant roots. Because it works under a slight negative pressure to prevent any leakage, it has potential for use in microgravity.

Accomplishments

(1) *Photosynthetic CO₂ uptake increased in pea plants and respiratory CO₂ evolution decreased in maize plants grown on a horizontal clinostat compared to vertically rotated or stationary control plants.* The net effect of this was an increase in the photosynthesis/respiration ratio in both species due to horizontal clinorotation (Figure 1).

(2) *Dark-grown soybeans produce starch in their cotyledons during the first week of growth. In six-day-old soybean, the concentration of starch was lower in the cotyledons of plants grown on the horizontal clinostat and higher in the cotyledons of plants grown on the centrifuge relative to the vertically rotated control plants* (Figure 2). Measurements of sucrose concentrations were higher in the cotyledons of the horizontally rotated plants.

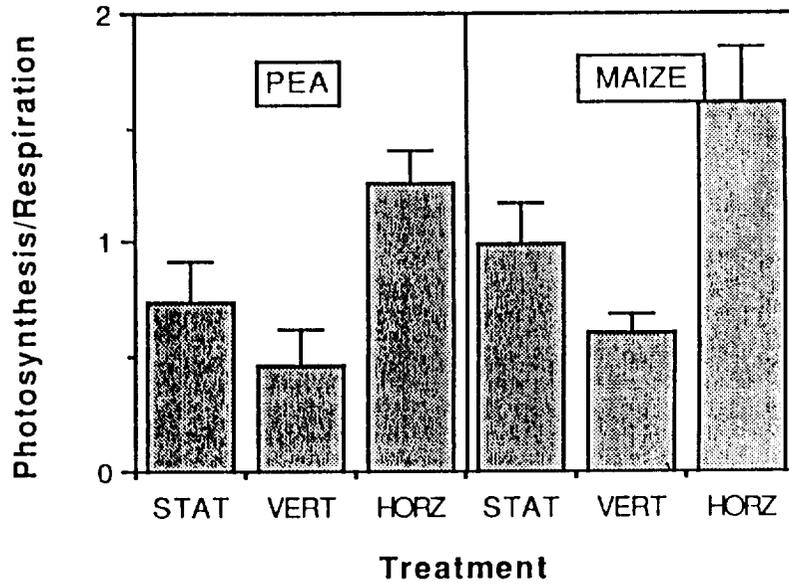


Figure 1. Whole plant photosynthesis/respiration ratio in two-week-old pea and maize seedlings. Plants were grown from seed in the stationary mode or while rotating either vertically or horizontally at 1 rpm. Each bar represents the mean of four replications and the standard deviation is shown.

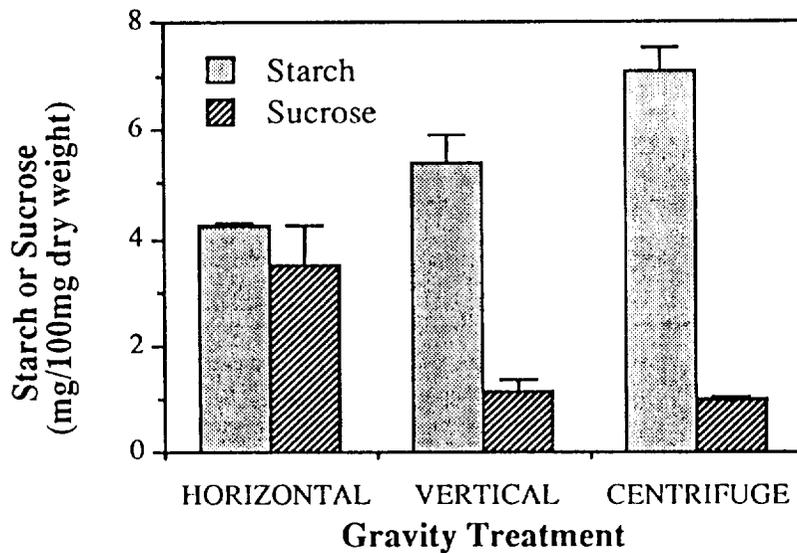


Figure 2. Carbohydrate concentrations in cotyledons of six-day-old etiolated soybeans. Plants were grown from seed on a horizontally rotating clinostat (HORIZONTAL), on a vertically rotating clinostat (VERTICAL) as a control, or in a constantly spinning centrifuge (CENTRIFUGE), which supplied a force of approximately 5 g to the seedlings. The clinorotation treatments were rotating at 1 rpm and the centrifuge was spinning at 175 rpm. Each bar represents the mean of two replications.

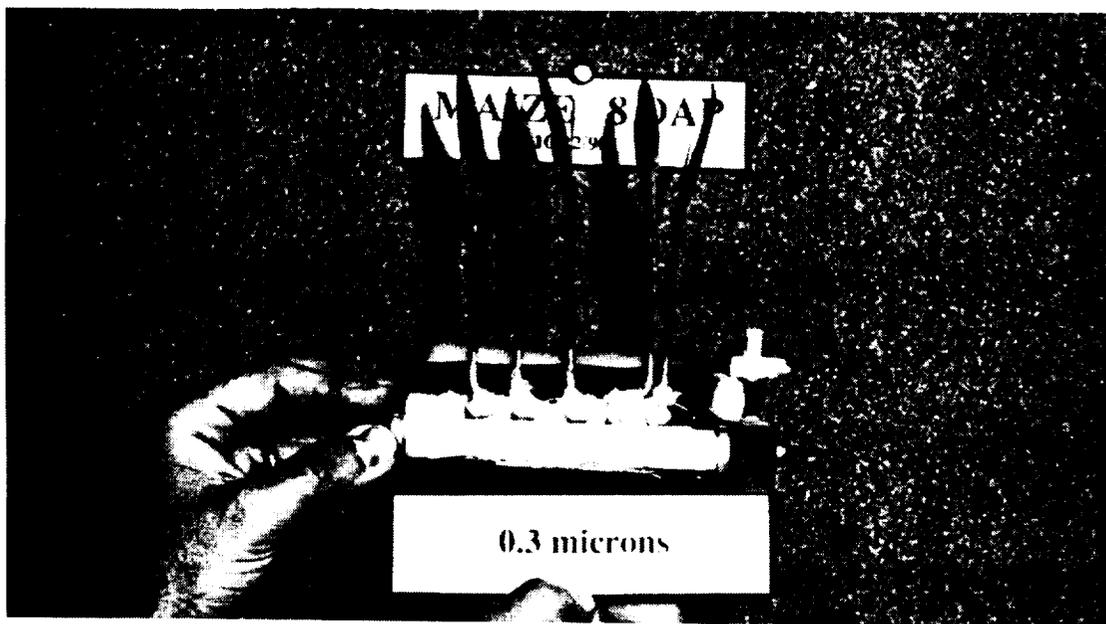


Figure 3. Eight-day-old maize plants germinated and grown on the porous tube nutrient delivery system, which has been modified to fit within the Plant Growth Facility-2. Water was supplied to the roots at a slight negative pressure and the pore size of the tube was 0.3 micron.

(3) *The porous tube nutrient delivery system was miniaturized to fit and function within the confines of the PGF-2 (Plant Growth Facility-2, a mock-up of the Shuttle mid-deck locker Plant Growth Unit). This version of the porous tube nutrient delivery system supported growth of a number of different seedlings, including maize (Figure 3), wheat, soybean, pea, and *Brassica campestris*.*

(4) A clinostat large enough to accommodate two of the PGF-2 units was developed and utilized (Figure 4).

Significance of the Accomplishments

Finding #1: Clinorotation appears to have an effect on the carbon exchange rate of plants. Whether this change is due to a mechanical disturbance in the plant's structure or to a biochemical change is not known at present. Whether plants grown in true microgravity will exhibit similar changes also remains an open question. Answers to these questions are critical to understanding how plants will function in the space environment.

Finding #2: Changes in the concentrations of starch and sucrose were noted in plants grown in altered gravity conditions. As sucrose is the primary form of translocated carbon in most plants and starch is both a transient (diurnal leaf starch synthesis and degradation) and a permanent (potato tuber and maize kernel) form of storage carbohydrate, alterations in the metabolism of these could have profound effects on growth and yield. The underlying mechanism for these gravity-induced changes is an area of current investigation in our laboratory.

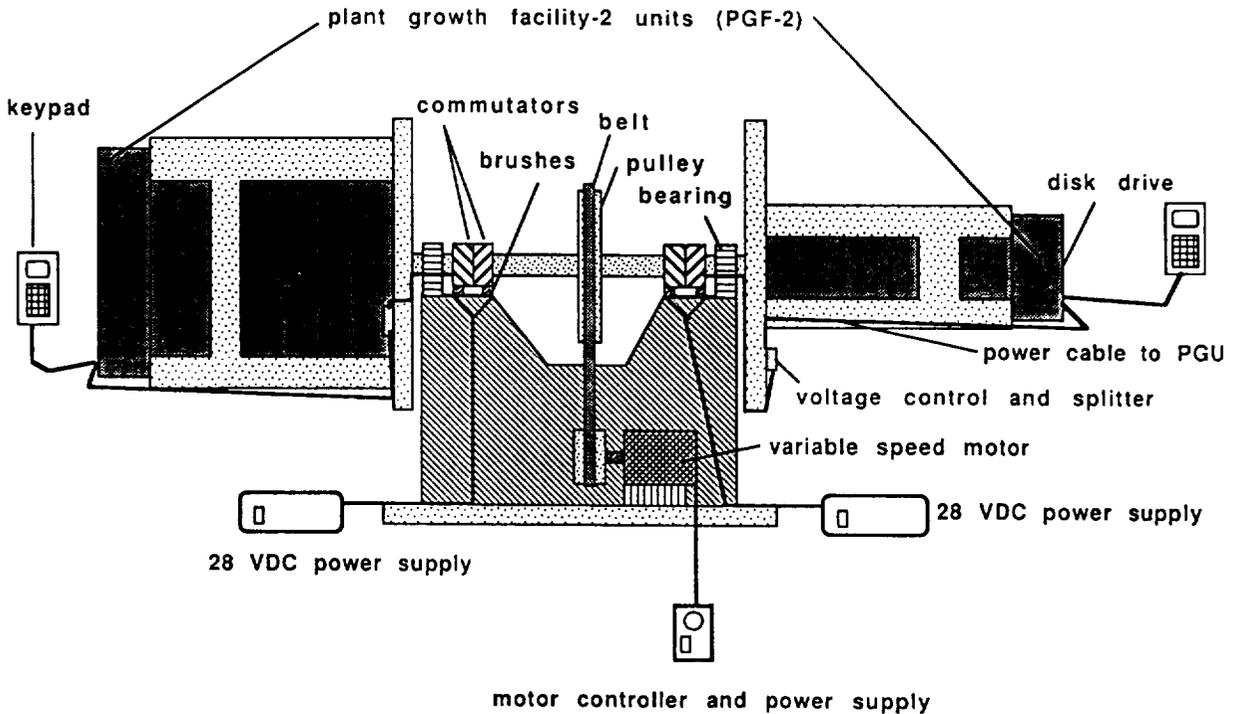


Figure 4. Schematic design of the clinostat for rotating the Plant Growth Facility-2. Constant power (28 VDC) is supplied to the units using a system of commutators and brushes. The clinostat can rotate from 0.3 - 1.8 rpm.

Finding #3: Plants which have been grown in space or on clinostats potentially experience root environments which lack control of water and nutrient flow. Possibilities for nutrient imbalance, changes in nutrient availability and problems with oxygen diffusion raise questions as to the health of the plant, irrespective of imposed gravitational effects. The development of a nutrient delivery system with the ability to control water and nutrients, as well as to fit the rigid constraints of spaceflight, is essential for plant experimentation in microgravity.

Finding #4: Development of the PGF-2 clinostat will allow us to conduct experiments under growth conditions similar to those available onboard the space shuttle while imposing the altered gravity conditions of clinorotation. In this way, we hope to determine the effects of these conditions on photosynthesis and carbohydrate metabolism while at the same time avoiding many of the pitfalls encountered in trying to adapt Earth-bound experiments to the relatively strict guidelines of spaceflight.

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THE ROLE OF EXTRACELLULAR CALCIUM IN PLANT GRAVITROPISM

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Description of Research

Gravitropic curvature in plants occurs when there is an unequal rate of cell elongation on the two sides of a non-vertical plant organ, e.g., in stems the lower side grows faster than the upper side with the result that the stem curves upwards. Our goal is to determine the mechanisms involved in this differential growth response. One of the current theories states that the differential growth is due, in some way, to a redistribution of the hormone auxin from the upper to the lower side and of extracellular calcium (Ca^{2+}) from the lower to the upper sides of stems. Because auxin causes cells to excrete protons, walls on the lower side of horizontal stems will be more acidic than on the upper side. Most of the extracellular calcium is bound to cell wall carboxyl groups, where it is believed to stiffen the walls. However, protons can solubilize this calcium. It has been proposed that the increased acidity on the lower side results in solubilization of Ca^{2+} and thus its redistribution to the upper side.

The first objective of our research is to determine the role of extracellular Ca^{2+} in the mechanism of gravitropic curvature of dicot stems and monocot coleoptiles. In previous years we have demonstrated that the calcium bound to the wall does not stiffen the wall, and that an acidic wall solution cannot solubilize enough wall calcium to lead to increased growth. However, the possibility has not been ruled out that differences in the amount of free, unbound Ca^{2+} on the two sides of stems and coleoptiles might be important in the gravitropic mechanism.

Two sets of experiments were conducted to assess the role of extracellular Ca^{2+} in gravitropism and growth. In the first, sunflower seedlings were grown in excess calcium so as to increase the amount of extracellular calcium. If a differential in calcium between the upper and lower sides is important in gravitropism, excess calcium would be expected to swamp out the differential and block gravitropism. In the second set of experiments, the time-course of inhibition of growth of sunflower hypocotyl sections by calcium was determined. If the level of extracellular Ca^{2+} is important in controlling growth, the inhibition by Ca^{2+} should become progressively greater with time as the calcium penetrates the cell walls.

The second objective is to assess the role of auxin-induced wall acidification and the importance of the outer cell layers in growth and gravitropism. While there is strong evidence that the initiation of rapid cell elongation by auxin occurs by wall acidification (acid-growth), it is not clear that prolonged growth has the same mechanism. Several investigators have claimed that only the outer cell layers of stems and coleoptiles can respond to auxin, and that both growth and curvature must be regulated by the growth of just these cell layers.

Two sets of experiments have been conducted to examine the second objective. In the first, the pH optimum for auxin-induced growth of coleoptiles and stems has been determined

both in the period immediately after addition of auxin and later, when prolonged growth occurs. If the prolonged growth is due to a different mechanism than the initial growth response, the two growth stages should have different pH optima. In the second series, the ability of coleoptiles and stems to undergo normal auxin-induced growth and gravitropism was determined for sections from which the outer cell layers had been removed. If normal growth occurred, it would indicate that the control of growth is not restricted to the outer cell layers.

Accomplishments

(1) It has been shown that *sunflower seedlings grown in the presence of various levels of Ca^{2+} , from 0-50 mM, differ greatly in the amount of extracellular Ca^{2+} but have the same rate of gravitropic curvature.*

(2) It has been shown that *addition of exogenous calcium to sunflower hypocotyl sections results in a rapid and nearly total inhibition of growth for 30-120 min, after which the growth resumes at a reduced rate. The length of the strong inhibition and the final growth rate depend on the Ca^{2+} concentration.*

(3) It has been shown that *the pH optimum for growth of oat coleoptiles is different in the first 2 hrs after addition of auxin, where the optimum is < pH 4.5, as compared with the prolonged growth (2-12 hrs), where the optimum is pH 5.5.*

(4) It has been shown that *the outer layers are not required for auxin-induced growth of oat or corn coleoptiles or pea stems, and that gravitropic curvature of oat coleoptiles occurs without the epidermal layer.*

Significance of the Accomplishments

Finding #1 indicates that the amount of extracellular calcium is unimportant in the control of gravitropism.

Finding #2 indicates that free calcium does not inhibit growth by directly interfering with some step in normal growth, but must inhibit in some secondary way, such as by altering the Donnan Free Space pH.

Finding #3 indicates that while rapid changes in growth rate, such as occurs in gravitropism, are mediated by an acid-growth mechanism, the steady-state, prolonged growth response is in response to a different mechanism. This is a major finding in the area of plant growth.

Finding #4 indicates that the outer cell layers do not control either the growth or the gravitropic curvature of stems or coleoptiles. It indicates that other cell layers are capable of responding to auxin and are able to control the rate at which the organs grow.

The results of our investigations have shown that extracellular calcium is not an important factor in the actual gravitropic curvature mechanism. Calcium is, however, certainly important intracellularly in the gravireactive region and plays a role in the perception of gravity. These data indicate that it is unlikely that plants will undergo a loss of matrix calcium in microgravity, such as occurs in animal bones. They point out the significant differences that exist in calcium control mechanisms between plants and animals.

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MECHANISM OF DIFFERENTIAL GROWTH DURING STEM GRAVITROPISM

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Description of Research

Gravity exerts a major influence on the biochemical and biophysical processes that control the directionality of plant growth. Shortly after switching a plant from an upright to a horizontal position, growth asymmetries develop on the upper and lower sides of the plant, with the typical result that roots grow downwards and stems grow upwards. We are using this gravitropic response to gain insight into the fundamental control mechanisms of plant cell expansion and to learn the steps by which gravity influences these processes.

Our studies use young cucumber (*Cucumis sativus* L.) seedlings because they exhibit a rapid and vigorous gravitropic reorientation of their stems. This reorientation is accomplished by a transient cessation of cell expansion on the upper stem surface and a simultaneous doubling or tripling of expansion rate on the lower surface. We have found that this growth asymmetry begins to reverse itself well before the stems attain a vertical posture. Because the stem is only 1.5 mm in diameter, it is evident that the plant is able to execute very fine spatial as well as temporal control of cell expansion. By studying this gravity response, we hope to elucidate how plants integrate sensory stimuli and use this information to manipulate their growth.

Our studies have shown that cell turgor pressure and membrane hydraulic conductivity play a passive role — not a controlling role — for this gravitropic response. Indeed, we have used the growth asymmetry to assess the possibility that water transport limits growth as advocated by some studies. Our results showed that water transport poses an insignificant limitation to cell expansion in cucumber seedlings and that the major control point lies in the biophysical relaxation of the wall.

We are now studying the biochemical basis for wall relaxation, with the working hypothesis that wall enzymes catalyze the wall loosening that leads to wall relaxation. A major puzzle is the nature of these wall enzymes and the mechanism by which their activity may be regulated. We have attempted to address this problem by studying the extension of isolated cell walls.

Accomplishments

(1) *Isolated cell walls extend at much faster rates when wall thiols are reduced.* Thiols were reduced with 1 mM dithiothreitol, cysteine and related reagents. Thioredoxin, however, was not effective.

(2) Thiolyte MQ has been used to assess the redox state of protein thiols in the wall. This is a Calbiochem reagent that becomes fluorescent upon reaction with -SH groups. We found that Thiolyte MQ penetrates the cuticle of the cucumber stem and reacts with wall thiols, but is excluded from the cell. Thiolyte DB, on the other hand, penetrates the cells of cucumber stems and reacts with cytoplasmic as well as extra-cytoplasmic thiols. Interference from autofluorescence can be eliminated by washing and protein extraction of the walls or by a tissue printing method.

(3) ***Auxin induces a two-fold increase in the concentration of reduced thiols bound to macromolecular wall components, presumably protein.*** This was established by reacting stem segments, +/- auxin preincubation, in Thiolyte MQ, then isolating, washing and extracting the wall for ionically bound proteins, and then measuring Thiolyte fluorescence. Tissue printing techniques have been used to gauge the localization of Thiolyte fluorescence.

(4) Low concentration of exogenous thiol reducing reagent (dithiothreitol, DTT) does not stimulate growth by itself. However, in the presence of auxin, DTT does prolong cell expansion. This results in a 50% enhancement of the auxin-stimulated growth over 18-24 hrs.

(5) ***Proteins isolated from cucumber walls by salt extraction are able to induce inactivated (heat-treated) walls to extend.*** Protein fractionation by differential precipitation and by HPLC reveals two protein fractions which, by themselves, are sufficient to induce walls to extend. Permeation through gel sizing columns suggests a small molecular mass (< 10 kDa) for these proteins; however, when these fractions are analyzed by SDS PAGE, a major band of larger size shows up, suggesting that the protein binds to the gel and elutes late, thereby appearing to have a smaller size.

(6) ***Phloem unloading into the growing region of the hypocotyl becomes asymmetrical during the gravitropism response.*** We monitored phloem unloading by applying radiolabelled sucrose to the cotyledons and measuring its appearance in the growing region of the stem. A large asymmetry appears at the same time as the growth asymmetry.

Significance of the Accomplishments

Finding #1 ***suggests a new growth control mechanism, involving enzyme activation by thiol reduction of one or more proteins in the wall.*** A precedent for this would be the thioredoxin-regulation of photosynthetic enzymes. Finding #2 shows that it is technically feasible to assess the redox state of wall proteins. Finding #3 indicates that exogenous auxin leads to a net reduction of the protein thiols in growing walls. This finding provides partial support for the above hypothesis. Finding #4 indicates that thiol reduction alone is insufficient for stimulation of cell expansion, but that long-term growth is somehow influenced by thiol reducing reagents. These results indicate that a reducing environment in the wall may be important, perhaps regulatory, for wall loosening reactions.

Finding #5 ***provides the first direct evidence that walls contain enzymes that can induce extension of isolated cell walls.*** This extension activity is pH-dependent in a manner consistent with the acid-growth hypothesis, so we propose that this isolated protein is responsible for wall extension under conditions of low pH. The involvement of this protein in control of growth in living tissues, and its possible role in gravitropism, remain to be tested.

Finding #6 indicates that sugar unloading from the phloem is coordinated with cell expansion rates. We have no evidence that this change in phloem unloading is a causal mechanism for the growth rate change. Rather, we suggest that it is a secondary consequence of the altered pattern of growth. Experiments are underway to test this hypothesis.

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AN ATTEMPT TO LOCALIZE THE GRAVITY SENSING MECHANISM OF PLANTS: THE SPECIFIC CONDUCTANCE BETWEEN STELE AND CORTEX

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Description of Research

A plant stem, placed horizontally, responds to the gravity stimulus by growing back into a vertical orientation (Figure 1). For the stem to grow more on the lower than the upper side, more of the ions, hormones, and solutes required for growth must be transported out of the central vascular stele into the lower side of the horizontal stem (Figure 2).

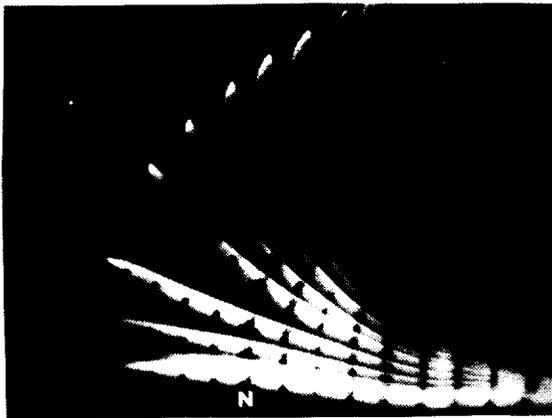


Figure 1. Time-lapse photography illustrating a horizontally placed plant stem responding to the gravity stimulus by curving back to its original vertical position.

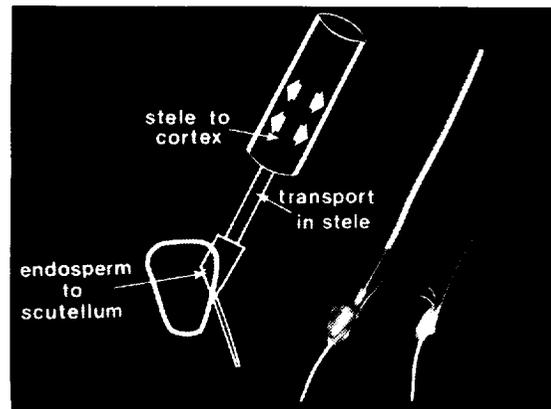


Figure 2. Diagram showing transport of plant growth substances out of the central vascular stele into the surrounding tissues, thus allowing asymmetric growth of the plant stem.

The objective of this research is to determine how the gravitational stimulus is transduced into a selective leakage of solutes from the central stele into the surrounding cortical tissues. Our working hypothesis is the Potential Gating Theory, which states that the gravity stimulus causes a perturbation in the plant's bioelectric field and that this perturbation opens and/or closes transport channels between stele and cortex. The resultant asymmetric solute movement permits asymmetric growth resulting in the response illustrated in Figure 1.

Accomplishments

We have measured the specific conductance of the tissues between the central vascular stele and the surrounding cortical tissues and found that the conductance changes as a function of the position of the stem relative to the gravity vector. This is shown in Figure 3.

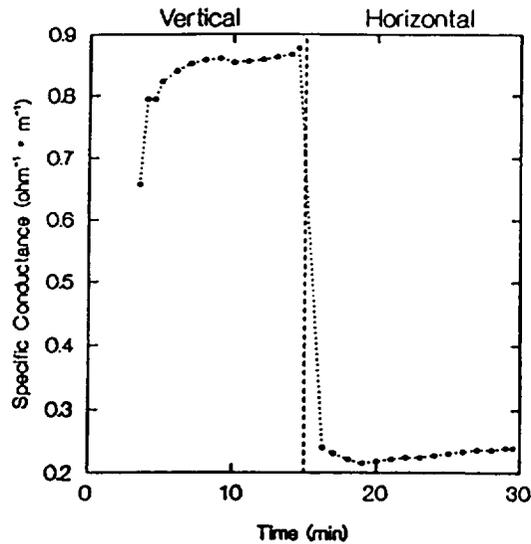


Figure 3. Graph showing changes in the specific conductance of the tissues between the central vascular stele and the surrounding cortical tissues of the plant stem. Conductance changes as a function of the position of the stem relative to the gravity vector. The vertical line indicates a change in the orientation of the tissue.

(1) Ag/AgCl electrodes having a tip diameter of 150 microns were constructed and held in a rigid jig in a micromanipulator apparatus such that one electrode could be inserted into the stele of a mesocotyl section and the second electrode inserted into the surrounding cortical tissue.

(2) An electronic apparatus was constructed such that a DC voltage of about 20 mV could be applied between stele and cortex and the resultant current measured.

(3) Many dozens of such measurements were made showing, almost without exception, the changes in conductance between stele and cortex illustrated in Figure 3.

(4) The apparatus is being modified such that initial rapid changes occurring in response to orientation changes may be measured.

Significance of the Accomplishments

Finding #1: *There is a change in the conductance of the tissue between stele and cortex of the mesocotyl of a corn seedling as a function of the orientation of the mesocotyl with respect to gravity.*

Finding #2: *The change is amazing both in magnitude and rapidity, changing from 0.85 ohms⁻¹ m⁻¹ to 0.22 ohms⁻¹ m⁻¹ in less than one minute.*

Finding #3: These changes in the conductance of the channels connecting stele to cortex may provide a biophysical basis for the asymmetric distribution of the growth hormone indole-3-acetic acid (IAA), previously described by this laboratory, and for the resultant asymmetric growth.

Finding #4: Success in monitoring the gravitropic response by a purely physical measurement, as opposed to complicated measurements of growth and hormone asymmetry, should permit progress in understanding how the plant perceives gravity and the initial transduction of gravity perception.

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THE GRAVITATIONAL RESPONSE OF *PHYCOMYCES* WITH RESPECT TO GADOLINIUM AND ION CHANNEL CHARACTERIZATION

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Description of Research

One of the simplest systems that 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 necessary for gravitropism — from detection of gravity to 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 indicate 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 effect of gadolinium on the kinetics of gravitropism in wild type and rapid gravitropic mutants and to survey ion channels existing in membrane patches from isolated membrane vesicles produced from sporangiophores, and to begin investigation of ion content and distribution in the gravitropic growth response zone and vacuolar region below. Much of this work has been done in conjunction with students Tawny Stecker and Vani Meesala and a postdoctorate, Girma Mitiku.

Accomplishments

Preliminary examination of sporangiophore kinetics last year indicated that gadolinium affects detection, transduction and growth phases of the gravitropic response; this was pursued more thoroughly using wild type and rapid gravitropic mutants. To avoid direct contact with the environmentally sensitive sporangiophore, indirect long-term gadolinium exposure of sporangiophores produced from 5-7 day-old mycelia grown on agar containing 1-250 micromolar gadolinium was examined. Sporangiophores were oriented horizontally and the kinetics of gravitropism were video-recorded and digitally analyzed. Fifty to 100 or more individual sporangiophore responses were needed to realize an average growth kinetic.

(1) *Gravitropic comparison of two mutants, C5 and C228, and of wild type Phycomyces sporangiophores has shown that wild type has an overall slow response with a 30 min delay to onset of positive gravicurvature, while C5 and C228 mutants show faster graviresponse with little to no delay between sensing gravity and response* (Figure 1A, C5 and wildtype). The "grand phase" of curvature required approximately 5 hrs for wild type with 20° curvature obtained in 2.5 hrs, while C228 required 4 hrs with 1 hr for the first 20°. The most rapid mutant, C5, required 3 hrs with 1 hr for the first 20°.

(2) *Gadolinium at 1-10 μM applied in the growth medium on which the mycelial mat grew and from which vertical sporangiophores arose had no effect on the kinetics of wild type* (Figure 1B) *or C228 sporangiophore gravitropism. C5 gravitropic response was slowed after 30 min, indicating gravity detection*

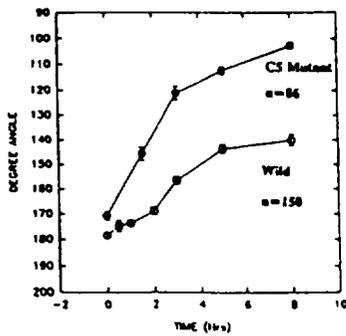


Figure 1A. Gravitropic curvature of wild and mutant sporangiophores.

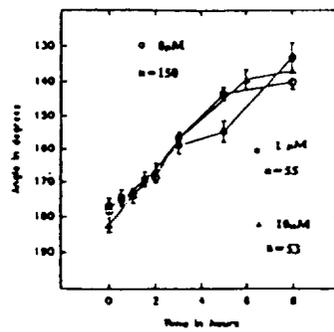


Figure 1B. Effect of gadolinium chloride on gravicurvature of wild sporangiophores.

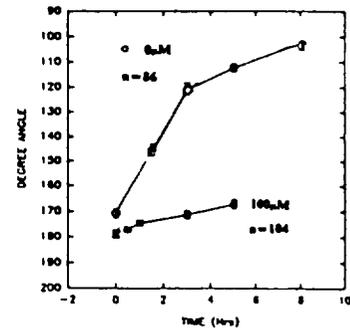


Figure 1C. Effect of gadolinium chloride on gravicurvature of mutant sporangiophores.

was not affected, but grand phase growth rate was reduced. Higher gadolinium concentrations in the growth medium, 100-250 μM , prevented any gravitropic response in C5 (Figure 1C) while not significantly affecting that of C228. Linear growth was not affected by gadolinium at any concentration in the C5 and wild type, but it was slowed slightly at high concentration in the C228 mutant.

Evidence in the literature has suggested that gadolinium might inhibit stretch-activated ion channels of membranes. Ion channels have not previously been characterized in *Phycomyces*. To access the membrane without enzymatic removal of the chitinous cell wall, membrane vesicles were produced by cutting sporangiophore tips and exuding cytoplasmic droplets. Most of the vesicles were membrane-bound, but the origin of the membrane is unknown: it is either vacuolar or plasma membrane. Ion channel observation by patch-clamping has been improved using vegetable shortening-coated coverslips, which immobilize sporangiophore membrane vesicles and increase successful patches to 90%.

(3) *Two relatively non-voltage dependent potassium channels have been preliminarily characterized from wild type sporangiophores*, passing both K^+ and Cl^- with a permeability ratio of 2.5 in excised patches. Sodium and nitrate are less permeable. The two channels may represent two different conductance states. With 75:150 KCl across the membrane, the slope conductances are 83.4 pS and 38.3 pS (Figure 2A, solid circles and triangles, respectively). The channel has a high frequency of distribution, as it is observed in nearly every membrane patch obtained (Figure 2B). Stretch-activated channels, on the other hand, are infrequently observed and are always short lived (< 1 min) even at low distortion levels of 1 - 4 in of water, making them difficult to characterize. Several possibilities exist to account for this fact, ranging from artifactual to molecular limitations. Other channels also have been observed, showing spike activity or small conductances.

(4) Preliminary X-ray microprobe scanning EM (EDAX) analysis of growing tip and vacuolar regions of the sporangiophore has shown high amounts of potassium, phosphorus, aluminum, sulphur and silicon.

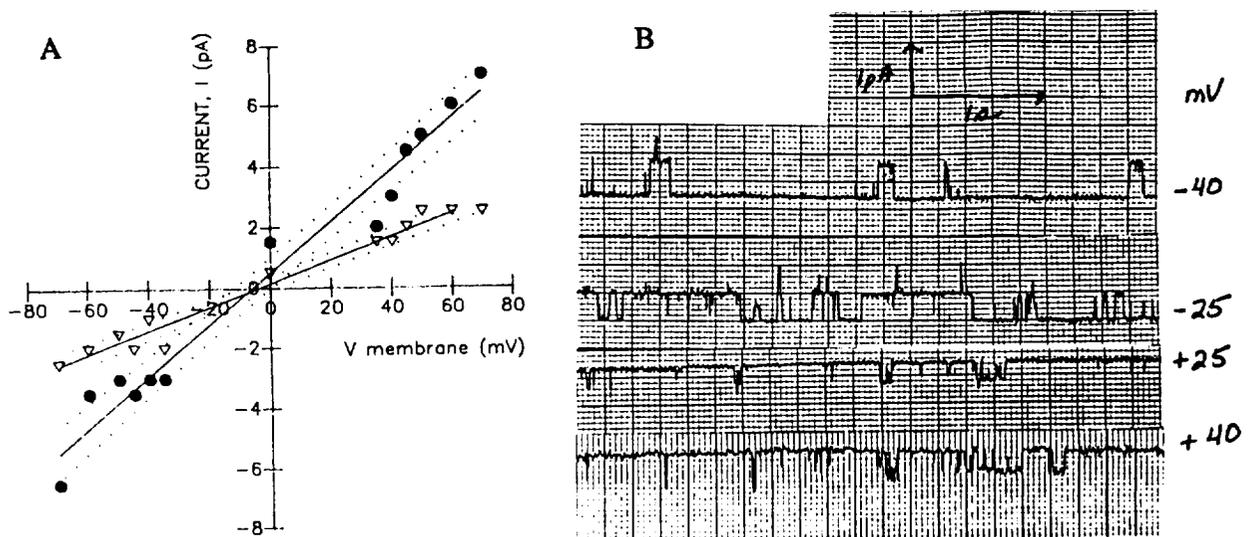


Figure 2. A. Slope conductances of two non-voltage dependent potassium channels in *Phycomyces* sporangiophores. B. Frequency of potassium channel conductance distribution in various membrane patches.

Significance of the Accomplishments

Gadolinium, known to inhibit higher plant gravitropism, also affects *Phycomyces* sporangiophores when produced from medium containing gadolinium. Evidence suggests that gadolinium affects the rate of gravity-induced curvature as well as the degree of curvature which could result from a detection system made less sensitive by gadolinium. At this point we do not have evidence that gadolinium directly affects ion channels as shown in animal cell systems. In past years we have shown that gadolinium is translocated through the cell wall and accumulates in the cytoplasm of the sporangiophore. Gadolinium is known to compete for calcium at calcium-binding proteins, and this action could produce slower response and lower sensitivity to gravity if such proteins are involved in sensing and transducing the gravitational signal or in the orchestration of asymmetrical growth. The rapid gravitropic mutants may have improved sensing and growth induction mechanisms. That one mutant is inhibited by gadolinium while the other is not suggests to us that the latter has protein modifications which prevent gadolinium binding. Comparison of such mutant growth kinetics, effects of calcium and cytoskeleton modulators, and ion channel behavior should yield information about gravity detection and induced curvature. We have shown that ion channels are present in the aerial sporangiophore. Such channels may exist in the vacuolar membrane or plasmalemma. Channels have been hypothesized to play a role in gravitropism and may be regulated by calcium or cytoskeletal proteins. We are now investigating these possibilities. We are using scanning EM X-ray microprobe analysis to investigate ion distribution in different regions along the sporangiophore. Limiting the analysis to calcium and gadolinium should yield information on their distribution at the growing tip and in the large vacuolar region which comprises the major mass of the sporangiophore.

Publications

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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 occur about 0.5 cm behind the tip. We are interested in the pathway of movement of signal from the cap to the elongation zone. We want to determine the interaction between stimulus-induced changes in the distribution of chemical signals that control growth (e.g., hormones) and accompanying changes in the sensitivity of the responding cells to hormonal or other growth modifying signals.

Our research has centered on the following: (1) Using antibodies that can immobilize hormones so that we can test the involvement of these hormones in the transmission of signals from the root tip to the growing zone; (2) Testing the responsiveness of the inner and outer cell layers to endogenous growth regulating factors such as hydrogen ions (acidic pH) and the hormone auxin; (3) Using a computerized video analysis system to determine the role in gravitropism of a specialized zone of cells located between the root cap and the elongation zone; (4) Direct testing of the ability of cells in the elongation zone of roots to adapt (change their sensitivity) to sudden changes in auxin level; and (5) Examining the kinetics of auxin action on seedling roots of mutants (*Arabidopsis*, tomato) with altered auxin or gravitropic sensitivity.

Accomplishments

(1) We used microsurgery to introduce a gap in the epidermis/cortical tissues of the root between the cap and the elongation zone. When this gap is filled with agar, the gravitropic signal traverses the gap. ***Filling the gap with antibodies to the plant hormones auxin or ABA does not prevent movement of the signal across the gap.***

(2) When vertically oriented roots of maize are cut longitudinally, bisecting the apical cm of the root, the two halves curve gradually toward the cut surface. This appears to result from more rapid growth of the outer cell layers relative to the inner cell layers. Low pH enhances the curvature, indicating that ***the outer cell layers show the strongest growth promotion in response to low pH.*** Auxin retards or reverses this curvature.

(3) Analysis of growth patterns in gravistimulated roots reveals that ***gravistimulation induces rapid cell elongation within the "PIG" (postmitotic isodiametric growth) zone, a region of the root near the tip in which cell division is absent and cell elongation is minimal.***

(4) We find that ***maize roots exhibit "adaptation" to inhibitory levels of auxin*** as indicated by their ability to resume rapid growth after the initial period of strong growth inhibition following application of the hormone. In addition, ***roots that have***

adapted to elevated auxin levels show excessively high (higher than normal) rates of elongation upon sudden withdrawal of the inhibitory hormone.

(5) We have improved the optics of our video digitizer system so that we can study root growth and gravitropic curvature in species with small primary roots (e.g., *Arabidopsis*, tomato). In preliminary experiments it appears that the latent period for auxin inhibition of growth in *Arabidopsis* roots is only a few minutes as compared with the 10-15 min lag reported for species with larger roots.

Significance of the Accomplishments

Finding #1: The finding that antibodies specific to IAA or ABA fail to retard the movement of the gravitropic signal from the cap to the elongation zone indicates that the signal is not traveling as either of these hormones. This discovery is significant because most popular models of root gravitropism propose that the signal moves as either IAA or ABA. Our results remain tentative until we can demonstrate that the kinetics of binding of the hormones to the antibodies is such that immobilization of the hormones can be expected under the conditions of these experiments.

Finding #2: The observation that the outer cell layers of the primary root of maize are the most responsive both to growth promoting and to growth inhibiting agents indicates that these are the most likely cells to be involved in establishing the differential growth pattern that leads to curvature in gravistimulated roots. Tests of the response phase of the gravitropism signal/response cascade need to be focused on the specific cell layer(s) involved in the response.

Finding #3: The observation that cell elongation is initiated in the "PIG" zone is significant because it represents a new mode of growth rate adjustment during the motor response of gravitropism. Until now attention has been paid almost exclusively to changes in rate in the existing elongation zone. This new finding draws attention to the possible "recruitment" of new cells in the adjustment of growth rates.

Finding #4: The finding that roots appear to reduce their sensitivity to auxin when exposed to elevated auxin levels is significant because (a) auxin levels are thought to change (e.g., increase along the lower side) during root gravitropism, and (b) the timing and spatial distribution along the root of the adaptation to elevated auxin levels matches the timing and pattern of growth rate adjustments during gravitropism. This indicates that time-dependent adjustments in auxin sensitivity play a role in the motor mechanism of root gravitropism.

Finding #5: The major significance of this finding is a technical one — we will now be able to use our video digitizer system to examine the wide variety of mutants available for gravitropism studies. Also, if *Arabidopsis* roots respond to auxin in only a few minutes, this would represent the most rapid growth response known, and it would cause us to rethink possible metabolic pathways involved in auxin action on growth.

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PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR PROCESSES ASSOCIATED WITH THE TRANSDUCTION OF GRAVITY IN ROOTS OF MAIZE

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Description of Research

Our research focuses on understanding the mechanisms that plants use (specifically the processes in roots) to convert the gravity stimulus into a growth response. For this effort we are using roots of a variety of corn (Merit) which require light in order for the roots to respond completely to gravity. If seeds of these varieties are germinated in total darkness the roots grow in a horizontal fashion. Illuminating the root — specifically the root cap — with red light initiates downward growth. It is hypothesized that the red light switches on some processes that are important in the complete processing of the gravity stimulus. Using these varieties of maize which require light for downward gravitropic curvature, we are trying to understand which processes are switched on, and for this effort have addressed two general questions. Both of these questions revolve around the hypothesis that the photomorphogenetic pigment phytochrome serves as the focus for transduction of the light requirement. Two areas of phytochrome physiology were investigated. First, we wanted to establish where phytochrome is localized in the root. Is it in high concentration within the cap, as would be predicted if the cap is the locus of perception of the light stimulus? Second, we wanted to understand how phytochrome could biochemically transduce the gravitropic stimulus.

Accomplishments

In order to analyze where phytochrome is localized within the root, and specifically within the root cap, we employed techniques involving localizing on a microscopic level processes associated with phytochrome. In particular, we felt it was not enough to show that the pigment was present in a tissue. Rather, it was important to show that the pigment was active — in other words, that after it absorbed light it actually caused a response. We asked where in the cap, if anywhere, phytochrome is active. We made use of earlier work showing that phytochrome regulates the level of its own messenger RNA. Using *in situ* hybridization techniques we showed that ***phytochrome is most active in the root cap, specifically within the central-most portion of the cap***, which is also the region of the cap containing the amyloplasts, the presumed sensors for gravity. Moreover, we were able to show that the ***phytochrome response was rapid enough to be considered consistent with its hypothesized role in transducing gravity***. Interestingly, the turnover of the mRNA for phytochrome parallels kinetics for root curvature.

The second aspect of our work involved asking how phytochrome may biochemically transduce gravity. We hypothesized that this molecule may work by activating or associating with specific protein kinases, hence leading to the phosphorylation of specific substrate proteins. We have taken a molecular approach for this work, and have used synthetic oligonucleotide probes to screen a maize root library for kinases which possibly could be associated with phytochrome. To date we have obtained clones for several putative kinases, have sequenced these kinases and are now trying to establish the mechanism of regulation for these kinases. We have shown that ***the isolated clones***

code for kinases that appear to be second-messenger regulated, indicating that gravity transduction in root caps may involve mechanisms found generally in microbial and mammalian systems.

Significance of the Accomplishments

During the past year we have concentrated on trying to unravel the role of the photomorphogenetic pigment phytochrome in root gravitropism. By deciphering this role we feel it might be possible to obtain insights into the biochemistry associated with converting the gravity stimulus into a chemical event, and ultimately into a developmental response. Hence, our ability to localize phytochrome activity to the central cells of the root cap is a considerable accomplishment. We have been able to show that phytochrome is active within the same cells in which it is hypothesized that gravity is perceived. This means that the gravity perception and signal processing mechanisms for roots are probably located within the same cells, suggesting that the two processes may be closely linked both developmentally and genetically. In addition, our ability to relate the kinetics of phytochrome processes to the actual gravity-induced curvature of the root provides further evidence for a role for phytochrome in the transduction of gravity.

Our second area of accomplishment is to show that elements of general signal transduction pathways, specifically protein kinases, are active in roots of maize. This is an important finding since it suggests that the biochemical processes in converting gravity from a physical to a developmental response involve elements found in other organisms — elements already well-characterized with regard to their involvement in the transduction of environmental stimuli.

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CIRCADIAN RHYTHMS PERSIST IN SPACE: RESULTS FROM "CHARACTERIZATION OF *NEUROSPORA* CIRCADIAN RHYTHMS IN SPACE"

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Description of Research

Shuttle flight STS-9 carried an experiment that examined the function of the circadian oscillator (i.e., pacemaker, which times biological events with periods approximately 24 hrs in length) in the microgravity environment of space. The experiment reexamined whether the drive of circadian oscillations is generated endogenously within the organism or imposed exogenously via environmental influences. The STS-9 results, while not conclusive, supported the endogenous generation of biological rhythms. Post-flight studies supported the hypothesis that the hypergravity of launch could have been responsible for the damped circadian rhythm. These studies suggest a gravity sensing system in the pacemaker of *Neurospora*.

To test the hypothesis that *Neurospora crassa* possesses an endogenously generated circadian rhythm and a gravity sensing system, we conducted an experiment on shuttle flight STS-32. Two culture strains, BND and CSP, were each divided into three experimental packages in the mid-deck locker. The "White" package, treated similarly to the STS-9 package, was exposed to ambient (mid-deck) white light on flight days 6, 9 and 10. The "Blue" package was marked in ambient light within 10 hrs after launch and then again on flight days 6, 9 and 10. The "Red" package was also marked within 10 hrs after launch and on flight days 6, 9 and 10. Tubes in this last package were wrapped with a red filter which allowed growth front marking in a light environment in which the *Neurospora* pacemaker appears insensitive.

Four flight back-up culture sets, each delayed 24 hrs, were used as synchronous 1 g ground controls. These controls were maintained in constant dark at 25°C. Asynchronous controls were run post-flight to delineate the effects of the mid-deck environment from the effects of gravity. The Orbital Environmental Simulator (OES), a computer-driven environmental chamber, read recorded calibrated ancillary flight data (see Figure 1) into controllers to simulate ambient environmental flight conditions (i.e., mimicking temporal variations in flight temperature, humidity and carbon dioxide concentrations). Flight temperatures within the mid-deck locker ranged from 20.8 - 29.7°C (mean \pm SEM, 26.6 \pm 0.1°C), carbon dioxide concentrations ranged from 683 - 4843 ppm (mean \pm SEM, 1957 \pm 33 ppm), and relative humidity ranged from 28.0 - 49.2% (mean \pm SEM, 36.7 \pm 0.2%). Two asynchronous control sets were run in the OES under flight conditions and a third asynchronous control set was run at 25°C in an incubator used for synchronous 25°C ground controls. All ground controls were marked in white light at the times defined by flight procedures.

Accomplishments

The conidiation rhythm of the BND cultures in flight was of quite high amplitude throughout flight. While there was a small decrease in rhythm amplitude in a significant percentage of the BND cultures in flight when compared to the 25°C controls,

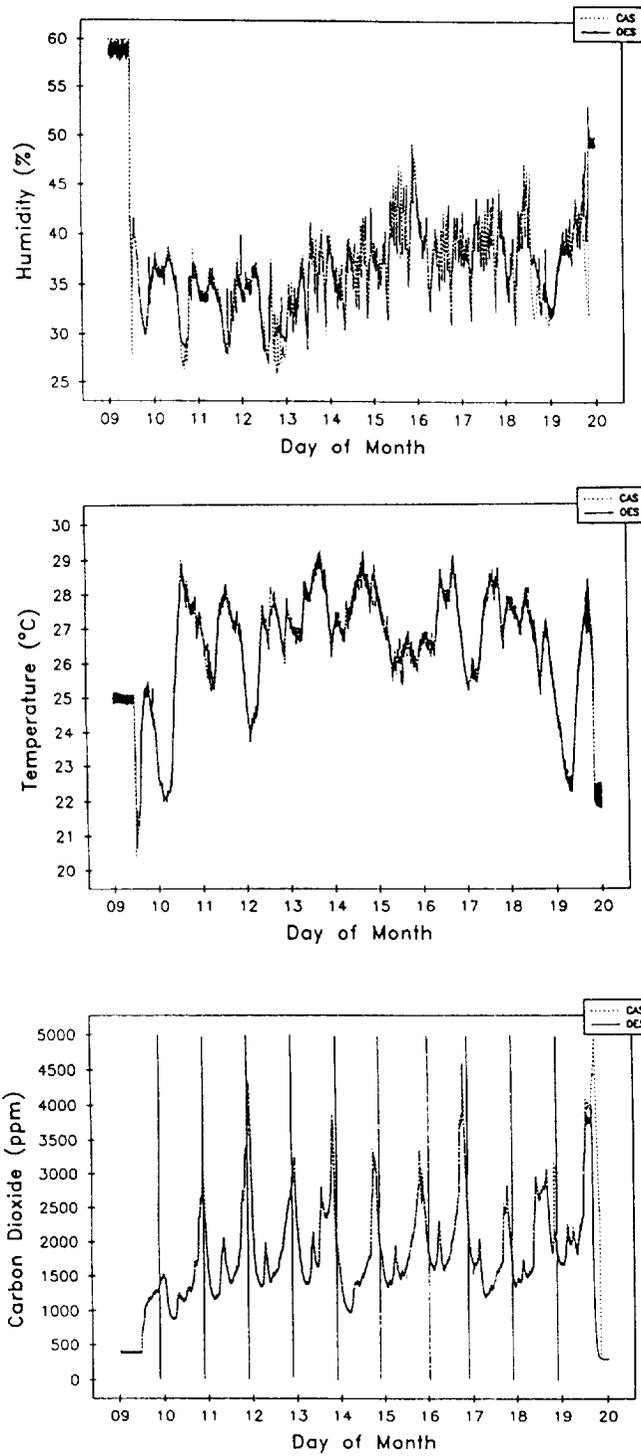


Figure 1. Mid-deck environmental profiles of relative humidity (%), temperature (°C) and carbon dioxide concentration (ppm) during flight. Flight temperatures within the mid-deck locker ranged from 20.8 - 29.7°C (mean \pm SEM, 26.6 \pm 0.1°C), carbon dioxide concentrations ranged from 683 - 4843 ppm (mean \pm SEM, 1957 \pm 33 ppm) and relative humidity ranged from 28.0 - 49.2% (mean \pm SEM, 36.7 \pm 0.2%). A diurnal pattern in these parameters is readily apparent. The periodicities of these diurnal environmental cycles were 23.9, 21.3, and 23.3 hrs, respectively, for temperature, humidity, and carbon dioxide concentration.

there was no significant diminution of rhythm amplitude in flight when compared to OES controls. A significant number of the flight CSP cultures, however, had rhythms with somewhat lower amplitude than CSP cultures in the 25°C or OES controls. A much smaller percentage of cultures was actually arrhythmic for any length of time. *There were no significant differences in the number of arrhythmic cultures between any of the experimental or control groups.*

Exposure of the BND cultures to spaceflight or similar mid-deck environment (OES controls) resulted in a significant and similar period lengthening when compared to the 25°C ground controls. There were no significant differences in period of the BND flight cultures when compared to the OES controls. Exposure of the BND cultures to spaceflight or the OES resulted in an average period lengthening of approximately 2.7 hrs and 2.8 hrs, respectively, when compared to the 25°C controls.

Exposure of the CSP strain to flight or OES significantly lengthened the free-running period when compared to 25°C controls. Exposure to space, however, attenuated the effects of OES-simulated conditions (i.e., the mean period length of the flight cultures, while longer than the 25°C controls, was shorter than the OES controls). Spaceflight increased the period length by approximately 0.8 hrs, while the mid-deck environment alone (simulated by the OES) resulted in an approximate 2.0 hr increase.

In more recent experiments, cultures of the two strains were placed in constant dark in low, high or a 24-hr high-low temperature cycle. The conidiation rhythm was consistently below 21.5 hrs in length in the BND strain and less than 22.0 hrs in the CSP strain in both the high and low temperature conditions, thus supporting the relative temperature compensation of the circadian clock. The cultures exposed to the high-low temperature cycle, however, had dramatically increased period lengths. While the period lengths greatly increased in the temperature cycle, they differed from the period of the temperature cycle. This result suggests that there are phase shifting effects of temperature transitions; however, entrainment to the temperature cycles was not attained. These findings support the observation of increased period length in space, without necessitating entrainment or environmental drive of endogenously generated rhythms.

Growth rate increased significantly in flight when compared to the 25°C controls. The increased growth rate in space is not exclusively due to higher in-flight temperatures. With only one exception, in both strains, in all three packages, the growth rate was significantly higher during spaceflight than in the OES. Overall, the increased OES (and flight) temperatures resulted in an approximate 0.15 mm/hr (21%) increase in growth rate. The temperature-independent effect of spaceflight on growth rate was an additional increase of approximately 0.12 mm/hr (17%).

Significance of the Accomplishments

If the hypergravity pulse of launch, and not the microgravity of space, caused damping on STS-9, the Blue package would display the greatest rhythm amplitude, since it was exposed to white light shortly after launch. Both the Red and White packages were hypothesized to remain damped in response to launch. Since the Red package was exposed to red light, to which the circadian system appears insensitive, it was expected to show less damping than the White package only if some aspect of the acceleration-induced *g* forces of handling was at least partially responsible for the reorganization of the rhythm. Therefore, the Blue package had a light control (Red) and a movement control (White) for the first mark. In both Blue and Red packages rhythm amplitude increased after the six day mark,

suggesting an involvement of acceleration, rather than white light, in the reorganization of the rhythm. The fact that the cultures in the White package were of the highest amplitude, however, complicates the issue.

It must be kept in mind that damping of the conidiation rhythm is not necessarily related to clock function. Conidiation is sensitive to input (e.g., carbon dioxide) that may not affect the underlying pacemaker. The finding that the BND flight and OES cultures, which had similarly high carbon dioxide concentrations, were equally damped suggests that the reduced rhythm amplitude of the BND cultures on STS-9 was due to increased carbon dioxide levels and not to the microgravitational environment of space.

The mean temperature in the mid-deck locker was approximately 1.6°C higher than the 25°C controls. The *Neurospora* pacemaker, however, is relatively temperature-compensated between 25 and 30°C (i.e., Q_{10} of the free-running period of BND is 1.2). Thus, Q_{10} cannot entirely predict the increased period length in the flight or OES temperature controls. Some environmental aspect of the mid-deck, however, accounted for the increased period length, since the OES increased the period at least as much as flight. The minor gravity-induced alterations in period were actually an attenuation of the non-gravitational effects observed in the OES controls.

Thus, the rhythm persisted quite normally in an environment devoid of usually occurring tonic and geophysical oscillations of gravity. While the flight experiment kept constant or significantly altered the periods of many known potential environmental zeitgebers, unanticipated diurnal oscillations were present in temperature, carbon dioxide concentrations and relative humidity (with periods of 23.9, 23.3 and 21.3 hrs, respectively). These oscillations were not observed during the STS-9 flight. Therefore, while exposure of cultures to spaceflight failed to stop or unacceptably alter circadian timekeeping, which supports the hypothesis of endogenous generation, it did not eliminate the possibility of exogenous genesis. While the conidiation rhythm of *Neurospora* has been shown to entrain to small amplitude temperature cycles, this possibility in the flight experiment is diminished by the fact that the periods of the conidiation rhythm of BND flight cultures and all OES cultures were at least 0.3 hrs longer than the period length of the temperature rhythm. The significantly shorter period length of the CSP cultures in flight even more strongly argues against entrainment.

That gravity may affect the circadian system was suggested by studies in Rhesus monkeys, which demonstrated external desynchronization in space, and by centrifugation studies in *Neurospora*. In the present study, however, the response to temperature was much greater than the response to gravity. In fact, microgravity attenuated the temperature response in the CSP strain. Thus, while gravity may affect the pacemaker, the strength of this zeitgeber appears substantially less than light or temperature.

Growth rate was significantly increased in response to spaceflight. The accelerated growth rate cannot be explained entirely through well-known thermal mechanisms. The Q_{10} of the growth rate of the BND strain in temperatures ranging from 25-30°C is about 2.2. Thus, a mean temperature rise of 1.6°C could only account for approximately 0.13 mm/hr of the difference observed between the OES and the 25°C controls which accurately predicts the increased growth rate of the OES controls. A similar thermally-induced acceleration of growth rate (approximately 0.13 mm/hr) could also have been predicted in flight; however, a substantial *additional* increase in growth rate (approximately 0.14 mm/hr) cannot be explained thermally. The response may suggest an altered metabolic rate in space. This hypothesis is supported by several studies of other species during spaceflight.

In conclusion, the findings of STS-32 Life Science Experiment CNCR-01 suggest that circadian rhythms can persist in space. The results 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. It appears that while temperature can, and did, have phasing effects on the circadian rhythm of conidiation in space, entrainment was not attained in orbit. Furthermore, the generation of the rhythm was endogenous to the organism and not likely a derivative of the environmental rhythms. Another potentially important finding is that of altered metabolic rates in space, evidenced by the non-thermal increase in growth rate. Both sets of findings 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 and/or habitation.

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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 unravel the mechanism of negative gravitropic curvature (upward bending) in pulvini (leaf-sheath bases) in lodged (prostrated) cereal grass shoots. In our research, we are trying to understand: (1) how gravisensing (gravity perception) occurs in gravistimulated pulvini; (2) how native hormones such as IAA (indole-3-acetic acid) and its conjugates and gibberellins become asymmetrically distributed in an upturning pulvinus during the transduction phase which follows gravity perception; and (3) the cascade of events that occur following transduction and that cause the gravistimulated pulvinus to grow upwards.

Our earlier studies have conclusively shown that starch-containing chloroplasts, located in parenchyma cells adjacent to vascular bundles, act as the gravisensing organelles in cereal grass shoot pulvini. However, we do not yet understand how these "heavy bodies" elicit signal transduction that leads to initiation of development of hormone asymmetry. We believe they could act as "pressure probes" to open hormone or ion channels or serve as information carriers (e.g., of Ca^{2+} or of hormone deconjugating enzymes).

Analyses for endogenous auxin (IAA) and gibberellins in top and bottom halves of graviresponding pulvini of cereal grasses indicate that both types of hormones develop a top/bottom asymmetry of ca. 1:2, as expressed in terms of amounts of free hormone present (nanogram levels) per mg fresh weight of tissue. We are currently determining when their asymmetry is first established and how it arises (through differential rates of synthesis, or through differential rates of release of free forms of IAA and GAs from their conjugates, or through differential rates of breakdown).

The asymmetric distribution of free IAA and free gibberellins in gravistimulated pulvini of cereal grass shoots has significance in that these chemical messengers may be the primary molecules which initiate the cascade of events leading to unequal growth (differential cell elongation) in an upturning pulvinus. We observed differential cell wall loosening (plastic extension), sucrose hydrolysis (mediated by invertase or β -fructosidase), cell wall biosynthesis (mediated by glucan synthase), and cell wall composition changes (notably, in β -O-glucan = mixed β -1,3- and β -1,4-linked glucan) in gravistimulated oat shoot pulvini. All of these metabolic changes are most likely under regulatory control by free IAA and/or free gibberellin. We are currently investigating this possibility at a molecular level, particularly in connection with differential invertase gene expression. The invertase (β -fructosidase) is important in that it results in release of D-glucose and D-fructose from sucrose, two hexoses which are important for cell wall biosynthesis and for maintenance of turgor pressure (the driving force for cell expansion).

