NASA Technical Memorandum 89809

1985-86 NASA Space/Gravitational Biology Accomplishments

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NASA Office of Space Science and Applications
Washington, D.C.
and
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National Aeronautics
and Space Administration

**Scientific and Technical
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Preface

Individual technical summaries of each research task within the Space/Gravitational Biology Program are presented in this publication. The summaries consist 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 this research during the past year. Accomplishments of particular significance in each task have been underlined. The summaries cover the period from January 1985 through April 1986.

The intent in compiling this publication is twofold. First, we wish to provide the scientific community with an annual summary of the accomplishments resulting from research pursued under the auspices of NASA's Space/Gravitational Biology Program. Secondly, we hope to stimulate an exchange of information and ideas among scientists working in the Program.

We would like to thank all of the participants in the Space/Gravitational Biology Program for their cooperative response to our requests for information. We would also like to thank F. Ronald Dutcher and Janet V. Powers for their editorial assistance and April Commodore Roy and Janice Susan Wallace for their technical assistance in the preparation of this report.

Thora W. Halstead
December 1986

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INTRODUCTION

THE NASA SPACE/GRAVITATIONAL BIOLOGY PROGRAM

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Introduction

One of the major features of the physical environment on the surface of Earth is the constant presence of the force of gravity. Terrestrial gravity has important biological consequences for organisms living on Earth. The phenomenon of weightlessness which is encountered on spacecraft provides an excellent biological research opportunity, both because of its uniqueness to space and because of the importance of gravity to life on Earth. Access to space provides an opportunity to manipulate gravity from its norm of one down to almost zero, effectively providing the full spectrum of gravitational research capability for the first time. This capability, combined with the stability and pervasiveness of gravity on Earth, its obvious impact on biological evolution, and its continuing effect on the morphology, physiology, and behavior of living organisms, has led the Space/Gravitational Biology Program to concentrate its efforts and resources on investigating the biological significance of gravity.

Program Goals

The goals of the Space/Gravitational Biology Program are to: use the unique characteristics of the space environment, particularly microgravity, as a tool to advance knowledge in the biological sciences; understand how gravity has shaped and affected life on Earth; and understand how the space environment affects both plant and animal species, thereby enhancing our capability to use and explore space.

Program Scope

Research in the Space/Gravitational Biology Program is divided into three broad areas:

1. Gravity sensing/perception. The objectives are to identify gravity receptors in organisms sensitive to gravity and determine their structure and function, and to elucidate the mechanisms by which gravitational stimuli are perceived and transmitted to a responsive site.

2. Developmental biology. The objectives are to determine the effects of gravity, and especially weightlessness, as provided by spaceflight, on the genetic integrity,

cellular differentiation, reproduction, development, growth, maturation, and senescence of living systems; and to examine the evolutionary importance of gravity as a determinant of the form and function of terrestrial life.

3. Biological adaptation. This area includes the use of gravity's physiological effects to explore biological problems; and achievement of an understanding of how gravity affects and controls the physiology, morphology, and behavior of organisms, of how gravity and other environmental stimuli and stresses interact in this control, and of the biological mechanism by which living systems respond and adapt to altered gravity, particularly that of the space environment.

Research Opportunities

With the proven feasibility of the Space Shuttle, we have the capability to perform biological experiments in space. The opportunity has arrived to use the locker space within the Shuttle orbiter on a continuing space available basis. This will provide a valuable augmentation to the ongoing ground-based research program.

Spaceflight will provide the validation for many experimental hypotheses developed in ground-based research, while gravitational experiments on Earth will continue to hone the questions, provide the necessary baseline data, and develop spaceflight experimental protocol.

The experimental approach of the ground-based studies in the Space Biology Program is to manipulate gravity on Earth and develop weightless simulation models to: (1) develop and test gravitational hypotheses, (2) identify gravity-sensitive biological systems and interacting environmental response mechanisms, (3) analyze biological systems and mechanisms known to be gravity-sensitive, (4) analyze flight experiment data and iteratively expand ground research capability, and (5) plan and design future space experiments. In addition, research is conducted to understand how the uncontrollable biodynamic factors of the spacecraft will affect the results of the various flight experiments.

Focus of Program

The current Program is focused on answering the following basic scientific questions:

1. What are the components of the gravity-sensing mechanisms of plants and animals? How do they perceive information? How is the information transmitted to evoke responses?

2. Does gravity influence fertilization and development of plants and animals, and can fertilization and development proceed normally in a near zero gravity environment? If gravity does affect fertilization and development, what are the sensitive physiological systems and how are they affected? If early development is affected by gravity, is it a result of an effect on the parent or a direct effect on the embryo itself?

3. What is the role of gravity in the formation of structural elements such as lignin, cellulose, silica, chitin, and bone calcium phosphates at the molecular level as well as at more complex organizational levels?

4. What role does gravity play in calcium-mediated physiological mechanisms and in calcium metabolism?

5. How does gravity as an environmental factor interact with other environmental factors to control the physiology, morphology, and behavior of organisms? Or, how do gravitational and other environmental stimuli interact in the control and direction of living forms? Can the action of gravity be replaced by different stimuli?

The research focus of the Space/Gravitational Biology program is dependent upon several dynamic factors: the requirements of NASA, the characteristics of flight experiment opportunities, the sensitivity of specific biological systems to gravity, the scientific value of the research, the state of knowledge and technology in the specific scientific areas, the interest of scientists in studying the biological questions, and the availability of funds to support the research.

Since Space Shuttle flights have been suspended until early 1988, new research proposals for flight experiments will not be funded until we know when we will have access to spaceflight.

ACCOMPLISHMENT HIGHLIGHTS

GRAVITATIONAL/SPACE BIOLOGY
ACCOMPLISHMENT HIGHLIGHTS

PLANT

Ground-Based

- A model for plant gravity perception involving calcium, auxin, and ion movement has been proposed.
- Calcium and low pH reduce auxin movement in the vascular stele.
- Calmodulin is associated with vacuoles, nuclei, and plastids, but not with cell walls.
- Calcium and calmodulin modulate the phosphorylation of nuclear proteins.
- In the root, calmodulin and calcium are involved in the sensing process, without auxin transport.
- Starch-containing organelles (chloroplasts) have been shown to be the graviperceptive bodies in cereal grass pulvini.
- Evidence is provided for describing a mechanotransductive mechanism in plant membranes for the transduction of physical stimuli, including gravity. This involves a pressure-sensitive channel which opens in response to very small changes in pressure applied to the inside of the plasma membrane.
- Tissue sensitivity to auxin appears to change in response to gravistimulation.
- In lazy corn mutants (which do not respond to gravistimulation) there is a lesion in the transduction process which results in a failure for hormones such as gibberellins to become asymmetrically distributed and synthesized.
- mRNA synthesis in the root cap is necessary for root gravitropism.
- The only pigment associated with the gravitropic response in corn roots is the red light absorbing pigment, phytochrome.
- During gravitropic bending, both sides of the stem react simultaneously - the upper surface ceases expansion or undergoes a small contraction while the lower surface more than doubles its growth rate.

- The changes in cell growth rate during gravitropic bending are due to changes in cell wall extensibility properties, not to changes in cellulose microfibril orientation or in properties governing cell water transport or water pressure.

- Ca^{++} chelation per se does not appear to result in cell wall loosening (and thus cell extension growth).

- Plants are generally more responsive to mechanical stresses when grown and treated at low light intensities than at high light intensities.

Spaceflight

- Corn seedlings grown in space on STS 61-C showed:

  - microgravity reduced root elongation and increased shoot elongation

  - microgravity may be required to regenerate root caps (i.e., the putative gravity-perceiving organ) of decapped roots

  - gravity may be necessary to distribute the endoplasmic reticulum in root cap cells

  - microgravity may cause putative statocytes to partition less volume to amyloplasts (i.e., putative statoliths)

  - microgravity may induce organelle-specific changes in cellular structure and patterns of cellular differentiation in root caps

- Roots of oat seedlings grown on Spacelab-2 showed:

  - fragmented chromosomes

  - severely retarded cell division

  - reduced metabolic activity

  - unusual root branching indicative of upset hormone balance

- Oat and mung bean seedlings grown on Spacelab-2 formed less lignin, a biochemical substance that with cellulose provides the supporting structure of plants.

- Observations of sunflower seedlings grown in space have proven that the ubiquitous spiral growth pattern of plants (called circumnutation) persists in the absence of a gravitational force.

ANIMAL

Ground-Based

- Sensory information in the vestibular gravity sensor (bioaccelerometer) of mammals is integrated on site as well as through the central nervous system.

- Within the mammalian gravity sensor, the otoconial crystals (test mass) are linked directly to innervated detector hair cells.

- Calcium is ubiquitously located on cell structures of the mammalian vestibular gravity sensing system.

- Jellyfish develop reduced numbers of statoliths when rotated to continually change the gravity vector.

- Disorientation with respect to the gravity vector affects animals as follows:

  - cell division and chromosome separation is inhibited in some mammalian (mouse) eggs

  - fertilization of mammalian eggs is normal

  - maturation of amphibian embryonic nerve and muscle cells is delayed

- In hypergravity centrifuge studies, embryonic mouse limb cells differentiate at an accelerated rate, resulting in shorter, less ossified bones.

- The complete proliferation and differentiation sequence for a primitive cell type to become a bone-forming cell (osteoblast) has been modeled.

- Skeletal unloading reduces the accumulation of dense, highly mineralized mature bone, in addition to reducing bone formation.

- Adrenalectomy does not prevent the inhibition of bone formation induced by skeletal unloading.

- Denervation induces significant changes of several enzymes measured in rat soleus muscle but not in EDL muscle, in contrast to the effect of unloading which induces similar changes in both muscles.

- Energy stores, which are necessary to trigger muscle contraction, are decreased as muscle mass is lost.

- Some brain cells mediate an increase in renin (a kidney compound which ultimately causes an increase in blood

pressure), while other types of brain cells mediate a decrease in renin. This could lead to regulation of blood pressure using compounds regulating these brain cells.