Accomplishments

(1) *Changes in levels of free indole-3-acetic acid (IAA) in graviresponding oat shoot pulvini.* Our first kinetic analysis of free IAA and total IAA (free IAA + IAA conjugates) in top and bottom halves of graviresponding oat shoot leaf-sheath pulvini was carried out by Shaorong Chong in Dr. Jerry Cohen's Plant Hormone Laboratory at BARC-West, Beltsville, MD, in 1990. Pulvini were excised from oat stem segments gravistimulated in the dark at 25°C for 3 to 24 hrs while being fed 0.1 M sucrose from the segment bases. Results are shown in Figures 1 and 2. Figure 1 shows time-course changes in free IAA and total IAA in top and bottom halves of graviresponding pulvini, and Figure 2 presents the typical kinetics for rate of upward bending in gravistimulated oat shoot pulvini. The following points can be made: (1) *asymmetry in free IAA levels starts to develop within 3 hrs after beginning gravistimulation;* (2) *full top/bottom free IAA asymmetry is manifested by 6 hrs following start of gravistimulation;* (3) *generally, free IAA and total IAA (free + conjugates) levels drop in these pulvini in both top and bottom halves over the 24 hrs of gravistimulation;* and (4) *the time-course changes in free IAA in top versus bottom pulvinus halves are closely correlated with rate of upward bending of the pulvinus over the 24 hr gravistimulation period.*

(2) *Graviresponse of oat shoot leaf-sheath pulvini at different pHs.* The effects of controlled pH on the gravitropic response of p-1 pulvinus-containing oat stem segments were assessed by measuring upward bending of the pulvini 24 hrs after initiation of gravistimulation treatment. (p-1 refers to the site of next-to-the-last-formed

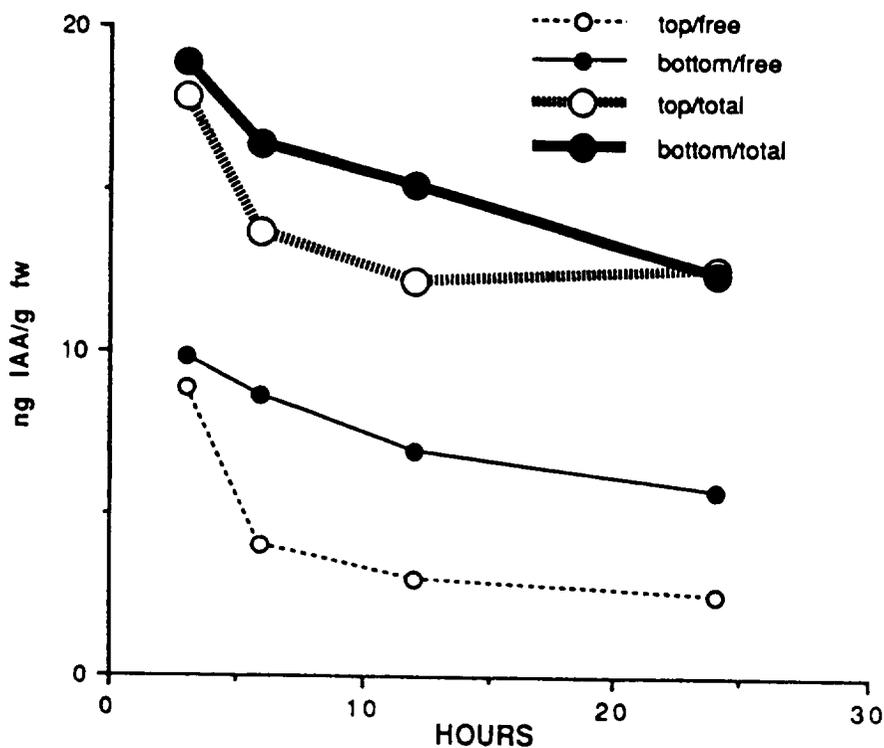


Figure 1. Time-course changes in free IAA in top and bottom halves of graviresponding oat shoot pulvini.

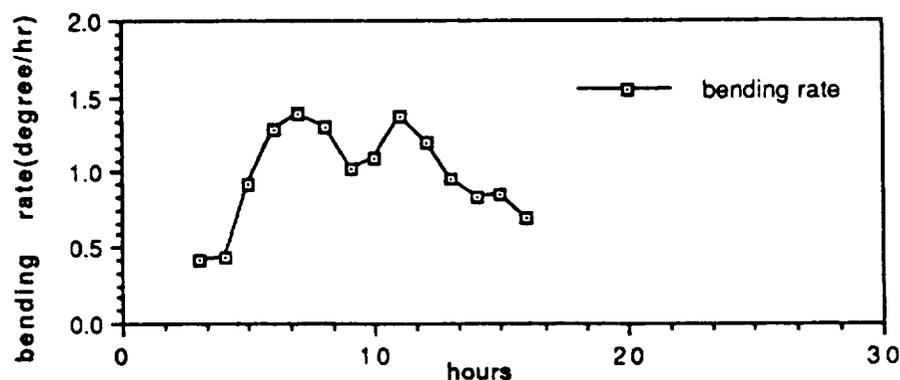


Figure 2. Typical kinetics for rate of upward bending in gravistimulated oat shoot pulvini.

node/pulvinus on a grass shoot. "p" is the last formed peduncular node/pulvinus of a grass shoot.) During the treatment, pulvinus segments were supplied with 0.1 M sucrose and 50 mM phosphate-citrate buffer (over a pH range of 5 to 8). A control experiment was carried out by using non-buffered 0.1 M sucrose solution. Figure 3 shows the difference in upward bending under different pH conditions. The following points can be made: (1) *when whole (intact) pulvinus-containing stem segments were gravistimulated, the graviresponse of the pulvini at pHs 7 and 8 was significantly lower (about 83% of the control curvature response) than that of non-buffered control pulvini and of pulvini at pHs 5 and 6;* (2) *when longitudinally pre-halved pulvinus segments were treated as above over a pH range of 5 to 8, the bottom half of the pulvinus showed 63% and the top half showed only 24% of full graviresponse that is manifested in non-buffered control pulvini.*

(3) *Changes in pH of the pulvinus during the gravitropic response.* Changes in extracellular pH of the pulvinus were measured during the gravitropic response of pulvinus-containing stem segments by using a combination pH microelectrode (Microelectrodes Inc., Model MI-4152). The microelectrode was inserted into the pulvinus prior to the initiation of gravistimulation. Changes in pH were continuously recorded during the gravitropic response of pulvinus by using X/Y-recorder. From the results presented in Figure 4, the following points can be made: (1) *the bottom part of the pulvinus shows transient alkalization followed by an enhanced rate of acidification compared to that of vertical control pulvini and to top pulvinus portions during the gravitropic response;* and (2) *the top part of the pulvinus shows virtually no difference in pH compared to that of the vertical control, and both show similar moderate rate pH drop after 200 min of gravistimulation.*

(4) *Invertase gene expression in oat pulvini tissue.* In our studies of the oat pulvinus response to gravity, we have attempted to isolate and sequence the invertase gene, following a comparison of the base sequence between carrot cell-wall β -fructosidase, yeast invertase, and sucrase from *Bacillus subtilis*. [Invertase is also called sucrase (old terminology) and β -fructosidase (correct biochemical terminology).] We saw two consensus regions. Between the two consensus regions, which gave a difference of 181 amino-acid equivalent to 543 base pairs, we designed two primers designated as OI-1 and OI-2. cDNA was then synthesized in our laboratory from total RNA as well as polyA mRNA. Employing the two primers, a polymerase chain reaction was performed with the

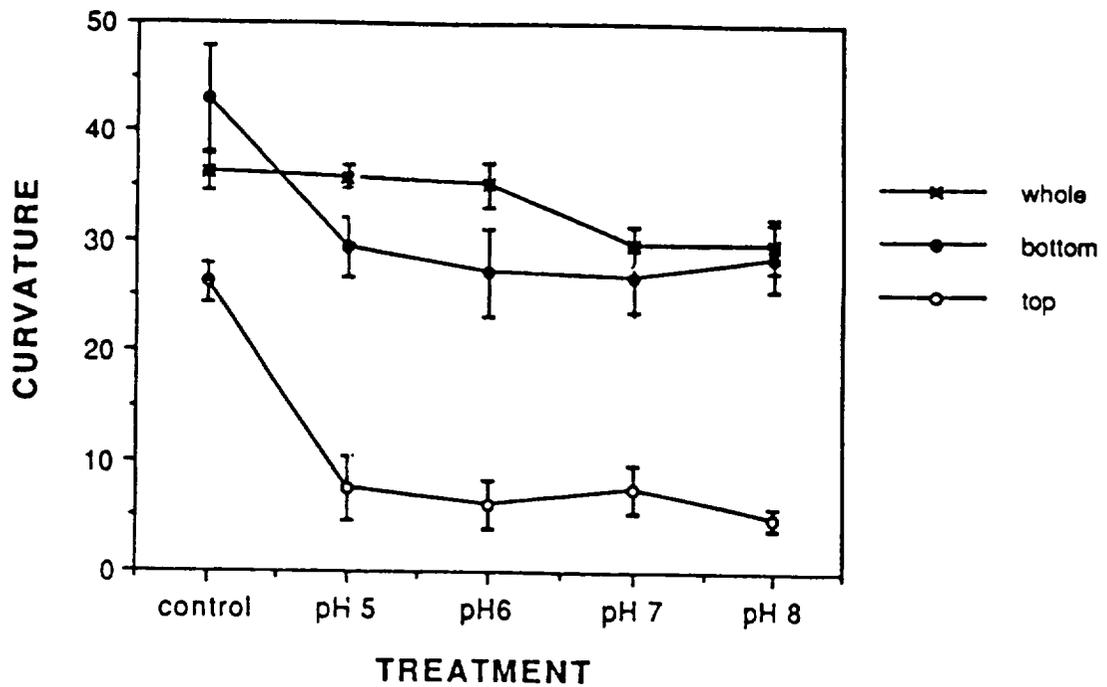


Figure 3. Differences in amount of upward bending (gravitropic response) in whole (intact oat shoot pulvini as compared with top and bottom pulvinus halves after 20 hrs under buffered (pHs 5, 6, 7, and 8) and non-buffered (control) culture conditions. p-1 (next-to-last) pulvinus-containing stem segments were fed 0.1 M sucrose under the different pH conditions in the dark at 30°C.

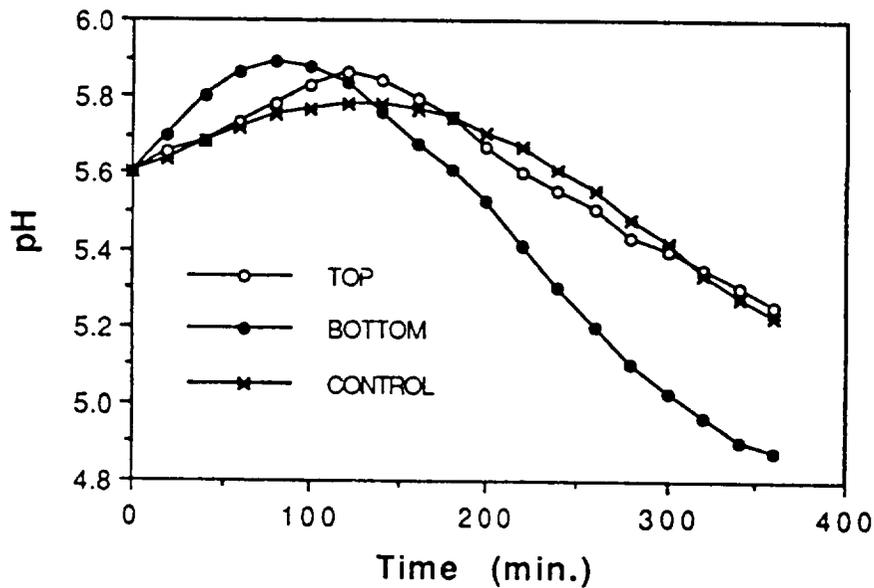


Figure 4. Changes in pH of vertical (control) versus gravistimulated top and bottom halves of the pulvinus during the gravitropic curvature response.

synthesized cDNA. The PCR products were then extracted with phenol/chloroform and ethanol precipitated. Following restriction digest and agarose electrophoresis, a **major band was observed with ~550 base pairs**. This band was subcloned into M13p18 sequencing vector. Employing the method of Sanger, we sequenced the DNA with sequence version 2 and compared its base sequence with the cell wall invertase of carrot. **The oat invertase showed a homology of 61%.**

Significance of the Accomplishments

Finding #1: We now know from analysis of free IAA in graviresponding oat shoot pulvinus upper and lower halves that full hormone asymmetry is established by 6 hrs after the initiation of gravistimulation treatment and that partial asymmetry is seen after 3 hrs of gravistimulation. Further, when the top/bottom asymmetry in free IAA levels also increases, and when it more or less reaches a steady-state rate of upward bending, the top/bottom asymmetry in free IAA is pretty much constant from the period of 6-24 hrs after initiation of gravistimulation treatment. Based on these results, we have reason to believe that initiation of free IAA top/bottom asymmetry starts earlier than 3 hrs after the initiation of gravistimulation of oat shoot pulvini.

How this asymmetry in free IAA arises is not yet resolved. This will await analysis of changes in amide- and ester-linked IAA as compared with free IAA during the course of upward bending in gravistimulated pulvini.

Findings #2 and #3: The importance of pH changes in cereal grass pulvinus tissue for the gravitropic response of the oat pulvinus has been demonstrated. Changes in pH of oat pulvinus tissue during the gravitropic response were measured for the first time. As expected, the bottom part of the pulvinus showed a faster rate of acidification than did the top part of the pulvinus. This result may be related to the asymmetric distribution of IAA during gravitropic response [see (1) above]. An interesting observation worth mentioning is the initial alkalization of the pulvinus preceding the acidification. Since our previous studies showed that upward bending of the oat pulvinus starts during this phase, the biochemical and/or physiological system responsible for the alkalization seems to be involved in the regulation of early steps in the process of upward bending of the pulvinus.

Finding #4: The cDNA ~550 base pairs will be used as a probe to pull out the full length cDNA from an oat pulvinus cDNA library. We will then sequence the cDNA and compare the similarity between the cDNAs for verification. Once this is achieved, we will construct a genomic library and pull out the oat invertase gene with the cDNA (full length) probe and sequence it. We will then assess how oat pulvinus invertase gene expression is regulated by gravity and by the two hormones, IAA and GA, that are involved in the transduction process.

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interpretability of results. Furthermore, it was found that cells accumulated on the apparatus windows and that window cleaning procedures require improvement.

(d) It was found that cell cultures deteriorate faster in the apparatus than in glass bottles. This effect could have been due to bacteria that ferment algal debris produced in the ball bearings of the belt system.

Conclusions. The fluid mechanical basic shear apparatus design is excellent. It could be improved with miniaturization and with facilities for sterilization. A new conceptual design has been developed in which the apparatus would use programmable linear motor driving

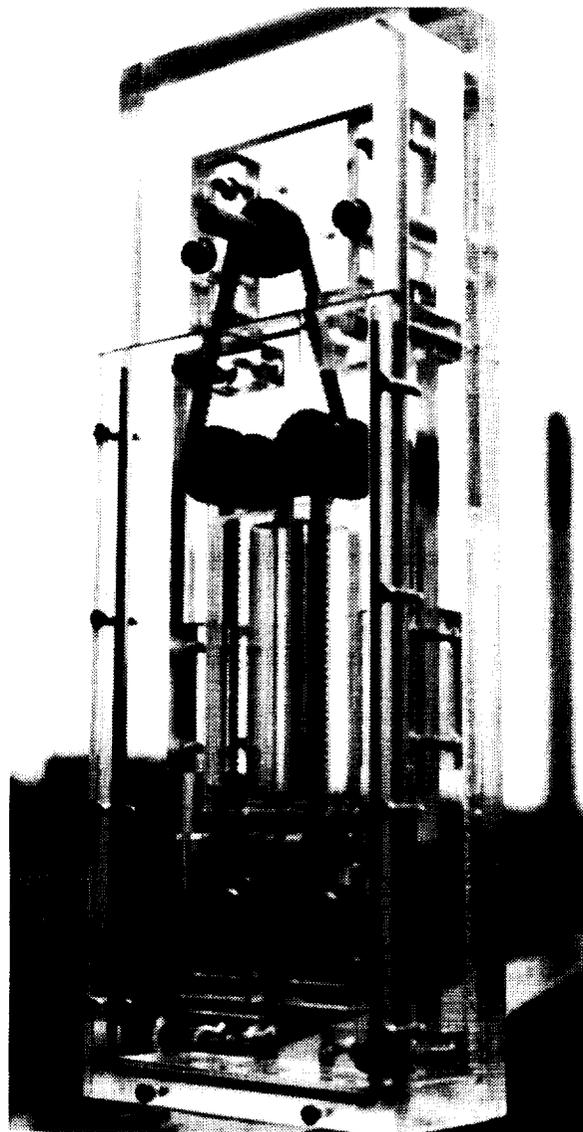


Figure 1. A shear apparatus was designed and built to study the effect of light, shear and \vec{g} on algal swimming orientation. The apparatus uses belts running in the cell culture medium to produce the shear. The variable speed motor is outside the fluid.

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ORIENTATION OF CELLS BY GRAVITY, ILLUMINATION AND SHEAR

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Description of Research

Many species of unicellular algae swim. Their direction of locomotion is strongly affected by the direction, intensity and color of the light they perceive. Gravity also exerts a very marked guiding influence on algal cells — most cells swim upward, especially in the dark. The cells' swimming direction depends not only on light and \vec{g} but also on shear, the local gradients of the streaming velocity of the fluid that surrounds the cells. Shear and gravity guidance were first demonstrated experimentally and theoretically by the Principal Investigator in previous research.

The overall objective of this research is to investigate the summation of these influences two at a time and all three at once. Shear and gravity are physical mechanisms that orient the swimming algal cells by the application of differential forces across the cell bodies, i.e., by torques. In contrast, the orientation of cells toward weak light is physiological. This photo-orientation is a mechanical response resulting from flagellar movements which are elicited by the physiological action of light on the organism. Phototaxis/photokinesis/photophobic accumulation are responses to that physiological action, and they involve the modulation of orientation and motility.

In the past, research on these locomotory photo-responses were essentially passive. "So many cells went this way, so many cells did that," etc. In contrast, the experiments undertaken in this research will make it possible to perform quantitative measurements using two or more control variables ($\vec{T}(v)$, shear, ω^2 , and g are all variable and controllable).

Long-range objectives of this research include: (1) Determining the rules of summation of: (a) shear vs. \vec{g}^* ; (b) photo- † vs. \vec{g} ; (c) photo- vs. shear; and (d) photo- + shear + \vec{g} . (2) Determining whether *physical* mechanisms that orient cells can be used to counteract (or "null out") physiological mechanisms, so as to permit measurement of the physiological effects, such as phototaxis, in physical units (e.g., dyne cm). (3) Deriving the results from the first two objectives, taking into account variability over the cell population.

A variety of experimental apparatus is required for reaching these objectives. Various conceptual designs for measurement systems, but no hardware, existed before the start of this research.

Current objectives of this research include: (1) Constructing an apparatus for generating a well-characterized, stable, quantitatively controllable shear flow of the fluid in which the cells are suspended. Such an apparatus will permit specific orientation of the flow relative to \vec{g} , permit illumination from various principal directions, and permit optical data

* \vec{g} can also be represented by $(\omega^2\vec{r} + \vec{g})$, the net acceleration vector.

† Wavelength and direction of light effects are included in "photo-."

acquisition of cell attitudes and trajectories. (2) Constructing optical systems for accomplishing the above objective. (3) Selecting and putting into service electronic data acquisition and analysis systems. (4) Building, acquiring or arranging for the use of centrifuge-type apparatus to be used in varying the net acceleration vector, $\omega^2\vec{r} + \vec{g}$.

The far long-range objective of this research is to turn \vec{g} off and re-investigate the conclusions reached in this research.

Most of the first year of research was spent in research laboratories in Europe. Research on the summation of photo- vs. \vec{g} was conducted in the laboratory of Professor Dr. D.P. Häder at the Phillips University, Erlangen, Germany, using Professor Häder's computerized trajectory analysis system in conjunction with the geometry suggested by the Principal Investigator. During the second portion of the year, at the University of Leeds, United Kingdom, the first prototype shear apparatus was built and tested. Some optical designs were tested, and a high speed video data acquisition system was used to investigate cells swimming in the shear apparatus.

Accomplishments

(1) *Photo- vs. \vec{g}* . It was shown that when illumination is incident horizontally, i.e., perpendicular to \vec{g} , upon a population of cells (*Euglena*) that normally have a strong tendency to swim upward, the combination trajectories depend strongly on the intensity of illumination. High intensity illumination elicits negative phototaxis (swimming away from the light source), whereas low intensity elicits positive phototaxis (swimming toward the light source). When illumination and gravity were perpendicular and the cell population was viewed from the side (a first-ever quantitative experiment for these orientations), it was found that ***high intensity produced a sharply peaked distribution of cell swimming directions, but low intensity produced extensive broadening.***

(2) *Shear apparatus*. A shear apparatus was designed and built (Figure 1). It uses belts running in the cell culture medium to produce the shear. The variable speed motor is outside the fluid. The apparatus can be placed with the belts running either vertically or horizontally. The first orientation applies shear perpendicular to \vec{g} , the second parallel to \vec{g} . The belts can also be tilted in the "vertical" orientation, so that the belt angle conforms to the mean angle of the algal cells' axis.

(a) It was shown that ***the algae's swimming trajectories conform to expectations*** — i.e., that cells are oriented as expected by the combination of gravity and the applied shear. ***They were found to swim transversely from the up-flowing to the down-flowing regions of the fluid in the apparatus*** (noting that at the center line the fluid velocity is zero).

(b) It was shown that ***the shear flow produced by a belt spacing ≤ 1 cm was stable***. It was further shown that impulsive flow reversal was smooth and did not result in an altered flow profile.

(c) It was shown that the apparatus dimensions and the clarity of the window systems were adequate for relatively low power (e.g., 100x) microscope observation of the cell trajectories. It was found that the optical design may not be adequate for high resolution measurements. Such measurements are not absolutely necessary to achieve all the objectives, but they would considerably enhance the

interpretability of results. Furthermore, it was found that cells accumulated on the apparatus windows and that window cleaning procedures require improvement.

(d) It was found that cell cultures deteriorate faster in the apparatus than in glass bottles. This effect could have been due to bacteria that ferment algal debris produced in the ball bearings of the belt system.

Conclusions. The fluid mechanical basic shear apparatus design is excellent. It could be improved with miniaturization and with facilities for sterilization. A new conceptual design has been developed in which the apparatus would use programmable linear motor driving

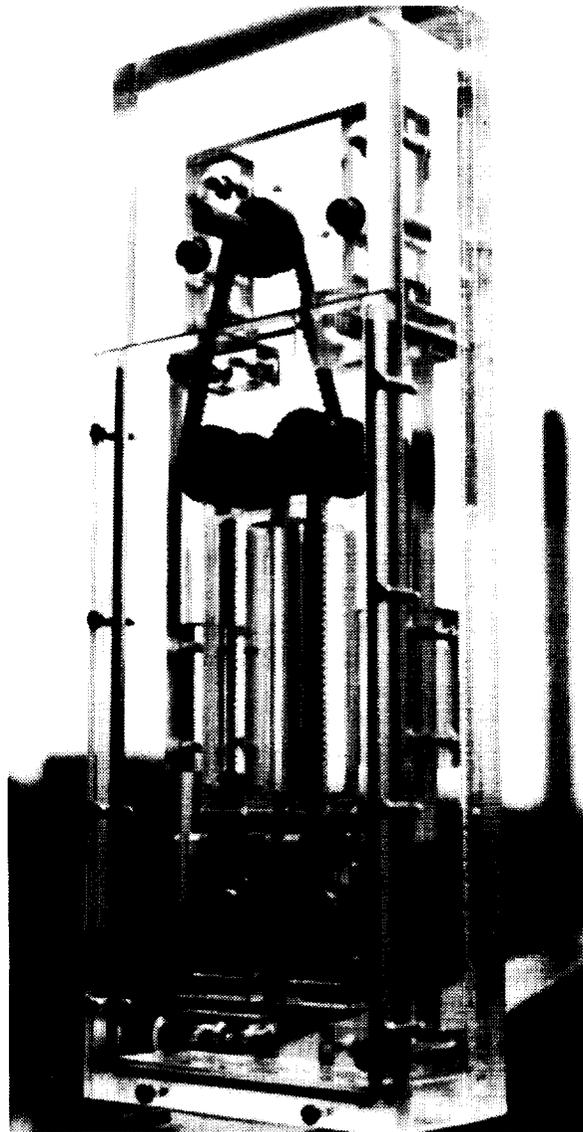


Figure 1. A shear apparatus was designed and built to study the effect of light, shear and \vec{g} on algal swimming orientation. The apparatus uses belts running in the cell culture medium to produce the shear. The variable speed motor is outside the fluid.

rods rather than belts. For vertical orientation, these motors could run outside the fluid; the whole apparatus could then be narrower (i.e., better optics) and less subject to fouling problems. The current apparatus would continue to be in use for the \vec{g} parallel to shear orientation.

Significance of the Accomplishments

The results obtained so far show that it will be possible to elicit and to analyze responses to orthogonal bimodal and trimodal directional stimuli. These results promise considerable advances in our understanding of the sensory and gravitational systems that guide motile microorganisms.

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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 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 fidelity of partitioning of the chromosomes of their cells during or after exposure to spaceflight.

The thrust of the investigations deals with: (1) the induction of active growth, cell proliferation and metabolism in otherwise mature quiescent cells as they exist *in situ*. This problem 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; (2) 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; (3) 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 temperatures) and nutritional; (4) the development of protocols that 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 (5) 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

Continued characterization of embryogenic cells for use in a flight context has been a top priority.

(1) We showed that "seeds" (mericarps) of carrot, after the germination of their zygotic embryo, as well as excised and mechanically wounded zygotic embryos, can produce somatic embryos on hormone-free medium. If this hormone-free medium contains 1 mM NH_4^+ as the sole nitrogen source and the pH is allowed to drop during the culture period, somatic globular stage embryos no longer mature into later embryo stages and gradually multiply only as preglobular stage embryos (PGSPs).

(2) PGSPs are maintainable for long periods without loss of embryogenic potential, i.e., they are able to continue their development into globular, heart, and torpedo and cotyledonary stage somatic embryos under the correct conditions, as long as the pH remains at or near 4 during most of the culture period.

(3) When PGSPs are cultured onto the exact same medium buffered at pH 4.5 - 6, PGSPs continue their development into later stage embryos.

(4) These cell cultures fulfill the commonly accepted definition of habituation in that they need no exogenous hormone for the multiplication of the PGSPs, yet they do not fulfill the classical definition because they require no exposure to exogenous hormone in order to initiate the embryos in the first place.

(5) We reasoned that if hormone-free medium near pH 4 provides an environment capable of permitting continued multiplication from these PGSPs, it might be possible that hormone-free medium at low pH could also support growth without further development of any hormone-derived, embryogenically competent cells, while the same hormone-free medium at pH 4.5 - 6 would not, but would support continued development into later embryo stages.

(6) We showed that embryogenically competent cells derived from seedling hypocotyls with the hormone 2,4-D (2,4-dichlorophenoxyacetic acid) are able to continue multiplication on a hormone-free medium with 1 mM NH_4^+ as the sole nitrogen source as long as the pH of the medium is maintained at or near 4. When these same tissues are cultured on medium of identical composition except the pH is buffered at or above 4.5, tissues are not maintainable without further growth into later developmental stages. *Low external pH does not, therefore, seem to have the inductive capacity of 2,4-D, but does mimic the ability of auxin to prevent PGSP development while maintaining PGSP multiplication.*

(7) We do not as yet know for certain whether it is the NH_4^+ or the fact that the pH of the medium drops as growth occurs on the NH_4Cl -containing medium, or both, that preferentially fosters production of embryonic globules rather than undifferentiated callus. There seems to be considerable flexibility within cell or clonal lines, but a given line is typified by one response type.

(8) With this information, "clean" cultures may be achieved by using hormones and then dropping out the hormone even as the pH is lowered; this comprises a "jump start" system. The system shows that there are various kinds of habituation and that the concept historically put forward is not always correct. An external pH of around 4 could be blocking continued embryonic development by a transient/reversible rise in internal hormone or by other means yet to be disclosed.

(9) *Competent cell cultures of daylily (Hemerocallis) have been shown to respond like the carrot system.* Unlike the carrot system, however, daylily cultures have to be exposed to hormone for at least 12 weeks before they can be "jump started" into the PGSP growth mode.

(10) The transition to the PGSP growth mode was accompanied by a number of intergradations of "correctness" of embryo morphology (called "neomorphs") before the pure PGSP state was sustained. The daylily PGSPs are capable of yielding later stage embryos when the pH is raised to pH 5.8.

Significance of the Accomplishments

The ability to generate embryogenic cultures in a predictable fashion for two key species in our work without a long lead time renders the systems amenable for the first time to reliable study at the free cell level as to mechanisms involved in their generation and manipulation.

Because both a monocotyledon and a dicotyledon respond in a similar fashion, we take this as strong evidence that we are dealing with a general phenomenon and controlling mechanism. pH may be likened to a second messenger in animal systems. Since the embryogenic cells/units are filterable to extremely small sizes, even down to the single cell level, it will be possible for the first time to attach embryogenically poised cells to a substratum in such a way as to test the effects of g vector "neutralization" (as by clinostating) or imposition of a g vector (up versus down; inversion of the vector, etc.) on embryogenesis in a higher plant system.

For instance, polarity levels can be tested from several viewpoints. From a simplistic point of view, low pH might not allow PGSP cells to act as a coordinated unit, i.e., the cells cannot form or are blocked from forming a polarity or gradient (via hyperpolarization) and therefore cannot develop into a polarized structure, i.e., a later stage embryo. It has been shown that there are well controlled, longitudinally oriented gradients of sugars, amino acids and NH_4^+ from the chalazal (radicle end of the zygote) to micropylar (shoot end of the zygote) end of the embryo sac and embryo during zygotic embryogenesis. These gradients may in fact influence the polarity and hence the first cell divisions of the zygote. There are no polarized external nutrient gradients in PGSP cultures; hence, if these gradients are important in directing the orientation of cell divisions, the lack of these gradients *in vitro* would leave cultured cells to divide without externally influenced direction.

But we have observed repeatedly that multiple somatic embryos, or clusters of somatic embryos that develop from aggregates of PGSPs, always develop with their shoot end outward from the aggregate, up and away from the solid medium. That is, multiple somatic embryos are always attached at their root end and the shoot ends extend away from the surface of the semi-solid medium. Being able to attach somatic embryos at the zygotic cell-equivalent stage will permit imposition of a number of conditions on the developing cell for study. Moreover, certain features of the immediate environment of the cells that permit them to continue their embryogenic development can be studied from a chemical perspective. All this means that embryogenic controls are ever-increasingly accessible to investigation for study on Earth and in microgravity.

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PERCEPTION AND TRANSDUCTION OF GRAVITROPISM IN PLANTS

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Description of Research

One sector of this research has aimed at unraveling the three partial processes of root gravitropism: sensing, transduction, and motor response. The existence of a requirement for calcium ions for normal ortho-gravitropism offers one analytical tool, and the existence of genetic materials which require red light for normal ortho-gravitropic response offers another. We have utilized both of these characteristics in an attempt to better define the partial processes involved in corn root gravitropism.

The other sector of this research has focused on gravity sensing by a single-cell system, the Characean algae. For several years it has been recognized that important progress could be made if we could find a single-celled system which responds well to gravity. The summary for the 1984 Gravity and Space Research Workshop stated, "information is only available for whole roots, and the reduction of this type of experiment to isolated organs or single cells is most attractive" (p. 28). In collaboration with Randy Wayne, we have established the Characean cell as just such an experimental device.

Accomplishments

In studies of the partial processes of root gravitropism, we have accumulated evidence that *the role of calcium may be in the transductive sector of root gravitropism*. And now more recently, evidence is accumulating to indicate that the step which is accelerated by red light is in the motor sector of root gravitropism. In addition, *the phenomenon of "springback," or the actual loss of tropistic bending when the gravity signal is withdrawn, appears to be related to the motor sector of gravitropism*.

Significance of the Accomplishments

Unraveling the sequential processes involved in gravity responses will be a major step forward in understanding gravity responses in plants. The experiments involved in defining aspects in sensing of gravity in single-celled systems permit a new basic concept of sensing mechanisms in plants. Evidence indicates that this new concept applies both to the single cell and to root gravity responses. The experiments on calcium, on red light, and on springback represent early probes into the sequence of transduction and motor phases of gravity responses.

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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 phenylpropanoid 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 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: (1) the rate of uptake and binding of specifically labelled lignin precursors; (2) the bonding patterns of lignin *in situ*; (3) the function, formation, and activities of specific peroxidase isozymes responsible for lignin formation; (4) cinnamyl alcohol dehydrogenase activity; and (5) development of vascular tissue.

In this reporting period, progress in three areas is described: (a) lignification in *Pinus taeda* cell suspension cultures, (b) stereoselectivity in phenylpropanoid coupling (i.e., during lignan biosynthesis), and (c) lignin contents and monomer composition of *Raphanus sativus* plants grown for 54 days in microgravity (MIR space station) and on Earth (1 g).

Accomplishments

Lignification in Pinus taeda Cell Suspension Cultures. In woody plants, lignification is an integral part of cell wall thickening, maturation and ultimate cell death. This maturation process occurs sequentially and cells, extending outward from the cambial zone to fully differentiated xylem, differ markedly in both lignin content and monomer composition. Typically, lignin amounts range from ~1% in primary walls to ~28-35% in fully differentiated xylem cells. In principle, it should be possible to obtain cell cultures at different points of cell wall maturation, and hence lignification, e.g., from the earliest stages of lignification to that of fully lignified cells. If this could be achieved, then factors affecting induction of lignification, differences in monomer composition and structure in different cell wall layers, etc., could be delineated.

In this report, two cell lines of *Pinus taeda* cell suspension cultures are described which differ in both cell wall ultrastructure and lignin composition. Both cell lines were maintained on modified Brown and Lawrence medium containing either 11.3 μ M 2,4-D (cell line 1) or 11.3 μ M NAA (cell line 2) as plant growth regulators. Table I gives a compilation of differences in key enzymatic activities, lignin amounts, and monomer composition for both cell types.

As can immediately be seen, the lignin contents of extractive-free cells, as measured by the acetyl bromide lignin determination, differ substantially, i.e., from 1.6% in the 2,4-D line to ca. 9.9% in the NAA line. The monomer composition (i.e., *p*-coumaryl or coniferyl alcohol derived) as determined by thioacidolysis also differs, i.e., 10:1 in the 2,4-D line to that of 6:1 NAA-grown cells. The differences in lignin contents are readily explicable on the basis of activities of both phenylalanine ammonia lyase and cinnamyl alcohol dehydrogenase.

Table I. Comparison of 2,4-D and NAA grown *P. taeda* cell suspension cultures.

ANALYSIS	2,4-D	NAA
Lignin Content (%) (Acetyl Bromide Method)	1.6	9.9
H:G ratio (Thioacidolysis Method)	10:1	6:1
Phloroglucinol Staining	faint	strongly red
Phenylalanine Ammonia Lyase Activity (pkat)	< 0.033	25
Cinnamyl Alcohol Dehydrogenase Activity (pkat)	288	2800

H = *p*-hydroxyphenyl (see Figure 4)

G = guaiacyl (see Figure 4)

Figure 1 shows electron micrographs of both 2,4-D and NAA cells, previously fixed in 2% glutaraldehyde and 2% formaldehyde, embedded in LR white resin and sectioned. As can be seen, the 2,4-D cells are approximately 0.2 μM thick which is consistent with essentially unligified primary cell walls. On the other hand, the NAA cell walls have undergone significant thickening (0.4 μM), roughly approximate to that of deposition of the S₁ layer of the secondary wall.

In order to establish whether the NAA cell line was truly lignified, the cells were individually administered [1-¹³C]-, [2-¹³C]- and [3-¹³C]-phenylalanines. The cells were allowed to metabolize for 14 days and were then harvested. Figures 2a-c show the solid-state ¹³C-NMR spectra of extractive-free cells from each experiment following subtraction of natural-abundance resonances. These spectra clearly reveal that the polymer formed is indeed lignin, and Figure 3 shows the main substructural types present. Assignments are given in Table II. *This represents the first proof of lignification in cell walls of cultured cells and the bonding patterns of a gymnosperm in situ.*

Stereoselectivity in phenylpropanoid coupling. Following monolignol formation, the next level of structural complexity in phenylpropanoid metabolism is the formation of dimeric lignans and neolignans. Several compounds of this class, in addition to monolignols, have been proposed as lignin precursors. Further, lignans and neolignans are frequently found in optically (enantiomerically) pure form, indicating that their formation cannot be explained on the basis of a typical non-specific peroxidase/H₂O₂ coupling reaction. For example, *Forsythia intermedia* contains only (-)-secoisolariciresinol and (-)-matairesinol; the (+)-forms are not detectable. We have established *in vivo* (whole plants) and *in vitro* (cell-free extracts) that the formation of (-)-secoisolariciresinol occurs via the stereoselective coupling of two coniferyl alcohol moieties in an enzymatic conversion requiring both NAD(P)H and H₂O₂ as cofactors. The corresponding (+)-enantiomer is not formed. This stereoselective conversion was demonstrated using both [8-¹⁴C] and [9,9-²H₂,OC²H₃] coniferyl alcohols as precursors, with product verification of (-)-secoisolariciresinol being obtained by both

ELECTRON MICROGRAPHS OF *PINUS TAEDA* CELLS

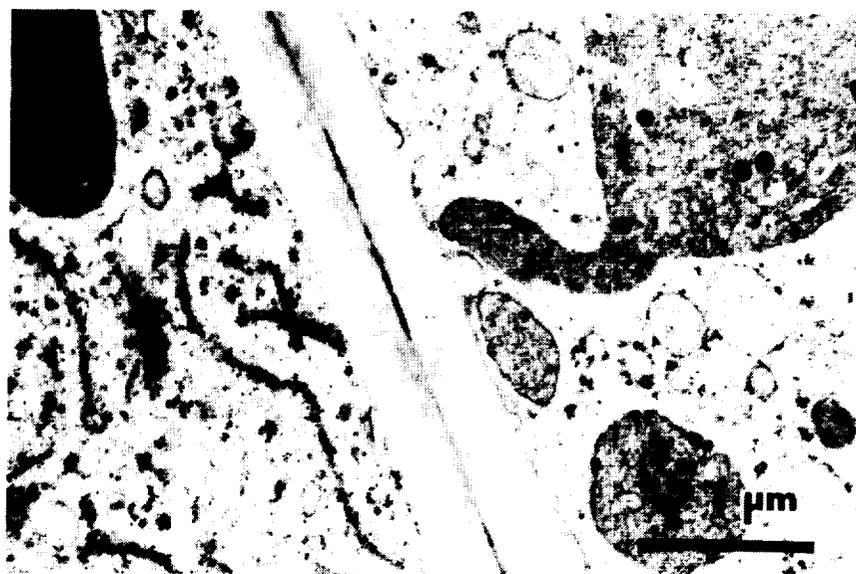
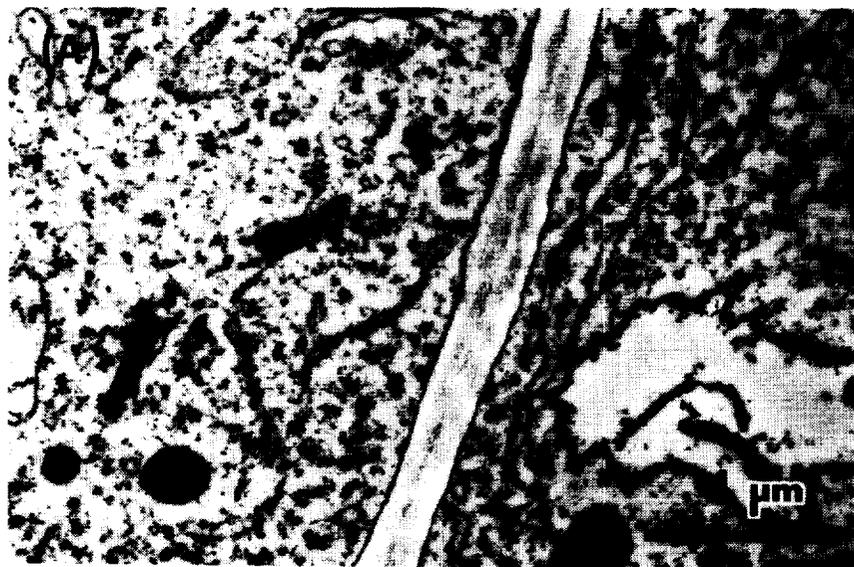


Figure 1. Electron micrographs of *Pinus taeda* suspension culture cell grown on (A) 2,4-D and (B) NAA.

^{13}C -NMR SPECTRA OF *PINUS TAEDA* CELLS

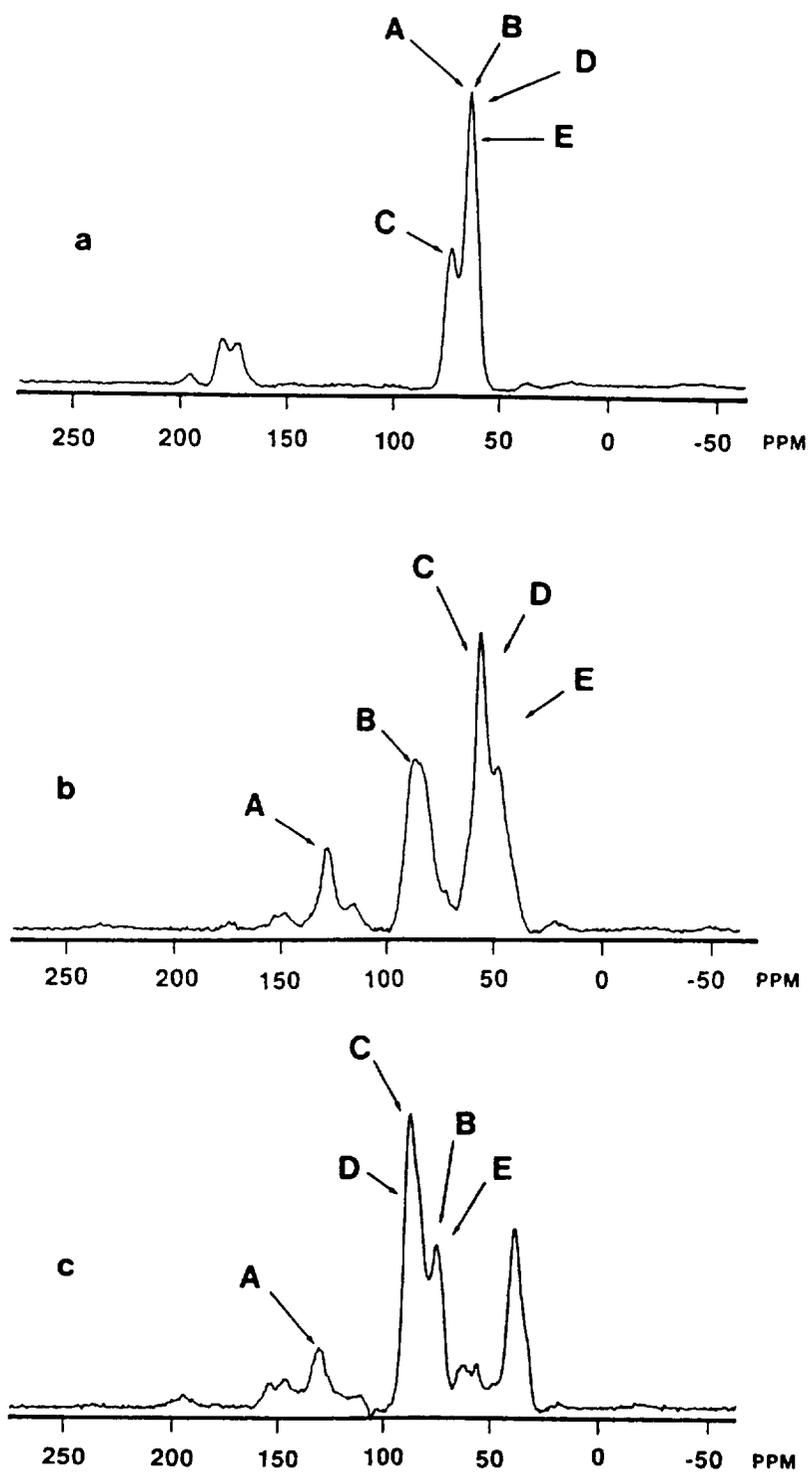


Figure 2. Solid-state ^{13}C NMR spectra of *Pinus taeda* extractive-free cells. Over a period of 14 days, cells were administered: (a) [1- ^{13}C]-Phenylalanine, (b) [2- ^{13}C]-Phenylalanine, and (c) [3- ^{13}C]-Phenylalanine.

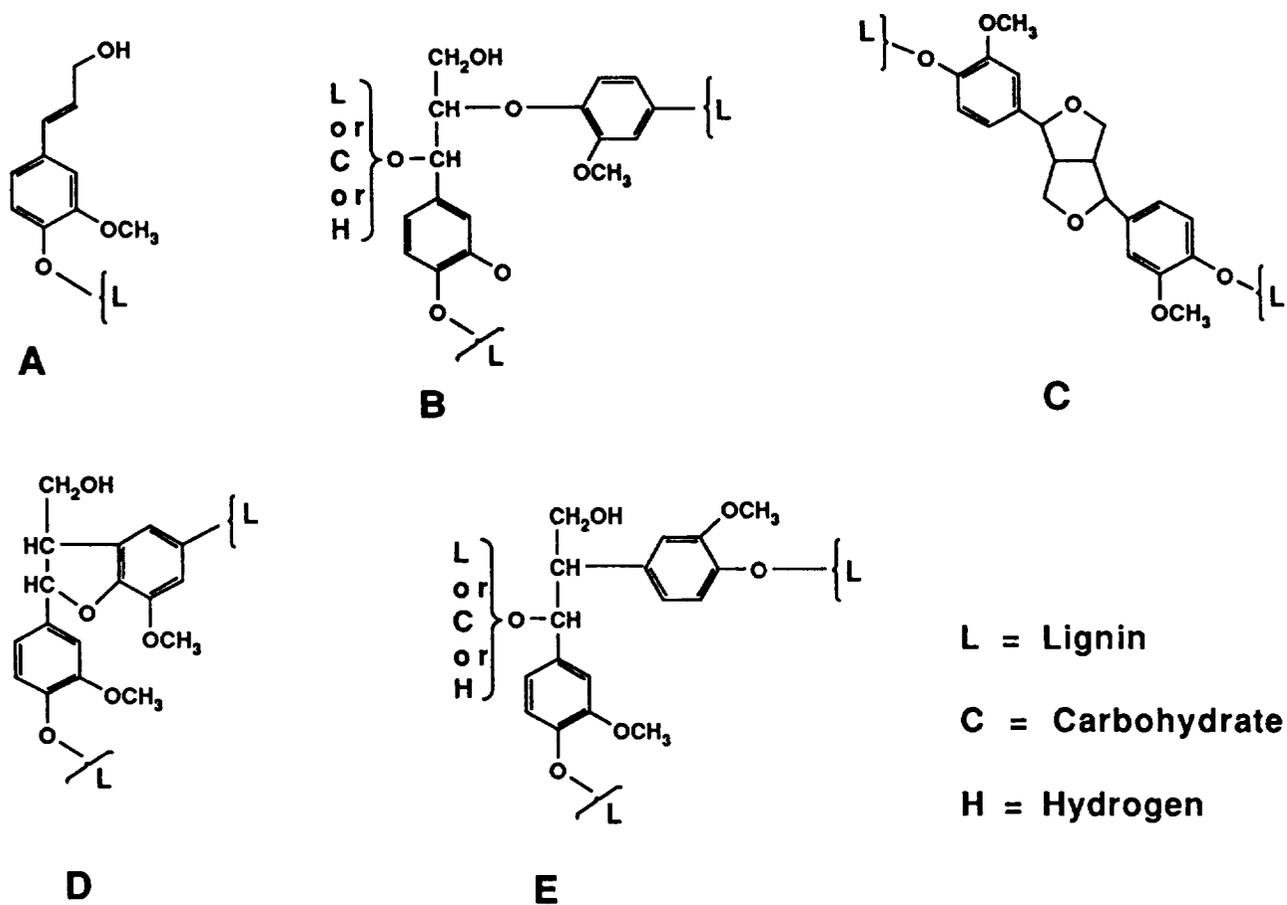


Figure 3. Main substructural bonding patterns in lignins.

Table II. Structural assignment of ^{13}C -enriched lignins.

Substructure	Chemical Shift (ppm)		
	1- ^{13}C	2- ^{13}C	3- ^{13}C
A	63.4	127.9	130.7
B	63.4	85.8	74.4
C	72.2	55.4	86.4
D	63.4	55.4	86.4
E	63.4	55.4	74.4

radiochemical and mass spectrometric analyses, respectively. Following incubation of *F. intermedia* cell-free extracts with pentadeuterated [9,9-²H₂,OC²H₃] coniferyl alcohol in the presence of NAD(P)H and H₂O₂, the resulting secoisolariciresinol obtained gave an m/z of 372 (corresponding to M⁺ + 10), 354 (M⁺ + 10-H₂O) and 140 (benzylic cleavage, with an OCD₃ group). Thus, deuterated (-)-secoisolariciresinol arises via stereoselective coupling of two intact pentadeuterated coniferyl alcohol molecules. In an analogous manner, the conversion of (-)-secoisolariciresinol into (-)-matairesinol requires NAD(P) as a cofactor. Note that the corresponding (+)-enantiomer was not converted into (+)-matairesinol. Thus, in this phase of the study, *we have discovered the first two enzymes in lignan formation, and have revealed both transformations to be stereoselective and stereospecific, respectively* (Figure 4).

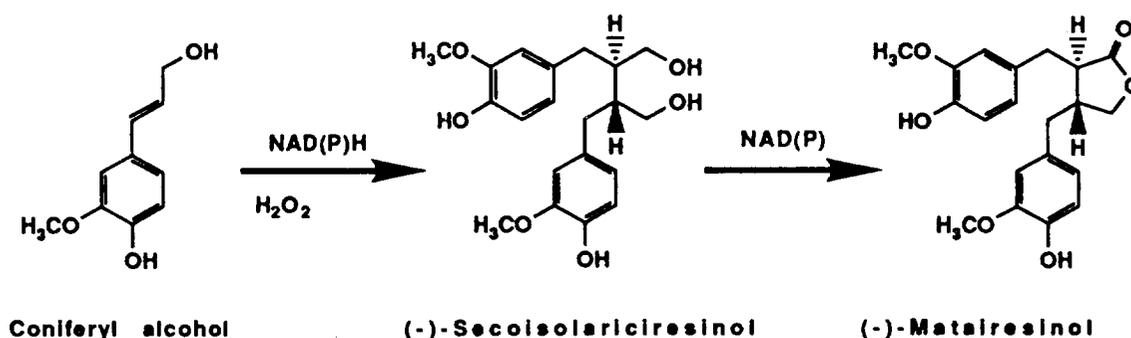


Figure 4. Stereoselective enzymatic conversion of *F. intermedia* cell-free extracts using coniferyl alcohol.

Cell-wall analyses of R. sativus plants. *Raphanus sativus* plants, grown on MIR for 54 days, were harvested and analyzed by solid-state ¹³C nuclear magnetic resonance spectroscopy, and their lignin amounts and monomer compositions, α-cellulose, pectin/hemicellulose and extractive contents determined. The results obtained were compared to 54-day-old Earth-grown plants which served as controls.

The acetyl bromide lignin content of the flight-grown stem tissue was slightly lower (8.8%) than the control (11.6%), whereas the reverse effect was observed for corresponding root tissue (4.5% vs. 2.5%). Interestingly, flight-grown stem tissue differed substantially in its lignin monomer composition as evidenced by thioacidolysis. The H:G:S ratios (Figure 5) were 3.3 : 89.6 : 7.1 (flight) versus 2.7 : 74.8 : 17.5 (control), i.e., the syringyl content of the polymer was markedly reduced in the flight-grown plants. It is also noteworthy that in the root tissue, the α-cellulose content (17.4% vs. 22%) as well as extractives (aqueous and organic solubles) were lower in the flight-grown plant than in the controls. Conversely, the pectin/hemicellulose contents were higher for root tissue (14.2% versus 9.2%) of flight-grown plants when compared with the corresponding controls. This was confirmed by analysis of the [¹³C]-solid state NMR spectra (MIR-Earth) which revealed mainly pectinaceous material (Figure 6). A similar trend was observed for stem tissue. From these data, it can be tentatively concluded that *flight-grown R. sativus plants produce more pectinaceous/non-cellulosic polysaccharide polymers, and that the cell walls have a lower α-cellulose content and a lignin of greatly reduced sinapyl alcohol moieties.*

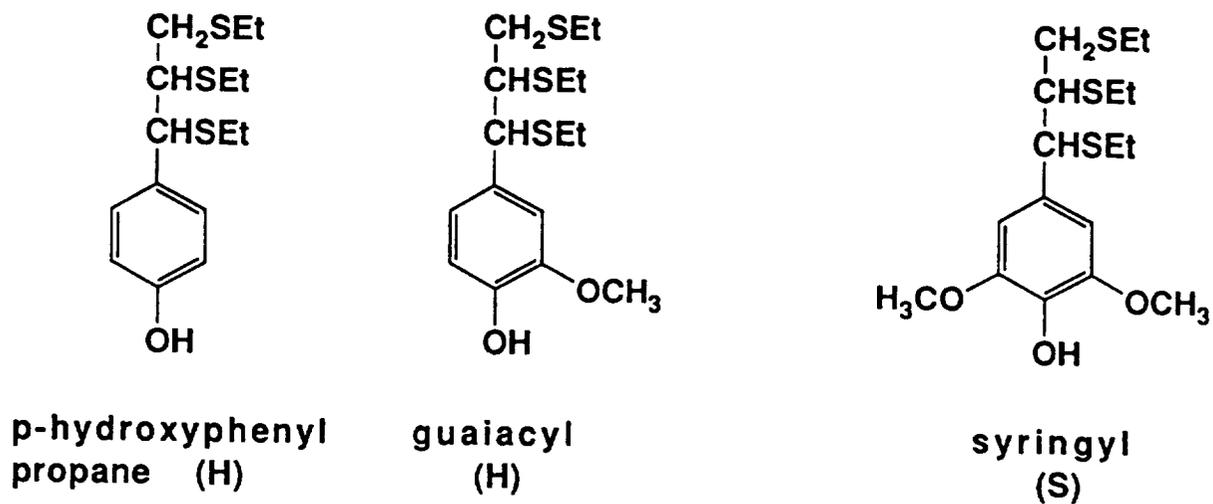


Figure 5. Thioacidolytic cleavage products from the three lignin constituents, derived from *p*-coumaryl, coniferyl and sinapyl alcohols.

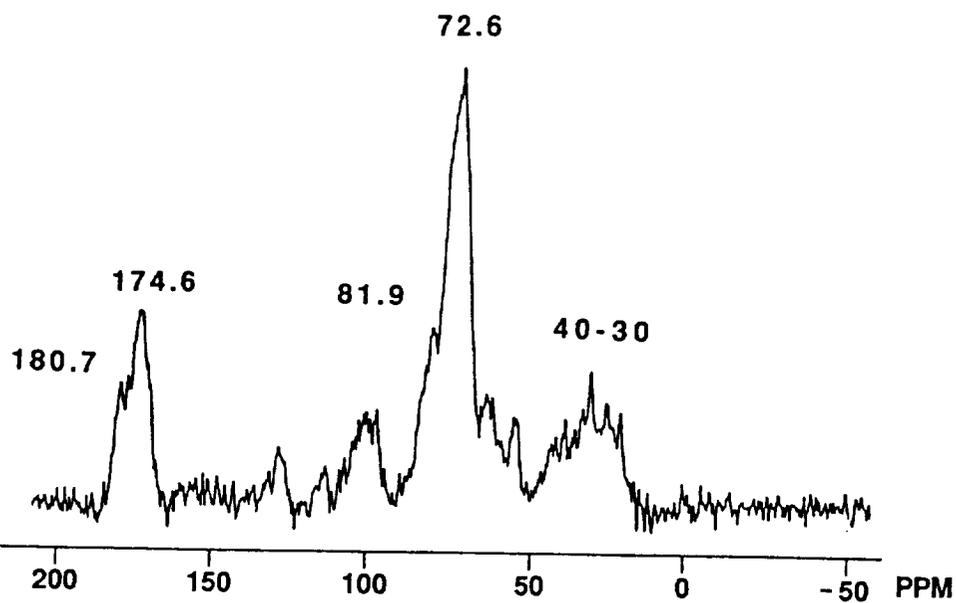


Figure 6. Solid-state ¹³C-NMR difference spectrum of *Raphanus sativus* root tissue (Mir-Earth grown, 54 days).

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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 Space Biology Program of NASA has identified "understanding the basic mechanisms whereby plants perceive, transduce, and respond to a gravitational force" as a research goal central to their mission. In addition, 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 (IAA). The goal of our research is to examine components of the plasma membrane (PM) that are involved in the gravitropic response of plants. The technical breakthrough that makes this study possible is the development of a method to obtain large quantities of highly purified PM from plant tissues. We adapted this technique to membrane preparations from zucchini (*Cucurbita pepo*) hypocotyls which yields PM vesicles of extremely high purity (95-98%). We are now using these vesicles to examine auxin transport carriers and receptors, G-proteins, and protein kinases, all of which have been implicated in the gravitropic response of plants. In addition, we are using a number of gravitropic mutants of tomato in a genetic dissection of the steps involved in gravitropism. Using this method, we hope to isolate previously unidentified components of the gravity response.

Accomplishments

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 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 PM vesicles from zucchini hypocotyls by photoaffinity labeling of polypeptides using ^3H -5N₃-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 (kDa), which displays properties consistent with those expected for an auxin receptor or transport protein. The polypeptides are (1) 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) present at low abundance in the PM; (3) saturated by increasing concentrations of IAA; and (4) 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 kDa) that 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.

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 that are dependent upon Ca^{2+} for autophosphorylation activity. In the past year, we have determined that these protein kinases are immunologically related to a unique class of protein kinases that has recently been identified in plants that contain both a catalytic domain and a Ca^{2+} -binding domain with high homology to calmodulin (a Ca^{2+} -binding protein) within a single polypeptide. The proteins we are studying may represent the first PM-bound members of this family of kinases. We are currently completing purification of the PM Ca^{2+} -stimulated protein kinases in order to generate specific antibodies that will allow us to study their *in vivo* involvement in the gravity response.

G-proteins. We are also examining the role of GTP-binding proteins (G-proteins) in auxin action and gravitropism. G-proteins play a leading role in signal transmission for many animal hormones, neurotransmitters, and responses to light; they function to link ligand-bound receptors in the PM with cellular responses. We first demonstrated that *there is GTP binding and hydrolysis in zucchini PM with characteristics similar to that found in animals and yeast: affinity; specificity for guanine nucleotides; stimulation by Mg^{2+} , detergents, and fluoride or aluminum ions; and a similar molecular weight (30 kDa)*. Recently, we have demonstrated that the *plant G-protein(s) can be stimulated by auxin, which may indicate that they are involved in plant signal transduction, especially in gravitropism*.