- Acclimation to hypergravity modifies the neural control system for temperature regulation in mammals.

- The presence of a hyperdynamic environment depresses primate body temperature for an extended period (more than 48 hours).

Spaceflight

- Muscle changes observed in rats flown on Spacelab-3 have validated the rat model.

- Insulin receptor and glucose mobilizing proteins on muscles are not affected when muscle wasting is due to unloading in space or by the rat model, but they are lost when wasting is caused by denervation.

- Mammalian (rat) bone-forming cells formed during spaceflight are reduced in size, suggesting decreased production of bone tissue.

- Fewer bone-forming cells develop during spaceflight.

- Bone growth in growing rats was significantly inhibited during the 7-day Spacelab-3 flight. Periosteal bone formation rate at the tibiofibular junction was significantly suppressed.

- Pituitary growth hormone cells from rats flown in space release less hormone, suggesting a direct effect of gravity on hormone release mechanisms.

- The microgravity of spaceflight has a significant influence on the circadian timekeeping system of rodents.

PLANT PROJECTS

AN ATTEMPT TO LOCALIZE AND IDENTIFY THE GRAVITY-SENSING MECHANISM OF PLANTS

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Description of Research

The overall objective of this research is to determine how, at the molecular level, a plant can perceive a gravitational stimulus and transduce this stimulus into the appropriate growth response.

We believe that the growth response of a plant to gravity may be too complex to permit an understanding of the perception and transduction of gravity at the molecular level. For that reason we have concentrated on a gravitationally induced chemical change--that is, the asymmetric distribution of the growth hormone, indole-3-acetic acid (IAA), and its ester conjugates. This chemical asymmetry occurs within 3 minutes after the gravitational stimulus and can be measured by accurate and specific physicochemical methods. Further, we have used young corn seedlings as our experimental plant, and with this plant it is possible to separate the vascular stele from the surrounding cortical and epidermal tissue so that it is possible to study IAA transport separately from IAA metabolism. Using this experimental system, we believe we can determine how the plant perceives gravity and then transduces that stimulus into a change in the amount of IAA and ester IAA in the cortical tissue.

As our experimental results and knowledge have accumulated, we have been able to formulate a working theory as to how a plant, without a complex nervous system, can perceive gravity and then translate that perception into a chemical change and even, finally, a growth change. The postulates of the theory are: that gravity causes the settling of charged cell organelles; that the movement of these organelles perturbs the cell's bioelectric field; that this perturbation opens and closes potassium and calcium ion transport channels; that this ion movement and accumulation amplifies the original stimulus, so as to voltage-gate the plasmodesmatal connections between cells in a manner analogous to animal gap junction cells; this allows IAA and its ester conjugates to move from vascular stele to the cortical cells, where metabolism of the hormone leads to asymmetric growth permitting the plant to grow back into its normal vertical orientation.

This is a working theory, but to our knowledge it is the first testable theory for a plant's response to gravity that integrates all that is known of the physiological and chemical changes induced by a gravitational stimulus. The theory accommodates the

geoelectric effect, the changes in potassium and calcium distribution, the changes in IAA distribution, and the growth changes.

Accomplishments

Progress has been made in understanding both the transport of the hormone and the metabolism of the hormone. Both kinds of knowledge are required since both transport and metabolism change the steady state amounts of IAA in a tissue.

(1) Hormone transport:

(a) We found that we can load the vascular stele of corn shoot tissue with IAA and then observe the movement of IAA out of the vascular stele and into the cortical cells. This finding may permit us to look for voltage-gating of hormone movement.

(b) We found that calcium and low pH reduces movement of IAA in the vascular stele. Calcium and low pH reduces gap-junction transport in animal systems.

(c) We have reproduced earlier experiments showing that applying an electrical potential to a plant with the tip of the plant negative will accelerate growth, whereas a positive potential at the tip will reduce growth. Completion of these studies will enable us to look for changes in IAA distribution following application of an electrical potential, thus testing our working hypothesis.

(2) Hormone metabolism:

(a) We have found that the pathway for IAA oxidation is from IAA to oxindole-3-acetic acid to 7-hydroxy-oxindole-3-acetic acid to 7-hydroxy-oxindole-3-acetic acid glucoside. The compound 7-hydroxy-oxindole-3-acetic acid and its glucoside had not previously been isolated nor synthesized so this is an important addition to knowledge of indole catabolism.

(b) We have shown that neither tryptophan nor tryptamine are important precursors of IAA in the corn seedlings.

(c) We have isolated and partially purified the enzyme that oxidizes IAA to oxindole-3-acetic acid and found that this enzyme uses linolenic or arachidonic acid as a co-substrate. This finding may link IAA oxidation to the metabolism of plant membrane components.

(d) We have developed methods for obtaining sufficient amounts of the enzyme which catalyzes the formation of IAA-glucose from IAA and UDPG. In the case of this enzyme and the enzyme that oxidizes IAA, it will be possible to purify these enzymes sufficiently to clone them and study their control mechanisms.

Significance of the Accomplishments

(1a) Learning how a hormone is transported within a plant will be of use in agricultural practice and may help in growing plants in microgravity. More immediately, this may well be the simplest system for studying transduction of the gravitational

stimulus by a biological system.

(1b) The finding of similarities between plant plasmodesmatal control and animal gap-junction control will enable transfer of knowledge from one system to another.

(1c) If cell to cell transport of a plant hormone does respond to an electrical potential, it will help explain the mechanism of a plant's response to gravity at the molecular level.

(2a) Knowing how a plant oxidizes its growth hormone will permit regulation of the plant's growth hormone level.

(2b) It is important to understand the mechanism of synthesis of IAA to understand how gravity changes growth hormone amount.

(2c) Linkage of IAA oxidation to lipid metabolism provides a clue as to the mechanism of action of the hormone.

(2d) Purifying any hormone-metabolizing enzyme to homogeneity provides a way of cloning the enzyme and studying how the activity of that enzyme is controlled by the gravitational stimulus.

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PLANT HORMONE DISTRIBUTION IN SPACE

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Description of Research

From ground-based experiments we know that displacement of a plant from its normal vertical orientation will cause a change in growth hormone IAA (indole-3-acetic acid) or distribution within 3 minutes following the orientation change. This is a remarkable example of transduction of the gravitational stimulus into a chemical change. Experiments done on Earth with the corn seedling system have already indicated that the gravity perturbation results in an altered distribution of both IAA and its ester conjugates. This has led us to believe that gravity-sensing leads to: a) altered IAA metabolic rates; and b) altered leakage of IAA from the plant's vascular system into the surrounding cortical cells. We postulate that the altered leakage is due to potential-gating occurring in the plant's vascular transport system. The experiments are leading us into an understanding of the electrophysiology of the plant and, possibly, to a real understanding of how the gravity stimulus is sensed.

Accomplishments

An experiment, designated NS/401, has been formulated and prepared for flight to examine the level of IAA in plants grown in microgravity. The experimental plants will be germinated and grown in the dark in special containers. The cannister containers must permit the plants to be frozen in flight without the use of significant crew time and without opening the cannisters.

The experiment is ready for flight. A total of 11 cannisters for growing the plant material have been constructed and experimental verification and biological verification tests have been performed. The filter paper growth-support medium and the plastic sleeves to contain and support the paper and germinating seeds have all been tested in the experimental and biological verification tests and are flight ready.

The major effort over the past year has been the design and construction of the cannisters and the testing of the cannisters as to their suitability for growing plants in microgravity. The cannisters have proven to be excellent and it is believed they will be suitable for many short-duration (5 to 7-day) flights in which 10 to 20 seedling plants are to be grown in darkness and frozen in flight. Since much plant hormone and plant metabolism

research is conducted on dark-grown seedling plants, the cannisters should have broad utility.

Each cannister consists of two anodized aluminum cans with a threaded screw cap. The cap of each can has four light-baffled holes to permit escape of carbon dioxide and ethylene and for oxygen renewal. The ethylene, carbon dioxide, and oxygen levels within a simulated cannister after 144 hours of growth of 14 corn seedlings were 0 to 0.5 ppm, 2% and 18%. These are reasonable levels.

A middeck locker can hold two cannisters and one LN2 freezer so that 4 x 14 seedlings, a total of 56, can be grown. Twenty-eight of these seedlings may then be frozen in flight, since the LN2 freezer will hold one cannister. The remaining 28 plants can be recovered upon landing or could be chemically fixed in flight.

Since each seedling is rolled in filter paper and each filter paper roll is encased in a Teflon sleeve, the plants may be individually recovered after freezing and without thawing for assay procedures.

Work in Progress and Planned

Although NS/401 is flight ready, we can use the flight delay time to improve our analytical capabilities and to do further biological verfication tests:

(1) Plant growth in the cannisters. Now that cannisters are available, we wish to redo the measurements of carbon dioxide, ethylene, and oxygen in the cannisters. In the simulated containers we approximated the 1-mm light-baffled holes, but it was not possible to exactly duplicate the gas exchange that would occur in a cannister. Thus, we plan to measure these three gases as a function of germination time and over the range of temperatures that might be encountered in the middeck. These will be baseline data. If oxygen depletion or carbon dioxide or ethylene accumulation is excessive, then a solution would be to reduce the number or size of plants, or to reduce experiment duration, or to introduce a solid carbon dioxide or ethylene absorbent. Data from the simulated containers indicate this will not be necessary, but it is a very important experimental condition.

(2) Recovery of frozen plant material. After living tissue is frozen, it is necessary that the enzymes in the tissue be inactivated. This can be done by thawing the tissue in a polar solvent such as acetone. It would, however, be desirable if the tissue could be dissected while still in the frozen state so that separate hormone assays could be performed on such tissues as vascular and cortical tissues. We wish to devise ways of "thawing" the tissue in, for example, glycerol or glycol, so that the tissue could be dissected without the temperature rising above $-5^{\circ}C$. The more finely we can dissect the tissue, the more information we can recover concerning the effects of microgravity on hormone content and movement within the plant.