Mutant analysis. The analysis of mutants is a powerful approach to dissecting the components of plant gravitropism. As described above, we have demonstrated that stem cells of *dgt*, an agravitropic mutant of tomato, display greatly diminished binding of IAA to PM proteins. For this reason, the *dgt* mutant provides an excellent experimental system with which to elucidate the role of these proteins in the gravitropic response of plants. In the coming year, we plan further experiments designed to test whether the auxin binding proteins are IAA receptors or transport proteins.

In addition to our continuing work on *dgt*, we have extended the genetic analysis to a second gravitropic mutant of tomato, *lazy-2*, which appears to be altered at another gene involved in the response process. Dark-grown *lazy-2* stems display a normal, upward gravitropic response, but upon exposure to light the stems grow downward in the direction of the gravity vector. In our initial characterization of this mutant, we have found that *light-grown lazy-2 plants are capable of perceiving gravity and achieving a normal asymmetric growth response*. *Lazy-2* plants have a wild-type phototropic response and *lazy-2* hypocotyls respond to exogenous auxin in a nearly wild-type fashion. In addition, the light-induced switch from negative to positive gravitropism is potentiated by red light, which may indicate a role for phytochrome in the gravitropic mechanism. These findings suggest that the *lazy-2* lesion lies in the signal transduction pathway between gravity perception and response.

Significance of the Accomplishments

In the past year we have made significant progress in characterizing the components of the PM that may be involved in the gravitropic response of plants. Especially important is the stimulation of G-proteins by auxin, which may indicate that this class of protein that is so important to animal signal transduction, also functions in the plant gravity response.

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 kDa polypeptides, but also associates that receptor with a specific set of morphological and physiological attributes that are known to be connected to auxin (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 kDa polypeptides to the *dgt* lesion.

The *lazy-2* mutant appears to be altered in another part of the gravity response mechanism of plants, signal transduction. This mutant has the enormous advantage of light regulation, which can be used as a molecular switch to dramatically alter (actually reverse) some aspect(s) of the gravitropic mechanism. Comparisons of *lazy-2* and *dgt* with each other and with wild-type tomato should provide a better understanding of the sequence of events occurring between gravistimulation and gravicurvature.

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MECHANICAL STRESS REGULATION OF PLANT GROWTH AND DEVELOPMENT

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Description of Research

Development and orientation of higher plants are drastically affected by physical forces in the growth environment. On Earth, gravity is a physical counterforce that shoots must overcome during normal development (negative gravitropism). A minimum amount of turgor pressure and cell wall reinforcement are required to generate the force and strength required to sustain growth and development against the static gravity vector. Other physical forces that plants encounter on Earth include random dynamic perturbations caused by wind, precipitation, and other living organisms. These "mechanical" forces, as they are called, are generally considered stressful to plants because they temporarily inhibit plant growth rates, delay and/or reduce reproductive development, and develop the resistance of the normal plant response to gravity. On the other hand, mechanical stresses tend to strengthen plant tissues to withstand even stronger physical perturbations and may toughen plants to better withstand other environmental stresses, including low temperature and drought.

In the absence of gravity, cells of growing plants will likely deposit less secondary wall material around them, resulting in structurally weaker tissues. Such plants might be readily damaged in space if subjected to sudden spacecraft accelerations or to perturbations resulting from astronaut activity. However, controlled mechanical vibrations of the right frequency, amplitude, duration, and orientation might substitute for gravity by enhancing the load-bearing properties of plant tissues. There is also a likelihood that programmed vibrations could be perceived by plants as pulses of gravity per se if the vibrations are oriented along the proper axes of plants. Accelerating plants abruptly along one axis with a very slow return to the starting position could be perceived as net acceleration in the "fast" direction, provided that cytoplasmic viscosity in microgravity is sufficient to serve as an inertial barrier to statolith displacement in the "slow" direction. The answers to such questions await plant vibration experiments that can be conducted under real spaceflight conditions.

The ground-based mechanical stress program of study with plants sponsored by the Space Biology Program has three objectives: (1) to characterize plant response to mechanical stress as completely as possible, (2) to determine the physiological mechanism of plant response to mechanical stress, and (3) to extend these models to real spaceflight conditions.

Accomplishments

The hypothesis that mechanical stresses condition or "harden" plants to better withstand the rigors of a more stressful growth environment was tested by pretreating soybean and eggplant seedlings with brief, periodic seismic (shaking) or thigmic (rubbing) stress in a wind-protected environment prior to transfer outdoors. Productivity (dry weight) and dimensional (leaf area and stem length) growth parameters were reduced more in a greenhouse or an outdoor wind-protected environment than in an unprotected outdoor area. Wind or mechanical stress pretreatments enhanced tissue density (specific weights) of

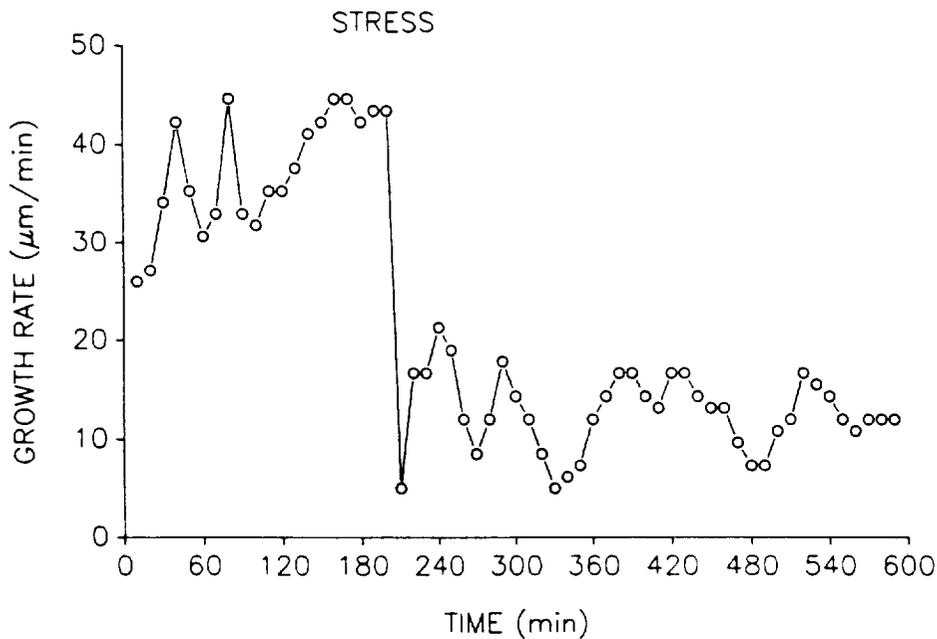


Figure 1. Real-time growth response of dark-grown soybean seedlings to thigmic or "mechanical" stress. There is an 88% inhibition of growth rate followed by a period of much reduced growth, which is sustained for at least 7 hrs. (Data of graduate student Laura Coe.)

stems and leaves, but reduced both internode diameter and internode length equally. Both abscisic acid and mechanical stress reduced relative growth rate and stem length but enhanced leaf specific chlorophyll content when applied to eggplant seedlings in a greenhouse. Both outdoor preconditioning agents reduced growth, but mechanical stress did not affect ABA content of leaves. Mechanical stress treatments increased leaf water potential up to 25% relative to undisturbed controls.

Studies of real-time growth responses to brief episodes of thigmic stress by dark-grown soybean seedlings indicate an immediate 88% inhibition of growth rate followed by a period of much reduced growth sustained for at least 7 hrs (Figure 1). A single mechanical stress episode such as this results in a 50% inhibition of cumulative stem elongation over a 24-hr period. Silver thiosulfate, an ethylene action inhibitor, applied to the hypocotyl hook of dark-grown soybean having just received thigmic stress, negated the typical growth inhibition response by 38%.

The Principal Investigator has been advising Mark Turner, an engineer in the Centrifuge Facility Project office at NASA Ames Research Center, regarding his ground-based vibration studies with dark-grown pea and corn seedlings. Turner found that neither continuous 10^{-3} nor 10^{-2} g-equivalent vibrations in the vertical plane had any consistent, significant effects on the growth of either species.

Significance of the Accomplishments

The fact that plants responded differently to mechanical stresses in different growth environments (e.g., indoor vs. outdoor) indicates that *the nature of the plant-growth environment is important in determining the extent and nature of plant response to physical stress. Dark-grown plants are most sensitive, but high light intensity negates plant response to mechanical stress, as shown in*

previous studies. *Mechanical stress preconditions plants to withstand even higher levels of physical stress.* Part of this response may be physiological, but another part is structural in that *the support tissues of mechanically stressed plants become stronger and more fibrous due to altered cell wall composition and deposition.* *The increased leaf water potential of mechanically stressed plants is due to stress-induced reduction in stomatal aperture,* as shown previously.

The rapid growth-rate changes measured for dark-grown seedlings in response to mechanical stress suggest that *turgor collapse may be an immediate response to physical disturbance by unhardened plants.* Later, sustained responses likely involve differential gene expression related to calcium and phytohormone changes, altered cell wall metabolism, etc.

Lack of plant response to 10^{-3} or 10^{-2} g vertical vibrations in a superimposed 1 g field is not surprising, considering that plants grown in Earth-normal gravity should be much stronger and resistant to low-level disturbance than would be μ g-grown plants. Furthermore, the Principal Investigator has previously reported that Earth-bound plants growing under conditions of continuous, horizontal, rotatory shaking (about 1 g equivalent) "harden off" to the stress, are not dwarfed, and sometimes are even mildly stimulated in growth. Rigorous testing for the threshold sensitivity of plants to periodic or continuous episodes of vibration awaits experimentation under real spaceflight conditions.

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Coe, L.L. and Mitchell, C.A. 1990. Role of auxin and ethylene in mechanical stress responses of soybean seedlings (Abstract). *ASGSB Bulletin* 4(1): 70. (GWU 12940)

Jones, R.S., Coe, L.L., Montgomery, L., and Mitchell, C.A. 1990. Seismic stress responses of soybean to different photosynthetic photon flux. *Annals of Botany* 66: 617-622. (GWU 12941)

DEVELOPMENTAL AND PHYSIOLOGICAL PROCESSES INFLUENCING SEED PRODUCTION IN MICROGRAVITY

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Description of Research

To date, the growth of plants in space has been poor. A particularly sensitive time in the life cycle of a plant growing in microgravity seems to be the transition from the vegetative to the reproductive phase. Most plants grown full term in space failed to produce any seed at all, and in a Soviet experiment in which seeds were produced, the seed quality was poor. Dosimetry readings taken in flight have failed to explain this ubiquitous sterility in terms of a radiation load; thus, some developmental failure seems to be triggered by the microgravity environment itself.

Reproductive events in angiosperms have a number of stages that could potentially be influenced directly by gravity. Pollen production, formation of the embryo sac, pollination and fertilization are all complex developmental events. No studies exist tracking the success of any of these processes in microgravity.

Aside from the possibility of direct effects of microgravity on seed set events, secondary effects are possible through changes in the space environment which may be brought about in microgravity. For example, poor root zone aeration caused by the lack of directed water movement in microgravity might restrict root respiration to such an extent that metabolites and hormones necessary for successful development during the reproductive cycle would not be available. As a second example, restricted gas exchange in the aerial portion of spaceflight plants might lead to a build-up of volatile products which could have a negative influence on reproduction. Simultaneous monitoring of developmental and physiological events during the reproductive cycle of plants in space is necessary to determine whether such a relationship might exist.

In this project, microgravity effects on developmental and physiological processes affecting seed set will be probed with a flight experiment. *Arabidopsis thaliana* (L.) Heynh., a cruciferous plant used in molecular biology experiments, has been selected for a flight experiment because of its short life cycle, minimal light and space requirements, and history of utilization in flight experiments by the Soviets. A full life cycle will not be possible on a 5-7 day Shuttle flight, so our initial research focuses on early events in seed production. Plants for the flight experiment will be loaded into the Plant Growth Unit at pre-flowering stage. During exposure to microgravity, pollen and embryo sac development, fertilization, and early embryogenesis will occur. The success of these processes in microgravity will be determined by comparison of the flight plants with ground control plants using ultrastructural and physiological analyses.

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Publications

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Jones, R.S., Coe, L.L., Montgomery, L., and Mitchell, C.A. 1990. Seismic stress responses of soybean to different photosynthetic photon flux. *Annals of Botany* 66: 617-622. (GWU 12941)

ROOT GRAVITROPISM: HOW GRAVITROPIC SIGNALS MOVE FROM THE ROOT CAP TO THE ROOT

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Description of Research

The objectives of our research are to determine the role of the root cap in root gravitropism, and to understand how signals that control gravitropism move in the tips of graviresponding roots. To meet these objectives we studied (1) the gravitropic responses of capless roots and roots of a newly discovered mutant of corn whose root caps are deformed and touch the root only at the extreme apex of the root; (2) the role of the apoplast and outer cell-layers of roots as potential pathways for stimulus transmission during root gravitropism; and (3) roots of the Ageotropic (and mucilage-lacking) cultivar of *Zea mays* to determine how the absence of mucilage affects root gravitropism. Roots of this cultivar of corn secrete negligible amounts of mucilage and are not responsive to gravity. We also tried to determine a cellular basis for the lack of gravitropic responsiveness in the agravitropic mutant.

Accomplishments

(1) Peripheral cells of caps of the agravitropic mutant have deformed dictyosomes. These dictyosomes produce but do not secrete mucilage. The absence of this mucilage along the cap junction of the root correlates positively with the lack of gravitropic sensitivity of the roots.

(2) Gravitropic effectors move in the mucilage of the agravitropic mutant of corn. The gravitropic response can be turned on and off by applying and removing exogenous mucilage.

(3) Disrupting the outer cell-layers at the tip of a root induces curvature away from the treated side of the root. Filling the disrupted tissue with a water-soluble jelly restores straight growth.

(4) The symplastic connections between the root cap and root are unnecessary for gravitropism in corn, bean, or onion.

(5) Primary roots of *Tristerix aphyllus* are graviresponsive and lack root caps. Therefore, root caps are unnecessary for root gravitropism in this species. A small group of cells approximately 2 mm behind the root tip contains amyloplasts that sediment in response to gravity. The sedimentation of these amyloplasts occurs before the onset of gravicurvature of these roots. To our knowledge, this is the only higher plant in which putative statocytes form in this position.

(6) A clinostat does not mimic the cellular effects of microgravity in roots of corn, mustard spinach, or onion.

Significance of the Accomplishments

Findings #1 and #2: Our studies of the agravitropic mutant show that gravitropic effectors move apoplastically.

Findings #3 and #4: Our studies of the path by which gravitropic signals move in graviresponding roots is important because it provides us with a relatively simple system for identifying the gravitropic effectors. Experiments aimed at identifying those effectors have been promising, but inconclusive.

Finding #5: Our studies of capless roots show that putative statocytes need not always occur in root caps. This system is unique in that putative statocytes in other higher plants (e.g., primary roots of *Zea*) occur in the root cap.

We now propose a cellular basis for agravitropism in the agravitropic mutant of corn. Peripheral cells of these rootcaps produce but do not secrete mucilage. Consequently, the peripheral cells are separated from protodermal cells of the root. Gravitropic effectors, which normally move from the root cap to the root in mucilage, cannot move from the cap to the root because of the lack of mucilage. As a result, the root grows straight, regardless of its orientation with respect to gravity.

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DEVELOPMENTAL AND PHYSIOLOGICAL PROCESSES INFLUENCING SEED PRODUCTION IN MICROGRAVITY

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Description of Research

To date, the growth of plants in space has been poor. A particularly sensitive time in the life cycle of a plant growing in microgravity seems to be the transition from the vegetative to the reproductive phase. Most plants grown full term in space failed to produce any seed at all, and in a Soviet experiment in which seeds were produced, the seed quality was poor. Dosimetry readings taken in flight have failed to explain this ubiquitous sterility in terms of a radiation load; thus, some developmental failure seems to be triggered by the microgravity environment itself.

Reproductive events in angiosperms have a number of stages that could potentially be influenced directly by gravity. Pollen production, formation of the embryo sac, pollination and fertilization are all complex developmental events. No studies exist tracking the success of any of these processes in microgravity.

Aside from the possibility of direct effects of microgravity on seed set events, secondary effects are possible through changes in the space environment which may be brought about in microgravity. For example, poor root zone aeration caused by the lack of directed water movement in microgravity might restrict root respiration to such an extent that metabolites and hormones necessary for successful development during the reproductive cycle would not be available. As a second example, restricted gas exchange in the aerial portion of spaceflight plants might lead to a build-up of volatile products which could have a negative influence on reproduction. Simultaneous monitoring of developmental and physiological events during the reproductive cycle of plants in space is necessary to determine whether such a relationship might exist.

In this project, microgravity effects on developmental and physiological processes affecting seed set will be probed with a flight experiment. *Arabidopsis thaliana* (L.) Heynh., a cruciferous plant used in molecular biology experiments, has been selected for a flight experiment because of its short life cycle, minimal light and space requirements, and history of utilization in flight experiments by the Soviets. A full life cycle will not be possible on a 5-7 day Shuttle flight, so our initial research focuses on early events in seed production. Plants for the flight experiment will be loaded into the Plant Growth Unit at pre-flowering stage. During exposure to microgravity, pollen and embryo sac development, fertilization, and early embryogenesis will occur. The success of these processes in microgravity will be determined by comparison of the flight plants with ground control plants using ultrastructural and physiological analyses.

Accomplishments

Work has focused on two main objectives: developing a set of procedures for studying embryo sac development in *Arabidopsis thaliana*, and determining an acceptable procedure for growing *Arabidopsis* aseptically to the pre-flowering stage which would also be amenable for use in the existing flight hardware, the Plant Growth Unit (PGU).

(1) *We successfully determined fixation procedures for use in studying embryo sac development in Arabidopsis thaliana.* Material is fixed in 2.5% glutaraldehyde in buffer, postfixed in buffered 1% OsO₄ and embedded in Spurr's resin. Other fixation techniques are currently being refined.

(2) *We completed a transmission electron microscopy (TEM) study on early embryo sac development.* The *Arabidopsis* embryo sac is the monosporic *Polygonum* type, tenuinucellate and bitegmic. Three walls protect the embryo sac, including an unusual inner callosic wall around the egg cell, synergids, and central cell, excluding the antipodals. Wall characteristics indicate that the central cell and antipodals as well as the synergids act as transfer cells.

(3) In a collaborative project with Huang Bing-Quan at the University of Oklahoma, we used immunofluorescence to study the microtubular cytoskeleton of isolated embryo sacs of *Arabidopsis*.

(4) We patterned a growing procedure for *Arabidopsis* in the Plant Growth Unit after the procedures used by Krikorian et al. for the CHROMEX-2 flight experiment. Plants can be grown full term utilizing nutrient agar plugs wrapped in spun polyester supported by a phenolic foam. Using this procedure, twelve plants can be grown in each Plant Growth Chamber (PGC). Six PGCs can fit into the Plant Growth Unit for a total of 72 plants.

Significance of the Accomplishments

It has recently been demonstrated that substantial changes occur in cell volume occupied by dictyosomes, plastids and lipid bodies when plant cells develop in microgravity. Generation of a protocol for examining the developmental events of an embryo sac in *Arabidopsis* will allow for the investigation of potential consequences of these changes in ultrastructure for embryo sac development in microgravity. Post-fertilization studies to follow will examine early embryo development. An exhaustive descriptive baseline is necessary to establish a point of comparison for the upcoming flight experiment.

By developing a growing procedure that permits the utilization of pre-flowering plants in the Plant Growth Unit, we have extended the flexibility of this flight hardware to use in all stages of the plant life cycle. It should now be possible to address microgravity effects on all stages of plant growth and development if the subject plant used is small and possesses a rapid enough life cycle.

Publications

Musgrave, M.E. 1990. Physiological and developmental aspects influencing seed production in hypoxically grown *Brassica campestris* (Abstract). *ASGSB Bulletin* 4(1): 54. (GWU 12890)

(2) Moreover, they almost certainly bring about some of the plant's responses to low temperature, due in considerable part, we believe, to Ca^{2+} entry through these channels.

(3) Based on the simple observation that mechanical stimulation of protoplasts can rapidly shift cell volume, as well as on the general behavior of the channel system, the channels appear to play a key role in regulating the turgor of the cells.

(4) During a variety of activities plants show rapid rhythmical activities. Rhythmic channel activity might underlie many or all of these rhythms.

(5) Gravitropism is merely one example of polarized activity of the cells in a plant tissue. It is currently believed that many of the basic cell polarities underlying plant morphogenesis have a mechanically generated component. We are accumulating evidence implicating the mechanosensory Ca^{2+} channels in the control of cell polarity, both during growth and during division. In particular, we hypothesize that the "gravitropic" transducer plays a key role in organizing microtubules and actin within the cell.

(6) We propose that the mechanosensory Ca^{2+} channel lies at the heart of a plasmalemmal control center which not only organizes the cytoskeleton and controls a variety of responses to mechanical, electrical, chemical and thermal stimuli, but also contains the blue light receptor system and perhaps a variety of ion pumps and channels.

Force Transmission. Before the channel system can transduce mechanical stress, that stress must be transmitted to the channel. The channel is in the cell membrane, but most of the mechanical stress experienced by the plant, including much of that due to gravity, is borne by the relatively rigid cell wall system. To a lesser though possibly important extent, stress is borne by the cytoskeleton as well. We have identified a wall-to-membrane linker that we believe to be essential for focusing stress borne by either the wall or the cytoskeleton onto the channel. We have visualized such linkers remaining on the surface of cells enzymatically stripped of the cellulose and pectin making up the fabric of the wall — that is, on protoplasts.

Integration of Technologies. This image of the linkers presented in the second illustration was obtained with a resolution far below the capabilities of the OSM. We plan to obtain images of much higher resolution. More importantly, visualizing the distribution of the linkers is merely a first step in our plan to explore the components and actions of the putative plasmalemmal control center which we believe contains the gravity transducer. We have embarked on a major program to utilize the tools of molecular biology as well as of conventional biochemistry and of a variety of biophysical disciplines to obtain relevant genes, proteins, and antibodies to the proteins. We will attempt to visualize the various antibodies with the OSM, using secondary fluorochrome-containing antibodies emitting light at certain specific wavelengths, and always simultaneously using the linker antibodies to make a reference map of the cell surface. We will use stains for cytoskeletal elements to study how their distributions relate to the linkers and to channel activity.

Thus, extending the application of this new technique, we hope to visualize the distribution of the channels themselves as well as their neighboring proteins, and to observe many of the effects of their action.

Accomplishments

Work has focused on two main objectives: developing a set of procedures for studying embryo sac development in *Arabidopsis thaliana*, and determining an acceptable procedure for growing *Arabidopsis* aseptically to the pre-flowering stage which would also be amenable for use in the existing flight hardware, the Plant Growth Unit (PGU).

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TRANSDUCTION OF THE GRAVITROPIC STIMULUS

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Description of Research

This project encompasses research in the field of space biology, although it is not funded through the NASA Space Biology Program. The research is funded through the NASA Office of Space Science and Applications Innovative Research Program. This program seeks to encourage creative, unique approaches to research by funding innovative research projects. Insofar as we have not recently reported on the research in the NASA Space Biology Accomplishments format, we will summarize two years' rather than one year's progress.

The short-range goals of our research proposal are: (1) to set up a versatile patch clamp system and identify a mechanosensory calcium channel in gravitropically sensitive and insensitive cell types; (2) to describe the channel in fair detail, and test the hypothesis that it is dramatically inhibited by a chemical that preferentially inhibits gravitropism and thigmotropism; and (3) to identify new inhibitors that are ineffective, further characterize the channel, and lay groundwork for channel protein purification and for studies of diverse roles of the channel system.

The long-range goals of our research program are: (1) to characterize the primary transducer of the gravitropic stimulus; (2) to test the hypothesis that this mechanotransducer plays multiple, important roles in plant development and maintenance; (3) to work out the structural, biochemical and physiological features of the macromolecular control system in which we believe the transducer is embedded; and (4) to spin off the technological facilities and expertise developed for this study to study related regulatory systems in plants, and to lay a foundation for new agricultural technology.

Accomplishments

Gravitropic Transduction. Working primarily with onion shoots, we have identified and characterized a mechanosensory calcium channel that we believe is responsible for gravitropic transduction. The channel has the general properties we have earlier predicted for the gravity detector, including high sensitivity to mechanical stimuli. The only three agents known to block gravitropic reception with reasonable specificity act as agonists of this channel at low concentrations and as antagonists of this channel at high concentrations.

We have studied single ion channel currents in patch clamped plasma membranes of representative plant cells from both growing, gravitropically sensitive tissue and from storage tissue.

(1) The channels are abundant. In the patch, they are exquisitely sensitive to membrane deformation, and we suspect they are at least equally sensitive in the walled cell. When strongly stimulated they pass relatively large currents. They accommodate to prolonged stimulation, thus tending to prevent damaging influxes of Ca^{2+} . Their large,

apparently dynamic, range of activity is suitable for guiding a cell to take different actions in response to weak versus strong stimuli as well as to brief versus prolonged stimuli.

(2) The channel is a complex of as many as eight cochannels or equivalent conductance units, for it manifests up to eight quantized, linked conductance levels. One possible benefit of having multiple linked channels is that they might pass a large amount of Ca^{2+} into the cell in response to a small signal such as that due to gravitropic stimulation.

(3) Expression of linkage is improved in the presence of mM Mg^{2+} and of μM Gd^{3+} and targeting herbicides. This fact, along with the kinetics of all agonist/antagonist actions studies to date, suggests that both agonism and antagonism occur cooperatively, perhaps at a central "gate" for the complex; additionally, less sensitive channel occlusion or "pore plugging" by the inorganic agonist/antagonists is not excluded as a secondary event. The influence of multivalent cations on linkage suggest that Ca^{2+} , the ion normally passed by the channel, itself regulates gating (opening and closing) patterns.

(4) Even without linking, the amount of Ca^{2+} passed by a cochannel per unit time is large in comparison with that passed by most known Ca^{2+} channels. This has been achieved by lowering the specificity with which the channel selects Ca^{2+} over other ions. The plant cell can afford to lower its selectivity, however, because in an ordinary situation the electrochemical driving force for Ca^{2+} typically favors inward passage of that ion above the movement of other ions such as K^+ .

(5) Mechanosensitive activity of the channels is strongly dependent on transmembrane potential, but only when the membrane is under tension. This means that the activities of ion pumps and of other ion channels can modulate mechanosensitivity via the membrane potential. It also means that, as long as the cells are under any tension (and healthy plant cells are generally under some tension), the channels may serve as voltage sensors.

(6) Mechanosensitive activity of the channels depends on temperature in a remarkable and sensitive way. Activity rises dramatically as temperature is lowered from room temperature to about 6°C , then, when the temperature is lowered one degree further, it plunges to a low level. This means that (again, as long as the cells are under any tension) the channels can serve as temperature sensors.

(7) Under some circumstances sets of channels synchronize activity in repetitive bursts, possibly promoting oscillation of cytosolic Ca^{2+} levels.

In seeking these channels, we have imagined from the beginning that gravitropic transduction is only one of a variety of critical sensory functions they perform for the plant. We shall list some of these, starting with more specialized functions and working toward more general ones.

(1) We have strong evidence that the mechanosensory Ca^{2+} channels transduce signals for several kinds of tropisms in addition to gravitropism, including thigmotropism, thermotropism, electrotropism and hydrotropism. They almost certainly bring about a number of morphological and physiological responses to mechanical stresses. These responses include transition to tougher, more stress-resistance morphology due to shifts in the pattern of cell expansion and in the polymers making up the walls.

(2) Moreover, they almost certainly bring about some of the plant's responses to low temperature, due in considerable part, we believe, to Ca^{2+} entry through these channels.

(3) Based on the simple observation that mechanical stimulation of protoplasts can rapidly shift cell volume, as well as on the general behavior of the channel system, the channels appear to play a key role in regulating the turgor of the cells.

(4) During a variety of activities plants show rapid rhythmical activities. Rhythmic channel activity might underlie many or all of these rhythms.

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Force Transmission. Before the channel system can transduce mechanical stress, that stress must be transmitted to the channel. The channel is in the cell membrane, but most of the mechanical stress experienced by the plant, including much of that due to gravity, is borne by the relatively rigid cell wall system. To a lesser though possibly important extent, stress is borne by the cytoskeleton as well. We have identified a wall-to-membrane linker that we believe to be essential for focusing stress borne by either the wall or the cytoskeleton onto the channel. We have visualized such linkers remaining on the surface of cells enzymatically stripped of the cellulose and pectin making up the fabric of the wall — that is, on protoplasts.

Integration of Technologies. This image of the linkers presented in the second illustration was obtained with a resolution far below the capabilities of the OSM. We plan to obtain images of much higher resolution. More importantly, visualizing the distribution of the linkers is merely a first step in our plan to explore the components and actions of the putative plasmalemmal control center which we believe contains the gravity transducer. We have embarked on a major program to utilize the tools of molecular biology as well as of conventional biochemistry and of a variety of biophysical disciplines to obtain relevant genes, proteins, and antibodies to the proteins. We will attempt to visualize the various antibodies with the OSM, using secondary fluorochrome-containing antibodies emitting light at certain specific wavelengths, and always simultaneously using the linker antibodies to make a reference map of the cell surface. We will use stains for cytoskeletal elements to study how their distributions relate to the linkers and to channel activity.

Thus, extending the application of this new technique, we hope to visualize the distribution of the channels themselves as well as their neighboring proteins, and to observe many of the effects of their action.

Significance of the Accomplishments

If our interpretations of the data are correct, systematic expansion of the gravity sensor program into the arena of molecular architecture and function should greatly broaden understanding of the plant cell and of the developmental processes and maintenance activities of plants.

Moreover, while studying how mechanoresponses are integrated into the entire spectrum of life activities of the plant, we anticipate the emergence of numerous ways of taking advantage of the plasmalemmal control center in order to control plant productivity in the field. The fact that one of the first mechanosensory channel agonists/antagonists we studied is a commercially important herbicide hints at the possibilities. We anticipate that other herbicides we want to screen will also prove to act via the mechanosensory Ca^{2+} channel, that these herbicides are purloining the site of action of an endogenous regulator (or regulators), and further that toxins of various disease organisms will prove to act via this and possibly other channel sites. By studying the channel with the tools of molecular biology, by considering the modulatory effects of cold, turgor, transmembrane potential, and mechanical stimulation on the channel, by examining the macromolecular architecture of the channel complex with the OSM and other emerging technologies, and by integrating these findings with a keen sense for natural history, we should be able to develop improved crops or, alternatively, to differentially manipulate crops and weeds to improve crop performance.

Publications

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Ding, J.P. and Pickard, B.G. 1990. Clustering of conductance units amplifies signal-to-force ratio for a putative gravitropic transducer channel (Abstract). *ASGSB Bulletin* 4(1): 106. (GWU 13383)

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Ding, J.P. and Pickard, B.G. 1990. Pulsed bursting of stretch and voltage activated Ca^{2+} channels: Model and possible basis for several ultradian phenomena (Abstract). *Plant Physiology* 93(1, Suppl.): 78. (GWU 12124)

Hong, B., Pickard, B.G., and Ho, T.-H.D. 1990. Is force focused on putative gravitropic channels by an ABA-induced amphipathic protein? (Abstract) *ASGSB Bulletin* 4(1): 100. (GWU 13380)

LaMotte, C.E. and Pickard, B.G. 1991. Control of root orientation by reversible conversion between gravitropic and agravitropic states: Roles of light, darkness and water stress (Abstract). *Plant Physiology* 96(1, Suppl.): 72. (GWU 13791)

MECHANISM AND CONTROL OF SHOOT GRAVITROPISM

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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 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. This information in turn will tell us a great deal about how plants integrate sensory stimuli and will eventually influence efforts to manipulate the growth and yield of plants on Earth and in the microgravity environment of space.

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.

Accomplishments

The diageotropica mutant (*dgt*) of tomato is insensitive to exogenously applied auxin and exhibits other symptoms of auxin insufficiency, such as diagravitropism, abnormal vascular tissue, and no lateral root branching. Thus, *dgt* is a particularly promising candidate for an analysis of auxin-regulated growth and development and has been the focus of much of my recent research.

(1) We performed northern blot analysis using RNA from wild type (VFN) and mutant (*dgt*) tomato hypocotyls treated with or without auxin, using as a probe a 59-mer oligonucleotide complementary to a portion of the published sequence of SAUR (Small Auxin Up Regulated gene) 15A. In wild-type tomato (which elongates in response to exogenous auxin), a transcript of size identical to that of SAUR 15A is strongly induced within one hour of auxin application. *In the auxin-insensitive dgt mutant, no auxin up regulated SAUR 15A homologue is expressed.*

(2) *The plant growth steroid brassinolide, which causes stem elongation independently of auxin in many systems, leads to elongation of hypocotyls in both VFN and dgt but does not induce SAUR 15A in either plant type.*

Significance of the Accomplishments

If our interpretations of the data are correct, systematic expansion of the gravity sensor program into the arena of molecular architecture and function should greatly broaden understanding of the plant cell and of the developmental processes and maintenance activities of plants.

Moreover, while studying how mechanoresponses are integrated into the entire spectrum of life activities of the plant, we anticipate the emergence of numerous ways of taking advantage of the plasmalemmal control center in order to control plant productivity in the field. The fact that one of the first mechanosensory channel agonists/antagonists we studied is a commercially important herbicide hints at the possibilities. We anticipate that other herbicides we want to screen will also prove to act via the mechanosensory Ca^{2+} channel, that these herbicides are purloining the site of action of an endogenous regulator (or regulators), and further that toxins of various disease organisms will prove to act via this and possibly other channel sites. By studying the channel with the tools of molecular biology, by considering the modulatory effects of cold, turgor, transmembrane potential, and mechanical stimulation on the channel, by examining the macromolecular architecture of the channel complex with the OSM and other emerging technologies, and by integrating these findings with a keen sense for natural history, we should be able to develop improved crops or, alternatively, to differentially manipulate crops and weeds to improve crop performance.

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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 different 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 used to isolate the DNA fragments of interest either by "walking" from the closest restriction fragment length polymorphism, or by genomic subtraction. As a consequence of this work, we expect to identify components in the gravity-sensing system of plants and to determine the mechanisms whereby gravity interacts with other environmental factors, such as light, to control the physiology and morphology of plants.

Accomplishments

(1) *Four mutant strains of Arabidopsis exhibiting the "random" phenotype have been genetically and physiologically characterized.* Based on data from reciprocal crosses, *these mutants carry single recessive nuclear mutations with respect to the gravitropism phenotype.* There is no correlation between the starch and gravitropism phenotypes. Thus, a loss of starch is not necessary for a loss of graviperception. In addition, the gravitropism phenotype appears to be repaired when the seedlings are not etiolated. That is, seedlings grown in white light prior to gravistimulation in darkness respond in a wild-type manner.

(2) In preparation for the use of the genomic subtraction procedure for DNA isolation, M2 seeds of *Arabidopsis* have been prepared following cobalt-irradiation and fast neutron mutagenesis. Gravitropism mutants screened from these seed collections should contain a high percentage of deletion mutants.

(3) Gravistimulated seedlings of *Arabidopsis* have been monitored as curvature develops in physiological "darkness" using an infrared-sensitive CCD camera. Curvature of the hypocotyl is followed by a small but significant degree of straightening. *Since straightening occurs both in stationary seedlings and in those on the clinostat, it follows that straightening is a consequence of curvature and not a consequence of the gravitational stimulus.*

(4) The thermotropic behavior of corn roots has been characterized. Since the magnitude and direction of curvature cannot be explained by either a temperature or humidity effect on root elongation, it is concluded that these roots sense temperature gradients in addition to sensing the gravitational force.

Significance of the Accomplishments

Finding that plants that exhibit the random phenotype in darkness change in light and exhibit the wild-type phenotype provides a new resource with which the interaction between the sensory responses to light and gravity can be studied.

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CALCIUM MESSENGER SYSTEM IN GRAVITROPIC RESPONSE IN PLANTS

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Description of Research

We have been investigating the role of calmodulin and calcium/calmodulin-dependent protein phosphorylation in gravity signal transduction in plants. Studies from our laboratory have shown that physical and chemical signals increase the expression of calmodulin mRNA. These results suggest that signal transduction in plants may involve changes in the concentration of both the receptor (calmodulin) and messenger (calcium). Calcium/calmodulin regulated protein phosphorylation is one of the mechanisms by which eukaryotic cells transduce extracellular signals to intracellular responses. Calcium and calcium/calmodulin-dependent protein phosphorylation has been demonstrated in a number of plant extracts, indicating the presence of calcium and calcium/calmodulin-dependent protein kinases in plants. The presence of calcium/calmodulin-dependent protein kinases similar to animal multifunctional CaM KII has not been reported so far in plants. We investigated the presence of CaM KII in corn roots and its possible role in gravity signal transduction. Accumulating evidence from animal systems indicates that there are multiple genes of calmodulin that are differentially regulated during development. We investigated whether multiple genes exist in plants and their role in the calcium messenger system.

Accomplishments

The involvement of calcium and protein phosphorylation in the transduction of the gravity signal was studied using corn roots of a light-insensitive variety (*Zea mays* L., cv. Patriot). The gravitropic response was calcium-dependent. Horizontal placement of roots preloaded with ^{32}P for 3 min resulted in changes in protein phosphorylation of polypeptides of 32 and 35 kDa. Calcium depletion resulted in decreased phosphorylation of these phosphoproteins and replenishment of calcium restored the phosphorylation.

We investigated the presence of CaM KII using (i) an affinity purified anti-peptide antibody produced against α -subunit of rat brain CaM KII and (ii) specific substrates for CaM KII. The CaM KII antibodies recognized a 56 kDa protein in soluble proteins of corn roots. The intensity of the crossreacting polypeptide is higher in the root tip as compared to the root base. Immunoblots with soluble extracts from different plants and tissues detected one or two bands of similar molecular weight (56-58 kDa). Two other anti-peptide antibodies raised against CaM KII also recognized these same proteins. The molecular weights of the plant proteins are similar to the mammalian CaM KII. *In vitro* phosphorylation and immunoprecipitation studies indicate that the protein recognized by CaM KII antibody is phosphorylated in a calcium/calmodulin-dependent manner. Soluble extract from corn roots phosphorylated synapsin I, a physiological substrate for mammalian calcium/calmodulin-dependent protein kinases, and this phosphorylation of synapsin I was stimulated by calcium and calmodulin. One-dimensional phosphopeptide mapping revealed that the site of enhanced phosphorylation of synapsin I was located within the 30 kDa fragment that is the known site of phosphorylation by CaM KII. BB40, a specific peptide

substrate corresponding to residues 281-291 of the α -subunit of CaM KII, was used to detect the CaM KII-like kinase activity in plants. Corn root soluble extract phosphorylated BB40 in a calcium/calmodulin-dependent manner. *These results suggest that plants contain a homolog of multifunctional CaM KII.*

We have isolated several cDNA clones from corn and *Arabidopsis* by screening the expression libraries with the antibody raised against CaM KII. These cDNAs are being characterized.

Our results also indicate that plants contain multiple genes of calmodulin and that they are developmentally regulated. To investigate the structure and expression of calmodulin genes, we have isolated several genomic clones from rice and potato. By restriction mapping and partial sequence analysis of some of these clones, we identified three different calmodulin genomic clones in rice. One of the genomic clones (cam-2) has been completely sequenced. The rice gene is very divergent from potato (pPCM-1). To study the activity of the calmodulin promoter, we fused the 5' region of the rice cam-2 genomic clone to a reporter gene, CAT, and transformed tobacco. *Analysis of the reporter gene expression has indicated that the activity of calmodulin promoter is developmentally regulated.*

Significance of the Accomplishments

Our results suggest that plants have multiple genes of calmodulin and that they are differentially regulated during development. In addition, we have been able to show the presence of calcium/calmodulin-dependent protein kinase II in plants. Our results suggest that calcium/calmodulin regulated changes in protein phosphorylation could play a pivotal role in amplifying and diversifying the action of the gravity signal.

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MECHANISM AND CONTROL OF SHOOT GRAVITROPISM

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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 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. This information in turn will tell us a great deal about how plants integrate sensory stimuli and will eventually influence efforts to manipulate the growth and yield of plants on Earth and in the microgravity environment of space.

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.

Accomplishments

The diageotropica mutant (*dgt*) of tomato is insensitive to exogenously applied auxin and exhibits other symptoms of auxin insufficiency, such as diageotropism, abnormal vascular tissue, and no lateral root branching. Thus, *dgt* is a particularly promising candidate for an analysis of auxin-regulated growth and development and has been the focus of much of my recent research.

(1) We performed northern blot analysis using RNA from wild type (VFN) and mutant (*dgt*) tomato hypocotyls treated with or without auxin, using as a probe a 59-mer oligonucleotide complementary to a portion of the published sequence of SAUR (Small Auxin Up Regulated gene) 15A. In wild-type tomato (which elongates in response to exogenous auxin), a transcript of size identical to that of SAUR 15A is strongly induced within one hour of auxin application. *In the auxin-insensitive dgt mutant, no auxin up regulated SAUR 15A homologue is expressed.*

(2) *The plant growth steroid brassinolide, which causes stem elongation independently of auxin in many systems, leads to elongation of hypocotyls in both VFN and dgt but does not induce SAUR 15A in either plant type.*

(3) Previous research by others has suggested that the epidermis of dicotyledonous stems is the primary site of auxin action in elongation growth. We have shown that for pea (*Pisum sativum* L.) epicotyl sections this hypothesis is incorrect. In buffer (pH 6.5), sections from which the outer cell layers were removed (peeled) elongated slowly and to the same extent as intact sections. Addition of 10 μ M indoleacetic acid to this incubation medium caused peeled sections to grow to the same extent and with the same kinetics as auxin-treated nonpeeled sections. *This indicates that both epidermal and cortical tissues have the same ability to respond to auxin.* Previous reports that peeled pea sections respond poorly to auxin may have resulted from an acid-extension of these sections due to the use of distilled water as the incubation medium.

Significance of the Accomplishments

Plant hormones can cause selective and rapid changes in the levels of specific mRNAs. A striking example is the group of Small Auxin Up Regulated (SAUR) genes isolated by McClure and Guilfoyle from soybean hypocotyl sections. Characterization of the regulation of SAUR genes in the auxin-insensitive mutant should facilitate studies directed toward establishing a causal relationship between molecular induction of specific mRNAs and a physiological process such as cell elongation or gravitropism. Our demonstration of SAUR induction in VFN and the lack of induction in *dgt* is a step in this direction.

There is an extensive body of information suggesting that the epidermis of dicotyledonous stems is the primary target for auxin action in cell elongation. If this conclusion is correct, such a tissue specific phenomenon would have important consequences for research on the biophysics of stem elongation and wall loosening, the location of auxin receptors, and tissues expressing auxin up- or down-regulated genes. Our demonstration that both the epidermis and cortical tissues have the same ability to respond to auxin suggests previous reports are in error. Our data further indicate that pea epicotyl sections have a maximum rate of extension that can be induced by either acidic solutions or by auxin. The two types of extension must have a common final step, since the extensions are not additive. These data further indicate that pea epicotyl tissues are capable of undergoing rapid elongation at the pHs expected to be present in the apoplast of auxin-treated tissues, i.e., pH 5.5 or below. They support the concept that the initial auxin-induced growth may be due to an acid-growth mechanism.

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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, and to thus clarify 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. Recent reports have demonstrated that both light and gravitropic stimuli induce changes in the concentration of cytosolic free calcium in responding cells. However, full confidence in the validity of this hypothesis will require that at least one additional piece of evidence be obtained: namely, the identification of specific targets of calcium action that have a clear and significant impact on growth. This 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 during evolution, 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. Last year we reported the partial purification and characterization of a 35 kDa annexin-like protein from peas, which we called p35. This year we completed the purification of p35, obtained partial primary sequence data on it, and localized it in pea seedlings. Our results, described here, appear quite relevant to current ideas on mechanisms of root gravitropism. We also obtained data implicating the involvement of a calcium-regulated protein kinase in the transduction sequence connecting a light stimulus with a germination growth response in fern spores.

Accomplishments

(1) *We have purified a calcium-binding annexin-like protein (p35) from peas and obtained the primary sequence of two peptides proteolytically derived from it.* The N-terminal end of p35 is blocked, so we digested p35 with a proteinase that cleaves at lysine residues, separated the peptides thus generated on reverse phase HPLC, and obtained the amino acid sequence of two of the purified peptides. Both of these sequences were highly similar to ones found in a conserved region in a tomato annexin.

(2) We have immunocytochemically localized p35, at both the ultrastructural and light-microscope level, in the root, stem and leaf cells of pea seedlings, and the results of

this study indicate that *p35*, like many animal annexins, may play an important role in the process of secretion. At the ultrastructural level, p35 appears to be mainly associated with Golgi vesicles and plasma membrane. At the light level, p35 appears to be most concentrated in the periphery of cells that are actively engaged in secretion, such as the outer root cap cells (Figure 1) and young developing tracheary elements. This localization is quite distinct from that of calmodulin, which is typically found concentrated in the cytoplasm rather than associated with secretory vesicles, and which is more abundant in the central columella cells of root caps and in the more mature tracheary elements of vascular tissue. Antibodies to p35 selectively recognize antigens at or near 35 kDa also in corn and oats.

(3) We have identified a protein kinase inhibitor, *staurosporine*, which is extremely effective against calcium-dependent protein kinases in both higher and lower plants (K_i less than $0.1 \mu\text{M}$), and have shown that this inhibitor blocks phytochrome-induced spore germination in the fern *Dryopteris*, specifically during the calcium-dependent phase of the germination process.

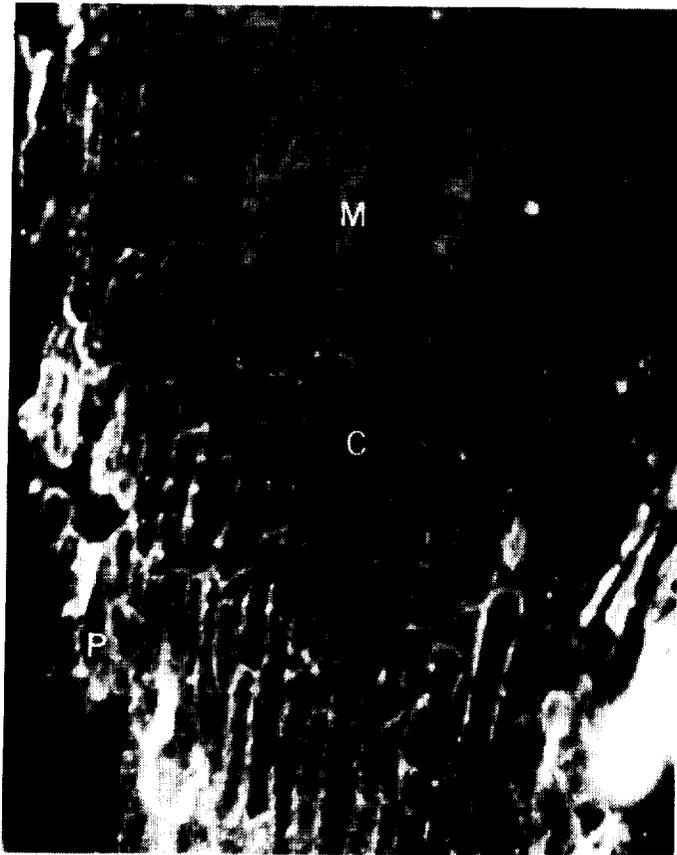


Figure 1. Fluorescence micrograph of a pea root cap in a longitudinal section stained with an antibody to the annexin-like protein, p35. Heaviest staining is seen toward the periphery of the outermost cap cells. 225x. M, position of mitotic region near the root apex; C, columella cells of the root cap; P, peripheral, mucilage-secreting cells of the root cap.

Significance of the Accomplishments

Findings #1 and #2: As yet there are only very few reports of annexin-like protein in plants, and no detailed localization studies. Our results provide more convincing evidence both that annexins are present in plants and that they may play a role in secretion, particularly in root caps where the secretion of mucilage has been proposed to be a critically important process in the control of root gravitropism. Also of possible significance, p35 is almost certainly a phosphoprotein, and a protein in root caps of the same size and pI as p35 has been reported to be selectively phosphorylated when roots are gravistimulated. Because antibodies to pea p35 recognize proteins of the same size in other species it should be possible to test whether annexin-like proteins are involved in secretory processes also in other plants. Annexins should be considered a possible target of calcium action in the control of root gravitropism.

Finding #3: That calcium plays a critical role in converting the light signal into a germination response in *Dryopteris* spores is well known. How it does so is unknown. In animal cells protein kinases have been shown to be important agents for amplifying the calcium signal into enzymatic activity changes, and they have been proposed to play the same role in plants. Because staurosporine has been shown to be a particularly potent inhibitor of calcium-dependent protein kinases in both higher and lower plants, Finding #3 lends support to the hypothesis that such kinases may be important targets of calcium action during light-induced germination.

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GRAVIPERCEPTION IN PLANTS

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Description of Research

Our long-term goal is to understand the mechanisms of gravitropic sensing in higher and lower plants. This involves identifying cells that sense gravity and determining the cellular mass that gravity acts upon to trigger sensing. We hope to learn which events occur during the transduction of a physical signal into a physiological signal that affects growth.

It is likely that starch-filled organelles called amyloplasts play a role in gravitropic sensing and are necessary for full sensitivity. However, reduced sensing can occur without starch in mutant plants, and therefore amyloplasts are not *required* for sensing in higher plants. Thus an "all or none" interpretation regarding the importance of starch is not warranted. There may be several cross-referencing systems in sensing, or perhaps a single (plastid-based) system can act at thresholds lower than previously imagined.

It is generally thought that gravitropic sensing in roots occurs in the central cells of the rootcap. However, various classical and contemporary data raise the question of whether additional sensing occurs apart from the cap in roots. In the course of writing an extensive review on "plant gravity sensing," several old (and apparently forgotten) German papers were found citing instances of amyloplast sedimentation other than in the rootcap. One such claim was reinvestigated.

Another system under investigation is the gravitropic tip cell of the moss *Ceratodon*, which grows up in the dark. We found that this cell appears to use amyloplasts that sediment in gravitropic sensing, and that sedimentation is followed by microtubule redistribution and then by upward curvature. Recently, we examined the tip cell by electron microscopy.

Accomplishments

(1) *Obvious sedimentation was found in the elongation zone as well as in the rootcap in gravitropic roots of Equisetum* (horsetail) (Figure 1).



Figure 1. Part of the elongation zone of a horizontal root of *Equisetum*. Gravity is towards the bottom of the figure, and the cells are elongating from the left to the right. The dark small structures are sedimented amyloplasts, and the large sphere in each cell is the nucleus, which is sedimented on top of the starch-filled amyloplasts. This stratification with respect to gravity suggests that this part of the root, as well as the rootcap, can sense the direction of gravity.

(2) *Nuclei as well as amyloplasts sedimented in both locations in Equisetum roots.* In the cap and young elongation zone, nuclei sedimented on top of amyloplasts in both vertical and horizontal roots.

(3) The zone in the tip cell of *Ceratodon* where microtubules concentrate by the lower wall is rich in Golgi.

Significance of the Accomplishments

Finding #1: This is the first modern report of significant sedimentation apart from the rootcap. While this does not prove that the elongation zone is capable of sensing, it does provide structural data that warrant a reinvestigation of the question of whether sensing is confined to the cap in all roots. Various other data support the idea that gravitropic sensing can occur away from the cap. For example, cells in the elongation zone of mung bean roots show rapid changes in electric properties depending upon the orientation of the roots. If sensing does occur in the elongation zone, this would not invalidate the classical view of the importance of the cap. Again, an "all or none" approach is uncalled for.

Finding #2: Nuclear position is often regulated in cells with sedimented amyloplasts, but as far as I know, this is the only report of the sedimentation of the nucleus in roots at 1 g. This finding demonstrates that the nucleus clearly has enough mass to sediment, but that in other plants its sedimentation appears to be prevented by the cytoskeleton since it is often at the top of central cap cells in vertical roots.

Finding #3: Assuming that amyloplast sedimentation and the redistribution of microtubules play a role in redirecting growth of the tip cell, it is intriguing that the region where microtubules become concentrated by the lower wall is also the region that is rich in wall-building organelles (Golgi complex). We therefore plan to investigate the hypotheses that microtubules influence the position of Golgi and that there is more Golgi in the lower than in the upper halves of horizontal protonemata.

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DEVELOPMENTAL STUDIES OF WHEAT IN MICROGRAVITY

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Description of Research

We are preparing for two flight experiments with a super-dwarf cultivar of wheat. One experiment will be in a modified Plant Growth Facility (PGF II) on a U.S. shuttle flight (approximately 10 days duration). The main objective is to measure CO₂ exchange as an indication of photosynthesis and respiration. We will also measure water vapor (calculate transpiration, stomatal conductance, and internal CO₂) and observe certain developmental stages in the life cycle of wheat, especially formation of tillers (axillary stems) and flowers. We also have the support of NASA and an invitation from Soviet scientists to carry out a seed-to-seed (life cycle) experiment (to be developed in conjunction with the Institute of Biomedical Problems in Moscow, USSR) in the Soviet space station Mir. Again, a primary objective is to measure photosynthesis and respiration (CO₂ exchange) along with transpiration and stomatal conductance. Final yield is also an indication of photosynthetic productivity, and all of the developmental stages of the life cycle will be observed if the plants succeed in growing from seed to seed in the space environment.

Ground-based research involves the development of hardware as well as the study of growth of plants under conditions simulating those to be encountered in the two flight experiment situations. There are several problems that have been encountered in previous experiments with plants in space. Primary among these are the amount of available light for photosynthesis, control of atmospheric composition, and provision of a suitable nutrient medium for plant roots. The PGF II has been upgraded from the earlier mid-deck locker plant growth facility, so sufficient light is now available to support adequate plant growth. Atmospheric composition is a serious problem, however, because CO₂ levels in both the shuttle and Mir are well above optimal levels for plants and may even be toxic. With no gravity to cause drainage, the nutrient medium also presents a serious challenge: roots easily become waterlogged unless special preventative measures are taken. A secondary problem concerns acquisition of data during the long-term experiment; photographic means will probably have to be developed, along with the usual recording of environmental factors and sampling of plant material. Our research includes development of hardware to solve these problems.

Accomplishments

Much time has been spent in planning for these two experiments. This has included travel within the United States and planned travel to the Soviet Union. We have developed detailed approaches to solving the three primary problems mentioned above, but so far we have not actually initiated hardware construction to test these proposed solutions. We hope to do so in the near future. In the meantime, we have been working with an advanced model of the PGF II and have learned to utilize its computer-driven system.

Although we have had several years of experience growing wheat (including the super-dwarf cultivar), this was always done under high irradiances that will be unavailable in the spaceflights. To gain experience at lower irradiances, we have recently grown the cultivar under a range of light levels (about 60 to 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$), down to and even below the

photosynthetic compensation point. Whereas our previous trials utilized sunlight supplemented with the red-rich light from high-pressure sodium lamps, our present studies utilize only light from cool-white fluorescent tubes. To our surprise (and chagrin), plants that were normally about 25 cm tall in the greenhouse or under high-pressure sodium light grew to 40+ cm under the fluorescent light, and plants growing at light levels below about 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ were considerably delayed in head emergence. We are investigating the factors that might be responsible for this, but the only obvious factor so far is the light environment. To maintain short plants with a short life cycle, it may be necessary to change the light sources in the PGF II and in its Soviet counterpart, the SVET apparatus.

Significance of the Accomplishments

If the growth phenomena described above prove to be caused by the light environment, this could be of considerable significance to plant photobiology. It has been stated as a general rule that blue light tends to inhibit stem growth, producing shorter plants, and that far-red wavelengths cause extensive elongation in many species. Yet light from cool-white fluorescent tubes is especially rich in blue light but contains virtually no far-red wavelengths, and indeed, most species are short instead of tall under fluorescent light. On the other hand, some plant responses are caused by both blue and far-red light, and it may be that wheat leaves and stems elongate in response to blue as though it were far-red light. The delayed head emergence is also of interest because we had previously studied leaf emergence and found it to be virtually unaffected by irradiance levels.

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AMINE OXIDASE AND PEROXIDASE REGULATION OF DIFFERENTIAL GROWTH IN GRAVISTIMULATED PLANTS

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Description of Research

Our long-term goal is to understand 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 those encountered during spaceflight.

A specific goal in our research program is to determine the extent to which peroxidase (POD) activities may regulate plant growth, particularly those specific POD isozymes whose activities are modulated in response to gravistimulation of the plant during resultant tropistic bending. It is generally believed that POD carry out various types of covalent cross-linking reactions (of cell wall polymers, for example) that may alter the growth of the plant. However, this has been difficult to demonstrate due to the existence of large numbers of POD isozymes in most plant tissues, coupled with a paucity of information regarding their localization and modes of physiological and biochemical regulation and regarding the specific types of cross-linking reactions catalyzed by these enzymes. Our lab is presently concentrating on the first two areas, namely, identifying which isozymes may play a role in growth regulation, focusing on cell wall-localized PODs, and examining mechanisms that control their activities. We have previously shown that diamine and polyamine oxidases, which produce H_2O_2 as a byproduct, are located in the plant cell wall. These enzymes could provide the H_2O_2 substrate required for POD-mediated cross-linking reactions in the wall and may contribute to the overall regulation of growth.

Accomplishments

(1) We have shown that *one anionic POD isozyme (A3) is induced by exogenous Ca^{2+} or IAA application to upright, non-gravistimulated corn coleoptiles in a manner that is consistent with growth inhibition. The activity of A3 also increases on the upper sides of horizontally gravistimulated coleoptiles within 30 min of stimulus onset.*

(2) IAA induction of A3 is blocked by the protein synthesis inhibitor cyclohexamide.

(3) We have partially purified the A3 isozyme.

(4) We have found that an "IAA oxidase" activity extracted in the ionic POD fraction increases 20% on the upper sides of coleoptiles gravistimulated for 3 hrs.

Significance of the Accomplishments

Finding #1: There have been few attempts to identify specific POD isozymes whose expression rapidly responds to environmental cues, such as gravity, which impact plant growth. The activity of POD isozyme A3 increases dramatically in upper side tissues of

gravistimulated coleoptiles within 30 min, prior to a visible bending response. A3 appears to be a cell wall-localized enzyme and increased A3 activities may promote localized wall cross-linking and "stiffening," contributing to the gravitropic bending response. Since asymmetric gradients of both IAA (auxin) and Ca^{2+} have been implicated in such differential growth responses, their effects may be mediated, in part, through their effects on POD expression.

Finding #2: Little is known about the regulation of POD activities *in vivo*. It appears that some POD isozymes which are synthesized intracellularly require Ca^{2+} for their secretion into the cell wall, where they may exert their effects on growth. Whether this secretion occurs co-translationally is unknown in most cases. By treating corn coleoptiles with cyclohexamide at various times before, during, or after IAA application, we have shown that POD isozyme A3 expression results from *de novo* synthesis of the enzyme.

Finding #3: Meaningful studies of POD isozyme A3 and its possible role in growth regulation will require the development of molecular probes such as specific antibodies and DNA clones that can be used to investigate temporal and spatial patterns of A3 expression. We have partially purified A3 from corn using a combination of ammonium sulfate precipitation, DEAE-Sepharose CL 6B ion exchange chromatography and Sephacryl S-200 gel filtration chromatography. On SDS-PAGE gels, the resultant 85-fold purified A3 preparation contains two major bands (20 and 26 kDa) and several minor bands between 55-70 kDa, one of which is likely to be A3. We are attempting to purify A3 further by preparative isoelectric focusing in order to produce A3 antibodies for future studies on the regulation of this enzyme.

Finding #4: In the process of characterizing various POD activities associated with the gravitropic bending process in corn coleoptiles, we discovered an IAA oxidase activity in an ionic POD fraction. This activity is stimulated by Mn^{2+} and *p*-coumaric acid in the absence of H_2O_2 , typical of peroxidative decarboxylation of IAA. While we have not yet identified which POD isozyme in this fraction exhibits IAA oxidase activity, we have observed a significant increase in IAA oxidase activities on the upper sides of coleoptiles gravistimulated for 3 hrs. The relatively high specific activity of this IAA oxidase (1.55 mmol IAA oxidized/h/mg protein) suggests that it might catabolize substantial amounts of IAA *in vivo*, resulting in decreased rate of growth in these tissues. We are further characterizing this activity and its possible role in the establishment of tropic curvature.

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GRAVITY-INDUCED POLARITY IN SINGLE CELLS OF *CHARA*

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Description of Research

The overall goal of our research program is to elucidate the gravireceptor and the biophysical events that the activated gravireceptor initiates in order to understand how cells perceive and respond to a gravitational stimulus. In order to accomplish our goals, we studied the effect of gravity in inducing a polarity in cytoplasmic streaming in the single internodal cells of characean algae.

As an outcome of our research, we have proposed a mechanism of gravisensing in which the entire protoplast experiences the force of gravity and settles within the extracellular matrix; consequently, membrane proteins experience compressive or tensile forces as they interact with the lower and upper cell wall, respectively. We consider the protoplast to be like a thin water-filled balloon. When it is held vertically, it bulges at the bottom and narrows at the top as a consequence of gravitational pressure. Inside the cell wall, however, gravitational pressure will cause a compression on the lower plasma membrane and a tension on the upper plasma membrane or, in two dimensions, a stretch on the plasma membrane at the bottom and a shrinkage of the plasma membrane at the top. The potential energy released is sufficient to open ion channels involved in signal transduction that are required for the realization of the graviresponse.

Accomplishments

(1) In order to quantitatively establish the validity of or to falsify the gravitational pressure model for gravisensing, it is important to make quantitative measurements of the densities of the various cellular compartments. Using interference microscopy, quantitative phase contrast microscopy, refractometry, chemical analysis and equilibrium sedimentation techniques, we have determined that the densities of the cell sap, endoplasm and cell wall of *Chara corallina* are 1004.98, 1013.9 and 1355.33 kg m⁻³ and 1006.89, 1016.7 and 1371.0 kg m⁻³ for *Nitellopsis obtusa*.

These values are similar to those found by Kamiya and Kuroda for *Nitella* (1957, Proceedings of the Japanese Academy of Sciences 33: 403-406), on which we based our gravitational pressure sensing model. Thus our values extend the early measurements of Kamiya and Kuroda and support the gravitational pressure model.

(2) We have previously shown that increasing the density of the external medium to one that is greater than the density of the protoplast tricks the protoplast so that the vector of gravity is perceived by the cell as being reversed; consequently, the endoplasm streams up faster than it streams down. In order to further characterize this effect, we treated the cells with a series of unrelated chemicals that affect the density of the external medium, the osmotic pressure of the external medium, or both. *The experiments showed that not only is the density of the external medium important for determining the direction of the vector of gravity during gravisensing, but also that the magnitude of the response depends upon the turgidity of the plasma*

membrane. That is, a turgid plasma membrane is a prerequisite for and not a hindrance to gravisensing in characean cells.

(3) To further test the gravitational pressure model of gravisensing, we subjected one end of the cell to a hydrostatic pressure in order to see if hydrostatic pressure is able to mimic the effect of gravity on cytoplasmic streaming. In fact, **hydrostatic pressure does induce a polarity in cytoplasmic streaming.** Like the gravitational pressure-induced polarity, hydrostatic pressure-induced polar streaming requires intact cell ends. Furthermore, the hydrostatic pressure-induced polarity, like the gravitational pressure-induced polarity, has a normal polarity when the external Ca^{2+} concentration is greater than $1 \mu\text{M}$ and a reversed response at lower concentrations. Other similarities include the effects of Neutral red, tetraethyl ammonium chloride (TEA) and external osmotic pressure. Moreover, **applied hydrostatic pressure can reverse, but not augment, the gravitational pressure-induced polarity of cytoplasmic streaming.** These data strongly support the gravitational pressure model of gravisensing.

(4) We have begun work to localize the gravireceptors by observing the effect of impermeant hydrolytic enzymes on the gravity-induced polarity of streaming in characean cells. Enzymes including Cellulysin, cellulase (Worthington), "Onozuka" cellulase, β -glucosidase, β -galactosidase, phospholipase D, proteinase K and thermolysin inhibit gravisensing in characean cells. By contrast, macerase and pectinase are ineffective. These data can only be explained by assuming that **either the gravireceptor or elements of the signal transduction chain are localized in the extracellular matrix** which includes the outer leaflet of the plasma membrane and the cell wall. We assume that a gravireceptor protein spans the distance between the plasma membrane and the cell wall.

(5) There is some question whether the gravitational pressure model of gravisensing plays any role whatsoever in the gravity sensing involved in gravitropism. In order to answer this question we have taken two tacks. Firstly, we have established that the internodal cells of *Chara corallina* are negatively gravitropic. Through differential growth they become oriented from the horizontal position to the vertical position within 45 hr. Gravitropic curvature is a "whole shoot" response: two or three of the internodal cells participate in curvature until the final angle is ninety degrees. Gravising takes place in plants that have had the statolith-containing rhizoids removed, indicating that the statoliths in the rhizoids are not involved in negative gravitropism. Ligation of the internodal cells prevents gravitropism just as it does the gravity-induced polarity of cytoplasmic streaming. Ligation does not inhibit the phototropic response of internodal cells, indicating that the receptors of the two responses are fundamentally different and that the motor system that causes differential growth is not adversely affected by ligation. We are currently measuring the dependence of the lag time and rate of curvature on the initial angle in order to localize the gravireceptors.

Secondly, we have tested the validity of the gravitational pressure model on root gravitropism by observing the effect of varying the density of the external medium on gravising in rice roots. When the density of the external medium is increased by the addition of sucrose, gravitropism is inhibited in a concentration-dependent manner (even though the osmotic pressure of the medium is maintained constant by the addition of ethylene glycol). Since a change in the density of the external medium has no effect on the sinking or rising of intracellular organelles but affects the sinking or rising of the protoplast itself, these data indicate that the plasma membrane (as predicted by the gravitational pressure model) and not intracellular particles (as predicted by the sedimenting amyloplast model) may be the gravireceptor in the cells of higher plants.

Significance of the Accomplishments

Finding #1: We have made measurements on the basic properties of state of cells required to quantitatively describe gravisensing.

Finding #2: We have determined that a turgid plasma membrane is a *sine qua non* for gravisensing in characean cells.

Finding #3: We have determined that hydrostatic pressure, applied to one end of an internodal cell, mimics the effect of gravity by inducing a polarity in cytoplasmic streaming.

Finding #4: We have shown that impermeant hydrolytic enzymes, including cellulases and proteinases, inhibit gravisensing, indicating that the gravireceptor may reside in the extracellular matrix, which includes the outer leaflet of the plasma membrane and the cell wall.

Finding #5: In order to test the validity of the gravitational pressure model on gravitropism, we have shown that *Chara* internodal cells are gravitropic and gravitropism of rice roots is inhibited by increasing the density of the surrounding medium.

All data to date are consistent with the explanation that the gravitational pressure model describes gravisensing in higher plant cells as well as in algal cells. Our gravitational pressure model, based on plant research, can also explain the effects of gravity and spaceflight on human and animal cells that do not contain otoliths. For example, our model can explain the observations that the mitogenic response of statolith-free, human lymphocytes cells must be attached to a substratum in order to respond to gravitational pressure.

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ANIMAL PROJECTS

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HAIR CELLS OF THE BULLFROG UTRICULUS

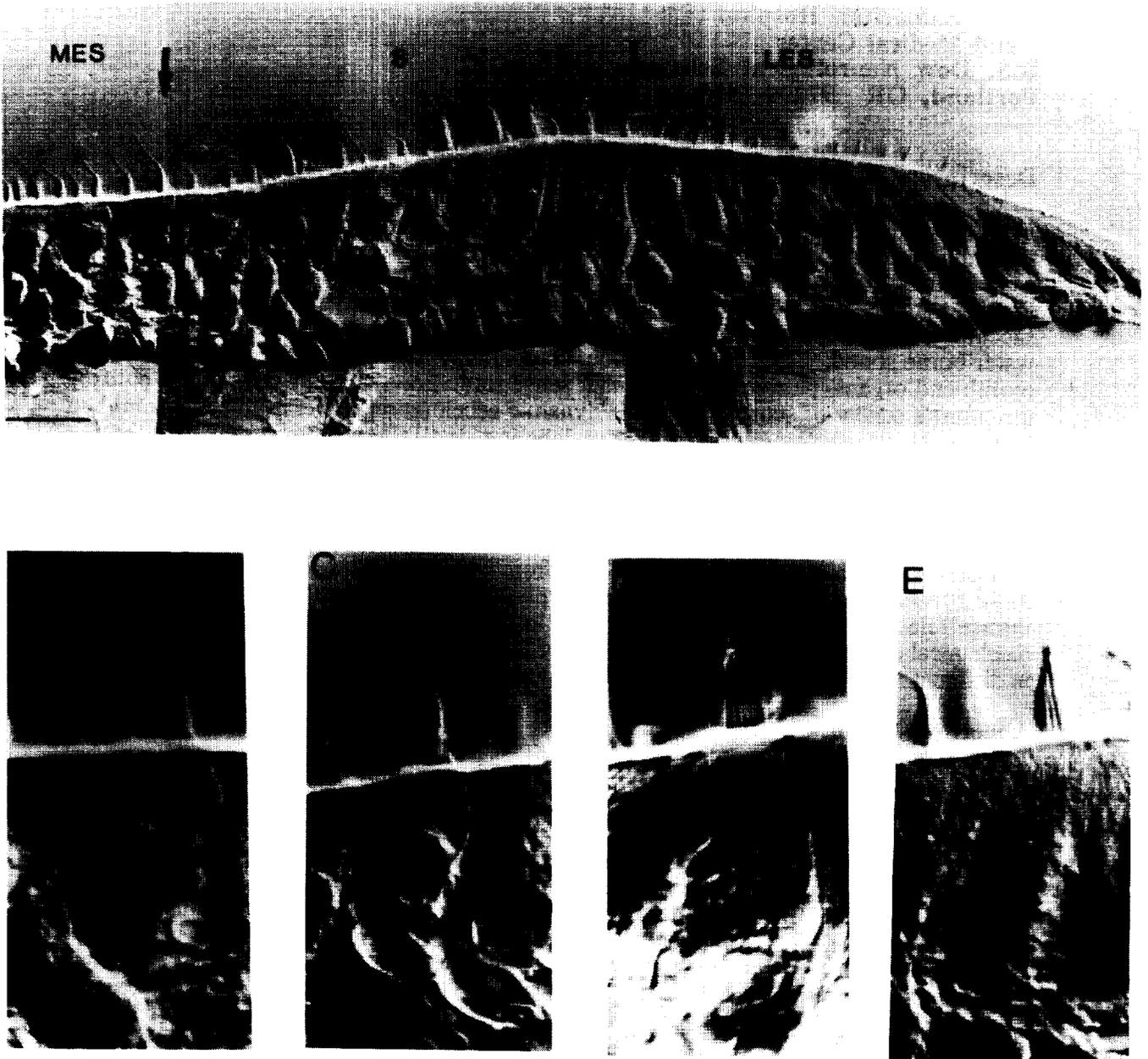


Figure 1. Photomicrographs of toluidine blue-stained cross-section of the utricular macula (top) and individual utricular hair cell types in the extrastriolar and striolar regions (bottom). Top: Outer arrows denote the medial (left) and lateral (right) borders of the striolar region; middle arrow marks the line dividing hair cells with opposing polarities. MES, medial extrastriola; S, striola; LES, lateral extrastriola. Bar = 25 μ m. Bottom: (B) Type B cells have small apical surfaces and short stereocilia, with kinocilia 3-4x as long as their longest stereocilia. (C) Type C hair cells resemble enlarged versions of type B hair cells. (F) Type F hair cells have kinocilia equal in length to their longest stereocilia. (E) Type E hair cells have bulbed kinocilia.

ANIMAL PROJECTS

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COMPARATIVE TRANSDUCTION MECHANISMS OF HAIR CELLS IN THE BULLFROG UTRICULUS

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Description of Research

The goal of our research is to understand the morphological and functional organization of the gravity-sensing end organs of the vertebrate inner ear. During the past year, we have examined the comparative transduction mechanisms of hair cells in the bullfrog utriculus. Previous studies of transduction in vestibular hair cells have largely been confined to the sacculus of amphibians and fish, sensors of high-frequency substrate-borne vibration and water-borne sound. Little is known about comparative transduction mechanisms in other otolith end organs, particularly those that sense static gravity. Hair cells in the bullfrog utriculus, a sensor of static gravity and dynamic acceleration, differ dramatically in their hair bundle morphology. Moreover, dye-labeling studies have shown that the response dynamics of utricular afferents are correlated with the macular location and hair bundle morphology of their innervated hair cells, implying that utricular hair cells exhibit regional variations in their transduction mechanisms.

To test this hypothesis, we selected hair cells from wholemount utricular maculae according to their macular location and hair bundle morphology. We then studied, with conventional intracellular recordings, the responses of selected hair cells to intracellular current and mechanical displacement. The primary aim of these studies was to see if utricular hair cells in different macular zones differed in their physiological response properties.

Accomplishments

(1) *Morphological properties of utricular hair cells.* Hair cells in the bullfrog utriculus differed dramatically in hair bundle morphology (Figure 1). Extrastriolar hair cells had small apical surfaces and short stereocilia, with kinocilia 3-4x as long as their longest stereocilia (type B). A small number of type B cells were also found throughout the striola region. The majority of striolar hair cells had larger apical surfaces and displayed a variety of hair bundle morphologies, with kinocilia and stereocilia markedly longer than those of type B hair cells. Hair cells on the outermost rows of the striola (type C) resembled enlarged versions of type B hair cells. Moving inward, these hair cells were gradually replaced by hair cells with kinocilia equal in length to their longest stereocilia (type F). Hair cells in the innermost striolar rows had bulbed kinocilia (type E).

(2) *Responses of utricular hair cells to intracellular current.* Utricular hair cells had similar resting potentials but differed in their steady-state current-voltage (I-V) relationships. Type C hair cells had low input resistances and linear I-V relations. The I-V relations of other hair cells were outwardly rectifying for depolarizing voltages. The I-V relations of utricular hair cells had no detectable "N-shape" at these voltages, suggesting that Ca^{2+} -activated potassium currents provide, at most, a small contribution to the membrane current of these cells. In type E hair cells, inward (anomalous) rectification was observed for hyperpolarizing voltages.

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HAIR CELLS OF THE BULLFROG UTRICULUS

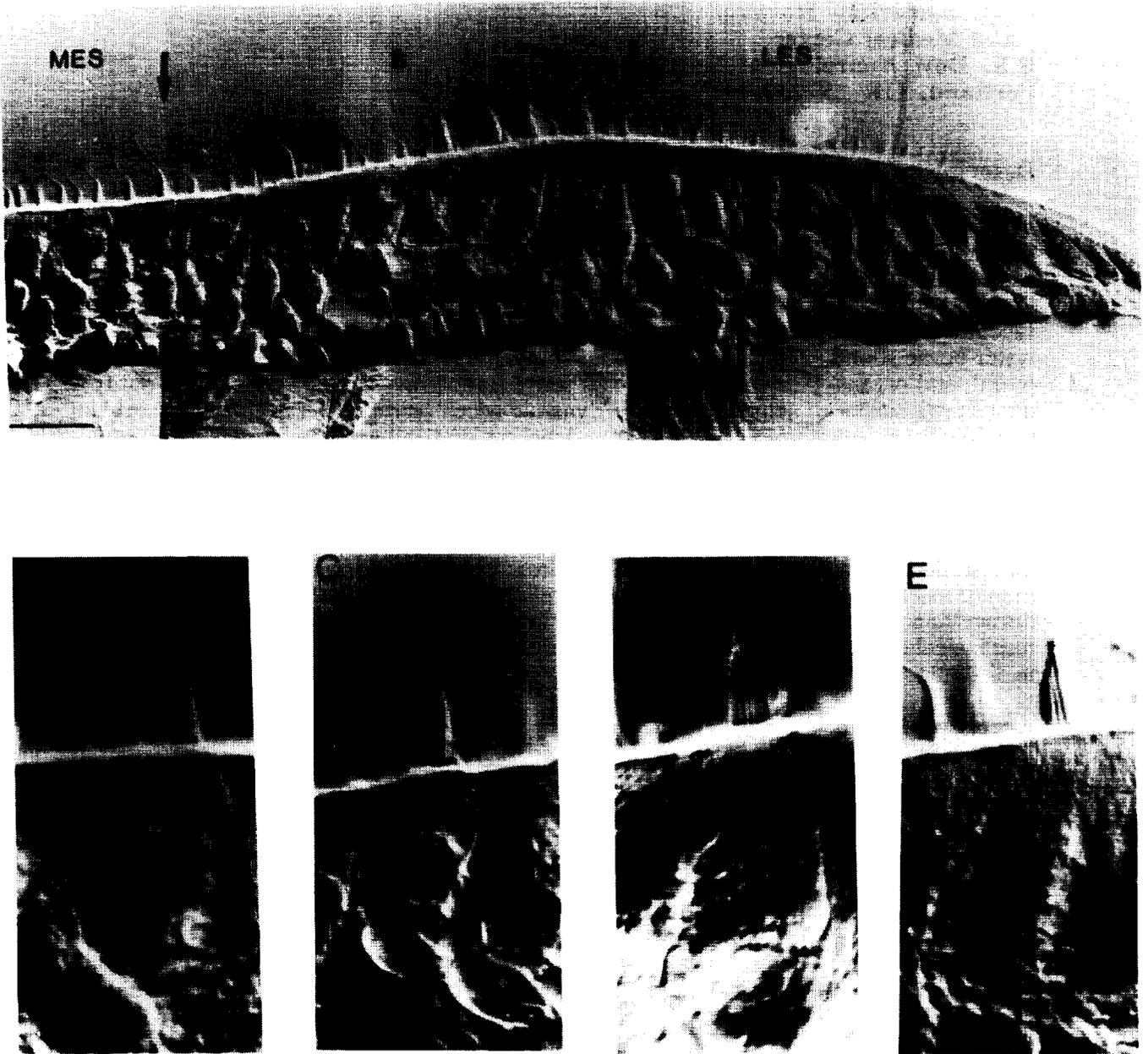


Figure 1. Photomicrographs of toluidine blue-stained cross-section of the utricular macula (top) and individual utricular hair cell types in the extrastriolar and striolar regions (bottom). Top: Outer arrows denote the medial (left) and lateral (right) borders of the striolar region; middle arrow marks the line dividing hair cells with opposing polarities. MES, medial extrastriola; S, striola; LES, lateral extrastriola. Bar = 25 μ m. Bottom: (B) Type B cells have small apical surfaces and short stereocilia, with kinocilia 3-4x as long as their longest stereocilia. (C) Type C hair cells resemble enlarged versions of type B hair cells. (F) Type F hair cells have kinocilia equal in length to their longest stereocilia. (E) Type E hair cells have bulbed kinocilia.

There were marked differences in the voltage responses of hair cells to intracellular current steps. In type B and type C hair cells, depolarizing and hyperpolarizing current steps produced merely a passive exponential change in membrane potential. In contrast, active potential changes were seen in types E and F hair cells within 25-75 ms following the onset of depolarizing and the cessation of hyperpolarizing current steps. Type E hair cells also exhibited an electrical resonance, depolarizing current producing small oscillatory potential changes from resting or hyperpolarized potentials. These oscillatory changes were smaller and more highly damped than those seen in saccular hair cells. The oscillations were not symmetric, as ringing was not seen at the termination of depolarizing currents. The amplitude and frequency of these oscillations increased with current amplitude.

(3) *Responses to mechanical displacement.* Utricular hair cells, when stimulated at the distal tips of their tallest stereocilia, differed in their sensitivity to mechanical stimulation. Type B hair cells, in both the striolar and extrastriolar regions, had markedly lower mechanical sensitivities than other hair cell types. These differences were not observed after mechanical sensitivity was normalized for input resistance and mean differences in kinociliary length.

Utricular hair cells, with the exception of type E cells, displayed low-pass filter characteristics in response to hair bundle displacement. Type B hair cells, in both the striolar and extrastriolar regions, had first order response dynamics and very low (< 1-5 Hz) bandwidths. Type C cells on the striolar border had second order response dynamics and much higher (35-50 Hz) bandwidths. These cells also had varying degrees of adaptation at low frequencies, exhibiting gain reductions and phase leads from 20° to 40° for frequencies less than 1 Hz. Type F hair cells had first order response dynamics and displayed little or no adaptation. Type E hair cells, unlike other utricular hair cells, had tuned filter characteristics, displaying highly damped resonances around 1-10 Hz at their resting membrane potential.

The low-frequency response dynamics of hair cells to mechanical stimulation were further examined with mechanical steps. Type B hair cells had slow, non-adapting responses to long-duration step displacements. The step responses of type C hair cells in the outer striola, on the other hand, adapted to near baseline levels within 200 ms. Type C hair cells located further inside the striola adapted more slowly and less completely. Types E and F hair cells had non-adapting step responses with rapid onsets and offsets.

The response dynamics of hair cells to mechanical stimulation differed in at least two ways from responses to intracellular current. First, the bandwidths of hair cells to mechanical stimulation, because of increased transducer conductance, were lower than those for intracellular current. Second, hair cells that adapted to mechanical stimulation did not adapt to intracellular current, suggesting that low-frequency differences between hair cells were determined by the nature of the adaptation process associated with transduction and not by voltage-sensitive conductances in the basolateral membrane.

(4) *Enzymatic dissociation of utricular hair cells.* Utricular hair cells were isolated by briefly immersing utricular maculae in collagenase followed by a longer incubation in papain and L-cysteine. Following enzyme treatment, end organs were mechanically triturated, depositing isolated hair cells to small glass-bottomed chambers. Isolated hair cells were observed to retain their cell body and hair bundle morphology.

(5) *Lectin receptors of utricular hair cells.* We used a battery of lectins with different carbohydrate specificities to see if utricular hair cells in different macular zones had distinctive lectin binding patterns. Some lectins, such as *UEA*, which binds to fucose residues, did not label any cells in the utricular macula. Other lectins labeled

complete cellular populations. For example, *CON A*, which binds mannose and glucose residues, and *RCA-I*, which binds galactose residues, labeled the hair bundles of all utricular hair cells. Still other lectins labeled subsets of utricular hair cells. *WGA*, which binds N-acetylglucosamine, labeled the hair bundles of extrastricular, but not striolar, hair cells.

Lectins were also applied to hair cells isolated from particular macular regions. *WGA* and *VVA*, as in wholemount material, labeled the apical surfaces and hair bundles of extrastricular, but not striolar, hair cells. We conclude that differences in lectin binding patterns can be used to infer the epithelial origin of hair cells isolated from the utricular macula.

Significance of the Accomplishments

The results of our studies have shown how specific classes of utricular hair cells are organized to extract static and dynamic information and have clarified the contribution of specific transduction mechanisms to the response properties of utricular nerve afferents. In particular, hair cells from different macular zones differ in their passive and active membrane conductances. Hair cells in different regions also differ in their sensitivity and response dynamics, particularly in the rate and extent of their adaptation to low-frequency mechanical stimulation. In future experiments we will examine in isolated hair cells the cellular mechanisms responsible for these regional variations. We have already demonstrated our ability to isolate and recognize individual hair cell types. In addition, we have demonstrated that the macular origin of isolated utricular hair cells can be inferred from their hair bundle morphology and lectin binding patterns.

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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 the vitamin D metabolites, parathyroid hormone, glucocorticoid hormones and growth hormone, 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 (IGF-1), transforming growth factors, and fibroblast growth factor 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.

Work conducted during the last year continued to focus on the roles of IGF-1 and $1,25(\text{OH})_2\text{D}$ in the inhibition of bone formation induced by skeletal unloading. cDNA probes for IGF-1/2, the receptors for IGF-1/2, and collagen were developed and used to measure changes in message levels during unloading. The earlier reported decrease in serum $1,25(\text{OH})_2\text{D}$ induced by unloading was further studied using kinetic analysis.

Accomplishments

(1) Demonstrated that $1,25(\text{OH})_2\text{D}$ has different effects on trabecular and cortical bone.

(2) *Demonstrated an (unexpected) increase in mRNA for IGF-1 in epiphyseal growth plates during the first 5 days of unloading. Message levels then return to normal by day 14.*

(3) *Demonstrated a decrease in mRNA for collagen during unloading.*

(4) *Demonstrated that the fall in serum $1,25(\text{OH})_2\text{D}$ associated with unloading is a consequence of changes in both production and metabolic clearance of the hormone.*

Significance of the Accomplishments

Preliminary evidence indicates that the concentration of IGF-1 in bone decreases during unloading, which may be the underlying cause of the decrease in bone formation in

unweighted animals. The availability of molecular probes for IGF-1/2 and their receptors will now permit us to assess whether the decrease in IGF in bone is a consequence of changes in message levels, as well as to examine the possibility that decreased bone sensitivity to IGF also contributes to the impairment of bone formation. The initial observation that mRNA for IGF increases during unloading is unexpected and must be confirmed. If message levels do indeed increase, it suggests that the decrease in IGF in the bone during unloading is not a consequence of decreased mRNA and other mechanisms must be sought. The decrease in mRNA for collagen confirms that bone formation is reduced during unloading.

The serum concentration of 1,25(OH)₂D decreases during skeletal unloading, a finding which has now been confirmed by other laboratories. Until our most recent experiments, however, the mechanism for this change was not known. Our data indicate that changes in both production and metabolic clearance of 1,25(OH)₂D are responsible. Whether these changes reflect the slight and sometimes insignificant rise in serum calcium induced by unloading (production of 1,25(OH)₂D) and/or cephalad shift of body fluids (clearance of 1,25(OH)₂D) needs to be investigated.

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

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Description of Research

This research has the long-term objective of examining the effects of microgravity on the physiology of the adult and developing mammalian gravity receptors. The research outlines ground-based studies to examine (1) the physiologic responses of otolith afferents in the adult rat and during postnatal development, and (2) the otolith organ contribution to the vertical vestibulo-ocular (VOR) and postural reflexes.

These experiments will provide important new information on the physiology of rat gravity receptors during development and in the adult. Otoconial demineralization may be associated with short- and long-term exposure to microgravity. Demineralization produces an unloading of the otolithic membrane and hair cells and may produce adaptive plasticity of the otolith-ocular and otolith-spinal reflexes. Therefore, experiments have examined 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 mature labyrinth.

Accomplishments

(1) *Analysis of otolith-dependent postural reflexes.* Behavioral experiments were conducted in Long-Evans rats to examine the early postnatal development of the antigravity postural reflexes including surface righting, negative geotaxis, swimming, head elevation and air righting. These reflexes were examined daily from postnatal day 1 (P1) to P28 in control animals and two experimental groups: (1) animals hemilabyrinthectomized on P4 or as adults, and (2) animals with defective (demineralized) otoconia produced by manganese-deficient diet. Female rats were placed on the Mn-deficient diet (Mn < 2.0 ppm) 3 wks prior to breeding. They were then mated and kept on this diet through embryonic day 20 (E20) before being returned to a control diet containing normal levels of manganese (45 ppm).

Early labyrinthectomy or Mn-deficiency produces a minimal effect on the time of onset of the antigravity reflexes, but produces major effects on response symmetry. Right-sided hemilabyrinthectomy caused animals to turn consistently away from the side of the lesion in surface and air righting and to roll in the same direction while swimming, i.e., lesion side up while on the back, or lesion side down in the prone position. The time course for compensation differed slightly between tests, but was generally complete within 1 wk after onset of the behavior. Mn-deficient animals could be divided into those showing no response asymmetry and those with labyrinthine deficits on the right or left. The response asymmetry in affected animals was variable in magnitude from animal to animal but in most cases was as severe as in the hemilabyrinthectomized group. Additionally, compensation in the Mn-deficient animals

occurred 4-5 days earlier than hemilabyrinthectomy in some tests (surface righting, geotaxis), at the same time (air righting), or in the case of swimming, several days later.

Results indicate that *the gravity receptors and vestibulospinal output are active at birth and exert powerful control over the antigravity postural reflexes.* Experiments with early hemilabyrinthectomy or Mn-deficient animals suggest that *adaptive mechanisms also develop during the first few postnatal days and are successful in completely reversing behavioral response asymmetry within 5-7 days.* These data, along with biochemical, physiological and morphological studies, will help to provide behavioral controls to determine the effects of microgravity on the developing and mature gravity receptors.

(2) *Physiology of otolith afferents in otoconial-demineralized rat.* Physiological experiments were conducted with adult rats that had been subjected to otoconial demineralization *in utero* produced by Mn-deficiency. These experiments were conducted at the University of Nebraska, Department of Dentistry, in collaboration with Dr. Tim Jones. Adult animals were anesthetized and surgically outfitted with a head holder secured to the parietal and bones. A recording electrode was then placed into the middle ear cavity and an indifferent electrode was placed on the vertex of the skull. Animals were then subjected to pulsed linear cranial acceleration along the x-axis (anterior-posterior axis). Far-field vestibular potentials were recorded, differentially between the vertex electrode and those placed in each labyrinth. Traditional signal averaging techniques were used to resolve responses. A series of pulsed linear acceleration protocols were delivered using intensities of 0.0312 - 1.0 g in 6 db steps in Mn-deficient animals and in two groups of controls. The first control group consisted of normal adult rats, and the second group consisted of control rats that were additionally subjected to chemical labyrinthectomy produced by injection of tetrodotoxin (TTX) into the middle ear of first one and then the other labyrinth. The entire stimulus protocol was repeated after each TTX treatment to determine the contribution of each labyrinth to the far-field potential, and after dispatching the animal to determine the contribution of mechanical artifact to the recordings.

At moderate intensities, the responses in control rats consisted of a series of 4-7 dominant peaks occurring within a period of 8 ms, having amplitudes of 0.5-15 μv peak-to-peak. The threshold of the responses ranged from 0.04-0.06 g. Latencies and amplitudes varied systematically as a function of stimulus intensity and met all criteria for biological signal (i.e., responses did not invert with stimulus inversion, declined with distance from the middle ear recording site, and disappeared with complete bilateral destruction of the labyrinth or upon death of the animal).

The Mn-deficient animals showed a clear departure from the responses in controls. First, the amplitudes of the first positive wave (P1), thought to be related to synaptic and action currents generated in the vestibular nerve, and all subsequent waveforms were reduced in peak-to-peak amplitude. Second, the Mn-deficient animals showed an approximate ten-fold elevation in thresholds (0.3-0.5 g) compared to controls (0.04-0.06 g). Third, the slope of the stimulus-response curve was steeper in treated animals, i.e., once the otolith receptors in the treated animals are activated they respond nonlinearly. Fourth, the vestibular responses in the Mn-deficient animals were very asymmetrical. Generally one ear was more severely affected than the other as judged by 1.5- to 2.2-fold differences in threshold for P1 (see below) between right and left labyrinths. Interestingly, there were also differences in the threshold and amplitude of P1 between the two labyrinths in controls, but these differences were considerably smaller. Fifth, the reduction in amplitude and threshold of the P1 potential was closely correlated with the severity of postural deficits (swimming, air righting, surface righting, and geotaxis) in the same animal. The

importance of the latter is that the postural deficits noted above can now be attributed to a disruption of the otolith organs, presumably due to loss of otolith mass.

Finally, the TTX treatment in controls and Mn-deficient animals were important in determining the source of the potentials. TTX blocks the voltage-dependent sodium channels involved in activation of the vestibular nerve. The potentials are abolished after treatment with TTX into both ears, establishing that they are biological in nature and related to activation of the VIIIth nerve. Unilateral treatment abolishes P1 and part of N1 and subsequent waves, indicating that *the P1 is derived entirely from activation of the ipsilateral VIIIth nerve whereas subsequent waves receive contributions from both sides of the head.*

Significance of the Accomplishments

In summary, research accomplishments this year included completing our analysis of otolith-dependent postural reflexes. In addition, we made major progress in analysis of the physiology of otolith afferents in Mn-deficient animals. The temporal bones from these Mn-deficient animals are now being processed to assess anatomically the loss of otoconia, so that we can eventually correlate anatomical findings with behavior and physiology. However, the Mn-deficient animals show remarkable abilities to adapt to significant loss of macular function. Our results indicate that *the behavior of animals with severely disrupted gravity receptor function*, as judged from their performance during pulsed linear acceleration, *is rapidly compensated* with a time course similar to that of hemilabyrinthectomy. *A significant factor in compensation is the animal's ability to see*, as evidenced by a correction in postural asymmetry at a developmental time when the eyes open. A knowledge of these developmental changes in gravity receptor function and the adaptation of the animal to loss of otoconial mass will be important in assessing the impact of microgravity on mammalian organisms in space.

BASIC GRAVITATIONAL REFLEXES IN THE LARVAL FROG

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Description of Research

Little is known about how vertebrates are able to sense gravity and how they process this information to generate appropriate motor responses. This investigation is designed to determine how a primitive vertebrate, the bullfrog tadpole, is able to sense and process gravitational stimuli. Because of the phylogenetic similarities in the vestibular systems in all vertebrates, the understanding of the gravitational reflexes in this relatively simple vertebrate should elucidate a skeletal framework on an elementary level, upon which the more elaborate reflexes of higher vertebrates may be constructed.

The tadpole manifests a powerful counter-rolling response of the eyes to static tilt of the head. In addition, the fact that amphibians are cold blooded means that their cellular metabolism is much lower than that of mammals. Consequently, the entire head can be maintained *in vitro* with the brain, sensory structures, and eye muscles exposed. The strong gravitational reflexes are still evident under these conditions and persist for several days. Such conditions permit detailed and reproducible electrophysiological and anatomical investigations of this reflexive behavior.

The focus of the experiments is threefold: (1) to understand from recordings of action potentials in the whole trochlear nerve how static and dynamic gravitational stimuli are processed by the nervous system; (2) to localize neuronal centers responsible for this processing through reversible, iontophoretic (i.e., ejection from a microelectrode) pharmacological ablation of these centers, while maintaining trochlear nerve recordings; and (3) to record intracellularly from individual neurons within these centers in order to define the single neuron's role in the overall processing of the center. This study will provide information on the mechanisms by which a primitive vertebrate, one with powerful gravitational reflexes, processes these reflexes.

Accomplishments

Quantification of the behavior. It was found that a suction electrode placed around the trochlear nerve is capable of detecting action potentials in the nerve directed towards controlling the superior oblique eye muscle. These action potentials are digitized for several minutes at a sampling rate of 50,000 points/sec. A computer algorithm written by the principal investigator is able to then detect and quantify these potentials in terms of frequency per unit time and in terms of shape parameters (such as amplitude). Such a system allows for the quantification of hundreds of thousands of such potentials over several minutes, thereby characterizing the motor output from the central nervous system to the muscle.

Responses to static tilt of the head. Typically, if the head is tilted nose up, trochlear activity increases and, correspondingly, if the head is tilted nose down, trochlear activity decreases. Units display both a phasic component during the tilt and a static component following the tilt. Larger amplitude units have a large phasic component, while smaller units have more of a static component.

Surgical lesions. Surgical lesions, conducted *during* the trochlear unit recordings, indicate that ***the source of the tilt sensitivity of the trochlear units is the inner ear***, since sectioning of the optic nerves and/or spinal cord does not abolish tilt sensitivity, while bilateral VIIIth nerve section eliminates all tilt sensitivity.

Reversible pharmacological ablation. Initial investigations attempting to localize further the neuronal components to this reflex have involved iontophoretic injections of synaptic antagonists into candidate brain regions and then assessing what blocking synaptic transmission in these regions does to the trochlear responses. ***Kynurenic acid (a glutamate synaptic receptor antagonist), iontophored into the trochlear nucleus, completely silences the motoneurons during the period of deposition. Following the cessation of the iontophoresis, motoneuronic activity returns to control values***, demonstrating the reversibility of these pharmacological ablations. Similar preliminary experiments have been attempted with success in the regions where the second order vestibular neurons are located.

Significance of the Accomplishments

In general, the above accomplishments provide a step toward the understanding of how these primitive vertebrates sense gravitational stimuli and how they process these stimuli and integrate them into reflex control. Furthermore, these first experiments demonstrate the feasibility of the entire project. The ability to rigorously quantitate the behavior through the trochlear nerve recordings provides information as to how the brain controls the eye muscles. The ability to tilt the head while maintaining the trochlear nerve recordings enables the characterization of the gravitationo-ocular reflex. That surgical lesions can be performed while maintaining trochlear recordings allows for the definition of brain areas involved in the reflex. The reversible pharmacological lesions can better pinpoint these regions and help determine what aspects of the reflex these regions control.

From these experiments, a skeletal framework of how the bullfrog tadpole senses and processes gravitational stimuli can begin to be assembled. Structures in the inner ear sense gravity. The central nervous system then passes this information via the vestibular nuclei onto the extraocular motoneurons. How this information is processed and the relevant contributions of the nuclei remain to be determined.

Accumulation and assimilation of this material should present a basic picture of how this reflex is organized in the larval amphibian. Such information can then provide a background for further investigations as to how the reflex organization changes as the aquatic, gravitationo-ocular reflexes present in the tadpole become modified to suit the terrestrial, gravitationo-postural reflexes that predominate in the adult, and also as to how such reflexes and their modifications may be altered in a microgravity environment.

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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 reduction of mechanical stress on weight-bearing bones results in a reduction in new bone formation. In previous studies of this effect, we compared results obtained following spaceflight to results from animals subjected to non-weightbearing on Earth (simulated weightlessness by the hindlimb unloading technique of Holton). The cells responsible for bone formation appear to be affected by both of these events. We have shown a reduction in bone cell activity by combining techniques of histochemistry, electron microscopy, and immunocytochemistry. We have described an additional response resulting from spaceflight in which the blood vessels within the weightbearing bones showed damage to the vascular lining cells and some vascular occlusion.

These changes at the cellular level are difficult to relate to the changes in whole animal physiology because (a) the cellular changes are difficult to quantitate and (b) these changes are variable depending on the anatomy and mechanical stress history of the bone under study. Recently, we have combined histochemistry and morphometric measurements to better understand, on a quantitative basis, the cellular responses to spaceflight and non-weightbearing.

Accomplishments

(1) We combined the morphometric determination of bone forming cells (i.e., osteoblasts) along the endosteal surface of compact bone with the histochemical determination of active Golgi synthetic activity (thymine pyrophosphatase activity). *Results from Cosmos 2044 (a 14-day spaceflight) showed that the vivarium and synchronous controls had 76.9% and 79.4% of their bone surface covered with cells containing an active Golgi region. A similar measurement on the spaceflight animals showed that only 52.2% of their surface showed similarly active bone forming cells.* However, the large standard deviation in this group prevented the measurement from showing a high significance. But this method does provide, for the first time, a means of comparing bone cell activity between animal groups and between identical anatomical sites within bone.

(2) Rats flown on 14-day spaceflights aboard the Soviet biosatellites Cosmos 1887 and Cosmos 2044 were studied by electron microscopy to determine the degree of vascular injury that occurred within the tibia. The damage described for Cosmos 1887 was much more severe than that found in Cosmos 2044. However, histochemistry and electron microscopy were combined to show that *the cells associated with the blood vessels in bone and the adjacent osteocytes contained cellular damage as a result of these flights.*

(3) We have combined histochemistry and detection of the resulting reaction product to individual cells using scanning electron microscopy coupled with a backscattered electron detector. Thus, relative metabolic activities of cells and tissues can be determined.

For example, it has been shown, using this new technique, that alkaline phosphatase activity of blood vessels in bone is reduced by as much as 300% as a result of spaceflight.

(4) The technical problems associated with the immunocytochemical localization of osteocalcin in bone cells have been solved. The localization of the Golgi complex of osteoblasts can be used as an important indicator of cellular differentiation and activity.

Significance of the Accomplishments

The reduction in new bone formation that occurs during spaceflight is an acute reproduction of the chronic disease of osteoporosis and the decrease of bone formation seen during skeletal aging. However, spaceflight seems to have specific effects on the weightbearing bones. We have shown that the compact bone of the tibia contains bone forming cells with reduced activity (Finding #1) and that the vascular supply in bone at these same anatomical sites is most affected by spaceflight (Finding #2). In fact, the cells associated with these particular blood vessels in bone often show degenerative changes as a result of the hypogravity environment. By combining several different techniques (Finding #3), we have determined relative cellular activities within small specific volumes of bone that are anatomically defined. This permits us to localize and quantitate bone cell activity using a variety of histochemical and immunocytochemical methods (Finding #4).

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EFFECT OF ALTERED G ON CHONDROCYTE DIFFERENTIATION

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Description of Research

The well-documented response of the skeleton system to μg includes the altered differentiation of bone, cartilage, and tendon, and the accompanying changes in matrix organization. Our laboratory projects address the question of pre- and postnatal skeletal development, with emphasis on cartilage formation and endochondral ossification.

Systems used in these studies include whole animal centrifugation and unloading as well as cell and organ culture systems designed to be used in spaceflight or at 1 g (centrifuges, clinostats, bioreactors). Cartilaginous growth plates from spaceflown rats (shuttle, Cosmos) and chickens are also being analyzed.

Accomplishments

Cell culture systems. (1) A Hardware Verification Test using preflight procedures and inflight operations was successfully completed for the first flight of cultured skeletal cells — the CELLS spaceflight project to be flown on International Microgravity Laboratory 1. This experiment will have the most extensive medium changes and fixations ever carried out in space. (2) Embryonic limb mesenchymal cells on various bead types were shown by light and electron microscopy to differentiate into cartilage cells. (3) *Pleurochrysis carterae*, a calcifying algae, was grown in culture bags.

Centrifugation, in vitro. (4) *No sex ratio difference was found in offspring of male "pilot" mice exposed to acute acceleration.* These mice were exposed to g forces similar to those experienced by fighter pilots. (5) *Significantly less Epidermal Growth Factor (EGF) was found in salivary glands from centrifuged male and female mice when compared to the salivary glands from controls.* This study was carried out in collaboration with Dr. Durban at the Dental Branch. (6) *In a countermeasure study using hindlimb unloading and centrifugation, growth plates from rats with osseous tibial defect did not exhibit normal plate thinning during the experiment.*

Spaceflight results. (7) *Increased vascularization (i.e., more open columns and more capillary sprouts) was found in growth plates of rats exposed to 7 days of weightlessness aboard Spacelab 3.* (8) Immunohistochemical studies were carried out on matrix proteoglycan of bones from the "Chix in Space" experiment, which was carried on STS-29 in March 1989.

Significance of the Accomplishments

Finding #1: The cell culture unit developed for this study has a gas permeable membrane, a deflector ring to control fluid forces, and a method of changing medium and adding fixative. Because it can now be cheaply manufactured, it should become easily available to any interested users.

Finding #2: Chondrocytes grown on beads can now be sent into space, which will allow us to determine which type of clinostat simulates μg best, and also to determine if effects on attached cells are different from those on beads. This should have some bearing on the question of direct vs. indirect effects of microgravity on cells.

Finding #3: Calcification and morphogenesis may be affected in this algae in the same manner in which they are affected in the growth plate. This would allow us to look for interspecies similarities, to determine effects on single cells, and to separate direct and indirect effects of μg exposure.

Finding #4: Of particular interest to the Air Force, this study also has implications for any situation involving acute centrifugation — for example, use of centrifugation as a countermeasure.

Finding #5: This is the first investigation of possible changes in growth factors in centrifuged animals. Lack of growth factors could lead to the observed problems with nursing behavior in centrifuged rats.

Finding #6: Previous studies showed that certain g forces and time may be beneficial to the maintenance of growth plate function, but that other g levels may be counterproductive. No significant differences were found in this study between cut hindlimb unloaded tibias exposed to 1.2 g centrifugation and the non-centrifuged cut controls. However, the normal reduction in growth plate height due to age was not seen in (loaded) cut controls, indicating an effect of healing (growth factors?) on the plate.

Finding #7: This finding is very important because it is one of very few studies of normal growth plate vascularization and the first using spaceflight tissue. Dr. Stephen Doty has previously noted some vascularity problems in osseous tissue.

Publications

Duke, J. and Little, B. 1990. Sex ratio in offspring of "pilot" mice (Abstract). *ASGSB Bulletin* 4(1): 25. (GWU 13352)

Duke, J. and Montufar-Solis, D. 1990. A preliminary report on histomorphometry of tibial epiphyseal plates from Cosmos 2044 rats (Abstract). *ASGSB Bulletin* 4(1): 35. (GWU 13354)

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Duke, P.J., Arizpe, J., and Montufar-Solis, D. 1991. Cartilage formation in the CELLS "double bubble" hardware. *Physiologist* 34(1, Suppl.): S76-S77. (GWU 12532)

Duke, P.J., Durnova, G., and Montufar-Solis, D. 1990. Altered cartilage differentiation in tibial epiphyseal plates of Cosmos rats. In: *Proceedings of the Fourth European Symposium on Life Sciences Research in Space*, Trieste, Italy, May 28 - June 1, 1990, p. 269-273. (ESA SP-307) (GWU 13145)

Montufar-Solis, D. and Duke, P.J. 1991. Effect of strain, diet and housing on rat growth plates: A Cosmos '87 - Spacelab-3 comparison. *Physiologist* 34(1, Suppl.): S183-S184. (GWU 13291)

Accomplishments

As part of this study, we previously demonstrated that the maturation of inner ear gravity receptors involves gradual ultrastructural refinements of both sensory (neurons and hair cells) and non-sensory elements (supporting cells, cartilage, and effector masses overlaying end organs). It is now known that *the amount of MAP and NF changes in developing VIIIth neurons before, during, and after synaptic genesis*. Since MAP and NF are essential for transporting nutrients and organelles and for determining axonal diameter, MAP and NF may very well be involved in the establishment of connections between hair cells and neurons. These connections convey information from the inner ear to the brain.

Staining of axon and soma MAP5 and NF200 proteins indicated that neurofilaments and microtubules seem to be differentially expressed in the different embryonic stages (Figure 2[1-8] and in the VIIIth nerve axons and

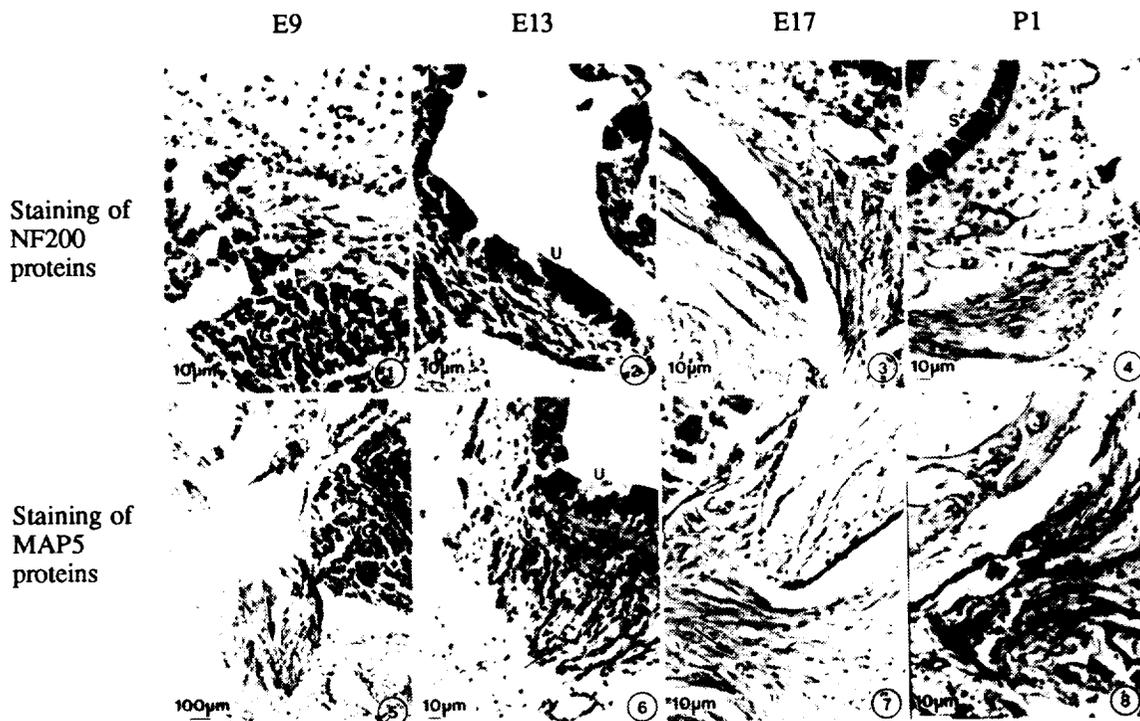


Figure 2. Black and white micrographs of color (red for positive and blue for counter-stain) reaction product using monoclonal antibodies against antigenetic determinants for MAP5 and NF200. Axons of four embryonic ages (E9, E13, E17, and P1) were processed in the same slide and at the same time under identical conditions to reduce preparatory artifacts. In plates 1-4 the relative concentration of NF200 is shown. Note the paucity of stain between E13-E17. Contrary to this pattern, MAP5 was more concentrated on E9 material and less in P1 (plates 5-8). Just as it is difficult to assess differences in protein concentration in these pictures with the naked eye, it is also difficult to analyze differences without color thresholding. Thus, development and application of technology used to assess these differences is an integral part of the investigation being conducted. Without this technology the investigator would have to base evaluation on subjective interpretations which carry no scientific validity for statistical comparisons.

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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 the vestibular epithelia of the utricle and saccule of the chick (*Gallus domesticus*) inner ear in order to: (1) delineate modifications that are known to change from the time that synapses form until they mature, and (2) investigate the relationship that may exist between refinements of cells and nerves, as well as changes occurring during the formation of effector masses overlaying vestibular epithelia.

Modified gravity induces changes in gravity sensors of the inner ear during embryonic development. Such modifications range from alterations of vestibular brain stem responses to ultrastructural modifications of cells and tissues. At each level, morphological and biochemical manifestations of the changes can be studied at critical periods of development, and the significance of the changes can be characterized. To identify changes that may occur in spaceflight, normal values from Earth-based experiments are needed.

Recent work published by other investigators demonstrates that microtubule associated proteins (MAP) and neurofilament proteins (NF) are essential for the survival and functioning of immature as well as mature neurons. Differential distribution of microtubules and neurofilaments at critical periods of development before, during, and after synapses are formed may contribute to maturation of connections, with profound effects on the onset of vestibular function (Figure 1). For this portion of the investigation two molecules known to be intimately associated with the transport of organelles from the neuronal body (soma) toward the growing ends of its fibers (axons and dendrites) were demonstrated, with highly sensitive alkaline phosphatase immunohistochemical staining, to be present in chick embryo gravitational organs. Results from this immunohistological work, from previous electron microscopy investigations, and from histological examination of embryonic chick inner ear will allow this investigator to define the important events that may be altered in environments with modified gravity, such as those encountered in spaceflights.

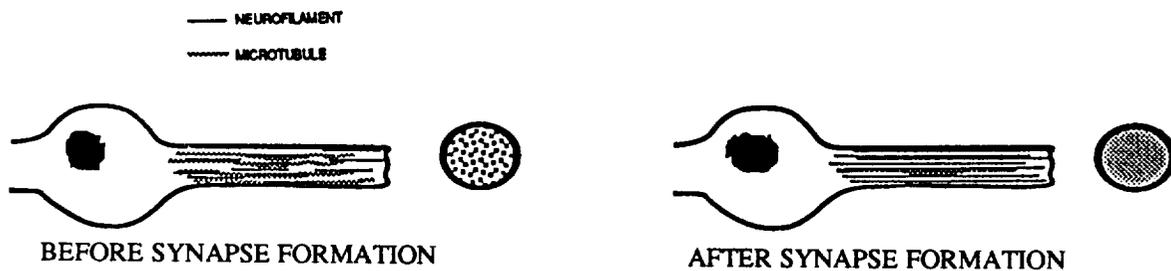


Figure 1. The ultrastructural distribution of microtubules and neurofilaments as seen at the electron microscopic level without immunohistochemical processing in soma and axons of the VIIIth nerve. Two neurons are shown with relative number and distribution of microtubules and neurofilaments before (left) and after (right) synapses are formed. A cross section of the axon is shown to the right of each neuron. Note that neurofilaments seem more abundant at the later stage of development.

Accomplishments

As part of this study, we previously demonstrated that the maturation of inner ear gravity receptors involves gradual ultrastructural refinements of both sensory (neurons and hair cells) and non-sensory elements (supporting cells, cartilage, and effector masses overlaying end organs). It is now known that *the amount of MAP and NF changes in developing VIIIth neurons before, during, and after synaptic genesis*. Since MAP and NF are essential for transporting nutrients and organelles and for determining axonal diameter, MAP and NF may very well be involved in the establishment of connections between hair cells and neurons. These connections convey information from the inner ear to the brain.

Staining of axon and soma MAP5 and NF200 proteins indicated that neurofilaments and microtubules seem to be differentially expressed in the different embryonic stages (Figure 2[1-8] and in the VIIIth nerve axons and

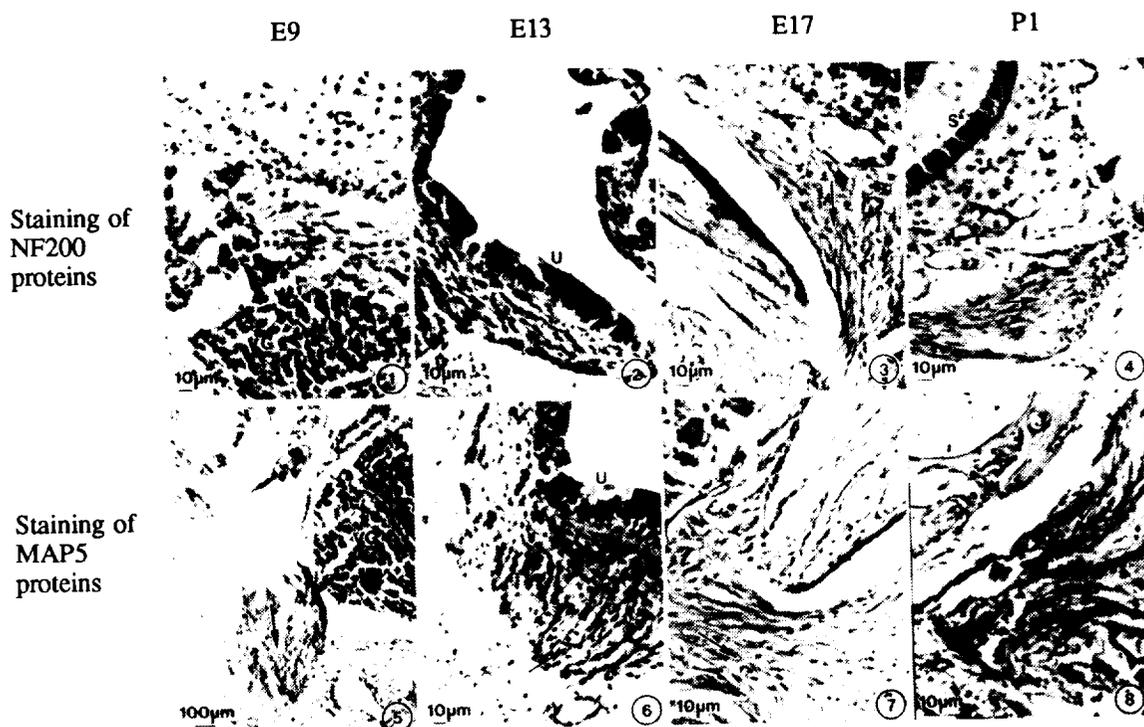
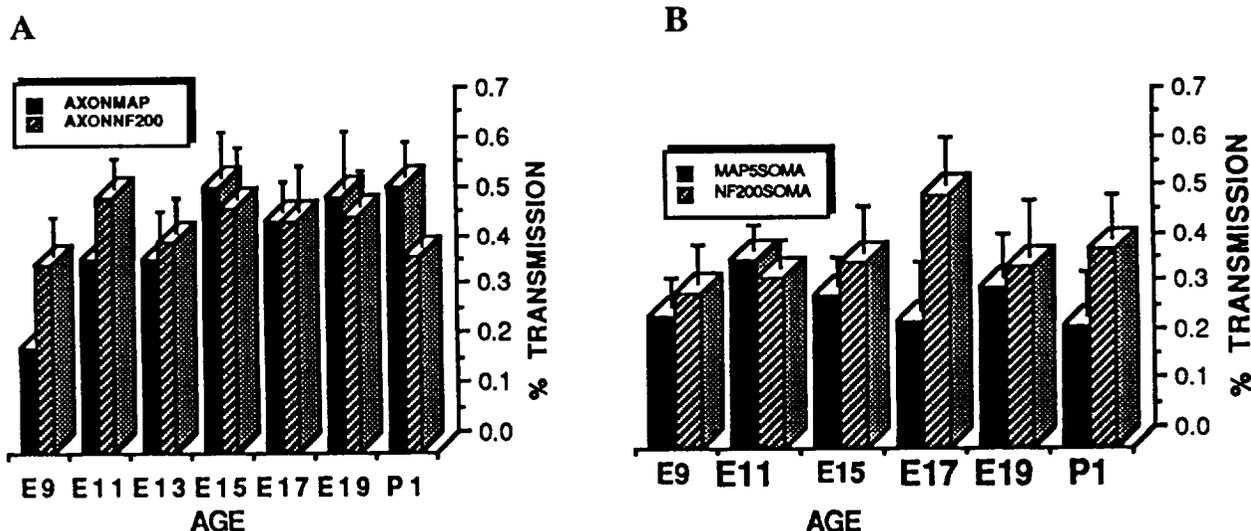


Figure 2. Black and white micrographs of color (red for positive and blue for counter-stain) reaction product using monoclonal antibodies against antigenetic determinants for MAP5 and NF200. Axons of four embryonic ages (E9, E13, E17, and P1) were processed in the same slide and at the same time under identical conditions to reduce preparatory artifacts. In plates 1-4 the relative concentration of NF200 is shown. Note the paucity of stain between E13-E17. Contrary to this pattern, MAP5 was more concentrated on E9 material and less in P1 (plates 5-8). Just as it is difficult to assess differences in protein concentration in these pictures with the naked eye, it is also difficult to analyze differences without color thresholding. Thus, development and application of technology used to assess these differences is an integral part of the investigation being conducted. Without this technology the investigator would have to base evaluation on subjective interpretations which carry no scientific validity for statistical comparisons.



BLUE THRESHOLD FOR E9-E15, RED THRESHOLD FOR E17-P1

Figure 3. The relative concentration of MAP5 and NF200 proteins of axons (A) and soma (B), which was almost unnoticeable in Figure 2(1-8), was objectively quantitated using color thresholding, which allows choosing area for measurements according to color. Relative concentration of MAP5 and NF200 (% transmission) at the ages shown is included with standard deviation.

soma (Figure 3). Relative concentration of these antibodies was determined with color thresholding for either the red hue of alkaline phosphatase (true density) or the blue hue of hematoxylin counter-stain (background and/or controls).