(3) Assay of IAA. This laboratory and that of our student J. Cohen has been responsible for the development of the mass spectrometric-gas chromatographic assays for IAA currently in use. We synthesized 4,5,6,7-tetradeutero-IAA, and Cohen has now synthesized $6C^{13}$ (ring)-labeled IAA for use as an internal standard. Thus, it is possible to assay for IAA with great certainty as to the identity of the IAA. With the use of a new mass spectrometer we intend to increase the sensitivity and precision of the IAA assay. The more sensitive we can make the assay, the more information can be obtained from the limited amounts of flight-grown material available.

(4) Effect of middeck conditions on IAA content. In addition to the possible effects of microgravity on the IAA content of the plant tissue, there will be certain effects of the varying middeck temperature and possible effects of the cannister gas environment. There has been no systematic study of the effect of these variables on the IAA tissue content. Since a 1-g centrifuge for use in a middeck locker is not available, we wish to study enough of these variables so that we can separate the effects of microgravity on IAA content from any other variable. We wish to be certain that our IAA levels will be independent of temperature over about a 10^{o} range and independent of fluctuations of 25% in the gas environment.

(5) A metabolic profile of microgravity-grown plants. Plant material grown in microgravity will be available in only a limited amount and at considerable cost. We wish to obtain every bit of chemical data that can be obtained from the material available. This is of value in that it is important that metabolic profiles be obtained for biological materials exposed to microgravity. Within the limits of flight-approved experiments and the needs of other investigators for flight material, it will be desirable to do such other assays as can be accomplished without compromising the planned experiment. For example, we can determine gross metabolic rates by measuring residual starch in the seeds of the plant material. We can look for lipid changes such as Professor Arthur Smith observed in chickens exposed to hypergravity. We will try to develop as many peripheral chemical assays as possible to provide a metabolic profile of changes occurring in hypogravity. We will pay particular attention to metabolic pathways operating in polyphasic systems, such as the cytoplasm and the mitochondria where the mitochondria have a density different from that of the cytoplasm.

RESEARCH IN GRAVITATIONAL PLANT PHYSIOLOGY

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Description of Research

The long-range goal of the research, in most general terms, is to improve our understanding of how gravity is important for plant development and physiological behavior. The unique advantage offered by experimental access to the protracted microgravity environment of spaceflight has made the exploitation for scientific purposes of flight opportunities the major but not exclusive operational objective of our research.

The short-range goals include:

(1) To derive the maximum amount of scientific information from data acquired from the Spacelab-1 HEFLEX Experiment.

(2) To evaluate, by experiments with clinostat and centrifuge, the validity of several kinds of hypogravity simulations. The favored test system is hypocotyl circumnutation of Helianthus annuus because we now have more extensive data on the g-functions of this system than on any other.

(3) To implement the objectives of GTHRES, a candidate flight experiment that will measure the kinetics of gravitropic responses of oat seedlings in near weightlessness, that is, without the complication of a significant background g force.

(4) To implement the objectives of the FOTRAN candidate flight experiment, which will measure responses of wheat seedlings to photic stimuli in near weightlessness, again without the complication of a background 1 g force.

(5) To implement objectives of a proposed AMYSED flight experiment, which will measure the relationship between plants' perception/response to an imposed g force stimulus and the extent of amyloplast sedimentation under a low g force (much less than unit g). Under those conditions, the times for plastid sedimentation and for evident g perception can be made much longer than at unit g and could yield results that may support (or may lead us to revise) current assumptions of the causal sequence: plastid sedimentation leading to perception and tropic response.

Accomplishments

(1) Further analyses of data from the SL-1 HEFLEX Experiment reinforced our earlier conclusion that a gravity force is not required for persistence of circumnutation. This important result disproved the theory which had interpreted circumnutation as a kind of gravitropic response. HEFLEX data enhancement was given consideration as a means of salvaging some video data that were not interpretable due to data recording problems of unknown origin. About 40% of the data were in that category. With the aid of personnel at the Jet Propulsion Lab Data Enhancement Laboratory we have examined methods that may enable us to retrieve as much as 30% more data from the HEFLEX Experiment. This effort is in progress although its chance of success remains uncertain.

(2) A serendipitous finding from the HEFLEX Experiment was that the kinetics of circumnutational growth movement were quantitatively not the same in true microgravity as in clinostat-simulated weightlessness. The unexpectedly large discrepancy required the conclusion that, at least for circumnutation, gravity compensation by the horizontal clinostat is not the equivalent of weightlessness. Complacency about the assumed validity of clinostat simulations is clearly not warranted -- a matter of far-reaching importance for some kinds of research in gravitational biology. HEFLEX data contradict the sometimes disingenuous suggestion that tests with clinostats could obviate the need for experiments on biological materials in orbiting vehicles.

(3) Stimulated by results from the HEFLEX Experiment, our laboratory along with others has renewed theoretical studies on possible driver/controller mechanisms that may account for circumnutation. An interesting "spin off" from those studies has been a possible explanation for why circumnutation is essentially ubiquitous for all elongating plant organs. It was shown that the plant organ's gravity sensor is more sensitive to axially imposed forces in the g range less than one g than it is at (or above) one g. If, by circumnutating, the plant organ can maintain, on average, a substantial departure from the plumb line (or other "preferred" growth direction), it should be able to determine that preferred direction with greater precision than if it did not circumnutate. Perhaps this simple advantage has been sufficient for it to be incorporated into the growth control systems of all plants in the course of their evolutionary history. The same reasoning suggests that the typical plant organ should be less sensitive to illumination that impinges on the growing tip almost directly in line with the organ's morphological axis than to illumination that is "off axis" -- in the extreme case, to light applied laterally (transverse to the organ's morphological axis). We have as yet no other general explanation for the universality of circumnutational growth behavior.

(4) Instrumentation: We are evaluating a GE TN2505 Video Camera. This recently developed, visible and infrared, surveillance-type camera is small, with many capabilities. We

have been evaluating its performance features with the hope of incorporating it into flight hardware to implement a proposed middeck locker experiment, AMYSED. This study is in progress; results so far are very encouraging.

A paired unit consisting of two variable speed centrifuges each with payload monitoring by video camera surveillance is being designed. The design concept was for the immediate purpose of implementing a proposed middeck locker experiment, AMYSED, but the potential applications for such a flight qualifiable centrifuge facility go far beyond AMYSED. We have been consulting with the personnel of other laboratories where we can identify potential users of the AMYSED centrifugation hardware for effective access to the full "g spectrum" so that we can incorporate into the development specifications payload accommodations that will be as versatile as possible. This design effort is at an advanced stage and is still in progress. An important advantage of this experiment facility is that it will enable tests to be carried out on small biological specimens in the Shuttle middeck, which is likely to be significantly more available for experimentation than the infrequently launched Spacelab.

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MICROGRAVITATIONAL EFFECTS ON CHROMOSOMAL BEHAVIOR

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Description of Research

The long-range goal of this research is to further understand the evolutive role of gravity on cell division. Its significance is to provide information on the potential genetic risk of the space environment to human health and reproduction.

The major thrust of the research activity, begun on October 1, 1985, has been the preparation of the biological specimens to be flown on Spacelab's Biorack. The specific objectives are: (a) to determine the effects of microgravity and cosmic radiation on chromosomal integrity, recombination, and transmission during mitotic and meiotic cell division; (b) to discriminate between chromosomal events sensitive to microgravity and radiation; and (c) to relate these findings to changes in the mating type (sex) of the cells and in the sporulation (gametogenesis) process. To accomplish these objectives we employ yeast cells as a eukaryotic model system.

Accomplishments

(1) Strain construction. A diploid strain marked with a multitude of mutations on all the chromosomes has been constructed. We are in the process of obtaining, by micromanipulation, haploid derivatives of this strain containing one extra copy of one chromosome.

(2) Fixation studies. We determined the cellular effects of prolongated potassium permanganate ($KMnO_4$) fixation. The results showed a remarkable degradation of the cell wall and membrane after only 24 hours of exposure. On the other hand, fixation with up to 5% of glutaraldehyde did not show any adverse effect on the cell structure.

(3) Oxygen consumption. We have quantitated the effect of limited oxygen supply on generation time, per cell. In a typical experiment, there is a delay of about 2 hours in reaching maximum cell density (saturation). The saturation level is 50% of the control when oxygen is limited.

(4) Sporulation delay. We have calculated the amount of glucose necessary to delay the cells before becoming committed to sporulation. The concentration of glucose in the sporulation medium has been titrated with respect to the delay time.

(5) We have conducted preliminary experiments with a Fluorescence-Activated Cell Sorter (FACS) to detect changes in chromosome number in viable cells. The results show that we are able to detect differences as little as only one chromosome.

(6) The effect of hypergravity on cell division has been

studied, and the results are striking. A level of 10 g is sufficient to kill 100% of the yeast cells. This dramatic effect is currently under investigation to determine the gravity threshold level and the minimum amount of time necessary for this level to affect cell viability.

Significance of the Accomplishments

Results from experiment 1 provide us with some of the yeast strains necessary to perform the genetic tests in space and on the ground.

Results of experiments 2-4 yielded essential data on the feasibility of the flight experiment as well as on the biocompatibility of the flight hardware environment. These data will be integrated into the postflight analysis of the experimental package.

The preliminary results of experiment 5 are very encouraging about the possibility of using the FACS system to quickly screen a large population of cells for chromosome loss and/or breakage. The use of this system could be extended to the chromosomal analysis of other cell types for basic as well as medically applied diagnostic purposes.

The results from experiment 6 are extremely important. If a precise correlation between hypergravity and alteration of cell division processes is confirmed, it would represent a major finding at the cellular level in gravitational biology. Chromosome behavior and dynamics will now be analyzed in particular to verify whether chromosomal segregation can be one of the genetic targets of gravity. This could lead to study of the potential genetic risk of cells and tissues undergoing centrifugal and/or gravitational stress.