Significance of the Accomplishments

In order to thoroughly understand the complicated processes that culminate with the end organs of the inner ear being capable of detecting gravity and angular acceleration, cellular, molecular and functional aspects of developmental processes must be characterized. Functional modifications detected by others (Jones, T., *ASGSB Bulletin*, 1990) in newly hatched chicks flown in space on the STS-29 shuttle mission have cellular and molecular counterparts that will probably contribute to the alterations of components required in mature synaptic contacts. While synaptic contacts are required for proper transmission of signal from the inner ear to the brain, they are not the sole determinants for generating signals that produce ideal balance and posture. Maintenance and recycling of molecules like those reported here is just as important. These results and others previously reported clearly show that a multidisciplinary approach combining traditional and state-of-the-art techniques is ideal for defining changes of normal development for later assessment of changes of gravity sensing structures of the inner ear.

In conclusion, this part of the project shows that: (1) MAP5 and NF200 are differentially distributed in soma and axons of the VIIIth nerve; (2) color thresholding is extremely useful for assessing objectively the amount of antibody present in tissue vs. amount of counter-stain; (3) counter-stain can add to actual density of antibody reaction and can offset relative concentration of antibody seen by intensity alone; and (4) objective quantification of these important proteins will contribute to our understanding of the role they play toward the establishment of mature synapses.

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HOMEOSTASIS IN PRIMATES IN HYPERDYNAMIC ENVIRONMENTS

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Description of Research

The ultimate goal of this research program is to understand the physiological influence of gravity on living organisms, particularly mammals. Of particular interest are the physiological mechanisms leading to adaptation of a mammalian organism to an altered gravitational environment. Included in this research program is the identification of receptors, pathways of information transfer (neural and endocrine), mechanisms of integration of information, and pathways and mechanisms affecting the organism's response to alterations in gravity.

The adaptation of homeostatic systems to changes in gravity are poorly understood. Such changes in animals exposed to hyperdynamic environments produced via centrifugation include depressed body temperature, alterations in circadian timekeeping, and changes in the level of arousal. To date, research interests in this laboratory have focused on the sensitivity of these and other homeostatic systems to alterations in gravity. This research has demonstrated these systems' responsiveness to gravity and has attempted to elucidate the underlying mechanisms of the responses. Further, this program has focused on the responses of the whole organism to further understand the interaction between the various physiological systems of interest. This research required the ability to alter the dynamic environment of the organism. At the Chronic Acceleration Research Unit at the University of California at Davis, four centrifuges (8-18 ft diameter) are available. These facilities provide for acute and chronic exposures of animals to acceleration fields ranging from 1 to 20 g.

Accomplishments

During the past year our research has primarily examined the effects of hyperdynamic fields on homeostasis and circadian rhythms of body temperature. The patterns of physiological and behavioral response to changes in gravity may be important for understanding many of the biomedical issues related to spaceflight. For example, alterations in circadian rhythms may play an important role in many conditions encountered during spaceflight, such as calcium loss and space adaptation syndrome.

Rats were implanted with biotelemetry units in order to constantly monitor body temperature and activity. A receiver under the cage floor relayed the data to a microcomputer for storage and analysis. The rats were placed on an 18 ft diameter centrifuge in order to expose the animals to a hyperdynamic environment (2 g). Several experiments were performed with the most significant findings as follows:

(1) The effect of long-term exposure to a 2 g hyperdynamic environment was examined during a constant lighting condition. During this time the pattern of body temperature was observed. *Body temperatures exhibited a significant decrease in the daily mean and a loss of circadian rhythmicity.* The recovery of mean daily body temperature preceded the recovery of circadian rhythmicity. Two important findings are that *mean body temperature never recovered to normal baseline levels*, and that *the freerunning period of body temperature circadian rhythms*

did not recover to baseline levels. The change in circadian period is very important because it suggests that the change in gravity had a significant effect on the circadian pacemaker (suprachiasmatic nucleus).

(2) Rats were also exposed to one hour pulses of 2 g every 24 hrs. This experiment was designed to test the potential for adaptation to short-term 2 g exposure and to examine the potential entrainment effects of gravity on circadian rhythms. During the 2 g pulses, the **body temperature of rats exhibited a significant decline of approximately 2°C. Although there were periods when body temperature exhibited less of a decline than others, an adaptation to the 2 g exposure never occurred. This daily response to the 2 g exposure also gave the appearance of an entrained circadian rhythm in body temperature.** When the rats were no longer exposed to the 2 g pulse they appeared to freerun from the point of 2 g release. These observations suggest that the circadian rhythm of body temperature was entrained by the hyperdynamic pulses. However, this observation was not always consistent and will require more research.

Significance of the Accomplishments

These studies performed over the past year have generated important advances in defining the role of gravity on the regulation of body temperature. Of particular significance are the roles of constant 2 g altering the freerunning period and of daily 2 g pulses exhibiting entrainment-like characteristics. These results are critical for establishing the initial steps in determining whether gravity has a direct effect on the physiological characteristics of the suprachiasmatic nucleus, the critical neural pacemaker that controls circadian rhythms.

Future studies are necessary to examine how changes in gravity affect circadian rhythmicity and the underlying neural mechanisms of rhythmicity. These studies may be critical for understanding the process of human adaptation to the space environment.

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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 the 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. One of our goals has been the determination of the specific parts of the hypothalamus and related areas that affect circulating renin and angiotensin. A second goal has been the determination of the pathways from this region to the renin-secreting cells in the kidney. A third objective has been the analysis of the importance of this region in the physiologic control of renin secretion. To study the role of the brain in the regulation of renin secretion, we have used stimuli that increase renin secretion in diverse ways. Emphasis has been placed on the gravitational stress of 45° and 60° head-up tilt in rats. In past research, we have demonstrated that discharge of serotonergic neurons in the dorsal raphe nucleus increased renin secretion, so we have also tested the effect of the serotonin-releasing drug p-chloroamphetamine (PCA). Additional standardized stimuli have included the psychological stress of immobilization, the chronic volume depletion stress of a low sodium diet, and the acute volume depletion stress of nonhypotensive hemorrhage. We are now adding to these methods the acute volume depletion produced by extravascular administration of polyethylene glycol, a hypertonic substance that draws fluid from the vascular system in a reproducible way. We are also studying the role of vasopressin-secreting neurons that connect the hypothalamus to the brainstem and spinal cord.

Accomplishments

(1) Demonstration that at least in the short term, the sympathetic nervous system plays a major role in the increase in renin secretion produced by a low sodium diet. Since the final common pathway by which sympathetic effects on renin secretion are produced is the action of norepinephrine on β_1 -adrenergic receptors on the renin-secreting juxtaglomerular cells of the kidneys, inhibition of the response by β -adrenergic blockade indicates that the pathway is sympathetic. During the past year, we completed studies demonstrating that *the β -adrenergic blocking drug propranolol markedly reduced renin secretion in rats fed a low sodium diet for 9 days.*

(2) Further research on the role of brain stem vasopressin and oxytocin in the regulation of renin secretion. In previous studies we demonstrated that in homozygous Brattleboro rats, which have no vasopressin in their brains or circulating blood, renin secretion is chronically increased and there is a chronic increase in sympathetic discharge. The increased sympathetic discharge does not appear to be peripheral in origin, since chronic subcutaneous infusion of vasopressin failed to restore renin secretion to normal. Vasopressin does not cross the bloodbrain barrier, so it seemed likely that the increased sympathetic discharge was due to vasopressin deficiency in the brain. Repeated direct injections of vasopressin in Brattleboro rats failed to inhibit renin secretion, but such injections are very sensitive to dosage and timing. Therefore, we have embarked on other

approaches to the same question. One of these, in collaboration with Dr. Lanny Keil, NASA, Ames Research Center, is measurement of oxytocin and vasopressin in the brainstem in conditions in which it is known that there is increased sympathetic discharge producing renin secretion. Other experiments involve intracerebral administration of vasopressin and oxytocin antagonists. Results of these experiments are as yet incomplete.

(3) Completion of our experiments on the cause of the reduction in plasma angiotensinogen produced by lesions of the paraventricular nuclei. Replacement experiments in which thyroxine or adrenocorticotrophic hormone (ACTH) was given to rats with paraventricular lesions have now been completed, and thyroxine was shown to restore plasma angiotensinogen to normal. Therefore, *the decrease in angiotensinogen is due to disruption of the thyroxine releasing hormone (TRH) secreting neurons in the nuclei.*

(4) Further study of the unique effect of stress and certain anesthetics on circulating angiotensinogen. In the course of experiments described in the preceding paragraph, we made the chance observation that 24 hrs after surgical stress, plasma angiotensinogen was elevated with little if any change in plasma renin activity. Since this effect is blocked by hypophysectomy or paraventricular lesions, we explored the possibility that it was due to increased secretion of a hormone from the anterior pituitary that was controlled by the paraventricular nuclei. No increase in ACTH, thyroid hormones, leutinizing hormone, or prolactin has been observed in animals in which the angiotensinogen is increased. However, *xylazine*, one of the anesthetics which produces this effect, *causes an increase in plasma oxytocin and vasopressin.* Experiments with antagonists to vasopressin and oxytocin are currently under way. In addition, we now know that *a decrease in thyroid hormone causes a sharp decrease in angiotensinogen secretion*, and it is possible that xylazine acts in some pituitary-independent fashion but simply cannot overcome the depressive effect of the thyroid deficiency produced by paraventricular lesions or hypophysectomy. This possibility is being explored.

(5) Further analysis of the role of the ventromedial nuclei in the regulation of renin secretion. In previous experiments, we demonstrated that bilateral destruction of the ventromedial nuclei does not lower circulating angiotensinogen but does prevent the increase in renin secretion produced by PCA, head-up tilt, immobilization, and a low sodium diet. In the past year, we tested the effect of *electrical stimulation of the ventromedial nuclei* and found that this *increased renin secretion.* However, in preliminary experiments, *microinjection of the excitatory amino acid dl-homocysteic acid failed to increase renin secretion.* Since this amino acid stimulates cell bodies but fails to stimulate fibers of passage, *it appears that the ventromedial nuclei are not integrating centers per se, but are part of a descending pathway.*

Significance of the Accomplishments

The experiments described above and the experiments conducted in previous years have done much to elucidate the role of the brain in the regulation of renin secretion. Postural changes and stressful stimuli, both of which occur in spaceflight, increase renin secretion, and renin via angiotensin II stimulates the secretion of the salt-retaining hormone aldosterone, affects water balance, and maintains blood pressure. In addition, our demonstration that brain lesions lower circulating angiotensinogen via a neuroendocrine mechanism involving thyroid hormone secretion is important because it demonstrates another way in which the brain can influence the amount of circulating angiotensin II and, consequently, influence its effects.

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PHYSIOLOGY OF THE VESTIBULAR SYSTEM

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Description of Research

There are two interrelated long-term goals to our research program: (1) to understand the cellular mechanisms determining the discharge of vestibular nerve afferents, and (2) to understand the operation of the vestibular labyrinth in terms of the overall operation of the vestibular system. There are specific groups of afferents innervating each end organ. The hypothesis guiding the research is that the various afferent groups differ in their transduction mechanisms and in the roles they play in the central processing of vestibular information. The research program extends from a biophysical analysis of hair cells to a study of the organization of reflex pathways in the alert primate.

Accomplishments

(1) The afferent innervation has been surveyed by the extracellular injections of horseradish peroxidase (HRP) into the vestibular nerve, which labels several fibers as far as their peripheral terminals. In describing results for the cristae, it has been useful to divide the neuroepithelium into central, intermediate and peripheral zones of equal areas (Figure 1A). Similarly, the utricular macula can be divided into a striola, a juxtastriola, and an extrastriola (Figure 1B). Three classes of afferents can be recognized in both the cristae and the macula. Calyx units are thick fibers seen in the central (striolar) zone. The axon is usually unbranched, giving rise to a single calyx ending surrounding almost the entire basolateral surfaces of 1-3 neighboring, flask-shaped type I hair cells. Bouton units, found in the peripheral (extrastrilar) zone, have thin axons, whose fine collateral branches provide bouton endings to several cylindrically shaped type II hair cells. Dimorphic units innervate all parts of the neuroepithelium and supply both kinds of hair cells. The medium-sized axons of dimorphs give rise to one or more relatively thick collateral branches terminating as calyx endings. In addition, bouton endings are present on thin collaterals that emerge from the parent axon, the thick branches, or the calyx endings. In much of the recent literature, *dimorphic units* have been considered rare. *They are, in fact, the most numerous kind of afferent, making up 70% of the fibers going to the cristae and more than 80% of those supplying the utricular macula.* To understand the contribution of dimorphic fibers, it has to be remembered that type I and type II hair cells are present throughout the neuroepithelium. Given the restricted distribution of calyx and bouton units, dimorphs must provide the main source of afferents to type II hair cells in the central (striolar) zone, to type I hair cells in the peripheral (extrastrilar) zone, and to both kinds of hair cells in the intermediate (juxtastrilar) zone.

(2) Do afferents with distinctive discharge properties differ in their branching patterns and in the kinds of hair cells they innervate? The question has recently been studied by intra-axonal labelling of physiologically characterized afferents in the cristae and utricular macula of the chinchilla. *Calyx units*, which supply central (striolar) zones, *are the most irregularly discharging and most phasic afferents found in either kind of end organ. The discharge properties of dimorphic units depend on the epithelial region they innervate.* Central (striolar) dimorphs are irregularly

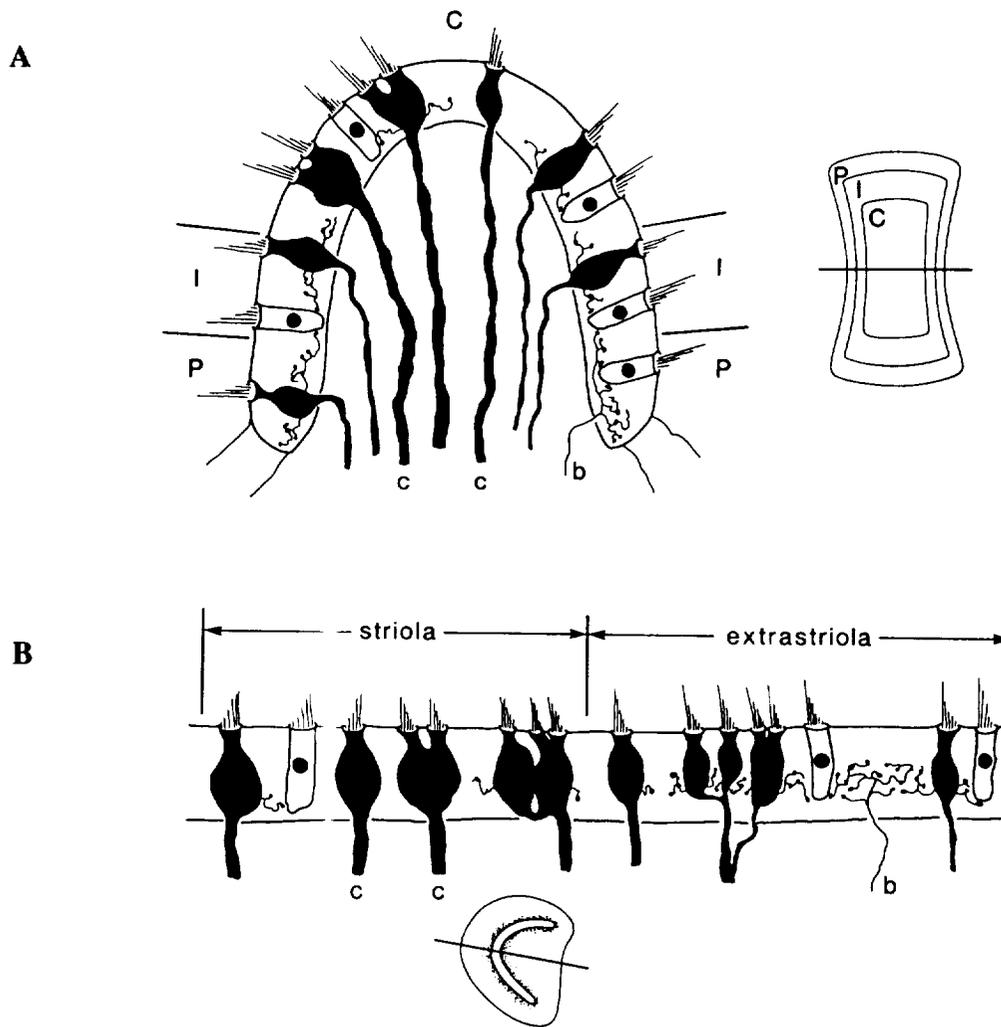


Figure 1. Afferent innervation patterns in the crista ampullaris (A) and utricular macula (B). The crista is divided into central (C), intermediate (I), and peripheral (P) zones. The macula is divided into three zones: a narrow, ribbon-shaped striola, a juxtastriola (indicated by stippling in the inset) immediately surrounding the striola, and a broad extrastriola. The section planes of the main figures are indicated. Fibers include bouton (b), calyx (c), and dimorphic (unlabelled) fibers.

discharging and phasic. Peripheral (extrastriolar) dimorphs are regularly discharging and tonic. Dimorphs innervating the two zones differ in their physiology, even in cases where afferent innervation patterns are similar. Only one physiologically characterized bouton unit was labelled, possibly because such units have thin axons and are difficult to impale. ***The labelled bouton unit supplied the peripheral zone of a crista and was regularly discharging and tonic.*** To obtain additional information on bouton units, extracellular recordings were done in the squirrel monkey and these units were identified by their slow conduction velocities (see Accomplishment #4).

The results, particularly those relating to dimorphic units, emphasize the importance of regional differences in determining afferent physiology. Presumably, there are regional variations in transduction mechanisms that are shared by type I and type II hair cells. Is there anything distinctive about the type I hair cell? Attention naturally turns to

calyx afferents, since they get most of their input from such hair cells. *In the mammalian cristae, the one feature that distinguishes calyx units from irregularly discharging dimorphs is their relatively low gains to head rotations.* As both types of units contact similar numbers of type I hair cells, it can be supposed that the higher gains of dimorphs are a result of the additional inputs they receive from type II hair cells. *The difference in gains can be used to estimate that, on average, a simple calyx ending gets three times the synaptic input of an afferent bouton. A similar conclusion is possible in the utricular macula* even though the linear-force gains can be similar for irregular dimorphs and for calyx units. A ratio of 3:1 in the effective synaptic weights of calyx and bouton endings can be compared to a 100:1 difference in the appositional contacts that each of the endings makes with its hair cell. Viewed in this light, *the calyx ending has a surprisingly low synaptic gain.*

(3) Our extracellular HRP studies show that there are differences in the innervation of central (striolar) and peripheral (extrastriolar) zones. Furthermore, units supplying the various zones differ in their physiological properties. This has led us to investigate the possibility that there are regional differences in synaptic organization. A quantitative ultrastructural study is being done in the chinchilla cristae. (a) *There are 2-3 times as many afferent boutons innervating individual type II hair cells in the peripheral zone as compared to the central zone.* Despite the fact that they are contacted by relatively few afferent endings, type II hair cells in the central zone have as many ribbon synapses as do those in the peripheral zone. One reason for this is that *many central boutons receive two or more synapses. In contrast, a single synapse is usually seen at the contact between a peripheral afferent bouton and its type II hair cell.* (b) Many type II hair cells in the central zone have large areas of apposition with the outer faces of neighboring calyx endings, and *ribbon synapses are usually seen between the two structures.* Quite a different arrangement occurs in the peripheral zone. Here, about half the type II hair cells do not contact calyx endings. The appositions that do occur are narrow, are typically interrupted by supporting-cell processes, and seldom show synaptic specializations. (c) *Ribbon synapses between type I hair cells and calyx inner faces are plentiful.* There are, on average, almost 20 such synapses per hair cell and the number is similar in all three zones. Another kind of specialized contact is also seen. It consists of an invagination of the calyx ending into the type I hair cell. Although the function of the invagination is obscure, the *invaginations are considerably more numerous in central, as compared to peripheral, type I hair cells.* (d) Highly vesiculated efferent boutons are in contact with the base of type II hair cells, the outside of calyx endings, afferent boutons, and unmyelinated nerve branches. The efferent innervation is similar in all parts of the neuroepithelium.

(4) Having completed our studies of the afferent innervation patterns in the chinchilla cristae, we decided to do a comparable study in monkey and expected that results would be similar in the two species. To our surprise, there are substantial differences. The most obvious of these differences concerns the proportion of type I and type II hair cells. *In the chinchilla, the two kinds of hair cells occur with approximately equal numbers throughout the end organ, while in the monkey, type I hair cells outnumber type II hair cells by a ratio averaging 3:1 for the entire crista and exceeding 5:1 in the central zone.*

How are the differences in hair-cell populations reflected in the afferent innervation patterns for the two species? To answer this question, afferents in the monkey were labelled extracellularly. Results were qualitatively similar to those for the chinchilla. In the squirrel monkey, as in the chinchilla, calyx units are concentrated in the central zone, bouton units

are largely restricted to the peripheral zone, and dimorphic units are seen in all parts of the epithelium. Two quantitative differences were noted: *(1) There are proportionally more calyx units and proportionately fewer bouton and dimorphic units in the monkey. (2) Dimorphic units in the central and intermediate zones of the monkey have relatively few bouton endings.* Both differences serve to match the afferent innervation with the complement of hair cells found in the two species. *The main interspecies difference is that there are proportionately more fibers innervating the central zone in the monkey. Most of these fibers should be calyx units.*

What are the functional implications of this latter difference? *Calyx units in the chinchilla cristae have a distinctive physiology: they are irregularly discharging, phasic afferents with relatively low rotational gains. Extracellular recordings in the monkey show that there is a comparable population of afferents in the monkey.* The identification of these afferents as calyx units was confirmed by their antidromic conduction velocities, which showed them to be among the largest fibers in the vestibular nerve. *Possible bouton units were identified by their low conduction velocities. They are regularly discharging tonic fibers* and thus resemble the peripheral dimorphs and the bouton units previously labelled in the chinchilla. *From a functional perspective, the most obvious difference between the two species is the presence of a considerably larger proportion of low-gain irregular afferents in the monkey.*

(5) Vestibular afferents differ in their discharge properties. What is the functional significance of this afferent diversity? One possibility is that the response dynamics of the various afferents might be matched to the dynamic requirements of the reflex pathways to which they contribute. To illustrate the situation, we can consider two canal-related pathways — the vestibulo-ocular (VOR) and the vestibulocollic (VCR) reflexes. The VOR is an open-loop reflex that controls the oculomotor plant, a mechanical load that is dominated by elastic forces at low frequencies and by viscous forces at high frequencies. In contrast, the VCR is a closed-loop reflex that stabilizes the head, whose mechanics at high frequencies are largely inertial. In addition, neck muscles are more sluggish than are extraocular muscles. Relatively simple control-systems calculations suggest that regularly discharging, tonic afferents would provide a better match to the requirements of the VOR than would irregularly discharging, phasic afferents. For the VCR, the reverse would be true.

The predictions have been tested in two ways. Both make use of differences in the galvanic sensitivity of regular and irregular afferents. The first way involves recording monosynaptic, vestibular-evoked (V_i) excitatory postsynaptic potentials (EPSPs) from secondary neurons in the vestibular nuclei of anesthetized animals. *As a class, secondary VOR neurons get a higher proportion of their monosynaptic V_i inputs from regular afferents than do VCR neurons.* At the same time, the segregation of inputs is far from complete, as is exemplified by the fact that *about one-third of the direct afferent input to VOR neurons is irregular.* The overlap of inputs is consistent with the finding that the two kinds of afferents project to the same regions of the vestibular nuclei. Certainly, we are not dealing with two parallel, independent channels. The second method uses anodal (inhibitory) electric currents to obtain a selective and reversible ablation of irregular afferents in alert animals. To date, the ablation method has been used only to study the contribution of irregular afferents to the overall operation of the VOR. Remarkably, *the results imply that the vestibular-nerve input to the reflex comes almost entirely, if not exclusively, from regular afferents.* At first glance, the two methods would seem to lead to contradictory

conclusions. One method shows that there is an appreciable *monosynaptic* irregular input to secondary VOR neurons; the second, that there is no *net* irregular input to the overall reflex. The two results can be reconciled by remembering that secondary neurons receive both direct and indirect inputs from the vestibular nerve. The indirect inputs could be used to effectively cancel the monosynaptic irregular input. The functional significance of this seemingly curious arrangement requires further study. One possibility is that the indirect pathways can be gated to provide the reflex with the flexibility to change its gain during voluntary cancellation or when gaze shifts from near to far targets.

Significance of the Accomplishments

The studies described above provide a detailed picture of the afferent innervation of the vestibular end organs in mammals and of the relation between the branching patterns of an afferent and its physiological properties. A baseline is provided by the quantitative ultrastructural studies for investigating the effects of various experimental procedures, including alterations of the gravitational environment, on the inner ear. While rodents remain convenient animals for studying the structure and function of the vestibular labyrinth, our comparative studies in the chinchilla and the squirrel monkey suggest that caution be used in extrapolating results from rodents to primates, including humans.

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SYNAPSE DEVELOPMENT IN VECTOR-AVERAGED GRAVITY: A PREDICTIVE MODEL FOR THE EFFECTS OF SPACE MICROGRAVITY ON CELL DEVELOPMENT

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Description of Research

It is well established that a large variety of cells are sensitive to perturbations in their gravity field. In cases where cells (e.g., plant root cells) possess a gravisensor, the mechanisms whereby altered gravity fields influence cellular processes is relatively easy to understand mechanistically, and therefore the search for the details of mechanisms can be methodologically pursued. Most cells, however, do not have a specialized gravisensor and therefore a logical but "wide net" must be cast in order to "fish" for the correct mechanism. Another complication in studying the effects of perturbed gravity on cells is the restricted access to actual microgravity (as in space or rocket flights) and the availability of what appears to be a sole paradigm for simulating some properties of microgravity — the clinostat. In this device the microgravity of space is simulated, from the cells' vantage point, by the continuous averaging of the gravity vector. Also available are a number of other rigorous control paradigms which may subject cells to a perturbed gravity field at the opposite end of the gravity spectrum.

We are examining mechanistically well-defined interactions between nerve and muscle cells that normally lead to the formation of a synapse — the morphologic and functional specialization of those cells designed for intercellular communications (e.g., in the peripheral control of the musculature by the nervous system and in the myriad of interneuronal interactions which make up brain function). By studying a prototype synapse, the neuromuscular junction, we hope to uncover general mechanisms that may be affected by perturbations in the gravity field of the cells. We believe that this research will result in two predictive pathways. The first concerns the responses of cells to exposure to the real microgravity of space. This pathway should provide information that is relevant to the ontogenetic development of cellular systems, and hence organs, of embryos if their development were to take place in the microgravity of space. The first step toward the verification of such predictive behavior is the execution of experiments carried out in the clinostat with parallel experiments in spaceflight. The second step concerns the examination of an organismic development of embryonic systems (e.g., *Xenopus*, mice) in the microgravity of space with specific attention to cellular processes examined in the clinostat. The second predictive pathway relates to the potential ramifications of our findings of cellular responses to altered gravity to evolutionary processes in cellular properties that we presume were "shaped" to some extent by the omnipresence of gravity. Thus, we expect microgravity research to produce relevant findings and ideas concerning the role that gravity has played in evolution.

Our experimental system consists of co-culturing neurons and muscle cells in a slowly rotating clinostat. Using this system, we have shown that cell and intracellular organelle morphologies are significantly altered: myocyte size increases, striations are less well developed, the nucleus and its nucleolus enlarge, and cells assume unusual shapes. In nerve cells, we found that the neuritic shafts, which are the conductile elements of nerve cells that transmit information from neuron to neuron, develop swellings that appear to be

deformations in the structure of the shaft. Finally, we have found that neuron-myocyte interactions, which normally produce a cascade of transcellular events resulting in the formation of the neuromuscular synapse, are disturbed to the extent that receptor (acetylcholine receptor) aggregation is either reduced or fails to take place after clinostat rotation. We are now methodically probing a number of pathways to determine how the perturbed gravity field of the clinostat produces these effects. To this end, we are examining with immunocytochemistry the cytoskeletal structures responsible for the maintenance of cellular integrity and for the localized receptor capping. Similarly, we have begun a series of experiments in which we substituted polystyrene beads for neurons in the process of receptor aggregation. Using beads, whose inanimate behavior cannot be changed by the clinostat rotation, we asked how muscle cells respond to rotation in their ability to aggregate receptors.

These approaches pave the road to precise, rigorously controlled experiments which should be carried out in the real microgravity of space. The fundamental question, which our work is designed to address, is: Will embryonic development of the brain be normal if it takes place in the microgravity of space? By accepting the predictive capacity of clinostat experiments, we conclude that such development will be quite different from that which takes place in the 1 g terrestrial environment.

Accomplishments

Recent results show that when beads are substituted for neurons, receptor aggregation on the muscle cell at the point of contact, between the bead and the cell, is significantly diminished. Figure 1 illustrates this point: A control myocyte (a) is shown in phase contrast with beads firmly attached (see arrows). The corresponding distribution of acetylcholine receptors, visualized by use of a rhodamine bungarotoxin probe, is shown in (b). Most of the beads are associated with receptor patches which are large, detailed in structure and well-defined. The accompanying rotated myocyte (c) shows a similar number of beads stuck to the myocyte even after rotation. After 36 hrs of rotation at 1 rpm, begun 8 hrs after seeding beads on myocytes, the number of beads with receptors is significantly reduced (see arrows; d). Moreover, where receptors did accumulate after clinostat rotation, the structure of the assembly is less well defined and is spread over a smaller area. Table I summarizes results from several replicate experiments and demonstrates the statistical significance of these results. The data show that *clinostat rotation is associated with a 50% reduction in the number of beads associated with AChR clusters. Moreover, the data show that the fluorescence area, indicative of the number of receptors in clusters that did associate with beads, is significantly reduced.* This experiment is parallel to our previously reported

Table I. Polystyrene bead-induced AChR accumulation on myocytes.

	CONTROL	1 RPM	10 RPM
Total # Beads	1,841	2,206	1,857
Beads w/AChR	379	255	227
% w/AChR	20.7	10.4	12.3
% of CTL	100	50.2	59.4
Patch Area μm^2	12.5 \pm 0.4	10.0 \pm 0.3	10.8 \pm 0.4
Student t test		t = 4.58 *	t = 2.95 *

Data from 176 control, 163 1 rpm, and 84 10 rpm myocytes, and from three separate experiments. Means \pm SEM. * denotes statistical significance $p < 0.05$.

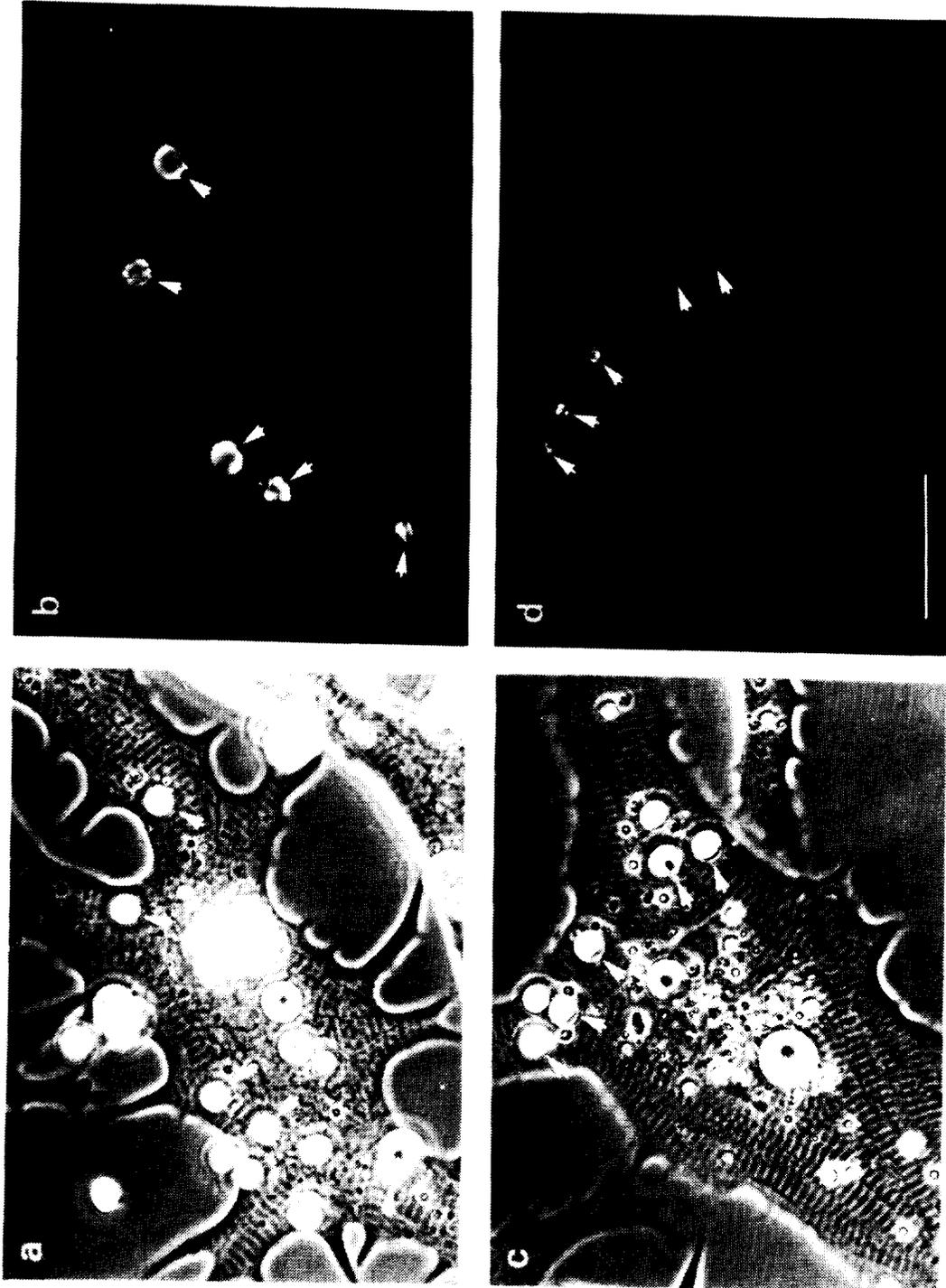


Figure 1. Phase contrast (a, c) micrographs of myocytes under control (a) and rotated (c; 1 rpm) conditions showing the presence of polystyrene beads used to cause receptor aggregation (bead diameter ca. 5 μm). Receptor aggregates are detected with rhodamine bungarotoxin and show that in the control cell (b) more beads are associated with receptor clusters than in the rotated myocyte (d), where the size of the clusters is also smaller than in the controls. Bar scale = 25 μm

experiment in which we showed that rotation of cocultured neurons and myocytes showed a significant reduction in receptor accumulation if the rotation began after nerve-muscle contact was established. We also showed that *if rotation begins before contact between nerve and muscle is established, receptor accumulation fails completely*. We are now performing the parallel experiment with beads by shortening to 30 min the time between bead seeding on the myocytes and the beginning of rotation. These data are consistent with the idea that the response of the muscle to the nerve signal is the root cause of its failure to accumulate receptors and therefore to form a functional synapse.

Significance of the Accomplishments

Our data continue to demonstrate that clinostat rotation is associated with specific alterations in cellular responses to perturbed gravity. It is impossible to state categorically that such changes will take place in the microgravity of space. But, taking into consideration findings from other systems (lymphocytes, oocytes, plant cells) where the concordance between clinostat and actual microgravity experiments has been very high, it is quite probable that the changes we have observed in cultured neurons and myocytes will be seen in spaceflight experiments. If that is the case, we predict that embryonic development of cells and therefore organisms is likely to be significantly different from development that takes place in the 1 g environment of Earth.

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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 the neuromodulator serotonin 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 7 days in space. In addition, a more recent study has shown that rats exposed to a hypergravic field of 2.0 g for 7 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.

The major focus of work conducted during the past year concerned the effects of hypergravity on different regions in the hippocampus. Marked differences were found in the response of granule cells in animals exposed to a 2 g field compared with Earth gravity control animals. A tentative model to account for the effect of gravity on this particular population of neurons is shown in Figure 1A. Fluid shifts evoked by the hypergravic field may affect the activity of hypothalamic neurons, the subsequent release of ACTH, and the production of adrenal glucocorticoids. The number of serotonin receptors has been reported to be inversely related to glucocorticoid levels, so that one would expect a down regulation of serotonergic receptors when the rat is exposed to a hypergravic field for 7 days. The long loop involving signals from the brain to the adrenal cortex and back to the brain is diagrammed in Figure 1B.

Accomplishments

(1) Perfusion of 5-HT (serotonin) over hippocampal slices leads to depression of the population spike in both the CA1 pyramidal cell region and dentate gyrus granule cell region of the rat hippocampus. However, ***10 mM 5-HT leads to a much greater decrease in spike amplitude in the CA1 region than in the dentate gyrus.*** In a single hippocampal slice from a rat, a submaximal population spike (53% of maximum possible spike amplitude) in the dentate gyrus decreased 18% in amplitude following perfusion of 10 mM 5-HT.

(2) A decrease in population spike amplitude in response to the administration of 10 mM 5-HT was observed in all recordings from the dentate gyrus. In 10 slices from animals exposed to 2 g hypergravic fields for 7 days, the dentate gyrus population spike amplitude was decreased an average of 24% during perfusion with 5-HT containing medium. However, in the dentate gyrus of control animals exposed to 1 g, the population spike was decreased an average of 36% in 10 slices. ***This 33% difference in response to 5-HT perfusion in animals exposed to 2 g and 1 g was***

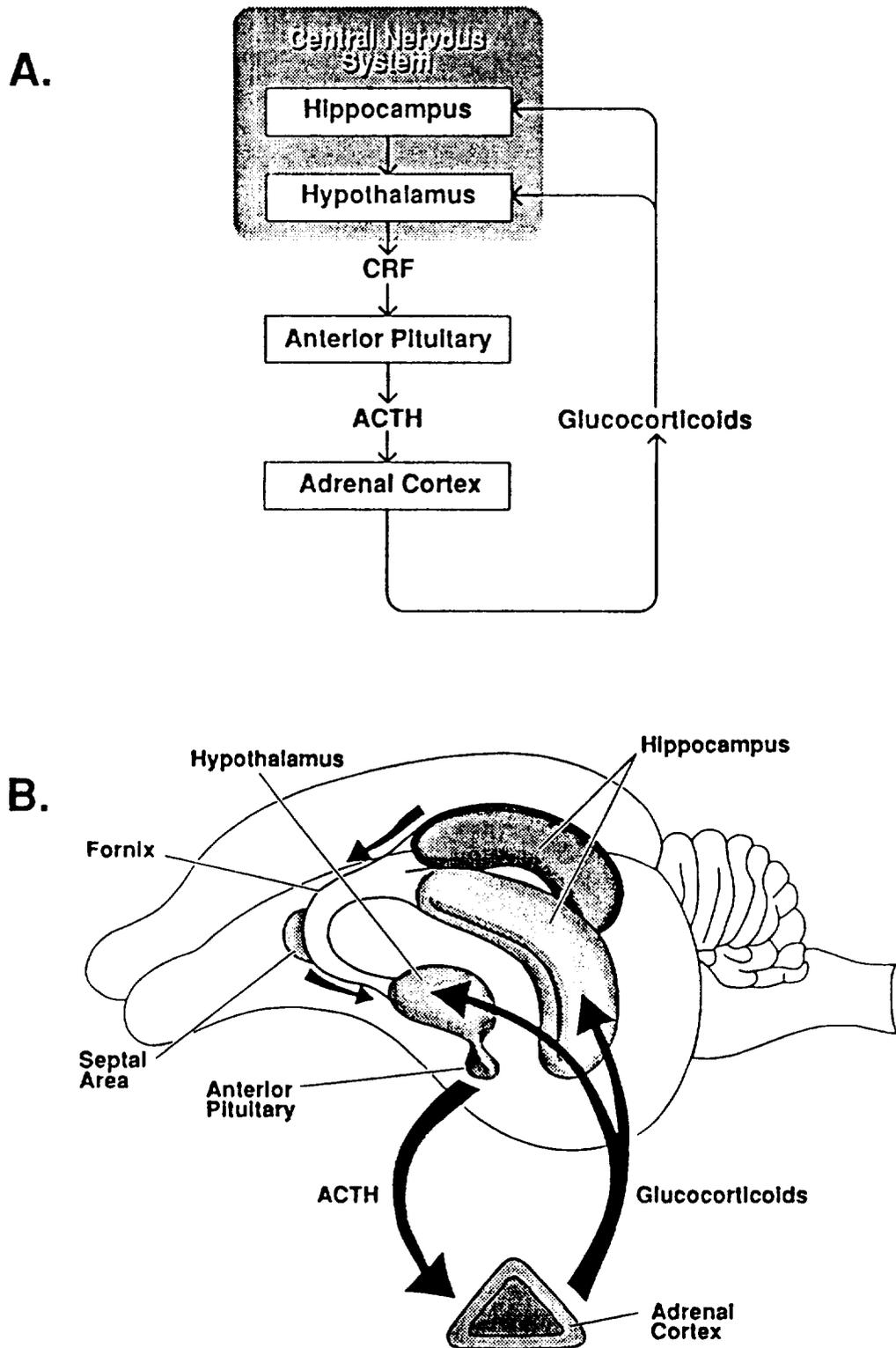


Figure 1. Long loop involving interaction between regions of the brain and the adrenal cortex. A. Relationship of releasing factors, hormones, and glucocorticoids to the loop. B. Sketch of anatomical regions involved in the loop.

statistically significant ($p < 0.05$). Hence, the inhibitory effect of 10 mM 5-HT on population spike amplitude was diminished in rats that had been exposed to 2 g conditions for 7 days.

(3) *The rebound of population spike amplitude following removal of 5-HT tended to be greater in the dentate gyrus population spikes than in CA1 population spikes.* In recordings of dentate gyrus population spikes, the spike amplitude returned to an average maximum of 123% of the levels seen prior to drug administration. The CA1 population spike amplitude returned to an average amplitude of 94% of control amplitude.

Significance of the Accomplishments

The principal finding was a specific difference in the response of granule cells in animals exposed to a 2 g field compared with control animals in a 1 g field. These findings are consistent with biochemical/anatomical studies indicating a change in receptor number in the hippocampus. Our results are the first report to show that changes in the modulatory effects of serotonin on electrical activity in hippocampal pyramidal cells are correlated with exposure to hypergravic fields. These results are significant in that they establish that changes in the gravitational effect is associated with a specific cell type, granule cells in the dentate gyrus, and with a particular neuromodulator, serotonin.

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MECHANISM OF CONTROL OF BONE GROWTH BY PROSTAGLANDINS

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Description of Research

The growth and repair of bone is a complex and poorly understood process. Over the past three decades, it has been noted that astronauts lose large amounts of calcium and bone when exposed to the microgravity of spaceflight. The mechanism by which bone growth is regulated is not known; however, clinical observations have demonstrated that increases of endogenous cortisol as seen in Cushing's syndrome are associated with bone loss and osteoporosis. Treatment of asthma and rheumatoid arthritis with glucocorticoids is also associated with poor bone formation in these patients. The bone loss associated with glucocorticoids involves the trabecular bone, and examination of patients treated with the synthetic glucocorticoid prednisone shows a reduction in bone formation, which is probably due to a direct inhibition of osteoblast function. In the Skylab missions, urinary cortisol of the nine crewmembers increased almost two-fold. These data suggest that the glucocorticoids could also play a role in the loss of bone during spaceflight.

This laboratory has previously shown in cell cultures that glucocorticoids cause a decrease in endogenous prostaglandin E₂ synthesis accompanied by a decrease in osteoblast growth and mineralization. Taken together, these observations suggest that glucocorticoids may regulate bone growth by inhibiting endogenous PGE₂ synthesis. The direct cause of glucocorticoid-induced bone loss is not known and an understanding of the relationship between bone growth and the eicosanoid synthetic pathway may help elucidate the regulation of bone growth. Using a cloned osteoblast cell line as a tool to study the cellular changes in growth, we have tested the hypothesis that endogenous prostaglandin E₂ synthesis plays a role in regulation by perturbing endogenous osteoblast synthesis of prostaglandins and by testing for alterations in growth and morphology.

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 E₂, and mineralized matrix containing collagen type I and hydroxyapatite. In these studies, we have used 30 μM indomethacin, a known inhibitor of cyclo-oxygenase, to block synthesis of the prostaglandins.

(1) When the indomethacin was added to the growing osteoblasts, cell growth as measured by DNA synthesis was decreased to 63% of control. Addition of exogenous PGE₂ to these inhibited cells resulted in renewed growth equal to or greater than control levels.

(2) Protein synthesis in the indomethacin-treated osteoblasts was decreased to 68% of control osteoblasts. Addition of exogenous PGE₂ to these indomethacin-treated cultures returned protein synthesis levels to that of the control cells.

(3) *Prostaglandin added to control osteoblast cultures increased DNA synthesis to 236% and increased protein synthesis to 118% of control cells.*

(4) Using epi-fluorescent microscopy, we noted changes in the f-actin cytoskeleton of the control and treated osteoblasts, with the treated osteoblast having a collapsed actin architecture. In addition, we found that the control osteoblasts had triangular morphology and that indomethacin treatment caused a change resulting in the treated cells having a spindle shaped morphology. Treatment of the indomethacin-treated cells with exogenous PGE₂ resulted in a recovery in actin cytoskeleton and osteoblast morphology to that of the control cells.

Significance of the Accomplishments

Biomedical studies of crewed spaceflight have consistently indicated a loss of weightbearing skeletal bone. Various lines of evidence from humans and animals suggest that this loss is due to a lack of bone formation in the absence of gravity. Biomechanical properties of both cortical and trabecular bones show strength deficits that appear to represent failure of normal increase in growth rather than an accelerated loss of bone. Joint US/USSR biosatellite flights of small animals have shown clear morphological evidence that bone formation is reduced during flight.

The cause of this loss of bone has been postulated to be related to the lack of biomechanical stimulation during flight and/or due to increases in systemic hormones associated with spaceflight. Urinary cortisols of crewmembers during the Skylab missions increased from a preflight value of 54 ± 4 $\mu\text{g/day}$ to 94 ± 5 $\mu\text{g/day}$ during flight, with individual crewmembers increasing from 1.2- to 2.8-fold during flight. Osteoporosis seen in Cushings patients is associated with elevated cortisols (2 to 5x control values), which suggests that the elevated cortisols seen in spaceflight may contribute to decreased bone mass. Since cortisol and the other glucocorticoids like dexamethasone cause a decrease in endogenous prostaglandin synthesis, prostaglandins have been implicated in the regulation of bone loss both in Cushings syndrome and in long-term spaceflight.

In order to test our hypothesis that prostaglandins play a role in the regulation of bone growth, we have used a more complete block of endogenous prostaglandin synthesis using the cyclo-oxygenase inhibitor indomethacin. In this study, we demonstrated that blocking endogenous eicosanoid production decreased osteoblast growth, DNA synthesis, and protein synthesis. Our results agree with studies by others which have demonstrated that therapeutic doses of indomethacin, aspirin, and ibuprofen can cause significant decreases in bone cell growth *in vivo*. Also, epi-fluorescent microscopic studies have demonstrated an alteration of actin cytoskeleton resulting in changes in cell morphology when blocking prostaglandin synthesis. In our studies we have completely reversed the action of indomethacin by adding back PGE₂, suggesting that indomethacin is acting through a PGE₂ mechanism. These data add to the growing evidence that prostaglandin E₂ is a natural regulator of osteoblast growth and that perturbations of PGE₂ play a pivotal role in bone cell formation.

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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 cells sense mechanical forces and transduce them into a biochemical response. The main objective of this study focuses on analysis of the process by which mechanical forces are conveyed by extracellular matrix (ECM) molecules, transmitted across the cell surface, and transduced into changes of cytoskeletal filament organization inside the cell. This approach is based on the hypothesis that cell shape, and thus the form of the cytoskeleton, is determined through a dynamic balance of mechanical forces that are generated within contractile actin-containing microfilaments and resisted both by internal cytoskeletal struts and by ECM attachment sites on the surface of the cell. This concept emerged out of studies with three dimensional "tensegrity" (tensional integrity) cell models constructed out of sticks and elastic string (Figure 1). If this type of tensegrity mechanism is used by cells, externally applied mechanical loads may affect complementary force interactions and change local thermodynamic parameters, thereby altering cytoskeletal filament assembly and distribution. Changes of cytoskeletal organization can, in turn, alter the distribution and hence function of much of the cell metabolic machinery, as well as the structure and function of the nucleus. Characterization of this process could have important implications for understanding the mechanism of gravity sensation in a wide variety of cells.

We have focused on the biomechanical mechanism by which actin polymerization is regulated. The specific aims of this research are: (1) to analyze the process by which cell-generated mechanical loads are transmitted across ECM receptors and are transduced into alterations of actin polymerization, and (2) to determine whether externally applied forces utilize a similar ECM-based mechanism for transmembrane mechanochemical coupling.

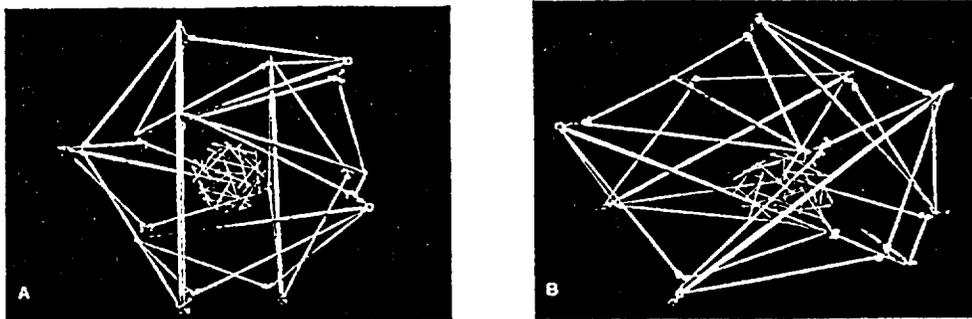


Figure 1. Nucleated Tensegrity Cell Models. (A) Unattached. (B) Adherent and spread on a rigid substrate. The nucleus is a geodesic sphere constructed out of applicator sticks and elastic string. The cell is composed of aluminum struts and elastic shock cord. The nucleus is connected to the cell surface by black elastic string which is not visible against the black background. Note that both the cell and nucleus spread in a coordinated fashion in the spreading "cell."

Accomplishments

(1) *Development of an in vitro system for controlling cell-ECM contact formation.* We have developed a simple *in vitro* system that can be used to vary the amount of tension that cells can exert on their ECM receptors. In this system, cell-ECM contact formation is controlled by varying the density of a single type of ECM molecule, such as fibronectin, which is coated on an otherwise non-adhesive culture surface. Using this system in combination with chemically defined medium, we have been able to clearly demonstrate that fibronectin controls cell growth based on its ability to support tension-dependent changes of cell and nuclear shape. We have also been able to demonstrate that the effects of fibronectin on cell form and function are mediated by binding interactions with specific ECM receptors ($\alpha 5\beta 1$ integrins). Furthermore, use of cytochalasin D, an inhibitor of microfilament assembly, revealed that actin polymerization is required for both cell spreading and progression through G1 phase of the cell cycle. Importantly, cytochalasin D had no effect when added at later times after cells had entered S phase.

(2) *Quantitation of actin polymerization within cells spreading on fibronectin.* We have used a fluorescent NBD-phalloidin binding assay to measure actin polymerization during the initial phases of cell attachment and spreading on fibronectin-coated dishes. We have found that monomeric actin shifts into a polymerized F-actin form and becomes associated with the cytoskeleton beginning approximately 10-20 min following cell binding to fibronectin. We can also control the amount of F-actin assembled by varying fibronectin coating densities, thereby altering cell tension.

(3) *Demonstration that cells use tensegrity architecture for their organization.* We have been able to demonstrate that ***cells and nuclei within membrane-permeabilized cells can be induced to physically retract and round over a period of minutes.*** This response is accomplished by permeabilizing endothelial cells with saponin. The cells are then incubated in a buffer containing calcium and ATP, which has been previously shown to support cytoskeletal tension generation. ***Changes of cell and nuclear shape can be inhibited in these non-muscle cells by adding synthetic myosin peptides,*** which interfere with acto-myosin interactions and inhibit both rigor complex formation and tension generation in skeletal muscle. ***Cell and nuclear rounding can be augmented and accelerated by adding soluble RGD-containing peptides,*** which dislodge cell surface integrin receptors from their fibronectin binding sites on the surface of the culture dish. ***These peptides have no effect when calcium and ATP are removed*** (i.e., in the absence of cytoskeletal tension). Using inhibitors of microfilament integrity (cytochalasin D) and microtubule assembly (nocodazole), either alone or in combination, we were also able to demonstrate that ***actin bundles and microtubules act as internal support struts in living cells, serving to transduce inward-directed tension into outward extension during cell spreading. While actin bundles are required for spreading, microtubules appear to be redundant support elements*** (they are not required if actin continuity is maintained).

Significance of the Accomplishments

Finding #1: The development of a system in which the amount of tension that cells exert on their adhesion sites can be controlled by varying cell-ECM contact formation should allow us to dissect the mechanism by which physical forces are transmitted across the cell surface. This system has already been used in our laboratory to demonstrate that tension-dependent changes of cell shape are responsible for control of cell growth and differentiation. Now we can use this model system to determine whether transmembrane

ECM receptors (i.e., integrins) mediate force transmission across the cell surface and to analyze how these forces affect cytoskeletal filament organization and polymerization inside the cell.

Finding #2: We now have a simple assay working in the laboratory that can be used to measure changes in actin polymerization in response to changes in ECM contact formation. This technique can now be used to determine whether externally applied forces (e.g., by stretching an elastic substratum) produce changes in actin assembly and whether the effects on actin can be modulated by varying the number of ECM contacts that can transmit force. We can also begin to relate changes in actin polymerization to alterations in cell and nuclear function (e.g., growth, or expression of differentiation-specific genes).

Finding #3: The demonstration that cells utilize a tensegrity mechanism for cytoskeletal organization is important for multiple reasons: (1) it clarifies that cells sense changes in a balance of pre-existing forces rather than recognize the presence or absence of a force (e.g., gravity); (2) it suggests that we should focus future studies on complementary force interactions between different cytoskeletal filaments and ECM receptors rather than searching for a single molecular force transducer; (3) it raises the possibility that mechanical forces may alter growth and gene expression based on their ability to be transmitted through the cytoskeleton and to the nucleus; and (4) it may provide a basic mechanism for force sensation and transduction that could be utilized by many adherent cells.

Publications

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SHORT LATENCY VESTIBULAR RESPONSES AND THE ONTOGENY OF VESTIBULAR FUNCTION

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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 Earth. Studies will be undertaken to examine how the gravitational vector strength and direction may modulate or influence normal responses to transient stimuli. Efforts will also be 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 an 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 in both anesthetized and unanesthetized animals for the first time. Salient features of these responses include short latencies (4 to 7 peaks occurring within 8 ms following the stimulus) and resistance to intense auditory masking (96 to 104 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. Pilot studies evaluating the effects of linear sine wave motion on response threshold support the hypothesis that responses are generated by otolith end organs. The research has demonstrated that responses to pulsed linear acceleration in birds are vestibular in origin and likely represent compound action potentials of the vestibular nerve and central relays. Although the precise vestibular generators are not yet known, our results suggest that the lagena contributes little to response components in birds.

Concurrent studies in rats demonstrated that similar responses could be obtained using pulsed linear acceleration stimuli. These responses occurred within 8 ms following the stimulus, had amplitudes less than $10 \mu v$, and were resistant to high intensity auditory masking (up to 105 dB SPL). As in birds, response latencies and amplitudes were systematically dependent upon stimulus intensity. Moreover, response threshold decreased as a function of the rate of change in acceleration (i.e., da/dt) in both species.

New research described in this report sought to: (1) establish an independent, $1.0 g$ laboratory control group for avian (Ross x Ross) microgravity studies and document hatchling maturation profile for the first 3 post-hatch weeks, (2) identify peripheral and central components of vestibular responses to pulsed linear acceleration in birds, and (3) demonstrate that responses to pulsed linear acceleration in rats depend exclusively on the VIIIth nerve by performing labyrinthectomies. The effects of selective cochlear destruction in rats remains a current research focus.

Accomplishments

Eighteen Ross x Ross chicks were studied over a 4 wk period after hatching. Vestibular response thresholds, onset latencies, and amplitudes were measured. Pilot studies in E19 embryos were also completed. Data are being processed to document functional changes during development.

Based on a number of findings, I have proposed that the earliest components of short latency vestibular responses in birds reflect the neural activity of vestibular primary afferents, whereas response peaks beyond P2 are likely of central origin. This hypothesis was explicitly tested by surgically isolating the central roots of primary afferents. Doing so virtually eliminated all peaks beyond P2, sparing P1 and N1. These studies clearly demonstrate that *the earliest vestibular response peaks (P1-N1) are generated by vestibular primary afferents and that the peaks therefore represent compound action potentials of the vestibular nerve of the bird.*

Complete bilateral neural blockage of the VIIIth nerve was accomplished in rats using the neurotoxin tetrodotoxin (TTX). *Noninverting components were abolished following complete bilateral blockade, thus demonstrating for the first time in rats that short latency responses to pulsed linear acceleration depend exclusively on VIIIth nerve neurons.*

Significance of the Accomplishments

We continue to study developmental changes in vestibular function using a new physiological test. It is critical that we definitively establish the vestibular origins of these response tests at all ages, both in mammals and in birds. The results presented here add significantly to our understanding of the origins of vestibular responses and to our understanding of vestibular development.

Publications

Fermin, C.D., Vellinger, J., Deuser, M., Craven, J., Martin, D., and Jones, T. 1990. Temporal bone analysis of E14 chicks flown in the STS-29 (Abstract). *ASGSB Bulletin* 4(1): 74. (GWU 13551)

Jones, T.A. 1990. Vestibular compound action potentials in unanesthetized animals (Abstract). *ASGSB Bulletin* 4(1): 93. (GWU 13494)

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Figure 1. A photomicrograph of a single sarcolemma intact slow (Type I) fiber from a soleus muscle stained with acrydine orange for nuclei. This micrograph was taken using a Leitz confocal microscope to permit measurement of nuclei through the depth of the muscle fiber. Mag. 500x.

by using ionizing radiation to preferentially ablate titin and nebulin from isolated myofilaments. Removal of these filaments causes the myofibril to become much less elastic and to 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. In the atrophic muscle, we found that the titin doublet moves during atrophy in conjunction with changes in Z band width. This movement of the doublet may temporarily weaken the myofibril during the phase of adaptation and predispose it to rupture during periods of increased load.

Finding #3: The metabolic, membrane, and structural proteins in muscle are regulated by the myonuclei scattered throughout the entire length of a fiber. Although the translational products assemble into consistently organized sarcomeric structures that function as a single cellular entity, little is known as to how this organization is maintained. Although conclusively unproven, it appears likely that the cytoplasmic domain that can be supplied by a single nucleus is limited. It was reported that mRNA from a nucleus remains close to that nucleus, a finding consistent with the concept of "nuclear control domains." However, little is known about how the overall demands of the cell are delegated to the appropriate myonuclei, or how these myonuclei communicate with each other in order to carry out the global requirements of the cell.

We have demonstrated a difference in the mean volume of nuclear domains in slow and fast muscle fibers in normal adult rats. Our current preliminary evidence also suggests that myonuclei within the same cell may exhibit delegated responsibilities and transcribe

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Significance of the Accomplishments

We continue to study developmental changes in vestibular function using a new physiological test. It is critical that we definitively establish the vestibular origins of these response tests at all ages, both in mammals and in birds. The results presented here add significantly to our understanding of the origins of vestibular responses and to our understanding of vestibular development.