While most of the past work provides the information necessary for the correct construction and performance of flight experiments, some of it has direct implications for the health and safety of the individuals who fly in space.

THE ROLE OF GRAVITY IN APICAL DOMINANCE

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Description of Research

Apical dominance, the control exerted by the shoot tip over the outgrowth of the side buds, determines to a large extent the branching patterns and the shape and form of herbaceous plants, shrubs, and trees. Since it has been demonstrated that apical dominance is sensitive to changes in the direction of gravity, it is important that the interaction between gravitational forces and the control mechanisms of bud outgrowth be elucidated. Shoot-inversion release of apical dominance (i.e., the outgrowth of the highest lateral bud) in Pharbitis nil provides a promising system for such a study. Our long-range objective is to enhance our understanding of the interaction between gravity and the regulatory mechanisms of bud growth on a precise biochemical basis. Our immediate objective is to investigate the role of the gaseous plant hormone ethylene in gravity-induced release of apical dominance during shoot inversion.

In plant shoots which were oriented upright, inverted, and/or rotating, analyses were carried out on bud growth, ethylene production and ACC synthase activity. Effects of gibberellin treatment and analyses of hydroxyproline, lignin, and peroxidase in upright and inverted shoots were also made.

Accomplishments

(1) Promotion of apical dominance release in plants vertically rotating about a horizontal axis of a clinostat was shown primarily to be due to gravity stress and not to other factors.

(2) Shoot inversion was demonstrated to induce ethylene production within 2-2.5 hours, a significant inhibition of extension growth within 3 hours (preliminary data) and a significant increase in stem radial expansion within 48 hours.

(3) Shoot-inversion induction of ethylene has been found to be accompanied by increases in ACC synthase activity.

(4) Rotation of plants with inverted shoots on clinostats and exogenous gibberellin treatments of inverted shoots of stationary plants counteracted the retarding effect of inversion on stem elongation and prevented the release of apical dominance. While the clinostating significantly reduced ethylene production in the inverted shoot, gibberellin treatments increased it. However, ethrel applications reversed the gibberellin effect.

(5) The growing region of the Pharbitis nil shoot has been found to be confined to the terminal 13 cm of the shoot (Figure 1). No further growth occurred below this region. Maximum

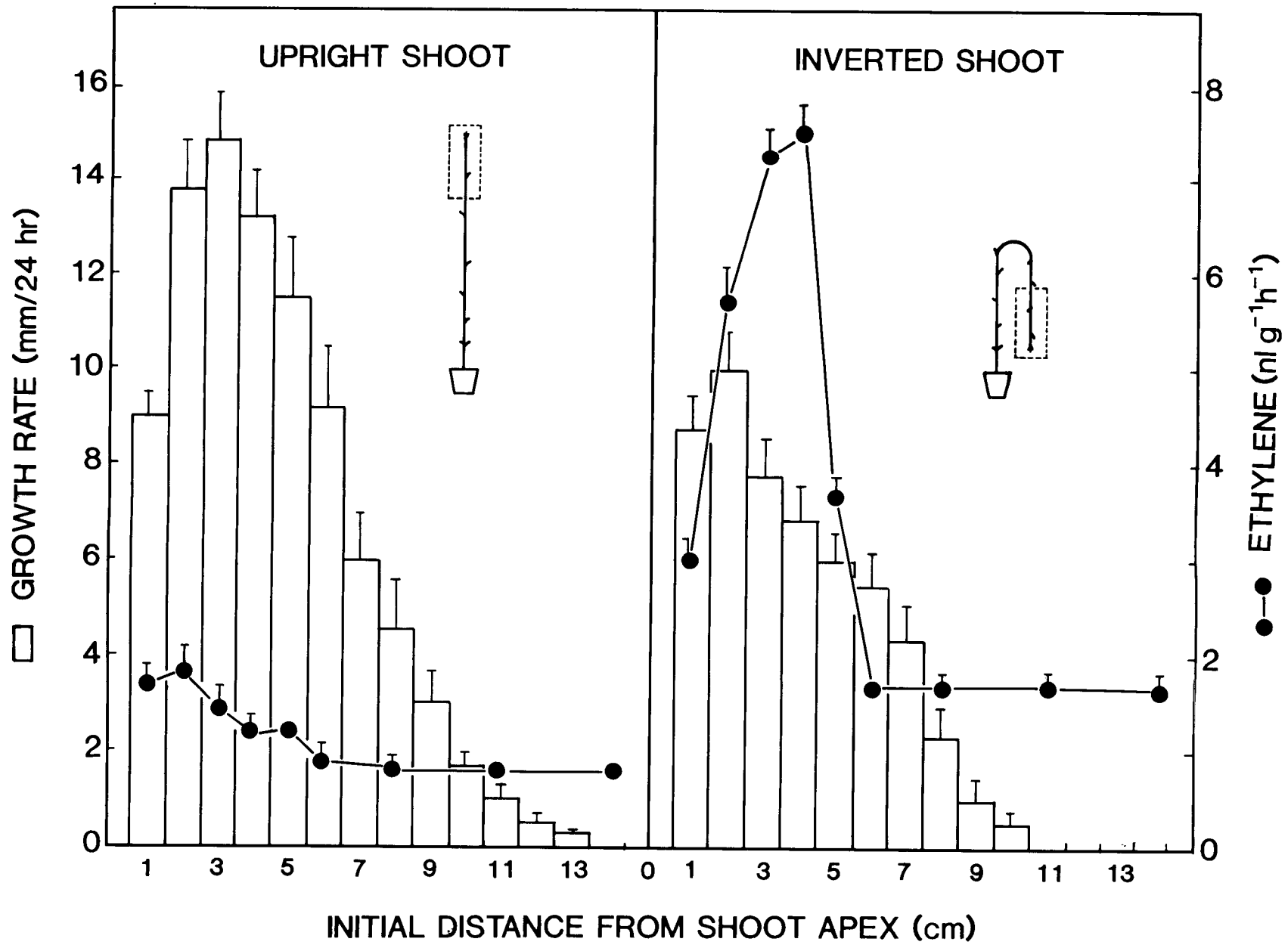

Figure 1. Localization of the growth region and the effects of endogenous ethylene production in upright and inverted Pharbitis shoots. Bar height represents growth during a 24-hour period at different distances from apex.

ethylene production in the inverted shoot also occurred in this region. Ethylene restriction of elongation in this growth region of our inverted shoot induced apical dominance release.

(6) Preliminary evidence indicates that endogenous levels of hydroxyproline, lignin, and peroxidase are higher in inverted shoots than in upright shoots.

Significance of the Accomplishments

Finding #1, that shoot-inversion promotion of lateral bud outgrowth was due to gravity stress and not to other factors, is of critical importance. Although a change in the direction of the gravity vector associated with inversion of the upper shoot would seem to be the obvious cause of apical dominance release, the possible effects of other factors such as stem-bending stress or changes in the light intensity impinging on the highest lateral bud cannot be ignored. Even though the clinostat treatment did not eliminate the presence of gravitational forces, the fact that the continual change in the direction of the gravitational vector upon the shoot (associated with clinostating) prevented the release of apical dominance (Finding # 4) would seem to eliminate these nongravity influences as major causal factors.

Finding #2, that the beginning of shoot-inversion-induced ethylene production was detected before significant inhibition of elongation of the inverted shoot was observed to occur, is consistent with a cause and effect relationship between the two.

In Finding #3, the fact that the gravity stress incurred by shoot inversion was followed by increased activity of ACC synthase and increased production of ethylene follows the trend of other types of stress-induced ethylene phenomena which have been found to be mediated by the activation of ACC synthase.

Finding #4, that clinostat and gibberellin treatments of this inverted shoot counteracted the retarding effect of inversion on stem growth and promoted elongation while preventing apical dominance release, is consistent with the hypothesis that restriction of growth of the inverted shoot is a necessary prerequisite for apical dominance release. Thus it appears that it is the extent of elongation of the terminal shoot which controls lateral bud outgrowth. The fact that clinostating also decreases ethylene production in the inverted shoot supports a body of previously accumulated data from our laboratory which strongly suggests that the above-mentioned restriction of growth in the inverted shoot is probably due to ethylene generated by the shoot during inversion. Hence, the role of ethylene in lateral bud outgrowth is indirect. It induces lateral bud outgrowth only to the extent that it inhibits extension growth of the shoot. The elongation-promoting effect of gibberellin on the inverted shoot is apparently strong enough to override the inhibitory effect of the endogenous ethylene whose production is stimulated by gibberellin. The implications of this result and

the fact that the gibberellin effect can be reversed by added ethrel require further study.

Finding #5, that the region of growth and maximum ethylene production was found to be located in the terminal 13 cm of the shoot and that the restriction of growth in this region induced the release of apical dominance, suggests, very significantly, that it is the entire terminal 13-cm growth region and not merely the tip of the shoot that controls apical dominance.

Finding #6, that shoot inversion stimulated an increase in endogenous levels of hydroxyproline, lignin, and peroxidase, suggests possible modes of action for shoot-inversion inhibition of elongation. Ridge et al. (Nature New Biology 229: 205) have proposed that ethylene may inhibit stem growth by increasing cell wall hydroxyproline.

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BIOPHYSICAL MECHANISM OF DIFFERENTIAL GROWTH DURING GRAVITROPISM OF STEMS

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Description of Research

Plant growth requires water absorption and irreversible wall expansion. When gravity induces a plant stem to grow upward during gravitropism, its effect on growth must be mediated via asymmetrical alteration of one or more of the cell parameters that control water transport or irreversible wall expansion. The goal of this project is to identify and quantify the physical properties altered by gravity when plant stems grow upward. This information will provide insight into the control mechanisms governing plant growth and how they are influenced by gravity.