Publications

Fermin, C.D., Vellinger, J., Deuser, M., Craven, J., Martin, D., and Jones, T. 1990. Temporal bone analysis of E14 chicks flown in the STS-29 (Abstract). *ASGSB Bulletin* 4(1): 74. (GWU 13551)

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Jones, T.A., Vellinger, J., Hester, P.Y., and Fermin, C. 1991. Weightlessness and the ontogeny of vestibular function: Evidence for persistent vestibular threshold shifts in chicks incubated in space. *Physiologist* 34 (1, Suppl.): S143-S144. (GWU 13303)

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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 role calcium ions and calcium-mediated physiologic mechanisms play in conjunction with gravity during hypokinesia.

Our work has focused on the atrophy-associated cellular changes that 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, and fiber debris in the intrafascicular and intrafibrillar spaces of the soleus.

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

Accomplishments

(1) *Spatial analysis of skeletal muscle during recovery from atrophy.* 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. We previously found that in normal muscles the fiber type pattern of actively interspersed fiber types persists in atrophic muscle following hindlimb unloading. Therefore, it is of interest to determine whether fiber types continue to be distributed randomly in the atrophic muscle during the period of exercised and non-exercised recovery from hypokinesia.

We sought to determine: (a) whether there were any nonrandom spatial patterns in exercised control adult rat soleus muscle, and (b) the spatial patterns of fiber types of exercised and non-exercised soleus muscle following 28 days of unloading relative to control. Specifically, our purpose was to determine whether fibers of any type tended to be adjacent to fibers of the same or any other type, and whether any fiber type tended towards non-random distribution in association with fascicle boundaries. Previous work demonstrated that Type IIC fibers appeared to cluster during the recovery period; however, this was a qualitative and not a quantitative description of the phenomena.

The frequencies at which muscle fibers of one type were adjacent to each other and to fibers of other types were tabulated and compared to expectations generated from Monte Carlo simulations. In the normal rat, there is a tendency for type I fibers to avoid each other, a tendency that persisted in the hindlimb unloaded group, despite the substantial shrinkage in

size of type I fibers. This tendency also persisted in the control exercise and the sedentary recovery groups. The results indicate that exercise during recovery following unloading induced a muscle fiber composition not seen in a sedentary recovery group or in control rats exposed to the same exercise regimen. We conclude that ***exercise of skeletal muscle during recovery following atrophy, unlike neurogenic pathologies, does not cause any remodeling, in the sense of changed adjacency relations among fiber types.***

(2) ***Titin banding patterns in atrophied skeletal muscle.*** Single fibers from control and atrophied soleus and plantaris muscles were dissected in a relaxing solution and were then mechanically peeled (sarcolemma removed), longitudinally split in half, and placed on calfskin coated coverslips. Stained titin doublets were measured in addition to sarcomere lengths. ***A ratio of titin spacing:sarcomere length revealed that there was a significant difference ($p \leq 0.05$) between control slow soleus and atrophied slow soleus muscle fibers, as well as between control fast plantaris and atrophied fast plantaris muscle fibers.*** Control slow soleus fibers were 0.380 ± 0.004 SEM, while atrophied slow soleus fibers were 0.340 ± 0.006 SEM. Control fast plantaris ratios were $0.290 \pm .004$ SEM, while atrophied fast plantaris fibers were 0.356 ± 0.009 SEM. Results are consistent with previous morphometric data from ultrastructural studies that have shown that the width of Z bands vary between control fiber types, with slow (type I) fibers the widest at 1445 Å and fast (type II) fibers narrower at 611-881 Å, and that Z band width changes as fiber type characteristics shift during adaptation of muscle.

(3) ***Nuclear control domains in skeletal muscle.*** Using the intact single skeletal muscle fiber technique, fiber segments from control soleus and plantaris muscles of adult female rats were mechanically dissected and analyzed for nuclear control domains by fiber type. Each fiber was typed as fast or slow either with histochemical myofibrillar adenosine triphosphatase (ATPase) staining or immunohistochemically with slow myosin heavy chain monoclonal antibodies. The longest fiber segment (3 mm long) was stained with hematoxylin and eosin or with acridine orange for determination of the number of myonuclei, the cytoplasmic volume, and the sarcomere spacing, using conventional and confocal microscopy (Figure 1). A relatively uniform distribution of myonuclei at lengths of up to 3 mm in the same fiber was found in 11 of 12 fibers, regardless of type. The one exception was a fast fiber possibly in the region of its endplate. In slow fibers, linear rows of usually more than eight, and in some cases more than thirty, myonuclei were observed. In fast fibers, rows of myonuclei were seen less often and a row usually consisted of only 2-5 myonuclei. In both slow and fast fibers, myonuclei in linear rows tended to be more ellipsoid and similar in shape than those myonuclei not arranged in rows. These results demonstrate that with respect to myosin type, ***the shape and spatial distribution of myonuclei are interdependent, suggesting some functional significance to their morphological features and transcriptional activities.***

Significance of the Accomplishments

Finding #1: Our findings indicate that the morphologic appearance of clustering produced by exercise during recovery following hindlimb unloading does not appear to be neurologically mediated, but seems due solely to myogenic variables.

Finding #2: Titin is the largest protein in skeletal muscle ($\geq 1 \mu\text{m}$ long and approximately 3 million daltons in mass). In the adult myofibril, titin molecules span from the M line to the Z line. This network forms a third filament system for the myofibril, which assists in sarcomeric alignment and elastic recoil. Direct evidence of this elastic role was described



Figure 1. A photomicrograph of a single sarcolemma intact slow (Type I) fiber from a soleus muscle stained with acrydine orange for nuclei. This micrograph was taken using a Leitz confocal microscope to permit measurement of nuclei through the depth of the muscle fiber. Mag. 500x.

by using ionizing radiation to preferentially ablate titin and nebulin from isolated myofilaments. Removal of these filaments causes the myofibril to become much less elastic and to 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. In the atrophic muscle, we found that the titin doublet moves during atrophy in conjunction with changes in Z band width. This movement of the doublet may temporarily weaken the myofibril during the phase of adaptation and predispose it to rupture during periods of increased load.

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We have demonstrated a difference in the mean volume of nuclear domains in slow and fast muscle fibers in normal adult rats. Our current preliminary evidence also suggests that myonuclei within the same cell may exhibit delegated responsibilities and transcribe

distinctly different mRNA species. It has been postulated that the nuclear matrix may play a critical role in the synthesis and processing of RNA. We extend these concepts to indicate that specific myonuclear shapes represent a unique chromatin arrangement and may indicate a particular genetic activity. Thus, observations of identically shaped myonuclei in the same row are believed to indicate identical chromatin organization, implying similar genetic functions. On the other hand, extra-row myonuclei having different shapes, i.e., those near the endplate, may be associated with different chromatin structures and different transcriptional activities. It is speculated that different myonuclear shapes could reflect different genetic duties of myonuclei, even within the same cell region.

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

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Description of Research

This research intends to describe the basic molecular biology and biochemistry, morphology, and ultrastructure of a bone cell culture system to be utilized at a later date for flight experiments under zero gravity conditions. We are currently characterizing the culture system under normal gravity. Cultures are derived from osteoblasts of chicken calvaria, and the growth and development of the bone cells and the onset and progression of mineralization in the culture extracellular matrix are studied. We are interested in 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. More recently, we have shown that the kinetics of collagen crosslink formation are found to correlate directly with increases in collagen fibril diameters in the culture extracellular matrices; the presence of remodeling or collagenase activity appears to occur in culture, affecting newly synthesized collagen molecules; osteopontin, a 66 kDa phosphoprotein found in our cultures, is associated with initial extracellular mineral foci in the system and contains an arginine-glycine-aspartic acid sequence among its amino acids; and strontium in low concentrations substitutes in a proportional manner for calcium in the extracellular matrix of the bone cell cultures.

Accomplishments

Most recently, studies with the culture system have provided the following results:

(1) We have examined the mineral deposits present in our bone cell cultures by a number of electron optical methods, including electron diffraction and topographic imaging. The latter technique provides a direct view of microscopic ultrastructure in three

dimensions and our studies with such imaging are the first reported in detail of biological material. The investigations show that *the mineral from the cultures is similar in its size, shape, surface texture, crystallographic properties, and relation with collagen as that determined from bone in vivo.*

(2) We have defined conditions for cell growth and development, hypertrophy, and mineralization of a chondrocyte cell culture system as an extrapolation of our work with bone cell cultures. The chondrocyte culture utilizes ascorbic acid and β -glycerophosphate in a nutrient-enriched medium and has been characterized by biochemical and microscopic means over a 21-day time period. We found that collagen types II and X synthesis increased during the first 2 days of culture and that thereafter type II synthesis decreased while type X synthesis rose. Measurement of collagen and proteoglycan message levels indicated pretranslational control of their respective syntheses. Electron microscopy of the cultures at various time points demonstrated the presence of a developing hypertrophic phenotype and a matrix consisting of type II collagen, proteoglycans, and vesicles. Mineral deposition was conspicuous by day 17 and occurred reproducibly in the cultures a few days earlier. Mineral was a poorly crystalline hydroxyapatite and was ultimately associated with the collagen fibrils. The data indicate that *this chondrocyte culture system assembles and mineralizes as does normal growth plate cartilage and should be useful as a model describing its developmental events.*

(3) We have reviewed certain aspects of mineral deposition in bone and other vertebrate calcifying tissues in order to describe physical, chemical, and biological factors important in the mineralization process. Mineralization is found to occur at spatially independent sites throughout the organic extracellular tissue matrices. Matrix vesicles and collagen fibrils each may serve as independent nucleation centers for mineral. Collagen fibril organization is suggested to be such that hole zones are aligned in three dimensions, creating extensive channels for mineral accommodation. Nucleation occurs initially in hole zones, and crystal growth leads to the development of plate-like mineral particles having particular orientation, disposition, and sizes within fibrils. Diffusion, crystallinity, and critical nucleation and growth events influence mineral deposition in bulk and local regions of tissue matrices.

(4) Mineralization of the bone culture system occurs in association with collagen, and the interaction, size, shape, and distribution of mineral crystals within fibrils have been studied at the NIH Biotechnology Resource Center in Albany, NY, by high voltage electron microscopy and three-dimensional computer graphic image reconstruction. We have newly developed some of the microscopic techniques for application to both tissue *in situ* and the bone culture system. The approaches afford a unique and direct three-dimensional view of ultrastructure. We have determined a number of characteristics that are important in describing the basic mechanism of vertebrate mineralization. Our results have shown definitively that the mineral crystals are thin platelets rather than rod-shaped crystals, arranged generally parallel to one another within collagen hole zones, with the region of the fibrils ostensibly serving as initial nucleation sites for mineral. Further, platelets develop in length (along their crystallographic *c*-axis) and width but not significantly in thickness, and larger crystals appear to form in a coplanar fashion from the fusion of smaller units. The size, shape, location, and arrangement of the mineral lead to the suggestion that adjacent collagen fibrils are well aligned with their hole zones in register, a situation which creates channels or grooves for mineral accommodation.

(5) Continuing collaboration with Dr. Marc Grynpas (Mt. Sinai Hospital and the University of Toronto, Ontario, Canada) has utilized strontium to mimic the action of calcium and to act as a measure of the kinetics and rate of mineral deposition in the culture

extracellular matrix. Cultures were grown in the presence of strontium in low concentrations (0.05 to 0.5 mM corresponding to 1:60 to 1:6 Sr:Ca ratios) and, after 30 days, measurements of elemental content were made by inductively-coupled plasma mass spectrometry and electron probe X-ray microanalysis. Strontium was found to substitute in a proportional manner for calcium in the extracellular regions of the bone cells, and the two elements were present in the same local sites of mineral deposition in the cultures. Moreover, the dose range was not toxic to the cells, and no effects on cell growth or mineralization were apparent. The results provide evidence that strontium may be used as a reliable and critical marker for determining the rate of mineral accumulation in collagen.

Significance of the Accomplishments

As we have noted before, our results are significant in characterizing a number of the physical, chemical, and biological events of mineralization in an *in vitro* system closely resembling the mineral deposition of normal calcified vertebrate tissues. Our findings provide insights into details of calcification still incompletely understood and otherwise unattainable with other models. These data, extensively describing the development and growth of bone cells, the assembly of their extracellular matrix, and the deposition of mineral in culture, may be compared with other information obtained from calcifying tissues themselves, and may help explain potential mechanisms of vertebrate mineralization. Our ground-based cultures also provide the experimental controls against which mineralization events may be analyzed from future inflight cultures exposed to zero gravity. From those results, it may be possible to understand the basis of skeletal mass loss and its related effects suffered by humans and experimental animals during spaceflight.

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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

Developing amphibian embryos show a natural gravity response. Initially, fertile eggs rotate with respect to the gravitational field such that their vegetal (lightly pigmented) hemisphere faces towards the center of the Earth. Subsequently, polarity of the future embryos can be influenced by experimental exposure to various force environments ranging from simulated weightlessness to hypergravity and by novel orientation, such as inversion, of the embryo with respect to the gravitational field. The large size of amphibian eggs, their external development, their polar distribution of cytoplasmic components, and their ease of experimental manipulation make early amphibian development a versatile and unique model system to study how embryos sense gravity and transduce gravity information.

In our investigations utilizing gravity as an experimental tool to understand the biology of developmental pattern formation, we made the discovery of an additional early amphibian natural gravity response. Early amphibian cleavage pattern (and therefore the size of the early blastomeres) is determined by the gravitational field. We have documented this phenomenon and its developmental consequence utilizing fertile *Xenopus laevis* eggs exposed to various experimental gravitational fields — simulated weightlessness (horizontal clinostat rotation) and simulated hypergravity (centrifugation).

Accomplishments

(1) *The location of the third (horizontal) cleavage furrow varies in direct relationship to the applied gravitational field.* We have developed a quantitative index for the location of the third horizontal cleavage furrow (animal vegetal cleavage ratio - AVCR). Embryos exposed to simulated weightlessness (μg) on horizontal clinostat rotation at 6 rpm had third cleavage furrows closer to the equator, and embryos exposed to hypergravity on a centrifuge (up to five times gravity — 5 g) had third cleavage furrows closer towards the animal pole than controls at one times gravity (1 g) (Figure 1). These results prove that under natural conditions the location of the third horizontal cleavage furrow is influenced by gravity. This gravity response is probably a universal amphibian phenomenon. Preliminary data showed a similar response to experimental gravitational fields among *Rana pipiens* and axolotl embryos.

(2) *Individual spawnings of eggs and individual eggs within a spawning show considerable variation in the location of the third horizontal cleavage furrow (AVCR).* Each spawning had a distinctive mean AVCR and a distinctive response to the applied gravitational field (amount of AVCR change in response to μg and 3 g exposure).

(3) *Gravity influences morphogenesis to the midblastula stage.* Fertile eggs exposed to μg , 1 g, and 3 g until the midblastula stage showed dramatic gravitational field-specific morphology. Location of the blastocoel, the thickness of and number of cell layers in the animal hemisphere cap, the size of animal and vegetal hemisphere blastomeres,

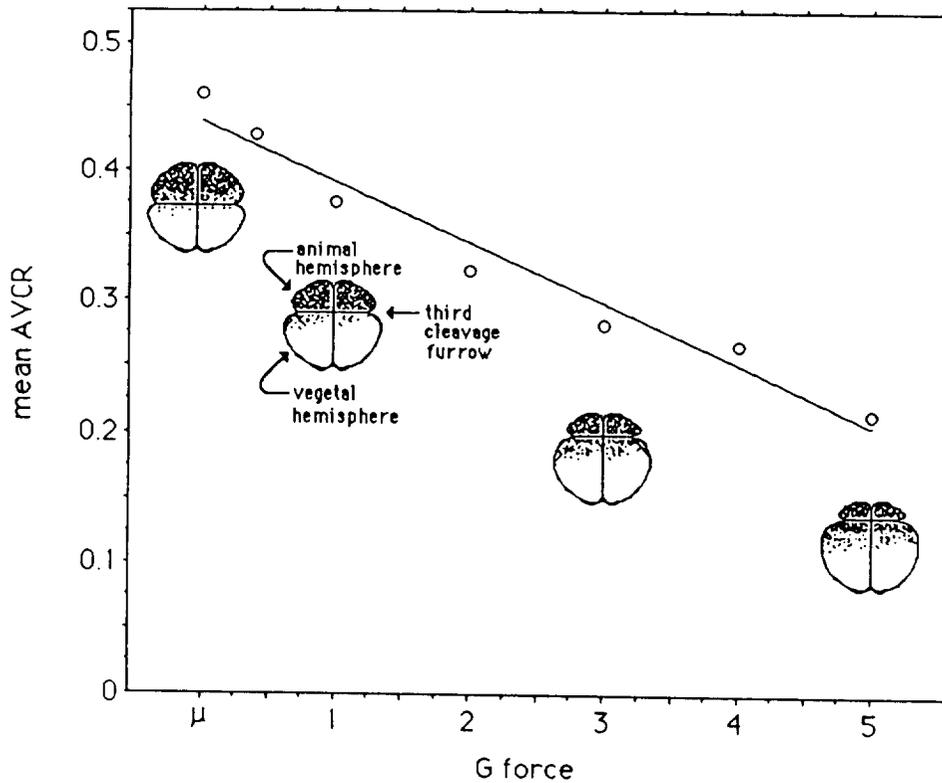


Figure 1. Direct relationship between the location of the third (horizontal) cleavage furrow (AVCR) and increasing strength of the gravitational field (μg to 5 g).

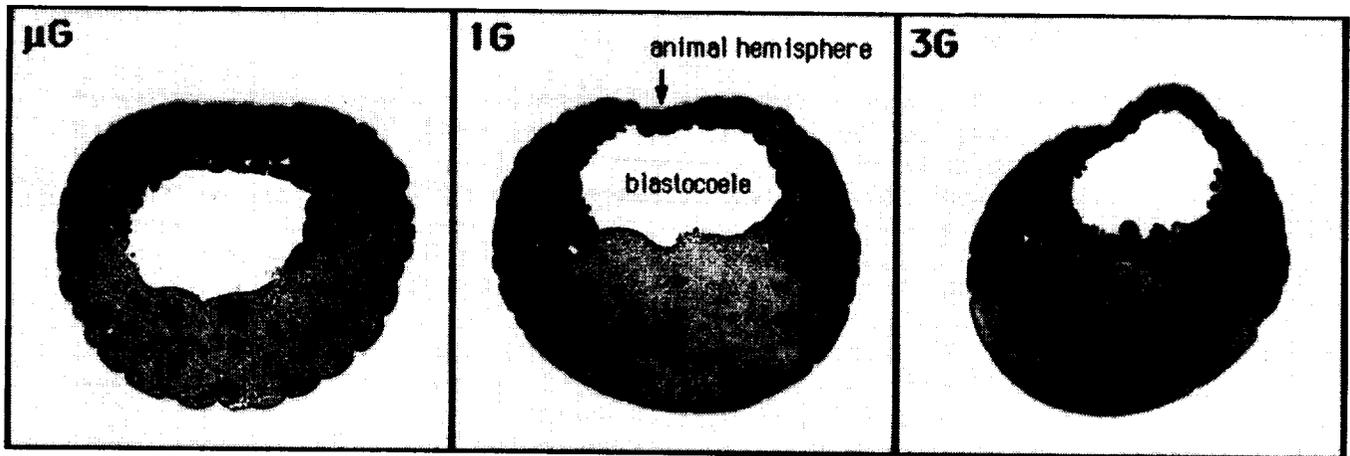


Figure 2. Exposure of fertile eggs to simulated weightlessness and to hypergravity to the midblastula stage dramatically alters their morphology. μg = simulated weightlessness on horizontal clinostat at 6 rpm; 1 g = desk top control; 3 g = hypergravity on centrifuge.

and the distribution of subcellular components such as yolk platelets was affected. Microgravity blastulas had more centrally located blastocoels and thicker and more cellular animal caps (~4 cell layers) than 1 g controls (~2 cell layers) and 3 g blastulas (~1 cell layer) (Figure 2).

(4) *The gravitational field-induced alteration in blastula morphogenesis becomes normal as development proceeds.* Fertile eggs exposed to μg , 1 g, and 3 g until the early gastrula stage showed distinctive gravitational field-influenced morphology. The location and shape of the dorsal lip of the blastopore was affected. Compared to controls (1 g), μg dorsal lips originated closer to the vegetal pole and 3 g dorsal lips originated closer to the equator. However, the number of cell layers in the animal caps of μg , 1 g, and 3 g gastrulas regulated toward the normal 2 cell layers. By the heartbeat/hatching stage only subtle differences in the anterior/posterior axis can be detected. For example, μg tadpoles have larger heads and larger eyes than 1 g and 3 g embryos, and μg , 1 g, and 3 g feeding tadpoles are grossly indistinguishable.

Significance of the Accomplishments

We have documented an additional natural gravity response during amphibian development (Findings #1 and #2). The fact that the location of the third (horizontal) cleavage furrow is gravity-sensitive means that gravity can be utilized as a tool to investigate pattern formation during early amphibian development. We have also made the important discovery that in a developmental sequence of events some of these events may be gravity-sensitive (Finding #3) whereas others may be gravity-insensitive (Finding #4). During the gravity-insensitive phase embryos regulate toward normal, which could mask gravity-sensitive events (Finding #4). This observation has implications for future gravitational studies on early embryogenesis. These studies will require consideration of the possibility of modular gravity sensitivity and will require an understanding of the physical and biochemical basis for this gravity sensitivity as well as an understanding of the physical and biochemical basis for regulation.

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EFFECTS OF MICROGRAVITY ON MICROBIAL PHYSIOLOGY AND ANTIBIOTIC SENSITIVITY

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Description of Research

The long-range goal of this research is to investigate the effects of extended microgravity during shuttle flight on a number of microbial physiological parameters, including sensitivity to antibiotics and other agents of therapeutic value. To achieve a successful inflight experiment, selection of appropriate technology, microbial strains, physiological parameters, antibiotics, and experimental conditions requiring minimal crew time is necessary. Research and developmental efforts to accomplish these objectives have been identified as Phase I, the current project brought to conclusion this past year. The second and final phase will be to conduct inflight studies designed using information gained in Phase I. Various physiological parameters targeted for study are microbial virulence factors, sensitivity to a battery of therapeutic antibiotics, and production of catabolic enzymes. These studies will be accomplished by incubating a viable suspension of microbes with various biochemical substrates during an STS shuttle flight with automated periodic monitoring of the resultant reactions. To eliminate extensive handling of viable microbes on-orbit, a method was developed during Phase I to inoculate these substrates on the ground and to subsequently stabilize all metabolic functions until incubation and automatic processing could take place in a microgravity environment.

The research involved selection of candidate microorganisms with proven physiological and metabolic stability following substrate inoculation. Commercial Vitek test cards containing either dehydrated biochemical substrates or antibiotics were inoculated with standardized microbial suspensions. The Vitek test card (9.0 x 2.5 x 0.3 cm) contains 30 biochemical substrates or 30 dilutions of therapeutic antibiotics. An inoculated test card is incubated in the AutoMicrobic System (AMS), and biochemical changes are automatically monitored by electro-optical sensors and compared through a computerized database for taxonomic delineation. Therapeutic drug sensitivity levels may also be established in the Vitek antibiotic test card by comparison of each test well optical density to that of a control well. A downsized AMS prototype model, the AMS II, has been successfully designed and tested in the laboratory and in simulated microgravity aboard KC-135 flights. This equipment will be used for processing the Vitek test cards during a shuttle flight. Completion of Phase II, inflight studies, will provide basic information about microbial growth and antibiotic sensitivity in microgravity while utilizing a technology intended for eventual use on Space Station Freedom.

To qualify for the shuttle experiment, it was realized that use of preinoculated cards could be successful only if the delineating physiological characteristics of the test strains were not adversely impacted during refrigerated storage at 4°C for at least 10 days. In-house laboratory testing has been ongoing to select candidate microorganisms that satisfy this qualification for the shuttle experiment. Refrigerated test card results have been compared with baseline data for the following parameters: (1) validity of organism identification; (2)

percent probability of the identification; (3) time required for final results; (4) individual substrate reaction evaluation; and (5) minimal inhibitory concentration (MIC) value of the antibiotic.

Eleven strains of reference microorganisms obtained from American Type Culture Collection (ATCC) and six clinical isolates were selected for extensive testing. For each organism, refrigerated card results had to agree with baseline AMS identification over at least a 10 day period. Individual substrate reactions were compared with baseline reactions for reproducibility over the refrigerated period.

Accomplishments

Of the seventeen microorganisms analyzed, eight ATCC microbes and three clinical isolates demonstrated stable enzymatic systems and metabolic functions after extended refrigerated storage. Correct categorization of these microorganisms to genus and species level was obtained upon processing the Vitek test cards. These microbes, commonly isolated on Earth from both environmental and clinical settings, are also representative of those expected to be found on Space Station Freedom. Microorganisms selected as candidates for Phase II, inflight studies, are listed in Table I.

Table I. Candidate microorganisms for inflight studies.

Microorganism	Number of Days in Cold-Storage without Significant Physiological Change
<i>Escherichia coli</i> (ATCC 25922)	16
<i>Pseudomonas aeruginosa</i> (ATCC 15442)	14
<i>Pseudomonas cepacia</i> (ATCC 25416)	16
<i>Staphylococcus epidermis</i> (ATCC 12228)	14
<i>Bacillus cereus</i> (ATCC 11778)	13
<i>Candida albicans</i> (ATCC 14053)	17
<i>Klebsiella oxytoca</i> (ATCC 33496)	17
<i>Staphylococcus aureus</i> (ATCC 25923)	16
<i>Staphylococcus aureus</i> (Clinical #910494)	16
<i>Candida albicans</i> (Clinical #39452)	14
<i>Klebsiella pneumoniae</i> (Clinical #39602)	10

Determination of the susceptibility of a microorganism to antimicrobial agents is of paramount importance for the selection of appropriate therapy. The effect of microgravity on the sensitivity of the microbe or on the action of the antibiotic cannot be predicted without extensive research. Nine antibiotics for gram positive organisms and five for gram negative organisms proved sufficiently stable under test conditions to qualify for inflight testing. Those antibiotics qualifying for Phase II inflight studies are listed in Table II.

Significance of the Accomplishments

The significance of selection of these candidate microorganisms and antibiotics is that it affords us the means by which we may evaluate microgravity-induced microbial physiological changes relevant to those obtained on the ground. Furthermore, we may

Table II. Antibiotics qualifying for inflight studies.

Gram Positive Antibiotics	Gram Negative Antibiotics
Ampicillin	Cefamandole
Cephalothin	Cefoxitin
Clindamycin	Cephalothin
Erythromycin	Chloramphenicol
Gentamicin	Trimethoprim-Sulfamethoxazole
Nitrofurantoin	
Vancomycin	
Oxacillin	
Trimethoprim-Sulfamethoxazole	

accomplish this seemingly complicated task automatically, with minimal crew time involvement, by using an instrument intended for Space Station Freedom. As a result of carefully planned and executed research methods, complete standardization has been achieved with the only experimental variable being the absence of gravity. The product of these second-phase inflight studies will be data concerning some 30-50 metabolic systems of 11 clinically and environmentally relevant microorganisms. The observed effect (e.g., failure to metabolize a particular substrate) may be caused by alteration(s) in any component of that metabolic system (e.g., gene replication, gene transcription, RNA translation, enzyme assembly, pathway regulation, or transport). The data may then serve as the starting point for more detailed examination of any metabolic system showing effects from growth in microgravity. The proposed study represents a necessary first step — the detection and identification of cellular "targets" influenced by microgravity.

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 flight 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 upon the length of exposure to unloading, (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; flight experiments will allow gathering of more information to support or negate the hypotheses. During this report period, ground-based studies focused on bone response to skeletal unloading, primarily on the correlation between bone mechanical properties and bone biochemistry during 1-4 wks of unloading. Flight activities included preparation for experiments on the first and second dedicated Spacelab Life Sciences missions and for a middeck experiment.

Accomplishments

(1) The effect of age/growth on bone chemistry and biomechanics in normal and unloaded limbs was investigated. Rats were unloaded at 5.5 wks of age; controls were weight and age matched. Animals were euthanized weekly beginning at 1 wk and concluding at 4 wks. *The data suggest that unloading alters bone metabolism more rapidly than biomechanics, that unloaded bones appear 1-2 wks younger than control bones, that both quality and quantity of bone are altered by unloading, and that cortical and cancellous bone respond differently to unloading.*

(2) Simulation of the SLS-1 tissue harvest was conducted at NASA-Kennedy Space Center and at NASA-Ames Dryden Flight Research Facility.

Significance of the Accomplishments

Finding #1 suggests that changes in bone metabolism precede changes in bone biomechanics in unloaded bones. Changes in bone metabolism were detectable within 1 wk whereas changes in bone biomechanics were not significant for at least 2 wks. Interestingly, the bones from unloaded animals were similar metabolically and biomechanically to control rats about 2 wks younger, suggesting delayed growth and metabolism in unloaded bones.

Finding #2 was necessary to familiarize investigators with both the facilities available and the details of multiple procedures that were being conducted simultaneously.

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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 humans and experimental animals under conditions of weightlessness are the loss of skeletal mass and the attendant hypercalciuria. These changes 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 proteins 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 radiolabelling of proteins, gel electrophoresis, ELISA assays and immunohistochemical staining, and subsequently at the RNA level 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, will provide some insight into how the process might be disrupted under conditions of zero gravity.

Hormonal regulation of collagen synthesis. We have examined the signal transduction pathways involved in the inhibition of collagen synthesis by parathyroid hormone (PTH). For this examination, we have used agents which mimic the action of second messengers. These agents have included the cAMP analogue 8-bromo-cAMP (8BrcAMP), the Ca²⁺ ionophore ionomycin (Iono), and the diacylglycerol analogue (or protein kinase C activator) phorbol myristate acetate (PMA). Our results demonstrate that 8BrcAMP most closely mimics the effects of PTH, suggesting that protein kinase A activation is the major route for PTH inhibition of collagen synthesis. However, Iono was also able to cause a reduction in synthesis of the protein, indicating that Ca²⁺ entry may also be important in the translation of collagen.

Purification of rat collagenase inhibitor(s). Working with Dr. Jeffrey, we have purified the rat collagenase inhibitory activities secreted by the UMR 106-01 cells and have shown them to be two tissue inhibitors of metalloproteinase (TIMPs) molecules of 20 and 30 kDa. Amino acid sequence of the inhibitor molecules from medium conditioned by the rat UMR cells showed that one of these molecules is clearly homologous to TIMP-1 in other species. The second and predominant inhibitor molecule produced by UMR 106-01 cells has been identified as rat TIMP-2, by virtue of its identity to human TIMP-2 in the amino terminal region. This protein is also capable of inhibiting both human and rat collagenases. Treatment with peptide:N-glycosidase-F resulted in an apparent shift of zymographically detected activity of the 30 kDa inhibitor to 20 kDa. No glycosidase-dependent change was observed in the apparent molecular weight of the 20 kDa inhibitor. These results indicate that while the 30 kDa rat inhibitor is glycosylated, the 20 kDa rat inhibitor is apparently not glycosylated. Thus, both molecules are composed of a peptide of approximately 20 kDa with considerable amino acid homology in the N-terminal region.

Regulation of collagenase mRNA. We previously demonstrated that stimulation of collagenase by PTH involved a substantial increase in collagenase mRNA via transcription. Northern blots and nuclear run-on assays were performed to further investigate the induction of collagenase by this hormone in the UMR 106-01 cells. Detectable amounts of collagenase mRNA were not apparent until 2 hrs of PTH treatment, showed the greatest abundance at 4 hrs, and declined to approximately 50% of the maximum by 12 hrs. The transcriptional activity of the collagenase gene in response to PTH exhibits a pattern similar to the steady state mRNA levels over the same period of time. After an initial lag period of between 1 and 2 hrs, collagenase transcription rates increased from nearly undetectable levels to a maximal response at 2 hrs, returning toward control levels by 10 hrs. The PTH-mediated induction of collagenase transcriptional activity was completely abolished by cycloheximide, while transcription of the β -actin gene was unaffected by the translation inhibitor. These data suggest that a protein factor(s) is required for PTH-mediated transcriptional induction of collagenase.

Since PTH increases intracellular levels of several potential second messengers, agents that mimic these substances were employed to determine which signal transduction pathway is predominant in the PTH-mediated induction of collagenase. PMA and Iono were ineffective at increasing the transcriptional activity of the collagenase gene, whereas 8BrcAMP could reproduce the PTH effect. PMA, but not Iono, augmented the increase in the transcription rate of collagenase produced by a sub-maximal dose of 8BrcAMP. We concluded that PTH induces collagenase transcription primarily through the cAMP-dependent pathway in rat osteosarcoma cells and requires the expression of other genes.

Our next goal is to isolate the regulatory regions of the rat collagenase gene. To do this, we have screened a rat Sprague-Dawley genomic library in Lambda DASH II using the HindIII fragment of UMRCase54, which contains the entire coding region of rat collagenase as the probe. We have used oligonucleotide probes corresponding to various regions of the cDNA clone to confirm and characterize two overlapping clones, 600-10 corresponding to all of the 5' cDNA sequence and 600-12 the 3' sequence. 600-10 includes approximately 7 kbp of sequence upstream of the transcriptional start site, which we expect to contain the hormone response elements and regulatory regions. At this time, a 2.2 kbp region of 5' upstream sequence is being characterized. This region will be subcloned upstream of the chloramphenicol acetyl transferase (CAT) gene and processive deletions will be made to determine the minimum amount of sequence necessary for PTH, 8BrcAMP, and 8BrcAMP plus PMA induction. We will also create stable transfectants which could be used in flight experiments to assess transcriptional rate.

Immunohistochemical detection of collagenase in sections of bone from Cosmos 2044 flight animals. The amount and distribution of collagenase was determined in calvaria of 107-131 day-old adult rats flown on Cosmos 2044 and of respective ground controls. The enzyme was detected in frozen sections by an immunohistochemical technique and cellular architecture was assessed by counterstaining with Mayer's hematoxylin. No difference was observed in the quantity or distribution of the enzyme, which was seen to be associated with cells lining the marrow spaces, lining the osteocytic canaliculi, and deposited in the matrix. This concurred with previous observations that collagenase is a product of osteoblastic cells. The recovery time may have been too great (8-11 hrs) and any differences in the flight animals may have been negated by this length of time. Morphologically, there appeared to be a difference in the calvaria of flight animals and hindlimb unweighted rats: in the first group the matrix seemed wider than in controls; in the second group, the marrow space appeared enlarged. It is possible that the shift in blood flow and the change in loading affects the morphology of the calvaria.

Accomplishments

(1) *Showed that protein kinase A activation is the major route for PTH inhibition of collagen synthesis.*

(2) *Purified and identified the rat TIMPs secreted by an osteoblastic cell.*

(3) *Showed that PTH induces collagenase transcription primarily through the cAMP-dependent pathway in rat osteosarcoma cells and requires the expression of other genes.*

(4) Isolated and mapped genomic clones for rat collagenase.

(5) *Showed a change in morphology of calvaria from flight animals.*

Significance of the Accomplishments

Finding #1 suggests that protein kinase A (PKA) activation is the primary mechanism for action of PTH in the osteoblast.

Finding #2: Rat osteoblastic cells synthesize two TIMPs that have close similarity to those identified in other species. The purification and amino acid sequence data gives us a means to prepare antibodies to the two inhibitors and to clone their genes.

Finding #3 supports our observations with collagen that PKA activation is the primary mechanism for action of PTH in the osteoblast, and suggests that other transacting genes are activated by this pathway. This finding will lead us to try to determine how nuclear proteins activated by PKA regulate expression of other DNA-binding proteins.

Finding #4: Isolation of the collagenase gene and upstream sequence will provide us with material to support our efforts to determine which transacting proteins regulate this gene.

Finding #5 suggests that the shift in blood flow and the change in loading that occur during flight lead to thickening of the matrix of the calvaria.

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GRAVITATIONAL EFFECTS ON EARLY DEVELOPMENT IN AMPHIBIAN EMBRYOS

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Description of Research

The long-range goal of this research is to identify the gravitationally sensitive mechanisms involved in amphibian development, concentrating on the establishment of pattern in the nervous system. This objective is being met by the development of molecular probes for early events associated with the process of differentiating cellular territories. Two types of molecular probes have been constructed. First, monoclonal antibodies were made that recognize a cell surface antigen expressed only on a subset of the ectoderm, the epidermal ectoderm. This monoclonal antibody, Epi 1, was used to study the source of inducing tissue and the mechanisms of signal delivery that normally establish the early general pattern of the nervous system. The second class of molecular probes are recombinant cDNA clones which represent RNAs localized to the dorsal blastopore lip area or RNAs synthesized only on the dorsal-most side of the embryo. These two molecular probes have allowed us to analyze the spatial rearrangements of a single species of molecule during the developmental period when the embryonic axes are being established. These probes have also allowed us to study the developmental consequences of the normal cytoplasmic rearrangements in a terrestrial environment, and they will allow us to study the developmental effects of cytoplasmic rearrangements in a non-terrestrial environment. The use of molecular probes, such as those described here, and the opportunities provided by the U.S. space program have enabled us to gain insights into the role of gravity during normal development and have enabled us to begin speculating about the role of gravity in the evolution of several species of animals.

The work originally proposed for these studies involved the amphibian *Xenopus laevis*. However, the recombinant cDNA probes representing localized RNAs are being tested for cross-reactivity in other species that are suspected of using gravity in establishing their early embryonic axes. Fish, birds, reptiles, and amphibians have all been shown to utilize gravity in establishing their early embryonic axes. Most notably, quail embryos are being tested for cross-reactivity with the recombinant clones for use in a potential collaboration with Russia.

Accomplishments

(1) *We have used the monoclonal antibody Epi 1 to demonstrate that neural induction occurs by at least three mechanisms*, including the mechanism which has been accepted as the only means of neural induction for the last 60 years. We have shown that a diffusible signal emanates from the dorsal blastopore lip region of a gastrulating embryo. This diffusible signal, which is part of the normal process of neural induction, establishes a prepattern for the development of the nervous system. Subsequent inducing events act on the prepatterned ectoderm to define specific cellular territories that will eventually form portions of the brain and spinal cord. The cells that are capable of sending the prepattern signal, the cells of the blastopore lip region, are determined by the cytoplasmic rearrangements occurring prior to the first cleavage cycle. It is these same cytoplasmic rearrangements that are sensitive to gravitational alterations in the environment.

(2) We have also constructed a recombinant cDNA library corresponding to RNAs isolated from the dorsal blastopore lip region during gastrulation. The dorsal blastopore lip library was screened, and clones representing RNAs either specific to the blastopore lip or highly enriched in the dorsal blastopore lip region were picked for further study. To date, over one million clones have been screened and 42 of these clones have been selected for further study. Developmental northern (developmental time course of RNA concentration) probed with these 42 clones indicate that *sixteen clones represent maternal RNA localized to the dorsal side of the embryo during the first cleavage cycles*. These clones provide excellent probes to study the movement of specific RNAs during the gravity driven cytoplasmic rearrangements. We are collaborating with Dr. Michael Danilchik, who has performed an *in situ* hybridization analysis with one of the cloned probes. *Clone #49 shows a very distinctive pattern of spatial rearrangements during oogenesis and early embryonic development*. We are in the process of doing *in situ* hybridization on embryos subjected to altered gravitational environments during the first cell cycle, when gravity influences the position of the dorsal-ventral axis. A collaboration on a flight mission with Dr. Steven Black is planned for a shuttle flight in which *Xenopus* embryos fertilized on the shuttle will be analyzed by *in situ* hybridization with as many of the cloned probes as we have studied by then.

Significance of the Accomplishments

Finding #1: The work with the monoclonal antibody Epi 1 has demonstrated that the process of neural induction is much more complex than previously believed. The mechanisms responsible for the neural induction process have a far more elaborate check and balance system than could have been predicted under the old model for neural induction. In addition, the Epi 1 probe has allowed us to demonstrate that the embryo has evolved at least one mechanism that is capable of detecting gravitationally influenced developmental abnormalities and of correcting for them up to a certain point. It is important to understand the level of developmental abnormalities caused by environmental stress that the embryo can detect and the mechanisms by which it can correct these abnormalities.

Finding #2: The second significance of these studies is that we have now developed a large array of recombinant cDNA probes which can be used to study the spatial rearrangements of specific molecules during critical developmental time periods. We have recently constructed molecular probes for RNAs localized to the blastopore lip, the cells which ultimately pattern the axes of the embryo. We have recently been able to use *in situ* hybridization to follow the spatial movements of these molecules as they become localized. We can now examine the spatial positions of RNAs, shown to be important in development, within embryos subjected to non-terrestrial gravitational environments. These probes will allow us to study the normal patterns of molecular localization and the mechanisms that are responsible for the localization process on Earth. These same probes will also allow us to begin to analyze, at the molecular level, the mechanistic problems encountered by embryos in altered gravitational environments.

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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. In addition, within hours following an animal's return to a 1 g environment, osteoblast histogenesis begins to recover toward normal levels. Recent evidence from tissue sampled ~55 hrs following return to 1 g showed that preosteoblast production actually exceeded normal levels at this time period — a "supercompensatory" phenomenon which may be likened to that seen in the recovery of muscle glycogen following acute exercise. To date, results from flight experiments (Cosmos 1129, Spacelab 3, and Cosmos 1187) have suggested that the recovery of osteogenic potential following spaceflight is of high physiological priority. The present goals and objectives for research in this area are to: (1) further define the cellular regulation of osteoblast production; (2) determine how this process is suppressed in microgravity; (3) continue to assess the recovery of osteoblast histogenesis following return to 1 g; and (4) assess preosteoblast differentiation with two new methodologies, immunohistochemical labeling and *in situ* hybridization for localizing specific mRNAs.

Previous studies utilized ³H-thymidine DNA labeling, mitotic activity, and nuclear size as indicators of the proliferation and differentiation aspects of osteoblast production. The following 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→A'→C→D→Ob. The increasing nuclear volume (A'→C) is believed to be a morphological manifestation of change in genomic expression (differentiation) and indicative of maturation of osteoblast cells. 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'→C) is stimulated by mechanical (orthodontic) force and inhibited following exposure to microgravity.

Research conducted in the past year has included both flight projects and ground-based preflight experimental development. This work has specifically focused on (1) examination of maxillary periodontal ligament (PDL) specimens from animals flown aboard Cosmos 2044, and (2) development of a nonradioactive, immunohistochemical labeling technique for cellular DNA synthesis.

Accomplishments

(1) Cosmos 2044 was a 13.8 day spaceflight with an 8.5 - 11 hr recovery period at 1 g. For fibroblastlike cells in the periodontal ligament (PDL), no significant differences

were observed in osteoblast precursor cells (A+A') or preosteoblasts (C+D) among the basal, synchronous, or vivarium control groups.

(2) *Comparison of flight (F) to the synchronous (SC) control groups showed no significant differences in the early osteoblast progenitor A+A' cells (F: 28.0 ± 3.7 vs. SC: 27.4 ± 2.2), nonosteogenic B cells (F: 33.1 ± 1.4 vs. SC: 32.4 ± 2.4) or C+D preosteoblast cells (F: 38.4 ± 4.5 vs. SC: 39.2 ± 1.6) cell compartments (mean \pm SEM, n = 5).*

(3) Preliminary studies of 5-bromo-2'-deoxyuridine as a nonradioactive, immunohistochemically locatable label for cellular DNA synthesis have provisionally indicated its suitability as a replacement for the traditional radioactive ^3H -thymidine method (Figure 1).



Figure 1. Photomicrograph of specific peroxidase staining of nuclei containing 5-bromo-2'-deoxyuridine (BDU) as located by immunohistochemical techniques. The stained PDL nuclei of an orthodontically stimulated molar were undergoing DNA synthesis at the time the BDU was given. The arrows indicate regions of BDU staining.

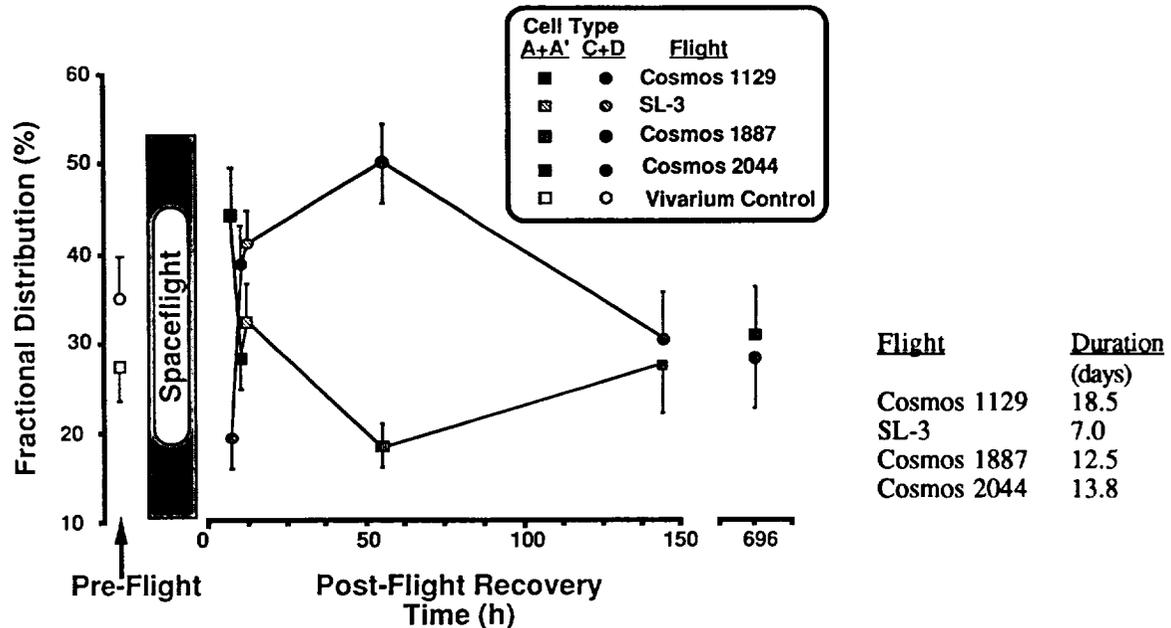


Figure 2. Comparison of all recent spaceflight experiments showing the rapid time-dependent recovery of osteoblast histogenesis. Data are expressed as mean \pm SEM for n = 4-5 animals per data point.

Significance of the Accomplishments

Finding #1: An important result is that no differences were observed between preflight (basal), age matched (vivarium), or flight simulation controls. This indicates there are no confounding variables complicating the assessment of recovery from microgravity.

Finding #2: Cosmos 2044 flight results fit the predicted recovery pattern derived from previous spaceflight experiments (Figure 2). These are important data because they confirm the approximate time-course of recovery established from previous flight experiments.

Finding #3: Development of 5-bromo-2'-deoxyuridine as a nonradioactive label for cells synthesizing DNA is a major technical improvement for assessing cell kinetics of simulated and actual weightlessness. This immunohistochemical method will reduce the risk and clean-up costs of cell kinetic experiments requiring identification of S-phase cells. Furthermore, it will not interfere with analysis of radioactive labels administered for other purposes.

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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 30 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 nerve fibers and cells in three different long series have been mapped. The computer reconstructions are based on digitized tracings of objects from long series of thin sections (570 sections in one series cut at 10 nm, 175 sections in another series cut at 20 nm) that are reassembled as shaded solid objects in a Silicon Graphics IRIS high performance workstation. Additionally, a study was conducted counting synapses in shorter series (50 sections cut at 20 nm) from maculas of rats centrifuged at 2 g for 2 wks, of ground control rats, and of on-center centrifuge control rats. All this work serves as a basis for interpretation of any observed changes in microstructure that may have occurred in maculas of rats flown in June, 1991, on SLS-1, and that may occur in rats to be flown two years later on SLS-2.

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. The question arises as to whether the macula is a continuous network. That is, do synapses occur across the striola, an irregular line that follows the contours of the macula and defines *a pars externa* from *a pars interna*? The stereociliary tufts of hair cells of the two parts have opposite directional polarizations. Our reconstructions and electron micrographs of synapses demonstrate that *there are synapses between type II hair cells and the nerve terminals that supply type I cells with opposing directional polarizations*. This finding is contrary to what many might have expected, but shows that *the neural network is continuous across the macula*.

Studies of serial sections through complete type II hair cells demonstrate that *all efferent-type nerve endings terminate near or opposite subsynaptic cisterns*. *The synapses correspond to c-synapses, described as inhibitory for many other kinds of neurons*. The synapses are thought to function by causing the release of calcium from the cisterns, raising intracellular calcium and causing the opening of potassium channels in the cell membrane. The net effect is that the cell is hyperpolarized, or inhibited.

Our further research indicates that hair cell endoplasmic reticulum (ER) stretches from the apical portion of the cell to its base, is continuous with the nuclear envelope and Nissl

substance, and is in communication (through vesicles) with the Golgi apparatus and with synapses. From place to place in both kinds of hair cells the ER becomes apposed to the cell membrane in subsurface cisterns.

With respect to calyceal and nerve fiber collateral-like processes, our findings continue to indicate that the intrinsic collaterals are significant in information processing. Members of my team are now simulating the electrical responses in processes whose measurements have been obtained from photographic negatives, electron micrographs, and reconstructions. Physiological responses are based upon findings reported in the literature. ***The simulations indicate that the geometry of the process is related to the electrical activity produced, as one would expect.***

A major finding comes from mathematical analysis of hair cell tufts, those bundles of stair-cased, filamentous processes called stereocilia that extend apically from the cell. Analysis of the hexagonal spacings and the angles between the stereocilia strongly suggest that the stereocilia are functionally analogous to man-made phased array antennas. ***Fourier analysis of the tufts shows that non-linearities in their responses to directional inputs as determined by direct experimentation are duplicated by this mathematical analysis.*** Moreover, this same kind of analysis indicates that ***stereociliary tufts of differing configurations also have differing non-linearities in directional tuning.***

Finally, our preliminary findings in maculas from centrifuged rats indicate that ***synapses in type I cells are comparable in size and in number to those in type I cells of both ground and on-centrifuge controls, but that synapses in type II cells are reduced by one-third in number in centrifuged animals.***

Theoretical Interpretations

A fundamental hypothesis that we continue to explore is that the basic organization of maculas corresponds to that in other neural tissue such as the retina and other parts of the brain. The results of the 3-D studies indicate that there are highly 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 but type II hair cells and the intrinsic collateral system comprise the distributed circuitry that modifies calyceal and vestibular nerve output. This interpretation brings maculas into line organizationally with other, more complex neural tissue. The distributed modifying circuit is itself subject to the continuous influence of the highly channeled circuit, and the timing of feedback of the second circuit to the nerve terminals may be critical in determining the coding of the message carried to the next neural station.

A third, non-specific circuit possibly should be separated from the distributed modifying one. Nerve fibers that are possibly of extra-macular origin have boutons that synapse with all neural elements of the macula except for the type I cells. The functioning of this circuit is likely determined by postsynaptic factors, so that asymmetric synapses on calyces and nerve fiber branches should be facilitatory functionally, while c-synapses should be inhibitory. This third circuit is interpreted to provide a background of activity that keeps the system in a ready state for information processing.

Our preliminary finding that synapses are decreased in maculas of centrifuged rats supports the conclusion that it is the distributed modifying circuitry that would be most subject to change in response to an altered g environment.

A new proposal is that the system of hair cell ER is functionally analogous to that occurring in muscle (the sarcoplasmic reticulum) to bring about voltage changes in all parts of the hair cell membrane rapidly in response to a signal. This same ER may be a mechanism for influencing nuclear responses to neurotrophic inputs to the cell.

It is the modeling effort combined with detailed studies of the morphological organization that drives the theoretical interpretations. In turn, because much of what we believe to be true of the functional organization of macular tissue is currently difficult to test electrophysiologically, computer-assisted simulations will continue to provide a test-bed to obtain insights into the validity or non-validity of the proposed theory.

Significance of the Accomplishments

The total effect of our research is that we have a solid base of morphological 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 and will provide a new conceptual framework for studying adaptation to altered gravitational environments. Centrifugation results already are indicating the correctness of the basic assumptions and, moreover, are demonstrating that gravitational force does have an effect on the functional organization of a mammalian gravity receptor end organ.

Publications

Mugler, D.H. and Ross, M.D. 1990. Phased array characteristics and the directional sensitivity of vestibular hair cells. *Proceedings of the International Conference of the IEEE Engineering in Medicine and Biology Society* 12: 1893-1894. (GWU 13576)

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GROWTH FACTOR INVOLVEMENT IN TENSION-INDUCED SKELETAL MUSCLE GROWTH

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Description of Research

Long-term crewed space travel will require a better understanding of skeletal muscle atrophy that results from microgravity. Astronaut strength and dexterity must be maintained for normal mission operations and for emergency situations. Although exercise in space slows the rate of muscle loss, it does not prevent it. A biochemical understanding of how gravity/tension/exercise helps to maintain muscle size by altering protein synthesis and/or degradation rate should ultimately allow pharmacological intervention to prevent muscle atrophy in microgravity.

The overall objective of this research project is to examine some of the basic biochemical processes involved in tension-induced muscle growth. Differentiated avian skeletal myofibers can be "exercised" in tissue culture using a newly developed dynamic mechanical cell stimulator device which simulates different muscle activity patterns. We have found patterns of mechanical activity that significantly stimulate muscle growth and alter myofiber metabolic characteristics. With this experimental *in vitro* system, we are examining the interaction of exogenous and endogenous muscle growth factors in mechanically stimulated muscle growth.

Exogenous growth factors found in serum, such as insulin, insulin-like growth factors, and glucocorticoids, modulate muscle protein turnover rates in mechanically induced muscle growth *in vitro*. Endogenous growth factors such as prostaglandins are synthesized and released into the culture medium when muscle cells are mechanically stimulated. This family of endogenous factors helps to regulate the rates of protein turnover in the muscle cells.

The study of the mechanisms by which muscle mechanical activity interacts with both exogenous and endogenous growth factors to regulate muscle protein turnover rates in this model system will be continued.

Accomplishments

(1) A defined, serum-free medium has been developed (muscle maintenance medium, mm medium) that maintains the myofibers in positive nitrogen balance for 3-5 days, based on myofiber diameter measurements and myosin heavy chain content.

(2) *Insulin and insulin-like growth factor 1, but not insulin-like growth factor 2, induce rapid and pronounced myofiber hypertrophy in mm medium. In 5 days, muscle fiber diameters increase by 51-98%.*

(3) *Mechanical stimulation of the muscle fibers in mm medium increases the sensitivity of the cells to insulin and insulin-like growth factor 1, based on a leftward shift of their dose/response curves for protein synthesis*

A new proposal is that the system of hair cell ER is functionally analogous to that occurring in muscle (the sarcoplasmic reticulum) to bring about voltage changes in all parts of the hair cell membrane rapidly in response to a signal. This same ER may be a mechanism for influencing nuclear responses to neurotrophic inputs to the cell.

It is the modeling effort combined with detailed studies of the morphological organization that drives the theoretical interpretations. In turn, because much of what we believe to be true of the functional organization of macular tissue is currently difficult to test electrophysiologically, computer-assisted simulations will continue to provide a test-bed to obtain insights into the validity or non-validity of the proposed theory.

Significance of the Accomplishments

The total effect of our research is that we have a solid base of morphological 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 and will provide a new conceptual framework for studying adaptation to altered gravitational environments. Centrifugation results already are indicating the correctness of the basic assumptions and, moreover, are demonstrating that gravitational force does have an effect on the functional organization of a mammalian gravity receptor end organ.

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Mugler, D.H. and Ross, M.D. 1990. Phased array characteristics and the directional sensitivity of vestibular hair cells. *Proceedings of the International Conference of the IEEE Engineering in Medicine and Biology Society* 12: 1893-1894. (GWU 13576)

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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, *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) system at ground level conditions in order to establish a data base that will serve as a comparison for 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. (2) Determine the effect 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

We have made considerable progress in preparing a databank "calendar" for the time of appearance and sites of localization of a variety of neuropeptides and neurotransmitters in two closely related species of *Xiphophorus*, *X. helleri* (swordtail) and *X. maculatus* (platyfish). Thus far in our immunocytochemical studies, we have utilized a variety of antisera to gonadotropin releasing hormone (GnRH), gonadotropin, and to approximately 12 other neuropeptides and neurotransmitters. Table I summarizes our most recent immunocytochemical data (some of which is based on preliminary studies) for the localization of these substances in sexually mature individuals of both species of *Xiphophorus*.

Significance of the Accomplishments

The substances listed in Table I, and others reported previously, have been selected for study because of their purported implication in reproductive system development and function in other vertebrates, and especially in mammals. The localization of these substances in regions of the brain and pituitary gland, indicated on Table I, suggests that these substances are involved in the neuroendocrine regulation of reproduction in *Xiphophorus* as well.

The analysis of data such as these will enable us to arrive at a comprehensive understanding of the brain-pituitary-gonad axis in *Xiphophorus* and of the influence of zero gravity on the neuroendocrine regulation of the reproductive system.

Table I. Localization of neuropeptides and neurotransmitters in mature *Xiphophorus*.

<u>ANTIGEN</u>	<u>SITE OF LOCALIZATION</u>	
	<u>Brain</u>	<u>Pituitary Gland</u>
Neuropeptide Y	T, VT, NOR, NPP, VC	NH, CPD, RPD, PI
Dynorphin	OB	RPD, PI
FMRF-amide	NOR	—
Galanin	NLT	NH, CPD, RPD, PI
Neurotensin	—	NH, RPD, PI
Vasoactive Intestinal Peptide	—	—
Bradykinin	—	—
Norepinephrine	—	—
Serotonin	PVO, NPO, VT, VC, NOR?, NPP, P, III	RPD, CPD, PI
Dopamine	NRL, NLT, P, PVO, NOR?	RPD, CPD
Tyrosine Hydroxylase	III, NPO	RPD, PI
Arginine Vasotocin	NPO, PVO, NPP	NH
Somatostatin	NLT, III, NRL	CPD

KEY

Brain Regions

NOR	nucleus olfactoretinalis
NLT	nucleus lateralis tuberis
NPP	nucleus preopticus periventricularis
VC	valvular cerebellum
VT	ventral tegmentum
T	telencephalon
OB	olfactory bulb
PVO	paraventricular organ
III	third ventricle
P	pineal
—	no localization

Pituitary Regions

NH	neurohypophysis
RPD	rostral pars distalis
CPD	caudal pars distalis
PI	pars intermedia
—	no localization

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EFFECTS OF HINDLIMB UNLOADING ON SKELETAL MUSCLE GROWTH

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Description of Research

Our studies have focused on how the weightless environment affects satellite cell behavior in immature growing animals. Satellite cells are important during skeletal muscle growth because they are associated with myofibers, which are responsible for the increase in myonuclei that accompanies myofiber growth. This myonuclear increase is accomplished by the continued mitotic activity of satellite cells during the postnatal growth period and the subsequent fusion of the cells with the enlarging myofibers. A second important function of the satellite cells is to provide a source of myoblasts during a regeneration response following injury. In this sense they are regarded as the stem cells of muscle regeneration. Both of the described functions of satellite cells in normal or injured muscle are dependent upon mitotic divisions of the cells to provide progeny for growth or regeneration. The long-term goal of our studies is to understand the manner in which the proliferative behavior of satellite cells in immature muscles, either in the intact growing system or during muscle regeneration, is altered by the weightless environment.