Our approach has been to measure the driving forces and physical coefficients that regulate cell wall extension and water uptake. Our working model is as follows: for cells to enlarge, the rigid wall surrounding each cell is biochemically loosened and consequently yields, or expands, irreversibly. The rate of wall expansion depends on a wall yield coefficient ("wall extensibility") and on the wall stress exceeding a minimum threshold. Wall stresses arise because of the high hydrostatic pressure (turgor) found within plant cells. Because water is incompressible, wall expansion tends to reduce turgor, and thereby induces an influx of water by osmosis. Growth may be altered either by a change in the wall-yielding properties or by a change in the water transport properties (osmotic pressure and hydraulic conductance) of the growing tissue. In the case of gravitropism, there are reports that implicate changes in wall extensibility, cell osmotic pressure, and other factors. We have set out to measure all of these factors in the gravitroping stem of young cucumber seedlings (Cucumis sativus L.).

Accomplishments

(1) The first step in this research was to characterize the spatial and temporal details of the growth response to gravity. We analyzed 15-minute time-lapse photographs of marked stems by digital image analysis and found that:

(a) Both sides of the stem react simultaneously: the upper surface ceases expansion entirely, while the lower surface more than doubles its growth rate.

(b) A growth differential occurs throughout the length of the stem, but is more pronounced in the apical region.

(c) At the peak rate of curvature, the upper surface undergoes a small but real contraction; at the same point in time, there is a 25% per hour differential in growth rate across

the stem.

(2) By use of angular displacement transducers resting on the apex of the horizontal stem, the lag before the start of curvature was determined to be 10 minutes.

(3) The contraction on the upper side of the stem is a passive process, caused by shear deformation of the bending stem. This was determined by stimulating expansion on one side of a vertical stem by local auxin application and measuring the change in the extension rate on the opposite side of the stem.

(4) The changes in growth rates on the upper and lower sides of the stem were not accompanied by changes in bulk osmotic pressure. This was determined by bisecting the stems before and during the period of most active bending and measuring cell sap osmolality.

(5) Direct pressure measurements with the pressure microprobe indicated nearly constant turgor on the upper and lower sides of the stem despite the large changes in extension rate.

(6) We have developed and tested a new technique for measuring the yielding properties of the cell wall. The method involves isolating a growing tissue from an external water supply. Continued wall loosening then induces stress relaxation in the cell walls and a concomitant reduction in the turgor pressure. Theoretical analysis shows that the rate of stress relaxation is controlled predominantly by the apparent wall extensibility and the volumetric elastic modulus of the cell. The final turgor pressure attained after completion of stress relaxation is the yield threshold.

Significance of the Accomplishments

Finding #1 shows that the upper and lower sides of the stem react at about the same time and to about the same extent, but in opposite directions. Any proposed mechanism for the mediation of gravitropism must account for the simultaneous suppression of growth on the upper surface and the doubling of growth on the lower surface. None of the proposed chemical mediators have yet been shown to be capable of accounting for this pattern, magnitude, and rate of growth modulation. However, they cannot be positively excluded on the basis of these data because we do not know enough about their action. The 10-minute lag time (Finding #2) is within the time of action of calcium, auxin, and other growth-affecting agents. Detailed kinetic studies of the action of some of these mediators would be useful to resolve this question.

Finding #3 points out that the mechanical linkage between the two surfaces of the stem needs to be considered when one interprets the pattern of expansion along the length of a bending organ. Findings #4 and #5 both indicate that the changes in growth rate during gravitropism are not due to changes in the properties governing water transport. While it is possible that changes in solute content occur in later stages of gravitropism, such changes are likely to be an indirect consequence, not cause, of

the differential growth.

These results also imply that the physical basis for the bending is entirely due to a change in the cell wall properties, such that they are "stiffer" on the top and "looser" on the bottom of the stem. The stress relaxation technique will allow us to determine whether this change is in the wall extensibility or in the yield threshold, or both. Work just published using this technique showed that short-term effects of auxin on growth were entirely due to changes in wall extensibility.

Finally, these results tell us something about the physical limitations on plant growth. The fact that growth rate could be either doubled or entirely suppressed without a substantial change in turgor pressure means that the water potential gradient supporting growth is small and thus not limiting cell expansion. This conclusion assumes that the hydraulic conductance of the stem is constant, an assumption which still needs a direct test.

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ROOT GRAVITROPISM: THE DETECTION OF GRAVITY AND THE BIOCHEMICAL TRANSLATION INTO A MODULATED GROWTH RESPONSE

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Description of Research

In general, most roots respond to gravity by orienting their growth, to some degree, to the direction of the gravitational field. This response is highly regulated and is initiated upon detection of a change in the gravitational field relative to the growing axis of the root. Although a large number of factors have been identified as ostensible mediators of the response, neither the mechanism of gravity detection nor the chemical/electrical sequence of events resulting in response are known. In this laboratory, research is focused on the mechanisms of gravity detection at the cell membrane of the root cap and, secondly, on the role of calcium in communication between cells of the root cap and cells in the region of response some distance behind.

The first half of the year was dedicated to elucidating the properties of cells along the root axis with respect to the processing of calcium. The second half has been focused on learning a potentially revolutionary technique for plant molecular physiology and obtaining preliminary evidence to support a model for the detection of gravity at the plasma membrane.

Calcium mobility in the root cap and elongation zone has been implicated in the coordination of root gravitropism. The distribution of total calcium along the axis of the root by atomic absorption spectroscopy coincides with the observed mobility. Calcium is concentrated in the root cap and in the elongation zone. Experiments detailing the uptake and efflux of radiolabeled calcium from cells of the root cap and those sequentially excised from the apical root behind the root cap revealed that maximum uptake and slowest efflux of calcium occurred with root cap and elongation zone cells. To determine whether calcium in these two regions had different functions, I examined the properties of uptake and efflux using auxin transport (NPA and TIBA) and calmodulin (C48/80 and W-7) inhibitors known to affect some calcium transport systems in plants. The plant hormone, auxin, is present in and implicated in the function of the root cap as detector and translator of the gravity signal and in the function of the elongation zone where downward curvature results. Calmodulin is a protein involved in some types of calcium transport and is required for a number of calcium-required biochemical "translator" events which are suspected in root gravitropism but have yet to be detailed. The

specific questions pursued are described in last year's report.

Although the above type of experiments are important for defining the molecular questions to be asked in regard to root gravitropism, they can provide only indirect evidence. However, direct mechanistic and biochemical evidence can be obtained from experiments at the molecular level. The objective of my sabbatical leave, therefore, has been to gain expertise in a molecular, electrophysiological approach for the examination of individual proteins which traverse membranes and thus are able to conduct ion flow into and out of the cell. Such proteins are called "channels" and the high resolution method for examining them in situ is the patch-clamp technique which, following attachment of a 1-2 micron area of membrane (the patch) to a fine-tipped glass electrode, allows the measurement of ion flow through the single channels in the patch area of the intact cell membrane or the excised membrane patch. The technique has been used extensively and successfully with animal cells, especially muscle, nerve, and red blood cells to understand calcium, potassium, sodium, and chloride regulation by voltage, hormones, and other effectors. However, there are only three laboratories to date which have reported data from membrane patches with plant cells. Plant cells are more difficult to "patch" than animal cells because the cell wall must be removed to access the cell membrane and the cells seem to be less pliable, perhaps because of the large vacuolar volume of plant cells. Also, the wall-less plant cells do not adhere to glass, as is often the case with animal cells, and thus they must be anchored by artificial means. Nonetheless, under the appropriate conditions plant cell membranes can be "patched" and the channel activity observed and characterized.

What potential does this technique hold for the elucidation of the detection of gravity in plants? For more than 40 years advancement in our understanding of how plant cells detect gravity as well as how other physical stimuli such as friction, flexure, turgor pressure, and vibration are sensed and transduced has been slow. The models for sensory physiology are not adequate because it has been impossible to imagine a mechanism at the molecular level which would detect the very small changes in energy just above that of molecular motion due to temperature (thermal noise). However, the basis for just such a model has been developed by Frederick Sachs at SUNY-Buffalo and is supported by patch-clamp data from chick embryonic muscle. Sachs and Guharay (1984, 1985) have characterized the first mechanotransductive protein. It is a channel which senses membrane stretch at the level of thermal noise and allows potassium ions to pass. Such a channel would be ineffective as a regulator or sensor if such sensitivity were the case for every channel. But Sachs hypothesizes that in muscle cells, the pressure-sensitive protein is linked to two submembrane protein networks engineered to reduce the tension borne by the phospholipid bilayer and to increase the area of membrane over which tension can be sensed. He feels that such stretch-

sensitive channels are generally distributed in animal cells and vary depending on the nature and engineering of the mechanical elements required for sensing. He expects them to be common for sensing, for example, acoustic vibration, proprioception, balance, and organ filling. Stretch-sensitive proteins could be the physical sensors in plant cells as well, detecting tension of the plasmamembrane resulting from pressure such as turgor or mechanically applied by structural mechanisms designed to detect gravity, friction, flexure, or vibration in specific cell types. Such sensors could even explain the determination of the plane of cell division or even plant morphogenesis.

To establish the presence of pressure-sensitive channels in plant cells quickly, we chose suspension-cultured tobacco stem cells which were available within the department and which had large protoplasts released from quickly digested thin cell walls. We expected these cells to have a pressure-sensitive osmoregulator. If these cells had pressure-sensitive channels, then we would be justified in looking for the gravity sensor in the plasmamembrane of the central cells of the root cap.

Patch-clamp data from tobacco cell protoplasts has been collected by our collaborator, Lee Falke, in Stan Misler's laboratory in the Washington University Medical School, and a preliminary characterization of a pressure-sensitive channel has been completed. We are presently in the process of setting up our own patch-clamp system so that rapid progress can be made on the gravity receptor.

Accomplishments

(1) A pressure-sensitive channel protein has been found in tobacco cell protoplasts and in excised membrane patches which opens in response to very small changes in pressure applied to the inside of the plasma membrane (Figure 1). For comparison, the channel is at least sensitive to an osmotic pressure change resulting from a 1 mM increase in internal ion concentration.