Our previous studies indicated that when young animals were placed in conditions of hindlimb unloading, muscle growth was markedly retarded. A prominent concurrent feature was a severe reduction in the proliferative activity of satellite cells that was most pronounced in the soleus muscle. The reduced mitotic activity of satellite cells was sustained and appeared to parallel the relative atrophy or reduced growth that was exhibited by the soleus muscle and the less severely affected extensor digitorum longus (EDL) muscle. However, the maximum reduction occurred quickly after initiation of unloading, suggesting that the cells in both the soleus and EDL muscles were responding to the unloading environment before any morphological atrophic changes were evident in the muscles. The goal of our most recent studies is to determine the rapidity of the satellite cell response by monitoring proliferative behavior of the cells at very short intervals after initiation of unloading.

Accomplishments

Immature, growing rats were unloaded at intervals of 1, 2, 3, 5, and 7 days. At the conclusion of each unloading period these animals and the weight-bearing controls received an injection of bromodeoxyuridine (BrdU), a thymidine analogue. One hour following injection the unloaded animals and their appropriate controls were dispatched. The EDL and soleus muscles were removed and processed for light or electron microscopy so the labeling pattern of the satellite cell population could be monitored through immunocytochemistry. A minimum of 1000 nuclei were counted per muscle. The opposite limbs were prepared for electron microscopic study so the changes in the number of satellite cells could be quantitated.

As observed in previous studies, a response of satellite cells to unloading was evident in both the EDL and the soleus muscles, but was always most pronounced in the soleus. Only data from the soleus are presented here. At all intervals, control animals had 5% labeled myofiber nuclei, indicating that satellite cells were dividing and providing

myonuclei to the growing myofibers. In the unloaded animals there was a significant reduction in the labeling of satellite cells within 24 hrs of the initiation of unloading, a time at which no significant changes in the weight of the soleus muscle had taken place. There was a continued reduction in the labeling of the satellite cell population, and by 3 days of unloading we were unable to find any labeled cells on fibers from unloaded soleus muscles. At 2 days, wet weight of the muscle was significantly reduced (9.3 mg vs. 12.1 mg for control). The present studies clearly demonstrate that ***hindlimb unloading results in a very rapid initial reduction and eventual complete shutdown of satellite cell proliferative activity. The initiation of this shutdown precedes any measurable changes in the soleus muscle***, suggesting that changes in satellite cell behavior are associated with the very early events of atrophy in immature muscles and can be useful and sensitive indicators of initial stages of atrophy in immature muscle.

The tissues processed for EM were used to document directly whether there is a reduction in the absolute and relative numbers of satellite cells as a result of exposing the animals to hindlimb unloading. A reduction in labeling following unloading might have been the result of not only a decrease in the mitotic activity of the cells but also in the loss of cells. EM quantitation at 3 days of unloading indicated there was an approximately 55% reduction compared to control in the relative and absolute number of satellite cells on soleus myofibers. Therefore, ***the reduced labeling during the initial period of hindlimb unloading is associated not only with a reduction in the number of cells mitotically dividing, but also with a reduction in the total number of cells***.

Significance of the Accomplishments

These results suggest that hindlimb unloading produces a profound and very rapid effect of satellite cell proliferative and fusion behavior. The initial reduction in satellite cell proliferative activity appears unrelated to any physical changes in the muscle. Rather, the response of satellite cells may be related to other factors associated with unloading, such as the sudden change in the level of electrical activity of the muscle at the start of unloading. The loss of satellite cells in the rapid manner observed could have serious implications with regard to the potential of the muscles to recover when weightbearing is started after a prolonged period of weightlessness. The key to understanding the long-term effects of weightlessness during the postnatal growth period is to know how unloading impacts not only the number of satellite cells but also the types of cells lost.

Based upon the results of other studies in our laboratory, we have proposed that the satellite cell population is comprised of two types of cells that arise from the same small population of slowly dividing progenitor cells that undergo asymmetric division: one daughter cell continues the progenitor line, while the other daughter cell gives rise to a lineage of cells that divide in a more rapid fashion. These latter cells constitute the larger population and after an unknown number of divisions, the cells terminally differentiate and become competent to fuse with their myofibers. These events are schematized in Figure 1.

Our primary hypothesis is that when unloading is initiated, mitotic divisions are completely stopped within 3 days of the initiation of unloading in progenitor cells and in cells that are intermediate between progenitor and fusion competent cells. However, during the same initial period before the fibers exhibit atrophy (atrophy in this system is relative; the fibers continue to grow during unloading, but at a slower rate than control), there is a continued demand for production of additional myonuclei and, consequently, for fusion of satellite cells. Our secondary hypothesis is that terminally differentiated cells are programmed to fuse with their myofibers, and that they do so despite the new environment imposed on the fibers. The result of either process is that the fused cells are lost from the satellite cell

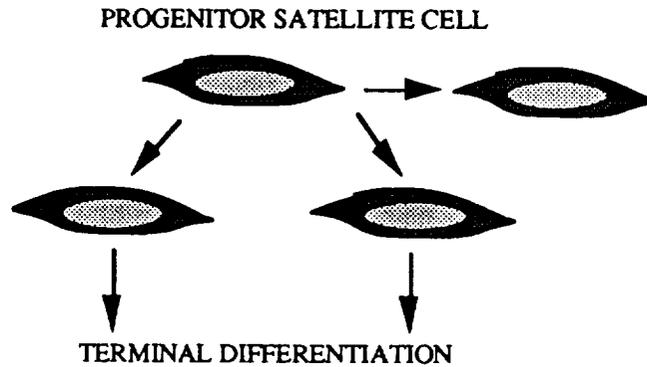


Figure 1. Terminally differentiated satellite cells are the only cells of the population that are available for fusion with myofibers. The number of mitotic divisions required between the progenitor cell and a fusion competent cell is unknown.

population. Because mitotic division of the satellite cell population ceases so rapidly and so completely, there is no production of daughter cells to replace the ones that have fused. The net result is a reduction in both the absolute and relative number of satellite cells within the initial 3-5 days of hindlimb unloading.

Will these perturbations in the satellite cell populations result in a lost capacity of the muscle to recover when weightbearing is reinstated? In the proposed scenario there would not be a reduced ability for "catch-up growth" because the progenitor cell population remains intact, suggesting that the ability of satellite cells to produce myonuclei during the recovery period would not be a limiting factor. However, if unloading depletes the progenitor population through fusion, then the ability of the muscle to undergo a catch-up growth phase would be severely limited. Likewise, the regeneration potential of the unloaded muscle would also be severely limited. We are presently attempting to determine the full impact of the unloading environment on muscle growth by examining the fate of the progenitor cells and their ability to participate in recovery of the muscle from unloading-induced growth retardation.

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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 were made with measurements in muscles whose nerve supply is interrupted. Our work this year has focused on aspects of carbohydrate and protein metabolism and on hormone binding and sensitivity.

We focused considerable attention on the beta-adrenergic receptor and post-receptor responses, extending our previous studies to *in vivo* analysis. Studies included hormone binding and intramuscular injections of hormone to test for *in vivo* biological response. We have also been investigating the differential responses of myofibrillar and sarcoplasmic proteins, though these results are not yet completed to report the final results.

Accomplishments

(1) *Hormone responses:*

(a) *Unweighting results in an increased isoproterenol (beta-adrenergic agonist) response of glycogen degradation and lactate production.*

(b) Isoproterenol has no direct effect on muscle glucose transport.

(c) *Intramuscular injection of isoproterenol produces a marked accumulation of cyclic AMP in the presence of theophylline, inhibiting phosphodiesterase.*

(d) *In vivo effects of isoproterenol are greater in unweighted muscle, in a similar proportion to that seen in vitro.*

(e) Intramuscular injection of insulin reduces the accumulation of cyclic AMP by theophylline-treated diabetic muscle.

(f) *Insulin intramuscular injection reduces cyclic AMP more so in unweighted than in weight-bearing diabetic muscle.*

(2) *Hormone binding:*

(a) *Binding of ^3H -dihydroalprenolol to muscle membrane fractions showed a similar dissociation constant in unweighted, denervated and weight-bearing soleus muscles, but a greater relative binding capacity in the unweighted muscle.*

(b) *Binding of ^{125}I -iodopindolol to muscle particulate fractions yielded similar results to those found with dihydroalprenolol.*

(3) *Other results:*

(a) Weanling rats were shown to grow normally when maintained on a space food bar diet in comparison to normal pellet rat chow.

(b) Muscles of rats, which were maintained on the space food bar diet, responded normally to hindlimb unweighting.

Significance of the Accomplishments

The hormone data has contributed to our understanding of the mechanisms for accelerated protein breakdown with muscle atrophy caused by unweighting. A limited role for the lysosome helps to spare membrane proteins with unweighting, since this organelle is the site for their degradation. Sparing of both the beta-adrenergic and the insulin receptors has now been demonstrated to a certain extent *in vivo*. Additional evidence supporting this hypothesis was shown by binding studies. The demonstration of increased effects of insulin *in vivo* indicates that this hormone may indeed limit the rate of muscle atrophy caused by unweighting relative to the rate of atrophy seen with denervation.

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GROWTH FACTOR INVOLVEMENT IN TENSION-INDUCED SKELETAL MUSCLE GROWTH

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Description of Research

Long-term crewed space travel will require a better understanding of skeletal muscle atrophy that results from microgravity. Astronaut strength and dexterity must be maintained for normal mission operations and for emergency situations. Although exercise in space slows the rate of muscle loss, it does not prevent it. A biochemical understanding of how gravity/tension/exercise helps to maintain muscle size by altering protein synthesis and/or degradation rate should ultimately allow pharmacological intervention to prevent muscle atrophy in microgravity.

The overall objective of this research project is to examine some of the basic biochemical processes involved in tension-induced muscle growth. Differentiated avian skeletal myofibers can be "exercised" in tissue culture using a newly developed dynamic mechanical cell stimulator device which simulates different muscle activity patterns. We have found patterns of mechanical activity that significantly stimulate muscle growth and alter myofiber metabolic characteristics. With this experimental *in vitro* system, we are examining the interaction of exogenous and endogenous muscle growth factors in mechanically stimulated muscle growth.

Exogenous growth factors found in serum, such as insulin, insulin-like growth factors, and glucocorticoids, modulate muscle protein turnover rates in mechanically induced muscle growth *in vitro*. Endogenous growth factors such as prostaglandins are synthesized and released into the culture medium when muscle cells are mechanically stimulated. This family of endogenous factors helps to regulate the rates of protein turnover in the muscle cells.

The study of the mechanisms by which muscle mechanical activity interacts with both exogenous and endogenous growth factors to regulate muscle protein turnover rates in this model system will be continued.

Accomplishments

(1) A defined, serum-free medium has been developed (muscle maintenance medium, mm medium) that maintains the myofibers in positive nitrogen balance for 3-5 days, based on myofiber diameter measurements and myosin heavy chain content.

(2) *Insulin and insulin-like growth factor 1, but not insulin-like growth factor 2, induce rapid and pronounced myofiber hypertrophy in mm medium. In 5 days, muscle fiber diameters increase by 51-98%.*

(3) *Mechanical stimulation of the muscle fibers in mm medium increases the sensitivity of the cells to insulin and insulin-like growth factor 1, based on a leftward shift of their dose/response curves for protein synthesis*

rates. Thus, one mechanism by which mechanical activity stimulates myofiber growth is by increasing the sensitivity of the cells to these growth factors.

(4) *The glucocorticoid dexamethasone at 10^{-8} M induces atrophy of the differentiated cultured myofibers after 3-5 days of incubation in mm medium. Mechanical stimulation of the muscle cells for 3-5 days significantly reduces this atrophic response, based on a 47% decrease in the dexamethasone-induced fall in protein:DNA ratios and myosin content.*

(5) A more physiological mechanical cell stimulator system (Model III) is under development for ground-based studies. Design changes have been made to reduce the size of the apparatus and to increase the number of sample wells (Figure 1). The new 36-well cell growth chamber is made entirely of stainless steel and is 50% smaller. A new compact power supply and motor controller, which are able to operate several mechanical cell stimulators at once, have also been designed. Work is continuing on the introduction of a perfusion system and environmental monitors for continuation of growth factor studies under more *in vivo*-like conditions. Automatic medium changes and real time monitoring of cellular metabolites will be possible soon (Figure 2).

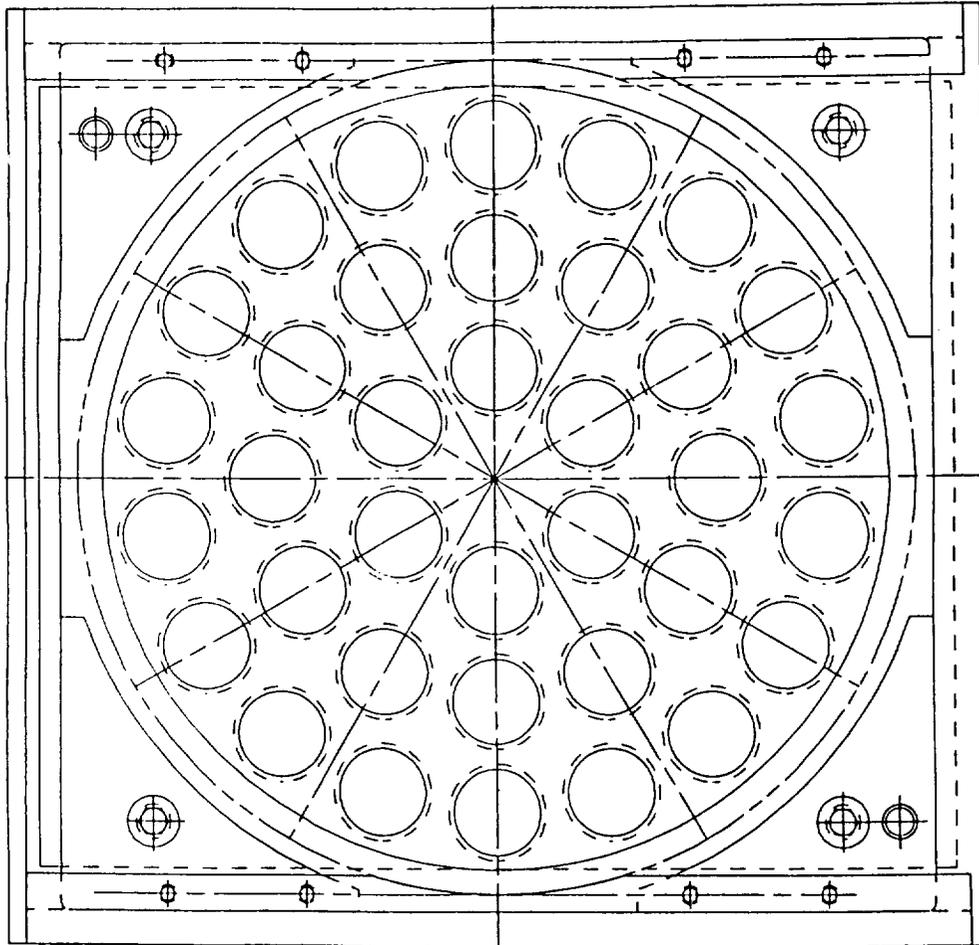


Figure 1. Cell growth chamber of mechanical cell stimulator. Top view of the 36 wells (16 mm diameter each) in the new Model III. Schematic is shown at full scale.

Significance of the Accomplishments

Finding #1: Development of a defined serum-free culture medium for maintaining differentiated skeletal myofibers in positive nitrogen balance will allow a better understanding of the complex interactions of growth factors and muscle tension in regulating skeletal muscle growth and in preventing microgravity-induced atrophy.

Finding #2: Insulin-like growth factor 1 at physiological concentrations has been shown to be an important stimulator of skeletal myofiber hypertrophy.

Finding #3: Mechanical stimulation of skeletal myofibers increases the cells' sensitivity to insulin and insulin-like growth factor 1 and is thus one example of the interaction of mechanical forces and growth factors in regulating muscle size.

Finding #4: The attenuation of glucocorticoid-induced myofiber atrophy by mechanical stimulation is another example of the similarities of our *in vitro* model system to exercise physiology studies *in vivo*, and is another example of the interactions of exercise and growth factors in regulating muscle size *in vivo*.

Finding #5: All of the hardware and software alterations in mechanical cell stimulator Model III are aimed at not only providing a better ground-based model system for studying muscle growth regulation, but will also allow use of the system in microgravity studies in the space shuttle and space station.

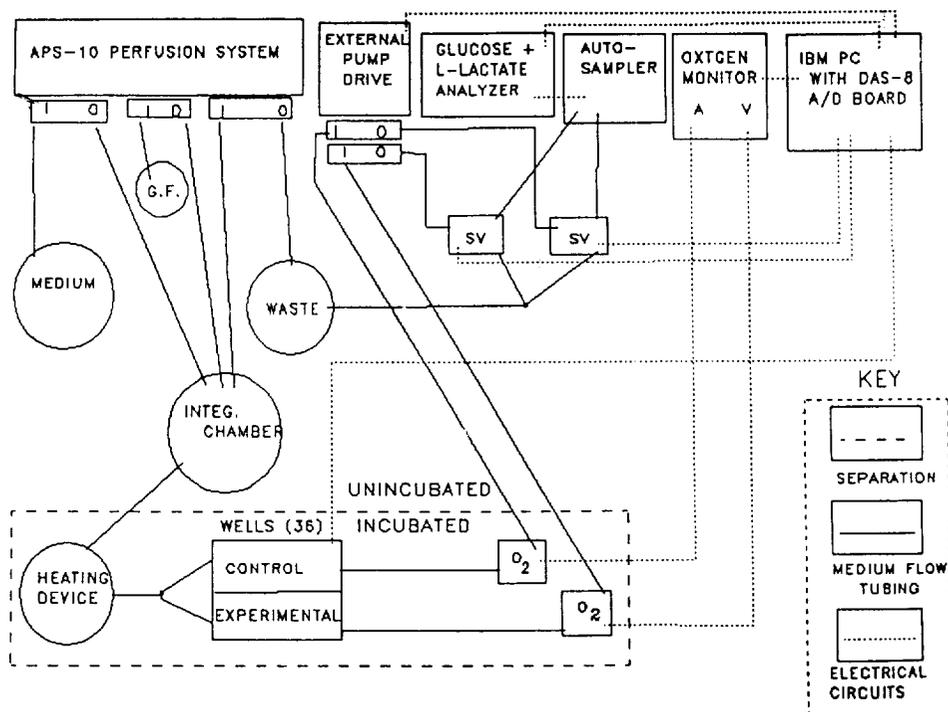


Figure. 2. Flow diagram of automatic perfusion and monitoring systems of mechanical cell stimulator (Model III). Abbreviations: G.F., growth factors; I, input; Integ., integration; O, output; SV, solenoid valves.

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DEVELOPMENT OF AN AMPHIBIAN GRAVITY RECEPTOR IN DIFFERENT GRAVITY FIELDS

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Description of Research

Our work over the past several years has focused on the development of gravity-sensing organs and on the mineralization of the stones (otoconia or statoconia) in those organs on which gravitational forces normally act. The long-range goal of this work is to understand the role of gravitational force itself in controlling development of gravity-sensing organs. If there is a regulatory mechanism controlling mineralization of the statoconia, based on the *weight* of the mass of stones, one would expect this regulation to be substantially modified in microgravity. If the test masses are abnormal when developed in microgravity, we would like to know if their development is normalized after first introduction to a 1 g environment. It is also important to determine whether exposure to partial gravity, upon intermittent exposure to hyper-g, can compensate for any detrimental effects of microgravity during development.

Most of our work has been carried out in the marine mollusc *Aplysia californica*. With the opportunity to study the development of gravity sensors in Japanese red-bellied newt embryos fertilized in microgravity, however, our emphasis is shifting to this animal. We have been developing a laboratory facility in which to raise and breed Japanese newts and have been coordinating with the Japanese research team that is planning experiments for the International Microgravity Laboratory 2 shuttle mission, scheduled for launch in 1994. We expect to receive our first newts this summer.

Accomplishments

A study of the normal developmental sequence in the form of the *Aplysia* statocyst has been completed. This study included growth of the statocyst from 4 days after fertilization through adult stages of 1 kg weight. A single statolith is present from day 4 through metamorphosis. When the statocyst reaches approximately 45 μm in diameter, the statolith can no longer be suspended by the cilia on the receptor cells making up the wall of the cyst. The statolith then falls and multiple statoconia begin to be formed in the supporting cells between the receptor cells. The production of statoconia continues throughout life, there being as many as 1000 in a 1 kg adult.

In order to quantify the rate of mineralization of statoconia we have begun work on marking the new calcification with the fluorescent calcium-binding dyes xylenol orange and tetracycline. Both dyes will deposit clearly labeled rings when introduced into the seawater in which the animals are growing for 24 hrs. We are currently working on fluorescent dyes that can be excited by the lasers available in the university's confocal laser-sectioning microscope. This system offers the great advantage of achieving very high resolution without having to section material. Using transmitted light in the laser sectioning microscope, with a gain of 16000x, we have been able to observe the internal structure of statoconia 10 μm in diameter (Figure 1). We have also developed a small centrifuge system to investigate the effects of hyper-g on statocyst development. Initial results indicate that *Aplysia embryos raised at 2 g have smaller statoliths than those reared at 1 g.*

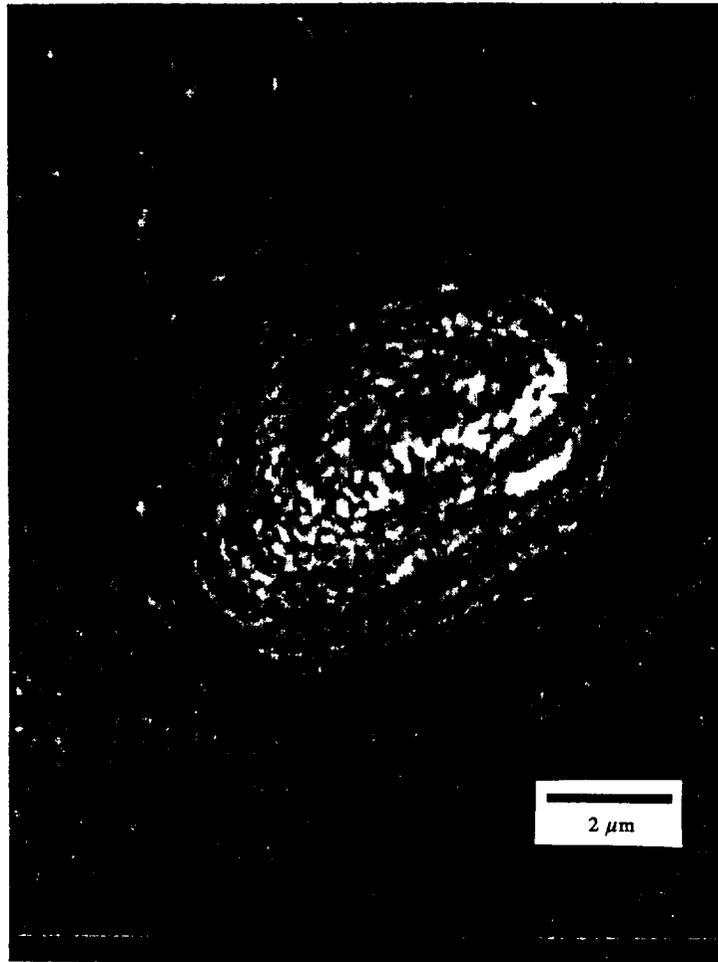


Figure 1. Computer-enhanced image of a single un-sectioned *Aplysia* statoconium obtained with the laser scanning microscope using the transmitted-light (nonconfocal) mode. The regular layering of mineralization is consistent with what we have previously seen in thin-sectioned material with the transmission electron microscope. They indicate that mineralization is deposited on successive protein layers spaced at approximately 160 nm. If we can successfully mark these layers with a calcium-binding label with fluorescence excited by one of the microscope lasers, we will be able to study the mineralization process with very high temporal and spatial resolution.

Significance of the Accomplishments

Our findings on *Aplysia* statocyst development suggest that the transition from a single statolith to production of multiple statoconia is dependent on the statolith falling to the bottom of the cyst lumen when the receptor-cell cilia can no longer support the lith in the center of the cyst lumen. If this is the case, this transition would be expected to be abnormal in the absence of gravity. Thus, this will be a very interesting preparation in which to study development of a gravity sensor in microgravity. We are currently exploring several containment methods in which to carry early post-metamorphic *Aplysia* on spacecraft in order to take advantage of any opportunities for flight of these specimens.

The *Aplysia* statolith and statoconia are both composed of calcium carbonate in the aragonite form, as are the newt otoconia. Thus, the optical techniques and dye-labeling schemes we develop using *Aplysia* will, in all likelihood, be transferrable to the Japanese red-bellied newt.

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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 to skeletal elements 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 *Strongylocentrotus purpuratus*. The calcite spicules of the *S. purpuratus* pluteus larva have an integral proteinaceous matrix. Our studies have centered on learning more about the proteins comprising the spicule matrix.

Several different approaches have been used to isolate and characterize these matrix proteins. One approach has used molecular biology techniques to isolate the genes that encode the spicule matrix proteins. During the past year, we have significantly extended our understanding of two of these genes and their encoded proteins. We have determined, in collaboration with Toru Higashinakagawa's group in Japan, that the *SM50* gene originally described a few years ago actually encodes a basic, non-glycosylated protein that does not fit the usual profile of integral matrix protein. It is conventionally believed that matrix proteins are most often acidic and glycosylated. This corrected structure of the *SM50* protein suggests that *SM50* may play a unique and important role in the structure of the spicule matrix. One possible role might be to act as a positively charged glue holding the various negatively charged matrix proteins together.

We have also acquired a detailed knowledge of the structure of the gene encoding a second spicule matrix protein designated *SM30*. This protein is a smaller, acidic, glycosylated protein which is more typical of integral matrix proteins. *SM30* is now under intensive study to compare the regulation of its synthesis with that of *SM50*.

Other approaches for study of the spicule matrix proteins include detailed biochemical analysis of the matrix proteins. Two-dimensional gel analysis has been done to determine more precisely the number of different matrix proteins. These studies show there are at least 20 spicule matrix proteins, which is about twice as complex as was originally believed. The vast majority of these proteins are acidic as expected. Other studies comparing the structure of *SM50* proteins in two distantly related sea urchins show a conserved region of the carboxyl end of the *SM50* protein. This finding supports the idea that this region of the protein is important in biomineralization.

Accomplishments

(1) The nucleotide sequence of the *SM50* gene and the biochemistry of the SM50 protein have been re-examined. These studies have found that *the SM50 protein has an alkaline pI and is not glycosylated.*

(2) A gene homologous to *SM50* has been isolated from another distantly related species of sea urchin, *Lytechinus pictus*, and the fundamental characteristics of the encoded homologous protein are very similar to those found in *S. purpuratus*. *The SM50 protein and its homologue in L. pictus have a proline rich region, both have alkaline isoelectric points, neither are glycosylated, both are secreted proteins, and both have a similar repeated conserved amino acid motif at the carboxyl terminus.*

(3) Two-dimensional gel electrophoresis analysis has been carried out on total spicule matrix proteins. The vast majority of spicule matrix proteins are acidic, SM50 being one of the few exceptions. *There are 10 major spicule matrix proteins and a dozen or so minor proteins.* This number of proteins is approximately twice that resolved by one-dimensional gels.

(4) A continuing effort to isolate more spicule matrix proteins has resulted in the isolation of a primary mesenchyme specific cDNA. This cDNA, however, probably does not encode a spicule matrix protein. We are continuing to isolate new cDNA clones and are hopeful that they will encode other spicule matrix proteins.

(5) *A gene that encodes a second spicule matrix protein, designated SM30, has been isolated and characterized from S. purpuratus.* The homologous gene in *L. pictus* is now being isolated. A detailed study of the structure of the SM30 protein and the expression of the gene has been carried out. Studies have shown that *treatment of S. purpuratus embryos with agents that disrupt the integrity of the extracellular matrix also inhibit the expression of SM30 while not affecting SM50 expression. These results point to a difference in regulation of the SM30 and SM50 genes and further implies that there are different functions for their encoded proteins.*

Significance of the Accomplishments

Finding #1: The proteins that constitute the integral matrix of mineralized tissues, ranging from teeth and bones to shells and tests, are highly acidic proteins that are usually glycosylated. But few of the individual proteins that comprise the integral matrices extracted from hard tissues have been characterized. The finding that the very first protein isolated from sea urchin spicule matrix is an exception is interesting. Although we do not yet understand the role of SM50 in spicule formation, without this basic information we cannot expect to progress in our understanding of how mineralization is controlled. The mineralization process is disturbed in microgravity and knowledge of basic biology and biochemistry of mineralization is essential if men and women are to live in space for prolonged periods of time.

Finding #2: Related to the above consideration is the comparison of homologous proteins in two widely divergent species. This type of analysis gives clues to the function of various parts of the protein. Thus, comparison of SM50 proteins in two species of sea urchin that diverged sixty million years ago may give functional clues for portions of the SM50 protein. It is interesting that the broad domains of the two homologous SM50 proteins are conserved.

Finding #3: The matrix proteins of mineralized tissues are a difficult material with which to work. There exists a large literature on the amino acid composition of total integral matrix proteins; however, the fractionation and characterization of individual matrix proteins have lagged behind other areas of biochemistry and protein chemistry. We have developed methods to overcome some of these problems and for the first time can get a better glimpse of the complexity of the sea urchin spicule matrix proteins. This, in turn, gives us an idea of how difficult the job will be to unravel structure/function relationships at the molecular level. The complexity of spicule matrix proteins is about twice as complex as we thought. It is, however, not extremely complex. This observation suggests it will be likely that we will be able to assign structure/function relationships to spicule matrix proteins.

Finding #4: The isolation of a cDNA encoding what is most likely a contaminant of our spicule preparation points out a difficulty in our strategy to isolate other spicule matrix protein genes. The methods are so sensitive that contaminants may easily surface, as they have this past year. The discovery of this clone indicates that a more specific antiserum is needed to continue this approach. Steps to correct this have been taken and new cDNA clones are being isolated.

Finding #5: The isolation and characterization of a second spicule matrix gene, SM30, allows a more powerful study of the function of these genes. Comparative studies will allow us to better understand at a molecular and cellular level the unifying mechanisms as well as the diversity of mechanisms involved in biomineralization.

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EFFECTS OF MICROGRAVITY ON MAMMALIAN DEVELOPMENT AND DIFFERENTIATION

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Description of Research

Our research program is directed towards understanding the effects of altered gravitational environments on mammalian germ cell development and early embryogenesis at the cellular and molecular level. Our goal is to understand how cells and organisms respond and adapt to altered environmental conditions, including altered gravitational environments. We have had a particular emphasis on identifying sensitive molecular markers of cellular perturbation. To this end, we have characterized the expression of members of cellular stress protein gene families. Cellular stress genes or heat shock protein genes (*hsp*) are synthesized in response to a number of environmental assaults and stimuli. Our studies have focused on establishing the pattern of expression of these gene families during normal development and differentiation and then examining their expression in tissues that have been subjected to altered environments. Two sources of materials for these studies have been obtained: male rats from a hindlimb unloading model for muscle atrophy (generously supplied by Dr. Emily Morey-Holton), and testes from rats that were flown aboard a Soviet spaceflight.

Accomplishments

(1) One series of experiments focused on characterizing the *hsp* 70 gene products synthesized in male mammalian germ cells at specific stages of spermatogenic development. In particular, the goal was to determine whether *hsp* 70 gene products in germ cells are produced only in response to developmental cues and/or whether they could be induced by external stresses as well. The properties and inducibility of the *hsp* 70 gene products were examined by one- and two-dimensional gel electrophoresis and immunoblotting. Low levels of the 72 and 73 kDa heat shock proteins normally found in mouse cell lines were detected in the mouse testis. ***A novel isoform with a relative molecular weight of 73 kDa (called 73T) was also observed, in the presence or absence of heat shock.*** 73T was shown to be produced by germ cells since it was not detected in testes from mutant mice devoid of germ cells. Furthermore, 73T was found only in adult mouse testicular cells, not in testes from animals which lack meiotic germ cells. 73T was synthesized in enriched cell populations of both meiotic prophase and post-meiotic cells, but was not inducible by *in vitro* heat shock. In the adult testis, low levels of the *bona fide* 72 kDa heat inducible *hsp* 72 were induced in response to elevated temperatures. In contrast, in testes from animals in which only somatic cells and pre-meiotic germ cells were present, there was a substantial induction of *hsp* 72. It is suggested that ***hsp* 72 is inducible in the somatic compartment and possibly in the pre-meiotic germ cells, but not in germ cells that have entered meiosis and that are expressing members of the hsp 70 gene family in a developmentally regulated fashion.**

(2) The developmental regulation of two *hsp* 90 genes in the mouse testis was examined at the level of mRNA and, in preliminary experiments, at the level of protein as well. The larger and more abundant *hsp* 90 transcript of ~3.2 kb in size was expressed at

high levels in the germinal compartment of the testis, particularly in germ cells in meiotic prophase. The smaller *hsp 90* transcript (~2.9 kb) was expressed predominantly in the somatic compartment of the testis. Expression of the two *hsp 90* genes, as well as of members of the *hsp 70* gene family, was seen in the testis of other species, including humans, indicating an evolutionary conservation of expression and potentially of function of *hsp* genes in mammalian germ cell development. Expression of both *hsp 90* genes was detected in the embryonic and extra-embryonic compartment of midgestation embryos, raising the possibility that these genes could be used as molecular markers of stress on normal embryonic development.

(3) 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. We have received testicular tissue from rodents used in ground-based studies on the effects of altered gravity, in collaboration with Dr. Emily Morey-Holton at NASA Ames Research Center. The hindlimb unloading 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 animals are allowed to move freely and to feed and drink *ad libitum*. Decreases in testis weight have been observed in these unweighted animals. Our preliminary observations at the morphological level indicate that ***the testicular weight loss correlated with a dramatic change in the population of spermatogenic cells present.*** RNA has been isolated from testes of rats that were unloaded for 2, 5, 7, and 11 days, plus their normally weighted controls. Our results to date indicated that ***there are changes in the expression of both hsp 70 and hsp 90 genes*** and that ***these changes correlate with the developmental stages of the spermatogenic cells that were affected.***

(4) We have received testicular tissues from rodents flown on a Soviet Cosmos Biosatellite as part of a collaborative effort to examine the effects of limited exposure to weightlessness on spermatogenesis. RNA was isolated from testes of spaceflight-exposed rats and was examined for the expression of *hsp 70* and *hsp 90* genes at the mRNA level. The samples were isolated from testes 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 from each of the rats unloaded at Ames plus their controls was fractionated by electrophoresis and transferred to Nytran membranes. Differences in the level of expression of both *hsp 70* and *hsp 90* were observed, but could not be correlated with spaceflight per se. This was because some of the control testes exhibited pre-existing abnormalities in testicular differentiation upon morphological examination. Nonetheless, an important finding was that ***we did not observe an obvious induction of hsp 70 or hsp 90 genes at the level of mRNA in the somatic compartment of the testis.*** This observation should be extended to examine testes obtained at shorter flight time intervals.

Significance of the Accomplishments

Findings #1 and #2: Members of the cellular stress gene families *hsp 70* and *hsp 90* are expressed in distinctive patterns during both normal development and cellular stress and can serve as selective and sensitive molecular markers of cellular perturbation in reproductive and embryonic tissues. Their expression is further correlated with the presence or absence of specific spermatogenic cell types, which in turn is affected in unloaded animals.

Finding #3: Cells in which *hsp 70* genes are normally expressed in response to developmental cues exhibit severely reduced capabilities to synthesize *hsp 70* genes in

response to exogenous stress such as elevated temperatures. This particular property suggests the possibility that cells in early stages of spermatogenesis and embryogenesis may be especially vulnerable to changes in their environment, including spaceflight.

Finding #4: At least at the level of Northern blot hybridization analysis of total testes samples, we did not observe an obvious induction of *hsp 70* or *hsp 90* genes at the RNA level in the somatic compartment of the testis in testes recovered from spaceflight animals. This is in contrast to our observations using elevated temperatures as the source of environmental stress, in which an induction of *hsp 70* genes in testicular somatic cells was observed.

Publications

Gruppi, C.M., Zakeri, Z.F., and Wolgemuth, D.J. 1991. Stage and lineage-regulated expression of two *HSP90* transcripts during mouse germ cell differentiation and embryogenesis. *Molecular Reproduction and Development* 28: 209-217. (GWU 12996)

Watrin, F., Scotto, L., Assoian, R.K., and Wolgemuth, D.J. 1991. Cell lineage specificity of expression of the murine transforming growth factor β_3 and transforming growth factor β_1 genes. *Cell Growth and Differentiation* 2(2): 77-83. (GWU 13342)

Wolgemuth, D.J. 1990. Expression and possible role during differentiation of the mouse homeobox gene *Hox-1.4* (Abstract). *FASEB Journal* 4(7): A2167. (GWU 12178)

Wolgemuth, D.J., Gruppi, C.M., and Grills, G.S. 1990. Effects of microgravity on mammalian development and differentiation (Abstract). *ASGSB Bulletin* 4(1): 27. (GWU 12912)

Zakeri, Z.F., Welch, W.J., and Wolgemuth, D.J. 1990. Characterization and inducibility of *hsp 70* proteins in the male mouse germ line. *Journal of Cell Biology* 111: 1785-1792. (GWU 12911)

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 interests. 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 young biological scientists by offering them Research Associate Awards. These awards offer opportunities to work on projects directly related to Space Biology in laboratories that provide the necessary facilities and a relevant research environment. It is anticipated that Research Associates will develop 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 1, 1991, 82 awards have been granted to 51 awardees, of whom 31 have received a second year of funding. Table I shows the variety of projects submitted and the institutions to which the 51 Research Associates have been assigned. These scientists represent different disciplines, including zoology, developmental biology, cellular and molecular biology, botany, and physiology (animal and plant). Currently there are 30 laboratories participating. Figure 1 illustrates the number of awards given each year to new recipients and to renewal candidates. A complete list of the 51 Research Associates, including the title of each research project, the host laboratory and director, and the current location of past Research Associates, follows Figure 1.

Many of the Research Associates have been asked to participate in NASA panels, national workshops and national meetings. There have been 146 publications in refereed journals and as many abstracts of papers presented at national and international meetings. Each year, in the fall, the Research Associates attend the annual meeting of the American Society for Gravitational and Space Biology (ASGSB), where they are active participants, presenting papers and posters along with their senior colleagues. The Research Associates are also 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 the private industry, and with NASA.

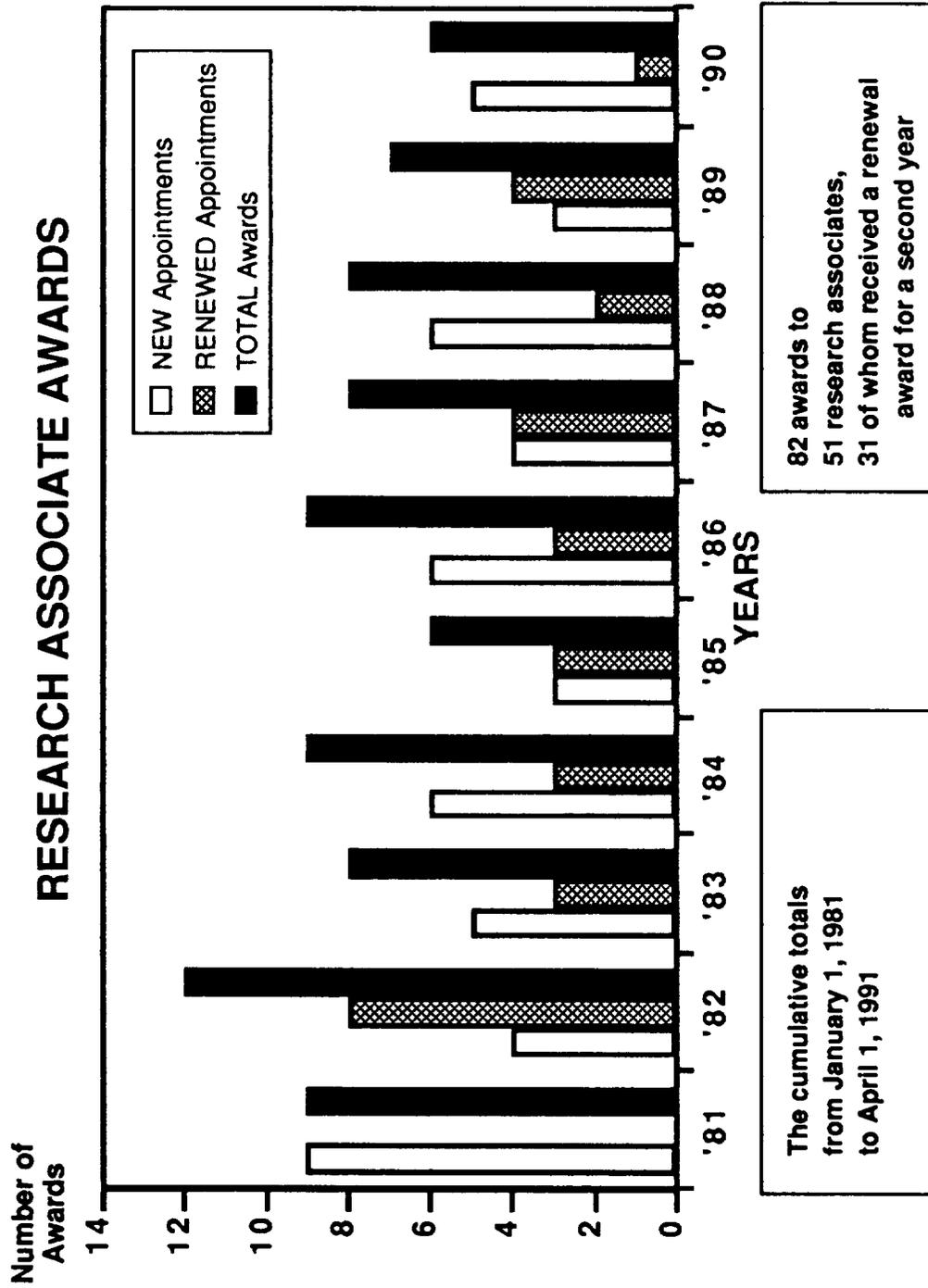
Table I. Space Biology Research Associates**ANIMAL PROJECTS**

<u>NAME</u>	<u>INSTITUTION</u>	<u>RESEARCH AREA</u>
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	NIH	Synaptogenesis/Neuropathology
D. CLOHISY	Washington U.	Osteoclastogenesis/Bone atrophy
M. COOPER	U. Cal., Berkeley	Osteoporosis/Bone atrophy
D. DICKMAN	U. Texas, Galveston	Semicircular canal fibers
S. GLOTZBACH	Stanford U.	Neurophysiology/Circadian rhythm
E. GOOLISH	U. Michigan	Weightlessness/Swimbladder function
C. GOULD	U. Louisville	Immunology/Interferon functions
M. GRAY	Tufts U.	Mechanical environment/Bone structure
E. GREENFIELD	Washington U.	Role of osteoblast/osteoclast activity
T. JONES	U. Cal., Davis	Neurophysiology/Brainstem potentials
T. KERR	Wayne State U.	Mammalian vestibular system
C. KIRBY	U. Texas, Houston	Exercise countermeasure/Muscle atrophy
D. KLIGMAN	NIMH	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
L. MINOR	U. Chicago	Responses of secondary vestibular neurons
D. MURAKAMI	U. Cal., Davis	Hyperdynamia in rat 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 of uvula
J. STEFFEN	U. Louisville	Glucocorticoid receptors/Muscle responses
J. SZILAGYI	Cleveland Clinic	Hypodynamic responses/Animal model
J. THOMPSON	Med. Col., WI	Muscle fibers following 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/Gravity sensing
T. BROCK	U. Michigan	Auxin and protein synthesis/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	Lignan biosynthesis in plant development
J. KISS	U. Colorado	Gravitropic response in <i>Chara</i> rhizoids
W. KROEN	NC State U.	CO ₂ enrichment and root/shoot ratios
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

Figure 1



RESEARCH ASSOCIATE AWARDEES

The 51 awardees are listed alphabetically, including their award terms (in parentheses after their name), host laboratory, and current locations. Current professional appointments are indicated in parentheses as: (F) Faculty; (I) Industry; (N) NASA facility; and (O) Other.

DR. STEVEN BAIN (6/1/88 - 5/30/90) worked 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. He is now Director of Biomedical Research at ZymoGenetics, Seattle, Washington. (I)

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 working for Chemical Abstracts in Columbus, Ohio. (I)

DR. WALLACE BERRY (7/1/88 - 6/30/90) worked on "Lymphokine Producing Capacity of Antiorthostatically Suspended Rats: Relationship to 1,25-dihydroxyvitamin D₃" in Dr. Gerald Sonnenfeld's laboratory at the University of Louisville, Louisville, Kentucky. He is now an Assistant Professor in the Boyd Research Center, University of Georgia, Athens, Georgia. (F)

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 a research fellow in the Pathology Department at Brown University, Providence, Rhode Island. (O)

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 now an Assistant Professor in the Department of Horticultural Sciences at Cornell University, Geneva, New York. (F)

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. (O,N)

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 now an Assistant Professor in the Department of Pathology at Washington University, St. Louis, Missouri. (F)

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. He is now a Research Associate in the Department of Human Genetics at the University of Michigan, Ann Arbor, Michigan. (O)

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. He is the project manager for the cardiovascular experiment

scheduled on Spacelab-4 and a Research Assistant Professor/Instructor in Clinical Medicine at the University of Texas Health Sciences Center, Dallas, Texas. (F)

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. (O)

DR. DENIS CLOHISY (7/1/86 - 6/30/87) worked on "Mechanisms of Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at 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. (O)

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. (O)

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. He is continuing to work with Dr. Bandurski at Michigan State University, East Lansing, Michigan. (O)

DR. J. DAVID DICKMAN (6/1/87 - 5/30/89) 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 now an Assistant Professor in the Department of Surgery, Division of Otolaryngology at the University of Mississippi, Jackson, Mississippi. (F)

DR. JOHN S. GARAVELLI (1/1/82 - 12/30/82) 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. (N)

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. (F)

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

DR. EDWARD GOOLISH (8/1/90 - 7/30/91) is working on "The Effects of Simulated Weightlessness on Swimbladder Function and Buoyancy Regulation in Fish" in Dr. Paul Webber's laboratory at the University of Michigan, Ann Arbor, Michigan.

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. (F)

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 Massachusetts Institute of Technology, Boston, Massachusetts. (F)

DR. EDWARD GREENFIELD (7/1/88 - 6/30/90) worked on "Regulations of Osteoclastic Bone Resorption by Osteoblasts" in Dr. Steven Teitelbaum's laboratory at Washington University, St. Louis, Missouri. He is now an Assistant Professor in the Department of Orthopaedics, Case Western Reserve University, Cleveland, Ohio. (F)

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. (F)

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 working at NASA-Ames Research Center, Moffett Field, California. (N)

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 Assistant Professor at the University of Nebraska, Lincoln, Nebraska. (F)

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 was an Assistant Professor at Wayne State University, Detroit, Michigan. (F)

DR. CHRISTOPHER KIRBY (11/1/90 - 10/30/91) is working on "Eccentric Exercise as a Countermeasure to Unweighting Atrophy" in Dr. Frank Booth's laboratory at the University of Texas Medical School, Houston, Texas.

DR. JOHN KISS (9/1/90 - 8/30/91) is working on "Gravitropism and Golgi Apparatus Function in *Chara* Rhizoids" in Dr. L. Andrew Straelin's laboratory at Ohio State University, Columbus, Ohio.

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. (O)

DR. WILLIAM KROEN (10/1/90 - 9/30/91) is working on "Balancing Reproductive and Root Demands for Carbohydrates and Nitrogen in Atmospheres with High CO₂ Concentrations" in Dr. Mary Peet's laboratory at North Carolina State University, Raleigh, North Carolina.

*deceased

DR. KONRAD KUZMANOFF (7/1/83 - 7/30/85) worked on "Isolation and Identification of β -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, Chicago, Illinois. (O)

DR. ANNA LYSAKOWSKI (7/1/89 - 6/30/91) is working on "Synaptic Relations of Type I and Type II Vestibular Hair Cells" in Dr. Jay Goldberg'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. (I)

DR. KENNETH MCLEOD (11/1/87 - 10/30/89) worked on "Regulation of Bone Remodeling Activity through the Control of Stress Generated Electric Fields" in Dr. Clinton Rubin's laboratory, SUNY, Stony Brook, New York. He is continuing to work in Dr. Rubin's laboratory at SUNY. (O)

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 is an Associate Professor at West Virginia School of Osteopathic Medicine, Lewisburg, West Virginia. (F)

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. He is now finishing his clinical training at the University of Chicago Medical Center, Chicago, Illinois. (O)

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, California. (O)

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. (F)

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 Assistant Professor in the Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah. (F)

DR. KENNETH POTE (6/1/88 - 5/30/90) worked 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. He is now an Assistant Professor in the Department of Otolaryngology, Harvard Medical School, Boston, Massachusetts. (F)

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. (O)

DR. DENNIS REINECKE (10/1/88 - 9/30/89) worked on "Does Indole-3-Acetic Acid Turnover Correlate with Topically-Induced Asymmetric Growth?" in Dr. Robert Bandurski's laboratory at Michigan State University, East Lansing, Michigan. He is now working as a Research Associate at the University of Minnesota, St. Paul, Minnesota. (O)

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. (O)

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. (F)

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. (F)

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 as a Research Associate at USDA, ARS, Beltsville, Maryland. (O)

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. (F)

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

DR. LAWRENCE TALBOTT (7/1/89 - 6/30/90) worked on "Actuation of Gravicurvature in Pea Stems by Alterations of Specific Wall Polymers" in Dr. Barbara Pickard's laboratory at Washington University, St. Louis, Missouri. He is now a Research Associate at the University of California, Los Angeles, California. (O)

DR. DONALD THOMASON (11/1/89 - 8/30/90) worked on "Mechanisms of Decreased Actin Synthesis during Rodent Hindlimb Unweighting" in Dr. Frank Booth's laboratory at the University of Texas Medical School, Department of Physiology and Cell Biology, Houston, Texas. He is now an Assistant Professor in the Department of Physiology and Biophysics at the University of Tennessee, Memphis, Tennessee. (F)

DR. JOYCE THOMPSON (7/1/90 - 6/30/91) is working on "Targetoid and Type IIC Fibers in Hypodynamic and Recovering Rat Muscle — Histochemistry, Ultrastructure and Myosin Isozyme Composition" in Dr. Danny Riley's laboratory at the Medical College of Wisconsin, Milwaukee, Wisconsin.

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, Irvine, California. (O)

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 the skeleton's mechanical environment to regulate bone remodeling and adaptation. 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 drive remodeling between genders, the skeletal changes in female astronauts may differ significantly and be potentially 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 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 the functionally isolated turkey ulna, an animal model of disuse osteoporosis. In this model the bone can be completely deprived of any mechanical stimuli without compromising the vasculature or innervation. In addition, through changes in hormonal status (endocrinopathy) or in age, the skeletal response to the superimposed effects of systemic factors can be quantified.

We have used the ulna model to address how bone responds to the absence of mechanical loading in each of the following systemic populations: (1) adult normal (healthy males, 1 year old, fused physes); (2) hormonally imbalanced (castrated adult males); (3) growing normal males (5 months of age); and (4) 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, growing, and aged populations, we have established a framework with which to evaluate the impact of metabolic status on the bone's ability to adapt to an altered mechanical environment.

Accomplishments

(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. In castrated males, the same 8-wk 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. 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.

(2) Comparisons of the intact ulnae of castrated and normal males show 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 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 male, total cross-sectional area was unaffected by the same loading protocol. Moreover, age significantly increased the resorption depth of remodeled osteons, leading to a concomitant elevation in the bone forming period (Figure 1). The reluctance of the bone to produce new tissue, coupled with the alterations in cortical remodeling dynamics, suggests that the cells in the aged animals are looking for a different (perhaps more intense) signal.

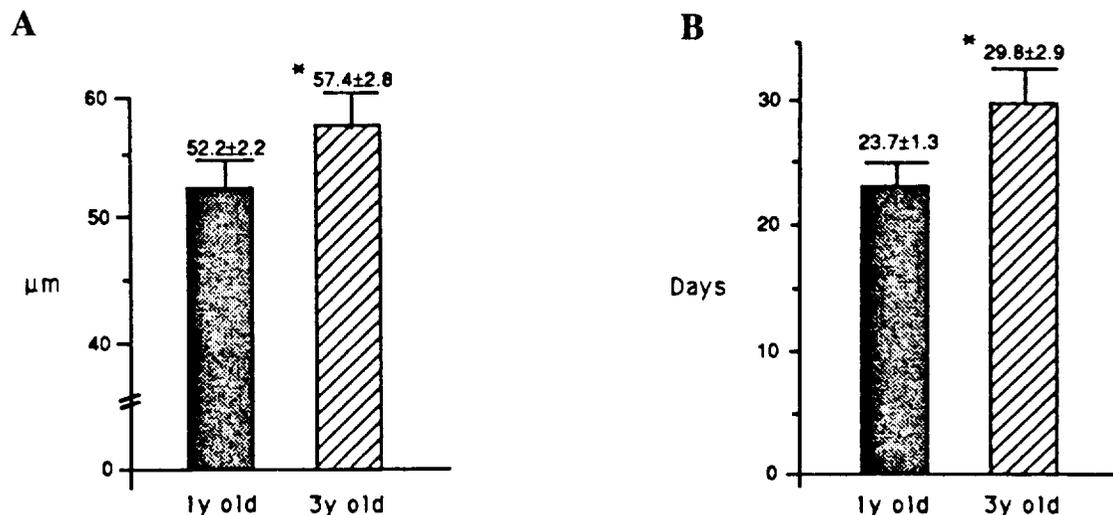


Figure 1. Effects of age on the resorption depth of remodeled osteons (A) and on the bone formation period, i.e., sigma formation, (B). Compared to the 1 year old animals, resorption depth and sigma formation are both significantly increased in the 3 year old males; $p < .05$.

Significance of the Accomplishments

It is apparent from these results that many of the morphologic and cellular aspects of adaptive 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 balance, growth, and aging as regulatory factors in the skeleton's response to disuse, a profile of the remodeling interactions 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, which will no doubt include scientists chosen not only for their physical fortitude, but also 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.

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EFFECTS OF THE UNLOADING MODEL ON DIHYDROXYVITAMIN D AND CELLULAR IMMUNITY

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Description of Research

There is evidence that immune cell responses are altered by spaceflight. Immune cells taken from humans and animals following spaceflight do not proliferate in response to a stimulus as well as before flight. Their ability to produce cytokine hormones may also be reduced. It is thought that these phenomena are due to "weightlessness" or "microgravity." The mechanisms accountable for the immunological effect of microgravity are unknown. However, it is possible that physiological changes due to musculoskeletal unloading interact with the immune system during spaceflight (Figure 1).

Recent studies demonstrate that the calcitropic hormone 1,25-dihydroxyvitamin D regulates immune cell activity. This finding 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 or whole body unloading 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.

In an effort to test this hypothesis, 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 harnessed with or without head-down tilt for periods of 1-14 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 stress.

Plasma concentrations of dihydroxyvitamin D were measured following each unloading period. In some experiments, the glucocorticoid antagonist RU-486 was administered to prevent glucocorticoid mediated stress inhibition of dihydroxyvitamin D production. In other experiments implanted osmotic pumps containing dihydroxyvitamin D were used to prevent changes in hormone levels during unloading.

Parameters of the cellular immune system were quantified in 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).

Accomplishments

(1) Plasma dihydroxyvitamin D levels were significantly reduced in all unloaded rats, regardless of the type of unloading used. Circulating 1,25(OH)₂D was reduced by more than 60% following 7 days of head-down unloading and by more than 70% following 14 days of head-down unloading as compared to pair-fed controls. Unloading without head-down tilt or restraint with full loadbearing produced results essentially

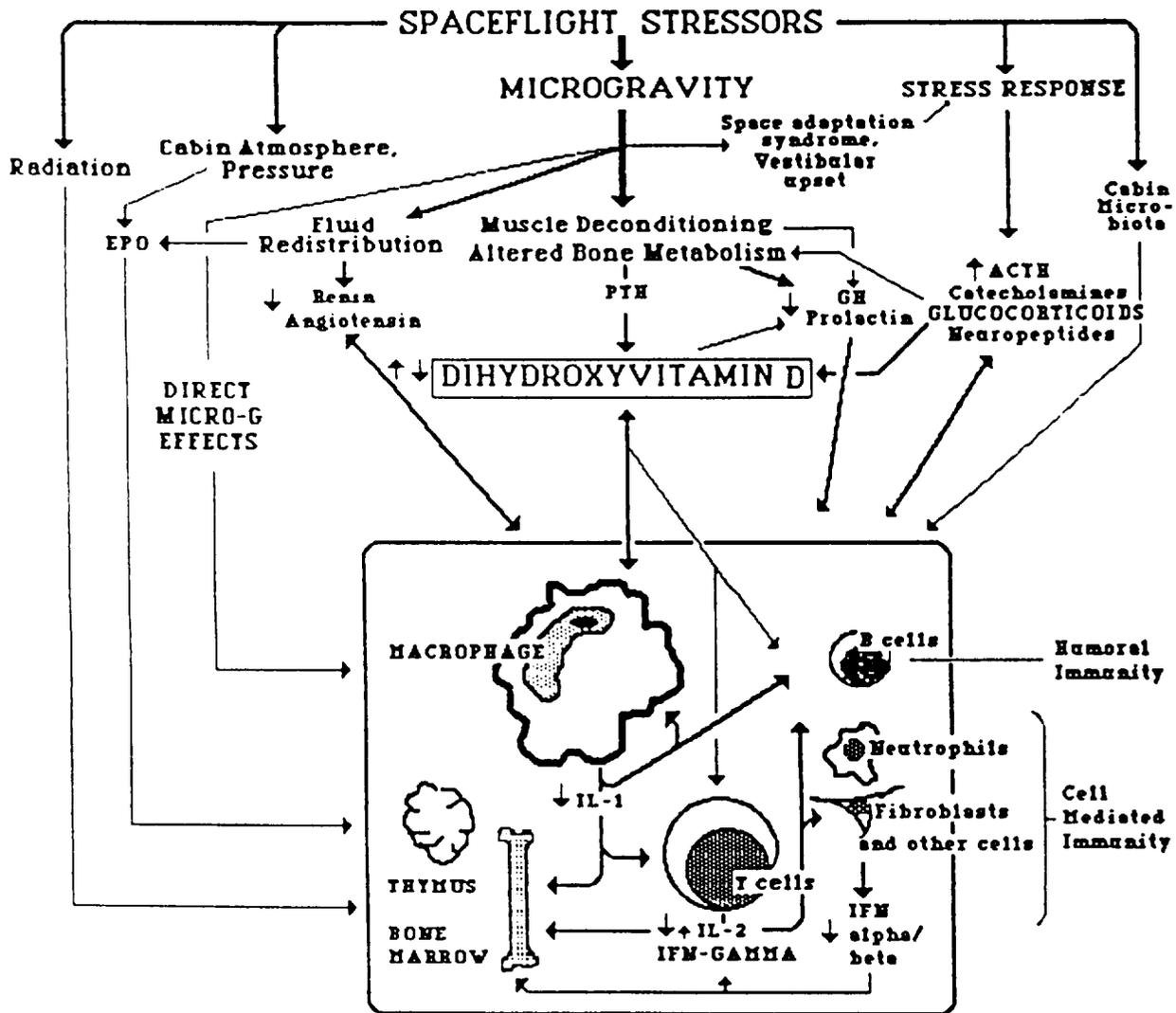


Figure 1. Possible interactions between physiological systems and the immune system during spaceflight. Abbreviations: PTH = parathyroid hormone, GH = growth hormone, ACTH = adrenocorticotrophic hormone, IL-1 = interleukin-1, IL-2 = interleukin-2, IFN-gamma = interferon-gamma, IFN-alpha/beta = interferon-alpha/beta,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

identical to those for head-down unloading. Plasma dihydroxyvitamin D levels returned to normal in all unloaded rats following 7 days of recovery in normal caging. *The glucocorticoid antagonist RU-486 partially protected dihydroxyvitamin D levels during unloading.* Unloaded rats receiving RU-486 during 7 days of unloading had plasma dihydroxyvitamin D concentrations approximately 30% higher than unloaded rats not receiving the compound. 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 7 or 14 days or restraint in the harness system with loadbearing 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 7 or 14 days of unloading with or without head-down tilt and also by harness restraint. Production of IL-1 was further suppressed following 2 days of recovery but returned to normal after 7 days of recovery. Unloaded animals receiving exogenous dihydroxyvitamin D had significantly increased IL-1 production compared to controls.