(2) This pressure-sensitive channel passes primarily anions such as chloride, but will also pass cations such as potassium and sodium as determined from excised membrane patches.

(3) Calcium is rapidly taken up by those tissues most active in root gravitropism and this uptake is pH sensitive, corresponding to the pH gradient along the root.

(4) The auxin transport inhibitor, NPA, enhanced calcium loss from cells and this might account for the inhibitory effect of NPA on calcium transport in the root cap.

(5) Three inhibitors of calmodulin did not produce predictable results, but the reported strongest inhibitor of animal calmodulin, C48/80, enhanced calcium uptake into cells. Calmodulin, therefore, may be involved in calcium efflux.

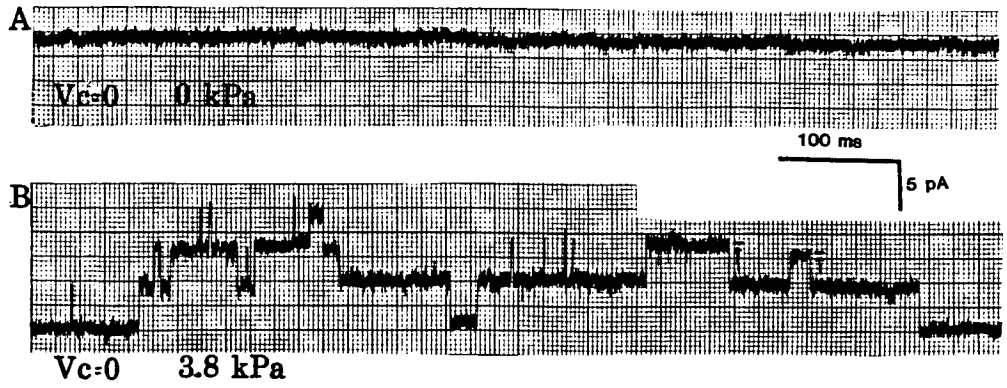

Figure 1. Electrical record of ion current activity from a plasma membrane patch on an intact protoplast of a cultured tobacco stem cell. (A) Background noise level where no channels are open and conducting ions, the voltage (V) across the cell is maintained at 0 volts, and there is no pressure (pascal units) differential across the membrane patch. (B) When suction is applied to the outside of the membrane patch (3.8 kPa pressure to the inside of the membrane patch), current activity is quickly realized. Each amplitude step increase (upward) or decrease (downward) indicates an individual protein channel opening or closing respectively. The amplitude of the step is indicative of channel type. Here two amplitude heights (arrows) are seen indicating two types of pressure sensitive channels. When a current vs voltage curve is plotted from the data, these two channels are very similar and likely represent the same channel in two different positions in the patch area. Those near where the membrane seals to the glass electrode are likely to show restricted activity.

Significance of the Accomplishments

Finding #1 provides the first evidence for describing a mechanotransductive mechanism in plant membranes for the transduction of physical stimuli. In this case it is a transmembrane protein that responds to small increases in pressure at the plasma membrane by altering its conformation such that ions can pass into or out of the cell depending on the chemiosmotic gradient. It is reasonable that a pressure-sensing mechanism evolved early in order to regulate cell volume. Modifications could have easily evolved with multicellular organisms and division of labor among cells such that different sets of parameters regulated the response of the protein and thereby its function and sensitivity to different physical stimuli like gravity, freezing, vibration, or even coupled with phytochrome for the setting of the biological clock. This breakthrough has the potential to revolutionize our thinking about plant sensory physiology, and using the patch-clamp technique will enable us to probe the molecular basis of these mechanisms in membranes.

Finding #2 characterizes the mechanotransductive protein as a probable osmoregulator. Order of magnitude calculations indicate

that activation of all pressure-sensitive channels in a cell would rapidly deplete the average plant cell of all cytoplasmic ions in less than 200 milliseconds. However, it is more plausible that only a few channels would initially respond with the flux of chloride or some other anion out of the cell. This flux would cause a depolarization of the cell membrane which would shift the ion preference to cations. Water would diffuse out following the loss of ions from the cell, and the cell volume would decrease, releasing the pressure at the plasma membrane and closing the channels. That this functional interpretation for these mechanotransductive channels is mathematically feasible leads us to strongly believe that such channels exist in most if not all living plant cells and that looking for the functional variant for gravity detection is now justified. We have a molecular model for the gravity mechanotransducer, which we expect will be selective for calcium or coupled to a calcium pump in the cell membrane.

Findings #3, #4, and #5, are pieces of the puzzle relating to how calcium function might vary in the tissues involved in gravitropism. These data suggest that those tissues most responsible for gravity detection and subsequent response have the most active mechanisms for uptake and retention of calcium. These mechanisms appear to be similar in the two tissues of primary interest, the root cap and the elongation zone, but the pH for optimal uptake varies with the pH of the cell walls of these tissues. The plant growth hormone auxin is believed to be coupled with the movement of calcium, and this appears to be the case at the cellular level where the auxin transport inhibitor NPA also reduces the efflux of calcium from all cells. The role of calmodulin in the movement of calcium in these tissues was not resolved because not all animal calmodulin inhibitors effected a response. But the permeabilities of these inhibitors into plant cells is not known. Nonetheless, the most sensitive inhibitor in animal cells, C48/80, did promote influx of calcium. Whether this is due to an effect of the inhibitor on membranes or on calmodulin has yet to be determined. If the effect is on calmodulin, the implication is that calmodulin is involved in calcium efflux and could be critical to the translation of gravity reception into the growth response.

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THE ROLE OF ACID AND CALCIUM GRADIENTS IN 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 and grow in the direction of gravity (gravitropism). The detection of gravity occurs at the tip of the root while the adjustments in growth rate occur in the growing region about 0.5 cm behind the tip. During the past two years we accumulated evidence that gravity-induced redistribution of calcium within the tip of the root plays a key role in linking gravity detection to the altered growth pattern causing reorientation of the root. We also obtained evidence that the primary cause of the altered growth pattern is gravity-induced asymmetric distribution (or activity) of the growth-inhibiting hormone, auxin, in the growing region of the root.

During 1985 our research centered on the following questions: (a) What is the overall distribution of calcium in roots and how does this relate to the potential role of calcium in gravity responses? (b) Since root caps are high in calmodulin, a protein thought to regulate calcium action, will application of calmodulin inhibitors to the root cap interfere with gravity responses? (c) What is the effect on root growth and gravitropism of metal ions related to calcium, and what does this tell us about the mechanism of the calcium effect? (d) The growth hormone, auxin, may ultimately mediate the differential curvature leading to responses to gravity. Since auxin induces the synthesis of ethylene, a second hormone known to affect root growth, to what extent is gravitropic curvature dependent upon formation of this second hormone? (e) What is the precise nature of the growth pattern shift that causes roots to curve in a gravitational field? Is this growth pattern controlled primarily by specific cell types in the root, or is it a general response?

Accomplishments

(1) Radioactive calcium applied to germinating corn grains is rapidly transported along the seedling root and accumulates in high concentrations in the root cap.

(2) Application of low concentrations of calmodulin inhibitors to the root cap strongly inhibits root responses to gravity without affecting growth.

(3) Gradients of aluminum applied across the root cap cause very strong curvature away from the aluminum. Gradients of copper cause curvature toward the copper (the copper experiments are a confirmation of earlier experiments by R. Moore).

(4) Auxin-induced biosynthesis of ethylene is strongly promoted by calcium (and certain other minerals).

(5) In roots oriented horizontally, the growing zone extends toward the basal part of the root.

(6) In horizontally oriented roots from which the epidermis has been removed from the upper and lower sides, the response of the root to gravity is strongly reduced. The roots regain the ability to respond to gravity after sufficient extension has occurred to form a new elongation zone with an intact epidermis. Horizontally oriented roots with the epidermis removed from the two sides but not from the top and bottom respond to gravity.

Significance of the Accomplishments

Finding #1: Calcium moves from the seed and accumulates in the cap. This indicates that calcium, a mineral thought to be rather immobile in plants, moves readily in seedling roots. The accumulation of calcium in the cap suggests the possibility of a physiologically important role for high levels of calcium in the cap.

Finding #2: Calmodulin inhibitors applied to the root cap prevent gravity responses. Our working hypothesis is that gravity-induced calcium gradients in the cap link gravity detection to the growth response. The ability to block the response with calmodulin inhibitors indicates that calmodulin may mediate the action of calcium either by controlling its distribution or by serving as a calcium-regulated activator of processes controlling growth in roots.

Finding #3: Aluminum induces strong curvature away from the site of application. Copper induces curvature toward the site of application. Until recently we thought that the induction of curvature by metal ions was specific for calcium (curvature toward), the ion known to activate calmodulin. Curvature induced by aluminum may be important to this hypothesis since roots curve away from aluminum and aluminum inactivates calmodulin. The ability of copper to mimic calcium is an enigma since copper is not known to activate calmodulin. This suggests either that the calmodulin model is wrong or that copper acts by an independent mechanism. Copper is known to potentiate the action of ethylene.

Finding #4: Auxin-induced ethylene biosynthesis is promoted by calcium. This finding raises the possibility that calcium gradients may influence the pattern of growth by enhancing the production of ethylene, a growth inhibitory compound in plants.

Finding #5: The growing zone shifts in roots responding to gravity. In considering how roots respond to reorientation in a gravitational field, most emphasis has been placed on changes in growth rate in a restricted zone of the root. Our data show that this zone is not fixed. Instead, gravity stimulation causes nongrowing cells to resume growth. This should be an important clue to the way that gravity stimulation affects the growth of

cells.

Finding #6: Removing epidermal cells prevents the response of roots to gravity. Although these are preliminary results, they indicate that the epidermis may be the key tissue controlling growth in roots. If so, these cells may be the site at which the major growth adjustments are made in response to gravistimulation. This finding could be very important in characterizing the gravity response since it will direct the search for potential causes of differential growth to the responding cells and avoid potentially misleading clues from cell types less directly involved.