(4) Spleen cell production of IL-2 was either unaffected or enhanced following 7 or 14 days of unloading with head-down tilt. Unloading without head-down tilt or restraint 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) Unloading with or without head-down tilt appeared to enhance IFN-gamma production. In contrast, harness restraint with loadbearing tended to suppress IFN-gamma production as did 2 days of recovery following unloading. Exogenous dihydroxyvitamin D suppressed the IFN-gamma response in unloaded and control animals.

(6) Whole body unloading 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 demonstrated enhanced production. Production of IFN-alpha/beta was lower in rats receiving exogenous dihydroxyvitamin D.

Significance of the Accomplishments

Finding #1: These studies confirm and expand on previous findings that whole body unloading has a negative effect on plasma dihydroxyvitamin D concentration. The finding that dihydroxyvitamin D levels were reduced regardless of tilt or loadbearing indicates that reduced movement and stress were the most important factors in low dihydroxyvitamin D during unloading. Experiments with RU-486 indicate that *glucocorticoid mediated stress accounted for approximately 30% of the decrease in dihydroxyvitamin D levels during harness unloading.*

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

Findings #2 and #3: Phagocytosis of pathogens and IL-1 production by macrophages are functions central to initiation and regulation of the immune response. Impairment of these functions during whole body unloading or spaceflight would be expected to result in a suppressed overall immune response.

Finding #4: Changes in IL-2 production could impair lymphocyte differentiation and function. As with IL-1, this impairment could lead to suppressed or inappropriate immune response. Reduced proliferation of lymphocytes after spaceflight could be due to low IL-2 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 and #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 has significant functions 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.

In summary, our studies indicate that whole body unloading reduces plasma dihydroxyvitamin D levels by a mechanism not directly dependent on musculoskeletal unloading or head-down tilt. Reduced movement and 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 during spaceflight. The results obtained for the other cytokines suggest that factors such as stress contribute to the immunological effects of musculoskeletal unloading. The recovery studies indicate that both the dihydroxyvitamin D changes and the immunological effects are reversible.

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THE EFFECT OF SIMULATED WEIGHTLESSNESS ON SWIMBLADDER FUNCTION AND BUOYANCY REGULATION IN FISH

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Description of Research

The objectives of this research are to (1) understand the mechanisms which fish use to regulate their buoyancy and to orient themselves vertically in the water column, and (2) use this information to predict how fish will behave in the weightless environment of space. The regulation of neutral buoyancy in most fish occurs through the use of a gas-filled dynamic swimbladder and complex accessory tissues. Atrophy of the swimbladder is likely in the weightless conditions of space since there would be no need for the lift force it provides nor for the physiological processes that regulate its volume.

Rates of gas secretion and resorption by the swimbladder of the killifish (*Fundulus heteroclitus*) were monitored under a variety of hydrostatic and hydrodynamic lift conditions. Weightlessness was simulated by attaching gas-filled tubes above the center of gravity with a lift force equal to the gas-free weight of the fish in water. Rotational forces were induced by adding lift (and/or weight) anterior or posterior to the center of gravity (Figure 1). We also studied changes in the volume of the swimbladder when fish are exposed to a current since the flow of water over the body of a fish is believed to generate lift.

Accomplishments

(1) *When artificial lift was sufficiently added to make the fish neutrally buoyant without swimbladder lift, approximately 7-10 days were required for the complete resorption of gases from the swimbladder (about 8% of body volume). Some fish were unable to decrease swimbladder gases below 50% of neutral volume. Five to seven days were needed to refill the swimbladder once the artificial lift was removed.*

(2) *Fish exposed to seven days of artificial lift displayed a slower rate of gas secretion into the swimbladder when the lift was removed than did control fish (gas removed by syringe). The rate of gas resorption was slightly faster the second time a group of fish was placed under artificial lift.*

(3) *Fish confined in clear cages on the bottom of their tank had larger swimbladder volume than fish similarly maintained near the surface of the water. Although statistically significant, the difference in volume was only 10%.*

(4) *Swimbladder volume decreased by approximately 30% when fish were forced to swim against a current. This effect was largely eliminated when the pectoral fins were removed. Pectoral fin removal did not affect gas resorption by fish under artificial lift.*

(5) *Twenty-five percent of swimbladder volume was resorbed when partial lift was attached anterior to and identical weight was attached posterior to the fish's center of gravity. When these forces were reversed,*

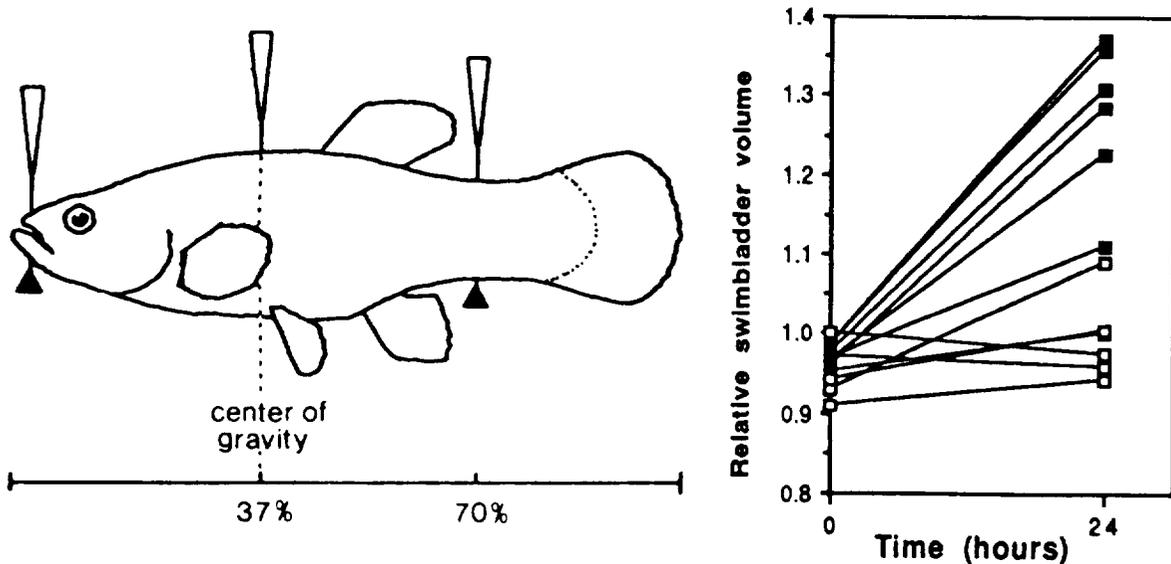


Figure 1. Sites of attachment for artificial lift (dorsal) or weight (ventral) relative to center of gravity. When lift is added at the anterior position and an equal amount of weight is added at the posterior position, no change in swimbladder volume occurs (□). With these forces reversed, swimbladder volume increases by 25% (■).

swimbladder volume increased by approximately 25%. When equal forces of artificial lift and weight were added, lift anterior and weight posterior, little or no change in swimbladder volume occurred. (Figure 1).

Significance of the Accomplishments

Finding #1: If the methods used here accurately simulate the effect of weightlessness on fish buoyancy, then one week of spaceflight would be sufficient to result in complete swimbladder resorption. The normal re-entry period would not be long enough for the fish to achieve neutral buoyancy and behavior would be affected upon return to 1 g.

Finding #2: The decrease in rate of gas secretion suggests that atrophy of the gas generating tissue has occurred after just one week of non-use. Similarly, the increased rate of resorption during a second exposure to artificial lift indicates that the physiological processes involved in volume regulation can be affected by demand.

Finding #3: The limited effect observed between tank locations indicates that perception of vertical position alone is not the major stimulus for swimbladder volume regulation.

Finding #4: The decrease in swimbladder volume which occurs in swimming fish and in fish exposed to a current is apparently the result of lift forces generated by the flow of water over the pectoral fins. However, forces acting on the pectoral fins do not appear to be the direct stimulus for gas secretion and resorption since fin removal did not affect the response to artificial lift.

Finding #5: These results demonstrate that the mechanism for regulating gas secretion and resorption is not simply dependent on maintaining a net hydrostatic lift force of zero. Rather, *the stimulus for regulation appears to be the rotational (i.e.,*

pitching) forces experienced by the fish; resistance to lifting the head stimulates gas secretion. Since resistance to swimming up or down is involved in regulation, and sensing up and down is dependent on vestibular function, fish in a weightless environment may lose their ability to regulate swimbladder volume.

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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 are 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.

(3) *The mechanism by which osteoblast-conditioned media maintains viability of osteoclast precursors does not involve prevention of apoptosis* (programmed cell death).

Significance of the Accomplishments

Osteoblast-derived factor(s) stimulate bone resorption by maintaining viability of osteoclast precursors rather than by activating mature osteoclasts. This finding is reminiscent of the action of a variety of hematopoietic factors. These factors include colony stimulation 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 has recently been shown to be the mechanism whereby a number of these factors maintain viability of their target cells. However, we found that the ability of osteoblast-conditioned media to maintain viability of osteoclast precursors does not involve prevention of apoptosis.

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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ECCENTRIC EXERCISE TRAINING AS A COUNTERMEASURE TO NON-WEIGHTBEARING MUSCLE ATROPHY

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Description of Research

The goal of this research is to examine the control of muscle gene expression during states of altered contractile activity (i.e., eccentric exercise and/or non-weightbearing). Our hypothesis is that soleus muscle atrophy associated with hindlimb non-weightbearing will be attenuated by the increased functional demand of eccentric exercise training. The specific aims of this research are: (1) to determine if eccentric exercise training can maintain normal protein content in non-weightbearing soleus, (2) to examine the influence of eccentric exercise on the quantity of total RNA and skeletal α -actin mRNA in non-weightbearing soleus, and (3) to investigate the effects of non-weightbearing and/or eccentric exercise on the expression of a skeletal α -actin promoter-chloramphenicol acetyltransferase reporter construct stably inserted into adult soleus muscle.

Muscle atrophy resulting from non-weightbearing is one of the principal alterations associated with space deconditioning. The countermeasure most often employed to combat non-weightbearing atrophy is exercise training. However, only partial success in attenuating the potentially deleterious effects of muscle loss has been achieved. Of the exercise protocols utilized, resistance training seems to be the most effective at reducing the loss of muscle protein. To date, only the adequacy of concentric (i.e., shortening muscle contractions) resistance exercise has been tested, leaving the efficacy of eccentric (i.e., lengthening muscle contractions) training to be determined. Normal locomotion, posture and activity incorporate a significant degree of eccentric contraction, which is absent during non-weightbearing. This provides a convincing rationale for including eccentric exercise as a component of any exercise countermeasure program for non-weightbearing (e.g., spaceflight).

Our experiments have focused on developing an optimal eccentric exercise training protocol. In addition, we have established several methods, new to the laboratory, which are necessary for inserting foreign genes into adult skeletal muscle.

Accomplishments

(1) *Insulin growth factor 1 (IGF-1) was found to increase 2-5 days following a single bout of eccentric exercise.*

(2) Rat skeletal muscle satellite cells have been successfully isolated and cultured.

(3) Skeletal muscle satellite cells have been transfected *in vitro* with a retrovirus containing the β -galactosidase reporter gene.

Significance of the Accomplishments

Finding #1: IGF-1 is known to play a role in muscle hypertrophy. Knowing the time course of increases in IGF-1 following a single bout of eccentric exercise is helpful in

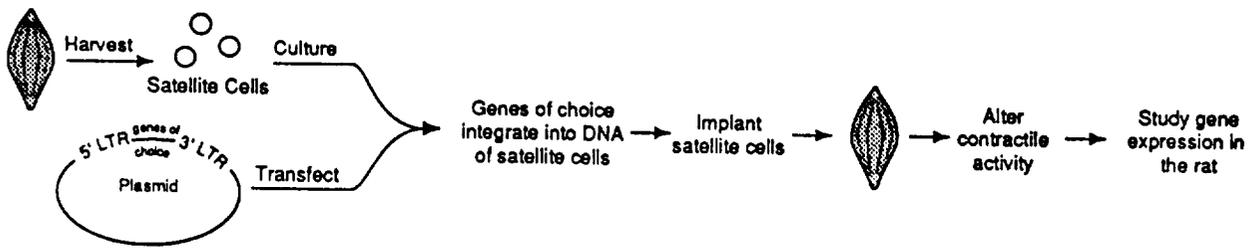


Figure 1. Integration of foreign genes into adult skeletal muscle.

determining the frequency of training required to reverse muscle atrophy during non-weightbearing.

Findings #2 and #3: Isolation and culture of rat satellite cells (skeletal muscle stem cells) are necessary for the transfer of large quantities of foreign DNA into adult skeletal muscle. Once in culture, these cells must be transfected with a retrovirus containing our genes of choice (i.e., skeletal α -actin promoter-chloramphenicol acetyltransferase reporter genes). Findings #2 and #3 represent the first two procedures required for the integration of foreign genes into adult skeletal muscle tissue. Following the implantation of transfected satellite cells (myoblast transfer), the effects of altered contractile activity (i.e., eccentric exercise and/or non-weightbearing) on muscle gene expression can be studied (Figure 1).

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GRAVITROPISM AND GOLGI APPARATUS FUNCTION IN *CHARA* RHIZOIDS

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Description of Research

The purpose of this research is: (1) to characterize the gravitropism pathway in rhizoids (rootlike extensions) of the lower plant *Chara*, and (2) to determine the role of the Golgi apparatus in gravitropism and asymmetrical cell wall growth in these rhizoids. In higher plants there is a cellular and spatial separation between gravity perception and response, and the signal must be transmitted over a relatively large distance. In certain lower plant groups, however, all phases of gravitropism occur in a single cell. One such system is the rhizoid of the green alga *Chara* (Figures 1, 2). In general, single-celled systems are excellent subjects for cell biological approaches because of their small size and relative simplicity. In addition to insights about gravitropism in single cells, the study of these systems may help in understanding gravitropism in higher plants and/or suggest better approaches for the subsequent analyses of the latter systems.

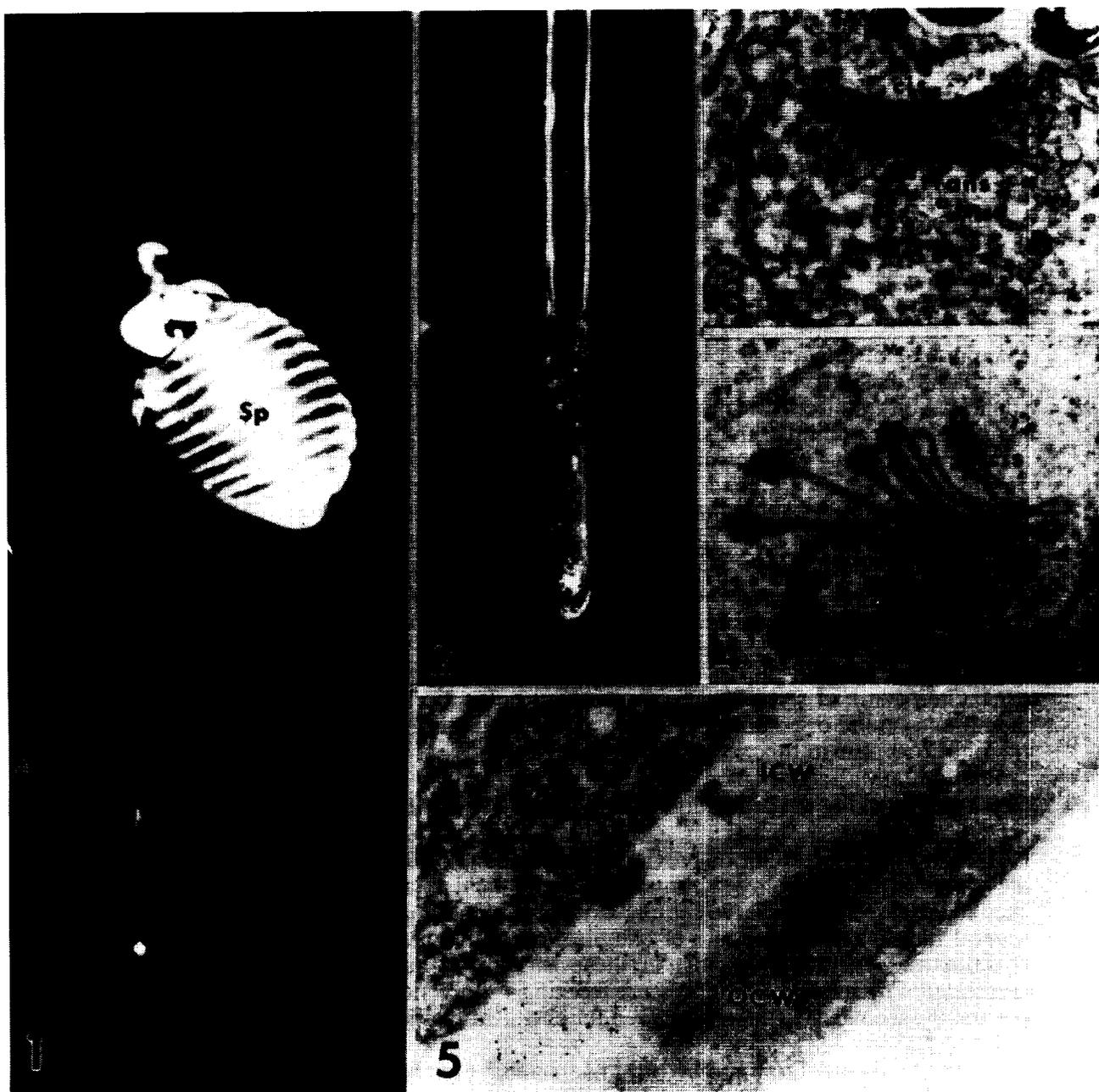
Gravity perception in plants is hypothesized to be mediated by the interaction of dense organelles (termed statoliths) with other cytoplasmic structures. In *Chara* rhizoids, membrane-bound organelles presumed to contain barium sulfate and located close to the rhizoid tip appear to function as statoliths (Figure 2). It has been hypothesized that after gravistimulation, sedimented statoliths inhibit growth in the new, lower cell wall by physically blocking the deposition of Golgi-derived vesicles that contain wall components.

However, a limiting factor of previous ultrastructural studies of the *Chara* rhizoid is that these studies have employed conventional chemical fixation techniques. The ability of chemical fixatives to preserve cellular structures in their natural configuration is limited by several factors (e.g., slow rate of chemical crosslinking, and the selective nature of the crosslinks). Fortunately, most of these limitations can be overcome by the use of cryofixation methods — high pressure freezing — in which cells are fixed in milliseconds (versus the seconds to minutes required for chemical fixation).

The first step needed to improve our understanding of the mechanism of gravitropism in *Chara* rhizoids is to obtain reliable, high resolution information on the structural organization of the rhizoid under a variety of experimental conditions. To this end, I have been using cryofixation techniques to refine our understanding of the architecture of *Chara* rhizoids and to thereby create a more reliable structural database for relating gravitational responses to structural parameters.

Accomplishments

(1) *The ultrastructure of cryofixed rhizoids is significantly different than that of conventionally fixed rhizoids.* For example, Golgi stacks in cryofixed cells (Figure 4) have more distinct *cis* to *trans* polarity than conventionally fixed samples (Figure 3). A gradient of condensation of material in Golgi vesicles (i.e., vesicles become progressively more electron dense towards the *trans* side) also was observed in cryofixed



Abbreviations. Cy = cytoplasm; ICW = inner cell wall; N = nucleus; OCW = outer cell wall; P = protonema; R = rhizoid, S = statoliths; Sp = spore; V = vacuole.

Figure 1. A germinated *Chara* oospore with a protonema and a rhizoid viewed with a dissecting microscope. 40x. Figure 2. Tip of *Chara* rhizoid viewed with differential-interference-contrast optics. Note the statoliths near the apex of the cell and the large vacuole. 230x. Figure 3. Electron micrograph of a Golgi stack from a rhizoid tip that was prepared by conventional chemical fixation. 28,000x. Figure 4. Electron micrograph of a Golgi stack from a rhizoid tip that was prepared by high pressure freezing/freeze substitution. There is a clear distinction between *cis* (widest, least stained) and *trans* cisternae as compared to the previous figure. Arrowheads indicate a series of Golgi vesicles whose contents appear to be undergoing a condensation reaction. Asterisk indicates a microtubule. 52,000x. Figure 5. Cell wall labeled (colloidal gold particles) with JIM 7 antibody which is against methyl-esterified pectin. Note the higher density of labeling in the inner cell wall. 27,000x.

samples (Figure 4). In addition, large vesicles with an electron dense core surrounded by less densely staining materials were seen budding from Golgi cisternae (Figure 4).

(2) ***Antibodies against complex polysaccharides of higher plant cell walls crossreact with cell walls of Chara.*** Results to date show that several antibodies against higher plant pectic polysaccharides crossreact with *Chara* cell wall molecules. These include a polyclonal antibody to polygalacturonic acid/rhamnogalacturonan I (PGA/RGI), a monoclonal antibody to methyl-esterified pectin (JIM 7), and a monoclonal antibody to de-esterified pectin (JIM 5). Using these antibodies in conjunction with immunocytochemical techniques, I have demonstrated that the *Chara* rhizoid cell wall has two distinct domains (Figure 5). The inner wall contains more esterified pectin (Figure 5) while the outer wall has more de-esterified pectin. Furthermore, a distinct class of apical vesicles can be labeled with the antibody against esterified pectin.

(3) ***Cryofixation helps preserve antigenicity in Chara rhizoids.*** An additional advantage of the cryofixation method is that it preserves not only structural features but also antigenic determinants better than conventional techniques. This is particularly noticeable for the JIM 7 antibodies, which label conventionally fixed samples weakly but label cryofixed samples very strongly (Figure 5).

Significance of the Accomplishments

Finding #1: The structural results obtained by high pressure freezing/freeze substitution reveal new details not detected in previous studies. These preliminary results confirm the need for a systematic reappraisal of the structural organization of the rhizoids by means of cryofixation methods. Furthermore, these data suggest that this new methodological approach will yield important insights into the role of the Golgi apparatus in differential growth of the cell wall in response to gravity.

Finding #2: The results obtained in these studies demonstrate that antibodies generated against higher plant molecules will be useful probes to study the cell wall and apical vesicles in *Chara* rhizoids during gravitropic curvature. These antibodies will also be useful in helping to determine the contents of the apical vesicles. In addition, these results are consistent with the model for pectin synthesis in higher plants in which pectins are synthesized in a methyl-esterified form and are subsequently de-esterified in the cell wall.

Finding #3: These results indicate that it is important to use cryofixation in conjunction with immunocytochemistry in order to better understand the distribution of cell wall polysaccharide antigens. For these reasons, I plan to use cryofixed material in my future structural and immunocytochemical studies.

Publications

Kiss, J.Z., Giddings, Th.H., Jr., Staehelin, L.A., and Sack, F.D. 1990. Comparison of the ultrastructure of conventionally fixed and high pressure frozen/freeze substituted root tips of *Nicotiana* and *Arabidopsis*. *Protoplasma* 157: 64-74. (GWU 12914)

Kiss, J.Z. and Sack, F.D. 1990. Severely reduced gravitropism in hypocotyls of a starch-deficient *Nicotiana* mutant (Abstract). *ASGSB Bulletin* 4(1): 100. (GWU 12915)

Kiss, J.Z. and Staehelin, L.A. 1991. Golgi-derived vesicles give rise to statoliths in *Chara* rhizoids (Abstract). *Plant Physiology* 96(1, Suppl.): 74. (GWU 12998)

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

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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 utricle, which detects gravity. Our study to date has concentrated primarily on the crista ampullaris, but preliminary data from the utricle 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 primarily in the central zone. Bouton fibers (20% of the total) are found primarily 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, depending on their location in the sensory epithelium. Dimorphic fibers 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 completed an ultrastructural study of serially sectioned material from four chinchilla end organs. This study utilized stereological (quantitative sampling) methods to examine semicircular canals from several animals. For each sample of 30 serial sections, 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, shape and location of all synaptic elements. These data were notated on the photomontages and compiled using a computer spreadsheet program. In the course of this study, profiles of 28,745 hair cells were examined in four longitudinal and six transverse samples taken through the entire sensory epithelium of cristae from four animals. In addition, we have used stereological point-counting methods to reconstruct the microarchitecture of the sensory epithelium by calculating the volume fractions of hair cells,

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Publications

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TOMATOES PARTITION CARBOHYDRATES AND NUTRIENTS TOWARDS FRUIT PRODUCTION AND AWAY FROM ROOT DEVELOPMENT WHEN GROWN AT HIGH CO₂

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Description of Research

Growth of plants at elevated CO₂ generally leads to increased growth and seed or fruit production. These increases occur despite only transient increases in photosynthetic carbon uptake; long-term effects may be due to initial increases in leaf area, thereby providing increased area for carbon exchange. Tomato plants grown under elevated CO₂ (approximately 1000 µl l⁻¹) were previously shown to have increased growth and fruit production, but suffered severe leaf curling, decreased foliar nutrient concentrations, and decreased root matter after 16 weeks of treatment. Decreased rooting ability was hypothesized to account for the nutrient deficiencies, which may also occur in fruit. Decreased root biomass and increased fruit weights would be beneficial in a CELSS system, since less non-edible material would be produced in the systems which are anticipated to run at high CO₂ concentrations.

Our experiments examined plant biomass, fruit production, photosynthetic carbon uptake, and nutrient and carbohydrate partitioning during 15 weeks of growth of two tomato varieties at normal (350 µl l⁻¹) and elevated (1000 µl l⁻¹) CO₂ concentrations. Only these two environments were possible because of the large number of plants required for biweekly destructive sampling. We concentrated on the relative partitioning of carbon and nutrients between fruit and the vegetative plant body, with particular attention to root systems and their ability to supply nutrients (particularly nitrogen) to the entire plant.

Accomplishments

(1) Growth of leaves, stems, and roots was increased by high CO₂ during the first two weeks in both varieties. Leaves and stems remained larger in the plants grown at high CO₂, though growth rate of plants in the two CO₂ treatments was similar after the first two weeks. *Fruit weight, but not necessarily number, was significantly increased at increased CO₂. Root growth slowed or stopped at the time of fruit set in plants at elevated CO₂, resulting in similar or reduced total root biomass relative to ambient-grown plants after fruit production slowed.*

(2) There was a massive buildup of starch in leaves of plants at high CO₂, though this reserve did not appear to be apportioned to stems or roots of these plants.

(3) Relative partitioning of nutrients was altered away from leaves in favor of the increased fruit production in plants grown at high CO₂.

(4) *When growth was reduced by tight spacing and root restriction, plants at high CO₂ were still larger than those at lower CO₂, and had increased fruit production compared to ambient-grown plants under the tight spacing*

regime. *Fruit production was lower in tightly-spaced plants than controls at both CO₂ levels.*

Significance of the Accomplishments

Finding #1: Tomato fruit production is increased by use of high CO₂ environments, though this is accompanied by increased leaf, stem, and root production. Root biomass, relative to non-enriched plants, was similar or higher until late in the experiments, so there is little hope of having relative "reductions" in root biomass until after fruit production is completed.

Finding #2: There are no substantial increases in short-term, area-based uptake of carbon dioxide by plants grown at high CO₂, though the larger plants might have a greater total carbon uptake than the smaller plants at lower CO₂. The dramatic increase in leaf starch at high CO₂, if it occurs in other crops (such as lettuce or spinach), might alter the nutritional value of these leaves for human consumption.

Finding #3: Fruit are such potent sinks for carbohydrates and nutrients that root growth slows markedly during fruit set and ripening. These changes, however, do not result in apparent nutrient deficiencies. The direct effect of elevated CO₂ on the nutritional value of tomato fruit is still unknown.

Finding #4: Tight spacing, though reducing fruit production compared to plants given ample room to grow, might allow for less vegetative growth relative to the return in fruit weight and number. This line of research needs to be further explored in reference to possible nutrient delivery systems and to their effect on root growth and viability.

Publications

Kroen, W.K. and Peet, M.M. 1991. High CO₂ concentration alters carbohydrate partitioning in favor of shoots in tomato (Abstract). *Plant Physiology* 96(1, Suppl.): 101. (GWU 14072)

Tripp, K.E., Peet, M.M., Pharr, D.M., Willits, D.H., and Nelson, P.V. 1991. CO₂-enhanced yield and foliar deformation among tomato genotypes in elevated CO₂ environments. *Plant Physiology* (in press).

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

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supporting cells, and afferent and efferent endings in the different regions. We have also determined the cell densities per mm^2 and the ratios of hair cells to supporting cells.

Recently, we have investigated the squirrel monkey cristae in light of the hitherto unexpected finding that the organization of the cristae in the monkey differs substantially from that in the chinchilla. We have also initiated comparative ultrastructural studies of the synaptic organization in the squirrel monkey cristae.

Accomplishments

(1) Hair cell counts, confirmed on the electron microscopic level, indicate that in the monkey *type I hair cells outnumber type II hair cells by a ratio of $\approx 3:1$. The ratio is higher in the central zone ($\approx 5:1$) than in the periphery ($\approx 1.5:1$). This difference can easily be seen by inspection of individual sections (Figure 1).*

(2) Following studies of the afferent innervation with extracellular injections of horseradish peroxidase, an afferent reconstruction (by means of calculations) of the innervation of both the chinchilla and squirrel monkey cristae was done. *Three differences were found to reflect the relative paucity of type II hair cells in the monkey: (a) the percentage of calyx units in the monkey is three times larger; (b) the percentage of bouton units is three times smaller; and (c) central dimorphic units have half as many bouton endings.*

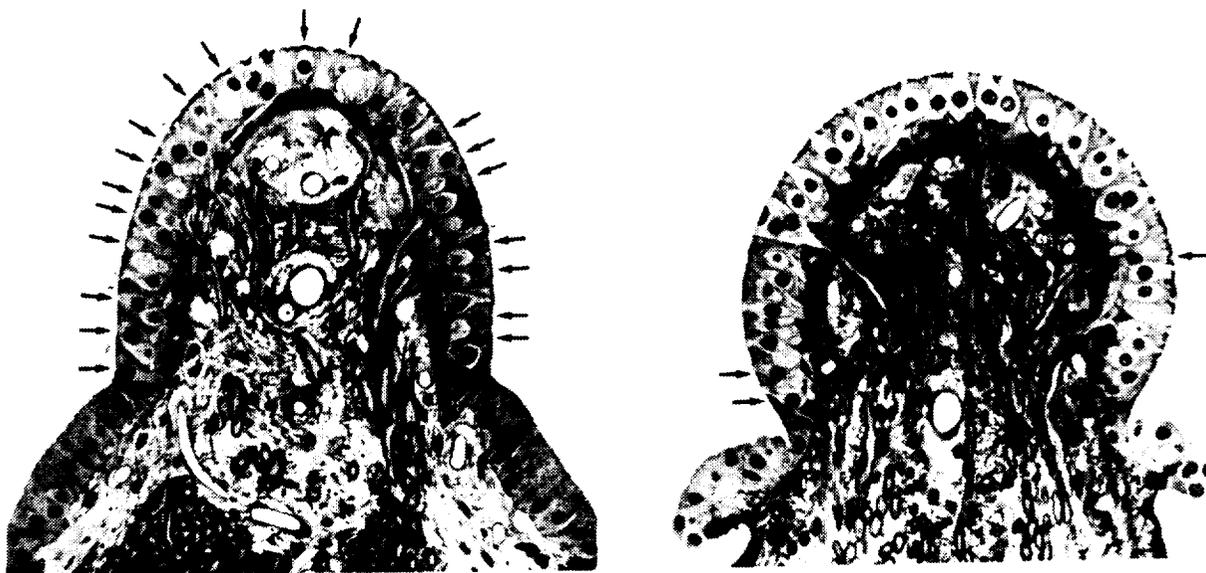


Figure 1. Transverse sections through the middle of the horizontal cristae in a chinchilla (left) and a squirrel monkey (right). In the neuroepithelium, supporting cells are located just above the basement membrane and are characterized by irregularly shaped nuclei. The two kinds of hair cells are easily distinguished because Type I hair cells are surrounded by calyx endings, whereas Type II hair cells are not. All Type II hair cells are indicated by arrows. Of the 35 hair cells with nuclei in each section, 19 type II hair cells are found in the chinchilla section and only three such hair cells are found in the squirrel monkey section. Plastic-embedded $2\ \mu\text{m}$ sections; Richardson's stain (1% azure II, 2% methylene blue).

(3) Preliminary ultrastructural results indicate that the squirrel monkey crista has a synaptic organization that is qualitatively similar to that in the chinchilla, i.e., *(a) peripheral type II hair cells are innervated by 30 or more boutons, each making a single synaptic contact, while central type II hair cells have fewer (5-10) afferent boutons, each of which receives several synaptic contacts; and (b) type II hair cells in the central zone make multiple synaptic contacts with the outer faces of calyx endings, while these type of synapses are rare in the periphery.*

(4) As part of an investigation of the microarchitecture of the chinchilla crista, the ratio of supporting cells to hair cells was found to be $\approx 1.8:1$ in the central zone compared to $\approx 1.4:1$ in the intermediate and peripheral zones. However, the volume fractions of each cell type are about equal among the regions, indicating that hair cells in the central zone are $\approx 25\%$ larger than in the peripheral zone.

Significance of the Accomplishments

Finding #1: This finding was unexpected. In the chinchilla, the two kinds of hair cells occur with approximately equal numbers throughout the end organ. We had expected that the results would be similar in the monkey. Instead, the situation is quite different and warranted examination of the afferent innervation pattern (Finding #2).

Finding #2: The central zone of the monkey was found to contain twice as many fibers as the central zone of the chinchilla. Two-thirds of these afferents are calyx units. Calyx units in the chinchilla cristae have a distinctive physiology: they are irregularly discharging, phasic afferents with relatively low rotational gains. The functional implication of this finding is that the monkey has a considerably larger proportion of low-gain irregular afferents.

Finding #3: Qualitatively, at least, the monkey crista has a synaptic organization similar to that in the chinchilla, indicating, perhaps, a common mammalian plan. However, further work needs to be done to quantify the differences, if any.

Finding #4: Our calculations indicate that even though the hair cell density is less in the central zone than in the periphery, central hair cells are larger and could therefore conceivably receive the same proportion of afferent boutons as peripheral or intermediate hair cells. In fact, however, central hair cells receive fewer afferent boutons than do hair cells in the periphery.

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.

Publications

Fernández, C., Lysakowski, A., and Goldberg, J.M. 1991. Comparison of hair-cell populations and afferent innervation patterns in the cristae of the squirrel monkey and the chinchilla (Abstract). *Abstracts of the 14th Annual ARO Midwinter Meeting*, p. 94.

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MECHANISMS FOR CONTROL OF PROTEIN SYNTHESIS IN MUSCLE DURING RODENT NON-WEIGHTBEARING

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Description of Research

The purpose of this research is to begin to define the mechanisms for the rapid decrease in protein synthesis observed in both skeletal and cardiac muscle during non-weightbearing. As a model for weightlessness, the rodent hindlimb unweighting model is useful because it induces skeletal muscle atrophy. Previous research conducted in this laboratory has shown that the skeletal muscle atrophy process is complex, with changes in gene expression regulated on different temporal scales transcriptionally, translationally, and post-translationally. The most rapid of these processes is the translational regulation, with a large decrease in protein synthesis occurring within the first few hours of non-weightbearing. Surprisingly, a similar downregulation of cardiac muscle protein synthesis occurs on the same time scale (Figure 1).

The working hypothesis of this research is that the external event of non-weightbearing signals the protein synthesis machinery in skeletal and cardiac muscle (i.e., ribosomes, mRNAs, initiation and elongation factors) to slow protein synthesis by slowing nascent protein chain elongation.

Accomplishments

(1) Using the novel electrophoretic polysome separation technique, it was demonstrated that *the same shift towards increasing polysome size observed for skeletal muscle after the onset of non-weightbearing also occurs in cardiac muscle* (Figure 2). These data indicate similar mechanisms for control of protein synthesis in a non-weightbearing environment.

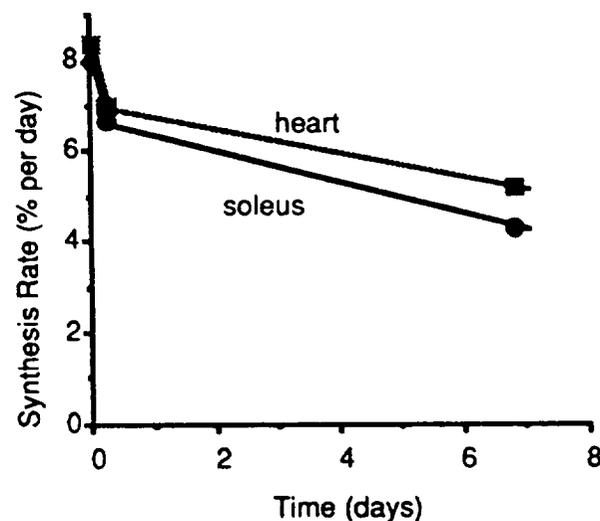


Figure 1. Rate of protein synthesis in cardiac and soleus muscle of rat during non-weightbearing periods.

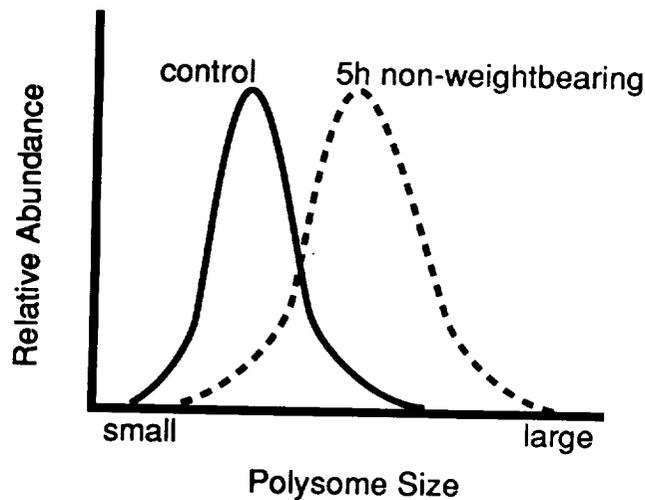


Figure 2. A shift towards increasing polysome size was observed for rat cardiac muscle exposed to non-weightbearing.

(2) Using molecular probes for α -actin mRNA, it was demonstrated that the mRNA is associated with the larger polysomes. From these data I have formulated a further hypothesis that the regulation of protein expression by a slowing of nascent polypeptide chain elongation may be specific for contractile proteins.

Significance of the Accomplishments

Finding #1: Atrophy of cardiac muscle in space, a previously unclear and not well substantiated phenomenon, has now been shown to have a biochemical basis. Although true atrophy may not occur in the heart because of a possible simultaneous downregulation of protein degradation, **significant biochemical alterations do occur in cardiac muscle**. These may, on a long-term basis, be debilitating or life-threatening.

Finding #2: If a specific regulation of a subset of genes occurs, the mechanotransduction mechanism is far more complex than a wholesale regulation of protein synthesis. This possibility offers potential pharmacologic interventions to prevent or slow the atrophy process. In addition, if the regulation is specific for contractile protein, there would be a significant functional impact of the decreased protein synthesis, especially for the heart.

Publications

Booth, F.W. and Thomason, D.B. 1991. Molecular and cellular adaptation of muscle in response to exercise: Perspectives of various models. *Physiological Reviews* 71(2): 541-585. (GWU 13346)

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SARCOMERE LESIONS IN ATROPHIED RELOADED ADDUCTOR LONGUS

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Description of Research

The overall goal of the current research is to investigate alterations in muscle structure and function during and following exposure to microgravity (space) or simulated microgravity (unloading hypodynamia/hypokinesia, HD/HK). It has been observed by our laboratory that the adductor longus (AL) from space-flown rats (Cosmos 2044), sampled 5-9 hrs following a 2-wk flight, exhibited a higher percentage of muscle fibers with sarcomere disruptions (46%; see Figure 1) than either the HD/HK exposed rats (15%) or the synchronous ground control animals (0%). The lesions seen in the flight rats are structurally similar to those found following eccentric contraction, downhill running or lengthening contractions, all of which produce higher tension on the muscle than isometric or concentric contractions. In view of the high g force experienced by the flight animals upon reentry, the differences in percentage of fibers affected by lesions between treatment groups suggest that antigravity muscles exposed to microgravity or simulated microgravity are more susceptible to high tension lengthening damage. Specifically, the present investigation tests the hypothesis that the HD/HK-treated AL is more susceptible to lengthening contractions than is the normal AL and attempts to define the mechanism of elevated sensitivity.



Figure 1. Longitudinal semithin section (0.5 μm) of adductor longus from a postflight rat (Cosmos 2044) demonstrating disruptions in the normal banding pattern. Bar = 100 μm .

Accomplishments

(1) In collaboration with Dr. Robert Fitts (Department of Biology, Marquette University, Milwaukee, WI), we developed a computer controlled transducer/servomotor system to produce eccentric contractions in the rat AL muscle at defined fractions of optimal length (L_0), while simultaneously recording tension (P) and dP/dt (Figure 2). Initial data indicates the following: (1) *a muscle undergoing eccentric contraction is under higher tension for a longer period than is the same muscle contracting isometrically or concentrically*; (2) *the magnitude and duration of this elevated tension are directly proportional to the lengthening rate*; and (3) *a 20% drop in the P_0 is seen following recovery from 5 min of lengthening contractions (10 mm/sec for 200 ms at 0.5 Hz), while no drop in P_0 is seen following an identical isometric bout.*

(2) We developed a surgical procedure for the isolation and reattachment of the AL distal tendon in surviving rats.

(3) We developed the immunocytochemical technique for localization of adenosine monophosphate deaminase (AMPda) isozymes and determined the cell specific and subcellular specific sites of distribution in rat soleus and plantaris muscles. Isozymes A, B

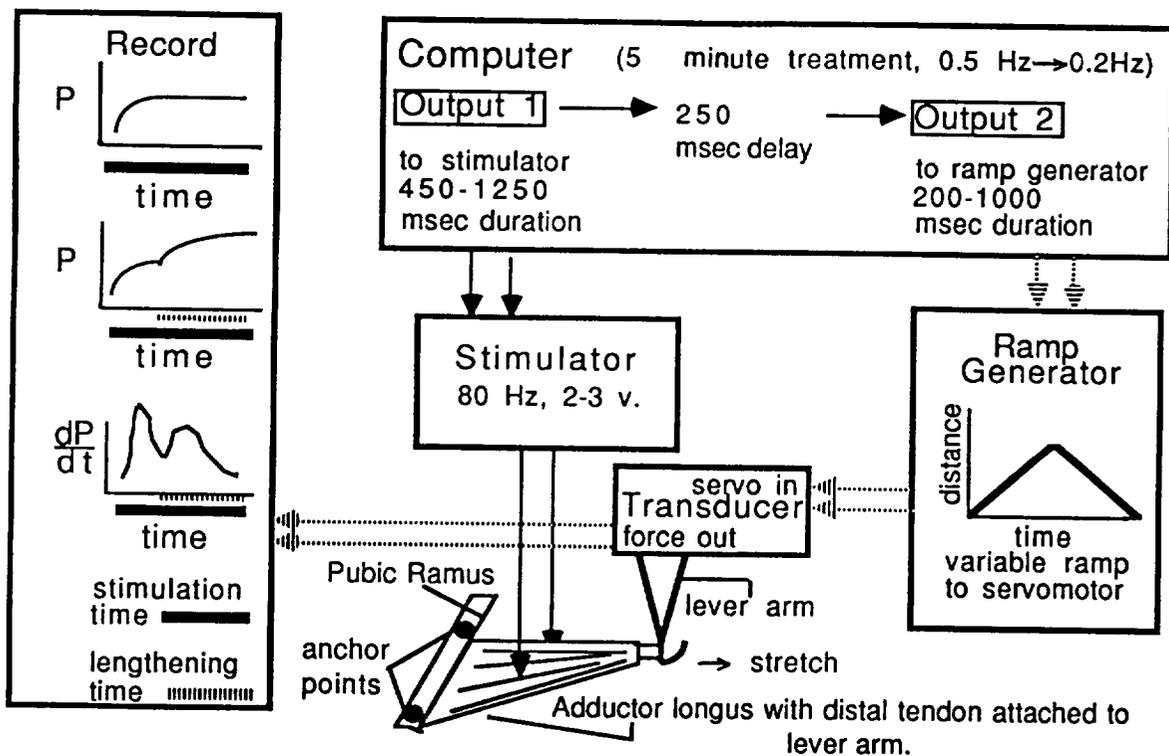


Figure 2. Schematic of system designed to produce lengthening contractions in the adductor longus. The computer controls the output to the muscle stimulator (Output 1) and after a specified delay activates the triangle wave generator (Output 2). The rate and duration of lengthening produced by the servomotor are determined by the slope of the triangle wave. Tension output from the force transducer/servomotor is recorded by the computer for later determination of P and dP/dt versus time plots.

and C can be isolated principally from muscle, kidney and heart, respectively; all catalyze the conversion of AMP to IMP and ammonia. *AMPda-A (muscle) is predominantly a myofiber enzyme localized in the subsarcolemmal and intermyofibrillar regions. AMPda-B (kidney) is principally associated with the connective tissue surrounding nerves and muscle spindles. AMPda-C (heart) is primarily associated with RBCs and vascular elements of muscle tissue; minor proportions are present subsarcolemmally in the muscle fibers.*

(4) We adapted the myofibril titin immunolocalization technique to cryostat sections of rat muscle and initiated further modification of the technique for methacrylate embedded sections and transmission electron microscopy (TEM). At the light microscopic (LM) level, the titin doublets were formed by staining of the ends of the A bands (thick filaments, principally myosin, confirmed by phase contrast microscopy).

(5) We demonstrated that the myoneural junctions of AL occur in the muscle midbelly, along a diagonal band which runs parallel to the proximal insertion (silver/silver cholinesterase staining of 98 μm thick cryostat sections).

(6) We demonstrated that the *lesions in the longitudinal sections of AL from rats flown onboard Cosmos 2044 were not randomly distributed, but were laterally grouped.*

Significance of the Accomplishments

Findings #1 and #2: Development of the "AL stretch model" will allow determination of the role that high-tension eccentric work plays in the production of sarcomere disruptions like those seen following the Cosmos flight, and will provide insight into the mechanism of increased susceptibility of HD/HK muscle to this type of damage. The observation of a drop in P_0 following a lengthening treatment indicates an effective lengthening rate to cause functional loss and sarcomere lesions. Used in combination with the survival tendon-reattachment, the stretch model will allow study of the time course of damage (lesion formation) and sarcomere repair, following eccentric work produced at precisely defined lengthening rates and tensions. This model is superior to eccentric exercise, i.e., downhill running on a treadmill, because in the stretch model the lengthening rate is precisely controlled and repeatable, and the parameters P and dP/dt are monitored.

Finding #3: The AMPda isozymes catalyze the following reaction: $\text{AMP} \rightarrow \text{IMP} + \text{ammonia}$. Functionally, AMPda isozymes are thought to stabilize fluctuations in the adenylate energy charge by shifting the myokinase equilibrium ($2 \text{ADP} \rightarrow \text{ATP} + \text{AMP}$). Additionally, due to differing kinetic properties these isozymes may have a role in regulation of microcirculation in the muscle. The enzyme 5'-nucleotidase catalyzes the conversion of AMP to adenosine, a potent vasodilator, and thus competes with AMPda for the substrate AMP. By diverting the flux of AMP to adenosine instead of IMP and ammonia, the levels of this vasodilator may be controlled. It is likely that the AMPda isozyme composition of the muscle may be key to the regulation of microcirculation in normal exercising and recovering muscle tissue. Changes due to HD/HK could alter this AMPda balance and hence microcirculation. Impaired microcirculation to muscle during atrophy and reloading has been implicated in causing muscle damage. Development of this isozyme discrimination technique will enable elucidation of the effects that altered AMPda isozyme ratios have on muscle metabolism and microcirculation, and the role that impairment of these processes has in lesion formation and repair.

Finding #4: Elevated tension is postulated to damage the muscle cytoskeleton. Immunolocalization of the cytoskeletal protein titin will assist in determining cytoskeletal involvement in lesion formation and myofibril repair. This process will allow serial staining of longitudinal semithin and ultrathin methacrylate sections for both lesion presence and titin staining abnormalities (diminished intensity, shifts in spacing or missing doublets). In this manner, the cytoskeletal integrity of the lesioned and non-lesioned areas may be evaluated at both the LM and TEM levels.

Finding #5: Localization of the endplate zones within the AL will aid TEM studies designed to distinguish the role of possible nerve damage from direct myofiber damage in the mechanism of lesion formation.

Finding #6: Grouping of lesions is consistent with the view that tension-induced lesions and weakening in a given region of one fiber increase the load on adjacent regions of neighboring fibers, thus making them more susceptible to similar tension-induced lesions. This implies a mechanically mediated fiber-fiber interaction. Since the fibers within a single motor unit show a random distribution, elevated tension rather than nerve damage seems a more likely candidate in the production of grouped lesions seen in Cosmos flight AL muscles.

Publications

Kasper, C.E. and Thompson, J.L. 1989. Limitations of histochemical identification in transitional single skeletal muscle fibers (Abstract). *Biophysical Journal* 55: 88a. (GWU 11860)

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Thompson, J.L., Sabina, R.L., and Riley, D.A. 1991. AMP-deaminase (AMP-da) isozymes in rat skeletal muscle (Abstract). *Medicine and Science in Sports and Exercise* 23(4, Suppl.): S57. (GWU 13820)

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 1,000 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, microgravity science, Earth science, astrophysics, space physics, solar system exploration, and communications and information systems. OSSA fellows carry out their research or a plan of study at their home universities. Each year they attend a 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, seven are in the space biology and biomedical research areas. These awardees' abstracts, which were compiled from the May 1991 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, Office of Space Science and Applications, Code S, NASA Headquarters, Washington, D.C. 20546. Mr. John T. Lynch, GSRP Program Manager, University Programs Branch, Educational Affairs Division, Office of External Relations, 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.

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) in both ground-based and space-flown animals. Hsp genes serve as molecular markers of both normal mammalian differentiation and the cellular response to stress. Our study has focused on the characterization of the hsp 90 gene family expression in the murine testis and embryo. Two different *hsp* 90 transcripts were detected in the mouse testis using two human 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 is expressed predominantly in the germinal compartment of the testis. The smaller transcript is expressed primarily in the somatic compartment of the testis. Expression of both *hsp* 90 transcripts was detected in the embryonic and extra embryonic compartment of the midgestation embryo. Monoclonal antibodies that recognize the *hsp* 90 proteins were used to detect *hsp* 90 proteins in tissue extracts of mouse testis and embryos. Immunoblotting detects the *hsp* 90 proteins in tissue extracts of mouse testis and the midgestation conceptus. Testis from animals exposed to space for two weeks and caudal-elevated animals were examined to evaluate the effects of altered gravity on mammalian development. We have used *hsp* 90 as well as a member of a second hsp family, the *hsp* 70 family, as molecular markers of stress in examining testis from these animals. Preliminary studies on these tissues showed no induction of *hsp* 90 and *hsp* 70 genes in the testis of space-flown and caudal-elevated animals. The effects of longer term exposure to spaceflight on reproductive tissues remain to be evaluated.

OSTEOBLAST-MEDIATED REGULATION OF SKELETAL COLLAGENASE

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The loss of skeletal mass is well documented in humans and experimental animals under conditions of weightlessness. This loss suggests that bone contains a mechanostat that senses changes in gravity, and then adjusts the mass of bone accordingly by altering the remodelling process. This process is normally an equilibrium between bone formation and bone resorption. Historically, bone formation was associated with the osteoblast and resorption with the osteoclast; however, recent data indicates that the osteoblast also takes an active role in the resorption process. This process possibly includes the role of the osteoblast as the target for a variety of resorption promoting agents, such as parathyroid hormone (PTH), and the capability of osteoblasts to produce neutral proteases, such as collagenase. We have shown that the osteoblastic cell line UMR 106-01 secretes collagenase following treatment with PTH (Partridge et al., *Endocrinology*, 1987); in addition, the present study shows that these cells remove the enzyme from the extracellular environment via a cell-mediated mechanism. Maximal concentrations of collagenase appeared in the extracellular medium 12-24 hrs after treatment with PTH but subsequently declined, becoming almost undetectable by 96 hrs. The disappearance of collagenase was not a result of enzymatic degradation or inherent instability, as purified enzyme was stable in both fresh and UMR 106-01 conditioned media for 72 hrs. The addition of increasing amounts of exogenous collagenase indicated that the removal of enzyme from the medium is a saturable mechanism. Using radioiodinated enzyme we then demonstrated the existence of a specific receptor for collagenase. We showed that appreciable amounts of collagenase were bound at 4°C within 10 min and reached equilibrium by approximately 2 hrs. Furthermore, using increasing amounts of labelled enzyme, we showed by Scatchard analysis that collagenase was bound with a high affinity ($K_d \sim 10^{-11}M$) and that there are approximately 10^4 receptors per cell. We therefore hypothesize that a cell surface receptor specific for collagenase might serve to regulate the amount of extracellular enzyme. An understanding of the regulation of extracellular enzyme may provide insight into the mechanism of skeletal bone loss in a weightless environment.

CIRCADIAN RHYTHMS OF BODY TEMPERATURE AND METABOLISM IN THE RHESUS MONKEY

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The circadian timing system regulates the time of day variation of many physiological, biochemical and behavioral systems. In mammals temperature regulation is not constant, but follows a daily rhythm in which, for example, it is elevated during the light period of the day in diurnally active species. The body temperature rhythm is controlled by rhythms in metabolic heat production and heat loss. The balance between these effectors determine body temperature at any time of day. Thermal balance in mammals is thus a dynamic process.

To examine the rhythms of heat production, body temperature, and physical activity in the Rhesus monkey, four adult male Rhesus monkeys were maintained in a 24-hr light-dark cycle (LD 12:12) in closed chambers with air circulated at a constant rate. Oxygen consumption was measured to determine metabolic heat production, corrected for respiratory quotient by simultaneously measuring carbon dioxide production. Body temperature and physical activity were measured by means of telemetry.

Body temperature, physical activity and heat production all followed a daily rhythm and were elevated during the period of lights on. Resting metabolism was approximately that predicted by allometric equations. Activity was mainly limited to the lights on period. Elevated activity following lights on was followed by elevated metabolism and a subsequent rise in body temperature. Respiratory quotient increased during the animals' active period; however, this increase occurred later than increased activity, metabolic rate, and body temperature. Variation in the timing of rhythm peaks suggests that Rhesus monkeys, like humans, may be either "early" or "late" individuals relative to the daily light-dark cycle.

INCREASED MUSCLE RESPONSES TO INSULIN FOR GLUCOSE UPTAKE AND METABOLISM IN RATS AFTER HINDLIMB UNLOADING

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This study was designed to measure rat hindlimb muscle glucose uptake, oxidation, and incorporation into glycogen after 2 wks of hindlimb unloading (simulated weightlessness). Adult male Sprague-Dawley rats (250-325 g) were assigned to either unloading in a head-down position (45°) so that hindlimbs were non-weightbearing (HS) or to control cages (CC) for 14 days. Immediately after HS or CC conditions, the rats were surgically prepared and their hindlimbs perfused for 45 min with a Krebs-Henseleit buffer containing aged-rejuvenated erythrocytes, 4% albumin, 6mM glucose, and D-[U-¹⁴C] glucose tracer. The insulin concentration of the perfusate was 0, 90 or 24,000 μ U/ml. Glucose uptake and oxidation was determined for the entire hindlimb preparation, while ¹⁴C-glucose incorporation into glycogen was measured in the soleus (SOL), plantaris (PL), and extensor digitorum longus (EDL) muscles as well as in a portion of the white gastrocnemius (GW). The results indicate that glucose uptake at 24,000 μ U/ml insulin (maximum insulin stimulation) was significantly ($p < 0.05$) greater in the HS rats than the CC rats by 18%, signifying an increased insulin responsiveness. In addition, glucose uptake at 90 μ U/ml insulin concentration (% of maximum response to insulin) was significantly greater in HS rats ($53 \pm 6\%$) than CC ($24 \pm 6\%$), indicating increased insulin sensitivity. Increased sensitivity to insulin was also observed for glucose incorporation into glycogen in the SOL, PL, and EDL muscles, but not in GW, after hindlimb unloading. Furthermore, hindlimb glucose oxidation was significantly elevated for the HS rats compared to CC rats at both 90 and 24,000 μ U/ml insulin. However, insulin sensitivity for glucose oxidation was not significantly different between the HS and CC groups. It remains uncertain whether these results are caused by the non-weightbearing conditions and consequent muscle length changes imposed upon the muscles during hindlimb unloading, or by a systemic factor related to the restraint of the animals. Lower body fat percentages and increased plasma catecholamine concentrations in hindlimb unloaded rats have been observed in our laboratory and are among the systemic factors that influence muscle glucose metabolism.

GRAVITROPISM IN THE MOSS *CERATODON*

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Dark-grown apical cells of protonemata of the moss *Ceratodon purpureus* (Hedw.) Brid. are negatively gravitropic (grow up) and appear to utilize amyloplasts in gravity sensing (i.e., they appear to function as statoliths). The kinetics of gravitropism and of amyloplast sedimentation support this hypothesis. After vertically growing protonema were reoriented to the horizontal, amyloplast sedimentation started within 15 min. This is within the 12-17 min presentation time for *Ceratodon*, and prior to upward curvature which occurs 1 hr after gravistimulation. The tip cells exhibited a characteristic amyloplast zonation, with a tip cluster of non-sedimenting amyloplasts, an amyloplast-free zone, and a zone with pronounced amyloplast sedimentation. This latter zone appears specialized more for lateral than for axial sedimentation since amyloplasts sediment to the lower wall in horizontal protonemata but do not fall to the basal wall in vertical protonemata.

Additional support for the role of amyloplasts as statoliths in gravity perception come from basipetal centrifugation experiments. Basipetal centrifugation displaces all amyloplasts in the apical cell to the end wall. In basipetally centrifuged protonema observed using infrared videomicroscopy, tip extension occurred with or without amyloplasts present in the apical dome. The initial return of upward curvature was always correlated with the return and sedimentation of amyloplasts in zone 3. Subsequent vigorous upward curvature was correlated with distinct amyloplast zonation and further sedimentation in zone 3. Initial downward ("wrong way") curvature, which often preceded upward curvature, correlated with the presence of amyloplasts in the apical dome (zone 1). These data support the hypotheses that non-sedimentating amyloplasts in zone 1 are necessary for initial downward curvature and that amyloplast sedimentation in zone 3 is necessary for upward curvature.

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