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GRAVITROPIC STIMULUS PRODUCTION IN ROOTS OF CORN

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Description of Research

In response to gravity, plant roots typically grow downward. Our long-term objective is to determine the biochemical processes associated with converting the physical stimulus of gravity into the downward growth response.

In roots, gravity is perceived and transduced within a specialized tissue of the root known as the root cap. If the cap is surgically excised from the root, the root is no longer able to respond to gravity. In some roots, even though the cap is present, the roots do not grow downward, but rather grow in a direction parallel to the surface of the soil. However, one can cause these roots to grow downward by illuminating the root. It is hypothesized that light initiates certain biochemical processes required for the downward growth of the root.

It is these light-stimulated processes which we are currently investigating. Our hope is that by determining what these processes are we might better understand the biochemistry involved with gravity transduction in the root cap.

Our accomplishments during the past year fall into the following categories:

(1) Elucidating the formation within the root cap and the movement from the root cap of two growth-regulating compounds, abscisic acid and xanthoxin.

(2) Discovering that light affects mRNA synthesis within the cap.

(3) Describing the photoreceptor(s) involved in the light-mediated gravitropic response.

Accomplishments

(1) A horizontal root curves downward because the upper and lower sides grow at different rates (with the upper side growing relatively faster that the lower side). This unequal growth is hypothesized to occur because of the preferential accumulation on the lower root surface of substances which inhibit growth. Our results show that two substances, abscisic acid and xanthoxin, with known growth inhibitor activity, are located or produced within the cap and, as a result of illumination, these substances are caused to move from the cap to a region of the horizontal root where downward bending occurs. Also, we have been able to show that when the level of one of these substances is artificially lowered, the roots respond differently to gravity

than do control roots. We conclude that the behavior of both abscisic acid and xanthoxin, following illumination of the root, is consistent with these compounds having a role in root gravitropism.

(2) Light stimulates mRNA synthesis within the cap. If this mRNA synthesis is chemically inhibited, roots will not respond to gravity when illuminated. Since the types of mRNA present in a tissue determine what proteins are produced, it is important to understand how light affects mRNA synthesis. This is especially relevant in connection with our finding that light causes a 3-4 fold increase in the level of certain mRNA species. The effect of light on mRNA levels occurs rapidly, within 15 minutes of illuminating the root cap. From this work we conclude that mRNA synthesis in the root cap is necessary for root gravitropism and that this synthesis may result in the production of unique and/or new proteins specifically required for the gravitropic response.

(3) Since light initiates downward bending in roots, we hoped that by characterizing the pigments involved with the absorption of light we might learn more about the gravity transduction process. During the past year we have established that in our roots a special pigment known as phytochrome is the sole pigment involved in absorbing light. Furthermore, the amount of light required to induce root bending is very small, and because of this low level is technically referred to as a "very low fluence response." Of particular interest is our demonstration that it is possible to shift the root's sensitivity to light and gravity, depending upon the pretreatment of the seed before it is germinated. If seeds are pretreated in the cold prior to germination, their roots need much less light to trigger the gravitropic response as compared with roots from seeds which were not cold pretreated. We consider this finding particularly significant because it suggests that roots may be made more or less sensitive to gravity depending upon environmental factors other than gravity. In summary, this portion of the work suggests that the response of roots to gravity may depend not only on the micro-g environment, but on other environmental factors as well. From this work we conclude that the only pigment associated with the gravitropic response in corn roots is the red light-absorbing pigment, phytochrome.

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EFFECTS OF SPACEFLIGHT ON CIRCADIAN RHYTHMS

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Description of Research

The fundamental question addressed in this project is: Do the conditions of space, especially the absence of the Earth's 24-hour geophysical and gravitational environment, affect the cellular processes responsible for timing circadian rhythms? Given the pervasiveness and significance of temporal organization in plant and animal physiology, it is important to document how the fundamental processes of cellular timekeeping function in space. The question of how well circadian clocks measure time in space is particularly relevant because: (a) the exogenous hypothesis for circadian clocks predicts that they will not function normally outside of the 24-hour geophysical environment of Earth, and (b) recent preliminary results from experiment 007 flown on STS-9 indicate some important alterations in circadian timing.

Because circadian rhythms can persist in conditions of constant temperature and constant light or darkness, they have been regarded by most investigators to be manifestations of an endogenous timekeeping system. This biological clock is capable of measuring time in the absence of environmental signals and provides the organism with temporal information. An alternative explanation is the proposal that the timing system is exogenous, that is, that subtle changes in geophysical variables provide temporal information to organisms in otherwise constant conditions. The organism perceives daily timing signals such as periodic fluctuations in air pressure or slight fluctuations in gravity associated with rotation of the Earth in relation to the sun and the moon. A test of this would be to determine how circadian rhythms persist outside of the Earth's environment.

Many investigators feel that because these daily rhythms are so pervasive in living systems, circadian clocks arose early in evolutionary history as an adaptation to the 24-hour periodicity of the Earth's environment. As a consequence, the fundamental cellular mechanism of circadian timekeeping may be similar in diverse species. Since circadian rhythms are an integral part of various processes ranging from plant growth and development to the sleep-wake cycle of humans, this study could have ramifications ranging far beyond resolution of the endogenous versus exogenous clock question.

The rhythmic patterns of Neurospora growth are due to a circadian periodicity in the production of conidia (asexual spores).

Because of inhibition of conidiation by CO_2, the wild type of Neurospora only expresses this rhythm under special growth conditions of aeration. A mutant strain, BND, is apparently less sensitive to CO_2, and nonaerated cultures show pronounced rhythmicity.

Accomplishments

To examine the issues outlined above, an experiment was developed which flew in a middeck locker on STS-9. The investigator was Dr. Frank Sulzman, at that time affiliated with SUNY Binghamton. This experiment studied the conidiation rhythm of the BND strain of Neurospora crassa monitored in constant darkness. In control experiments, the tubes were inoculated on one side, maintained in constant bright light for 24 to 48 hours, and then placed in constant darkness for 9 days. As the Neurospora grew along the tube, a band of conidiation (asexual spore formation) was produced at 21.5 to 22.0-hour intervals. Since the period of the conidiation rhythm was faster than 24 hours, 10 cycles of conidiation were completed during the 9-day experiment. After the ninth day, the tubes were left in constant light and the rhythm decayed.

On STS-9, 24 tubes of Neurospora were flown in constant darkness in a middeck locker. Before launch, the transition from constant light to constant darkness was set so that launch would be at CT 12 for tubes 1-12 and CT 15 for tubes 13-24. On mission day 7, the tubes were removed from the locker and the growth front of each tube was marked. This procedure took about 15 minutes to complete. After all the tubes were marked, they were restowed in the locker. Approximately 1 hour after landing on mission day 9, the package was recovered, marked and photographed. Some qualitative results were evident. In all of the tubes there was a reduction in the contrast between the bands and the interband intervals, and this reduction in amplitude progressed with the duration of the experiment. Marking the tubes of day 7 resulted in a 10-15 minute light pulse, and this produced a clear effect: after this pulse the rhythm sharpened, with the contrast between the bands and interband intervals becoming quite distinct. The period of the conidiation rhythm in the second phase of this experiment was similar to that seen on Earth.

To verify and extend these results, a similar experiment will be conducted on EOM 1/2. Several major experimental design changes have been made: (a) the package has been reconfigured in order to carry 45 race tubes; (b) two strains (BND and CSP) of Neurospora will be flown instead of just one (BND), (c) the package has been divided into three subdivisions: one set of tubes will remain undisturbed throughout the flight, one set will be exposed to light and marked similar to the STS-9 tubes, and the final set of tubes will be wrapped with a red filter and marked in the light; (d) the tubes are to be marked at T+33 hours instead of T+167 hours; and (e) gas syringes have been added to the package in order to take reference gas samples

during the marking procedure.

The logic behind these changes is as follows: the increase in sample size results from an attempt to decrease the variance and aid in statistical analysis. This is especially important due to the extended number of experimental groups. Each package will contain half BND strain and half CSP strain. The CSP mutation of the BND strain has been added since it possesses a tighter and more distinct banding pattern than the BND strain; furthermore, the period of the CSP strain is one hour shorter than BND, thus allowing more bands per tube. The BND strain will be flown as a control for the last flight. The first package will be treated similarly to the race tubes during STS-9 to verify the damping of the rhythm following liftoff. Since Neurospora are relatively insensitive to light in the red region (above 550 nm) of the visible spectrum (i.e., light of this wavelength will not alter the phase or velocity of the circadian oscillator) and the gelatin filter has less than 0.1% transmittance below 610 nm, it is possible to mark the race tubes in the light without affecting the conidiation rhythm of the Neurospora. The third package that is left undisturbed in darkness is a control to verify that the reinstatement of clear oscillations was not due to some other phenomena, such as merely time for accommodation to the new environment of space. The tubes are to be marked much earlier in the flight due to the damping and decreased amplitude of the rhythm between liftoff and the light exposure of marking during the STS-9 flight. By marking the tubes earlier we expect to have a greater number of clear oscillations (bands) in space. One of our working hypotheses is that it may be the positive g force of liftoff which caused the damping of the rhythm and not the lack of gravity or other geophysical factors; and furthermore that it was the light pulse during marking that corrected this problem. Therefore, marking the tubes and exposing them to light earlier in the flight will aid in our period analysis of the oscillator's velocity by increasing the number of bands after the light pulse. Lastly, the reasoning behind the gas sampling is to measure the amount of CO_2 in the microenvironment of the race tube. Carbon dioxide is known to damp oscillations in conidiating Neurospora; therefore, if there are high levels of CO_2 in the tube this may be a contributing factor to the STS-9 results.

This experiment should greatly extend our understanding of the circadian pacemaker, its timekeeping processes in the microgravity of space, the importance of exogenous geophysical influences upon the generation of the rhythm, and further elucidate the reason(s) for the unexpected damping of the conidiation rhythm encountered during STS-9.

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MECHANISMS OF GRAVIPERCEPTION AND TROPISTIC RESPONSE IN PEA SEEDLINGS

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Description of Research

Our aim is to understand how a plant stem senses the gravitational field of the Earth, and then reacts by curving upward. We are thus interested in the physics of graviperception and the chemistry of the early response mechanisms.

Two important chemical events are known to occur early in the response of stems to gravity. The accumulation of the plant growth hormone auxin on the underside of the organ has been well documented for at least 50 years. In recent years it has also been shown that calcium becomes relatively more abundant on the upper side of the organ. It has been suggested that the movement of these two substances is obligatorily coupled, and that the asymmetry in calcium is also involved in the tropistic response. The general idea is that auxin promotes growth on the underside, and that calcium in the walls inhibits growth on the upper side. Thus, a combination of a "push" and "pull" mechanism could account for the differential growth leading to curvature. Our aim has been to investigate whether calcium and auxin necessarily move together in opposite directions, and whether the calcium asymmetry is linked to tropistic response.

Our techniques have been to use well-known chemical inhibitors of auxin transport and of calcium transport, administered separately. This permits us to see whether inhibition of transport of the one substance necessarily leads to a concomitant inhibition of the movement of the other. The inhibitors of auxin transport used were: 2,3-5-triiodobenzoic acid (TIBA), and a "morphactin," 9-hydroxyfluorene acetic acid. Inhibitors of calcium transport are the new proprietary drugs nitrendipine, nisoldipine, and Bay 8644; we also employed a well-known calcium ionophore to open calcium channels to load the tissue with exogenously applied calcium. To facilitate the study of movement we used labeled auxin (^{3}H-IAA), and isotopic calcium, $^{45}Ca^{2+}$. We also sharpened the gradients between upper and lower tissues by removing the upper and lower epidermis after appropriate gravitational exposure. Finally, to facilitate the introduction of labeled materials, the surfaces of the pea stems were gently abraded with carborundum before exposure to the test material.

Accomplishments

(1) Pea stems loaded with ^{3}H-IAA or $^{45}Ca^{2+}$ and placed horizontally develop asymmetries of both auxin and calcium

rapidly and approximately simultaneously. Auxin accumulates below and calcium accumulates above. Under optimal conditions the asymmetry ratios are approximately 2:1 in each case.

(2) Inhibition of asymmetric auxin transport by TIBA or morphactin also inhibits the development of a calcium asymmetry (Table 1).

(3) Contrariwise, use of the calcium transport inhibitors nitrendipine, nisoldipine, or Bay 8644 has no effect on either calcium asymmetry or auxin asymmetry in etiolated pea epicotyls (Table 1).

(4) These inhibitors of calcium transport do inhibit the uptake of $^{45}Ca^{2+}$ into protoplasts (cells without walls), and also enhance the loss of $^{45}Ca^{2+}$ from preloaded protoplasts.

(5) Since these calcium transport inhibitors work well on membrane-mediated calcium transport, but not on pea stems during gravitropism, this suggests that most of the calcium in the stem is in the wall, and that the apparent movement occurs in the apoplast, external to the cell membrane, not in the symplast, inside the cell membrane.

(6) This hypothesis was confirmed by simple pH experiments. When filter paper strips buffered at pH 7 are placed symmetrically on both sides of a pea epicotyl, the $^{45}Ca^{2+}$ content is high and symmetrical. When similar filter papers at pH 3 are placed symmetrically on the pea epicotyl, the $^{45}Ca^{2+}$ content is much lower, but also symmetrical. When pH 3 is placed on one side and pH 7 on the other side, then a 2:1 asymmetry develops, with the high point on the side with the pH 7.

	Ratio of CPM in upper (U) to lower (L) epidermal peels		Curvature (degrees)
	^{3}H-IAA	$^{45}Ca^{2+}$	
Controls	0.71	1.22	31.5 ± 1.8
Nitrendipine (0.2mM)	0.95	1.27	26.7 ± 3.6
Nisoldipine (0.2mM)	0.71	1.57	22.7 ± 4.1
Bay K 8644 (0.2mM)	0.86	1.19	21.0 ± 2.8
A 23187 (50μM)	0.85	1.20	22.0 ± 2.0
TIBA (0.2mM)	1.46	0.82	8.0 ± 2.1
9-HFCA (0.2mM)	1.58	0.91	7.4 ± 1.0

Table 1. Effect of substances interfering with IAA and Ca^{2+} movement on asymmetric distribution of ^{3}H-IAA and $^{45}Ca^{2+}$ in pea epicotyls submitted to 90 minutes of gravistimulation.

(7) This suggests that the calcium asymmetry arises in the wall as a consequence of the development of an auxin asymmetry. The higher auxin level below activates a proton pump which secretes hydrogen ions into the walls, thus acidifying them. This releases bound calcium from the wall, which is thus able to move in the apoplast.

(8) Thus, calcium does not appear to be involved either in primary gravity perception or in the lateral transport of auxin. Neither does calcium move transversely. The $^{45}Ca^{2+}$ asymmetry is solely the result of its release from the acidified wall near the locus of high-auxin stimulated proton pump.

(9) Companion work with polyamines, organic bases, shows some interesting interactions with calcium.

Significance of the Accomplishments

This work casts doubt on recent theories implicating calcium as a primary actor in early tropistic response mechanisms. It also helps us to focus clearly on asymmetric auxin movement as the earliest chemical transduction event in gravitropism. It makes us realize that much of the calcium in stems which react to gravity is located in the wall, and that membrane-mediated transport processes are not involved in the development of a calcium gradient. This also raises the possibility of the control of gravitropic response by externally applied pH gradients. With particular individual plants on space vehicles, this could be a practical mechanism.

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GRAVITROPIC RESPONSES IN CEREAL GRASS SHOOTS: GRAVITY PERCEPTION, TRANSDUCTION, AND RESPONSE

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Description of Research

The primary objectives of our research are twofold: (a) to shed light on gravity perception, gravity transduction, and gravitropic response mechanisms in cereal grass shoot pulvini at 1-g, and (b) to determine how clinostat-simulated microgravity affects key elements of the perception and transduction processes and the gravity response mechanism in these pulvini.

We are attempting to unravel the mechanism of the upward-bending response (negative gravitropic curvature) in prostrated (lodged) cereal grass shoots under Earth's 1-g conditions and to explain the basis for the lack of an upward-bending response in shoots of these plants when they are grown under microgravity conditions on a clinostat (and later, in space). We are approaching this investigation by studying gravity perception, transduction, and the asymmetric growth response mechanism in prostrated (gravistimulated) oat, barley, and corn shoots under 1-g conditions. At the same time, we are trying to decipher what happens to the perception/transduction/response processes that result in failure for upward bending to occur in genetic mutants of corn whose shoots are "lazy" or prostrate, and likewise, in barley and oat plants subjected to 10^{-4} g (microgravity) on a clinostat rotating at 2 rpm.

In connection with gravity perception, we are looking at how long it takes for shoots to perceive a gravistimulus (presentation time), the nature of the gravireceptors (starch statoliths), and the kinetics for their descent in gravistimulated shoots. Relative to the transduction process, we are analyzing how long it takes for asymmetries in hormone levels to develop in top and bottom halves of gravistimulated pulvini (localized, swollen "joints" at nodes of grass shoots where graviperception and the upward-bending response take place). The two hormones we are studying are (a) indole-3-acetic acid (IAA) and its bound or conjugated forms and (b) gibberellins and their bound or conjugated forms. For these two hormones, we are trying to find out how they become unequally distributed in the pulvini (through downward transport, release from bound form, or enhanced hormone synthesis or a combination of these) and how the onset of hormone asymmetries correlates with the onset of upward-bending in gravistimulated pulvini. The asymmetric growth response mechanism is being examined through analyses of time-course

changes in the kinds and amounts of salt- and buffer-soluble proteins, cell wall-loosening enzymes, cell wall synthesis enzymes, and sucrose hydrolysis (by invertase) in upward-bending pulvini of gravistimulated oat and barley shoots.

Accomplishments

(1) Graviperception in Cereal Grass Pulvini

(a) The process is temperature-dependent, with a maximum upward-bending response at 30°C, and none at 0° and 40°C. This suggests that metabolic controls (enzyme reactions) are important in the gravitropic response mechanism.

(b) The presentation time in cereal grass shoots varies from 1 to 2 minutes for over 12 different genera of cereal grasses. This is significant because it agrees and correlates well with the time required for starch statoliths to fall in gravistimulated pulvini.

(c) Starch grains in pulvini of gravistimulated oats and barley begin to descend within 15 seconds, and all are lying at the bottoms of starch-containing cells (statocytes) within 2 minutes (Figure 1). This agrees well with the presentation time needed to obtain an upward-bending response in cereal grass shoot pulvini.

(d) Treatment of pulvini with alpha-amylase results in loss of starch in the graviperceptive organelles (statoliths) (Figure 2) and loss in the ability of the pulvini to respond to gravistimulation (Table 1); if, after such treatment, the pulvini are fed 0.1 M sucrose, starch grains are reconstituted, and graviresponse is restored. These observations clearly support the idea that the starch-containing organelles (actually, chloroplasts) are the graviperceptive bodies in cereal grass pulvini.

SOLUTION	DEGREE CURVATURE
Sucrose	65.4°
Low Temperature	58.1°
GA_3	2.6°
α-Amylase	0.0°

Table 1. Effects of various treatments on the gravitropic response of gravistimulated barley leaf-sheath pulvini after 24 hours of gravistimulation. Barley pulvini (45 days) were shaken in solution at 150 rpm for 24 hours at room temperature and gravistimulated for 24 hours in the dark at 30°C. Sucrose = 0.1M Sucrose, Low Temperature = 4°C, GA_3 = 30 μM GA_3, α-Amylase = 1mg/ml in 0.05M KPO_4 buffer (pH 7.0).

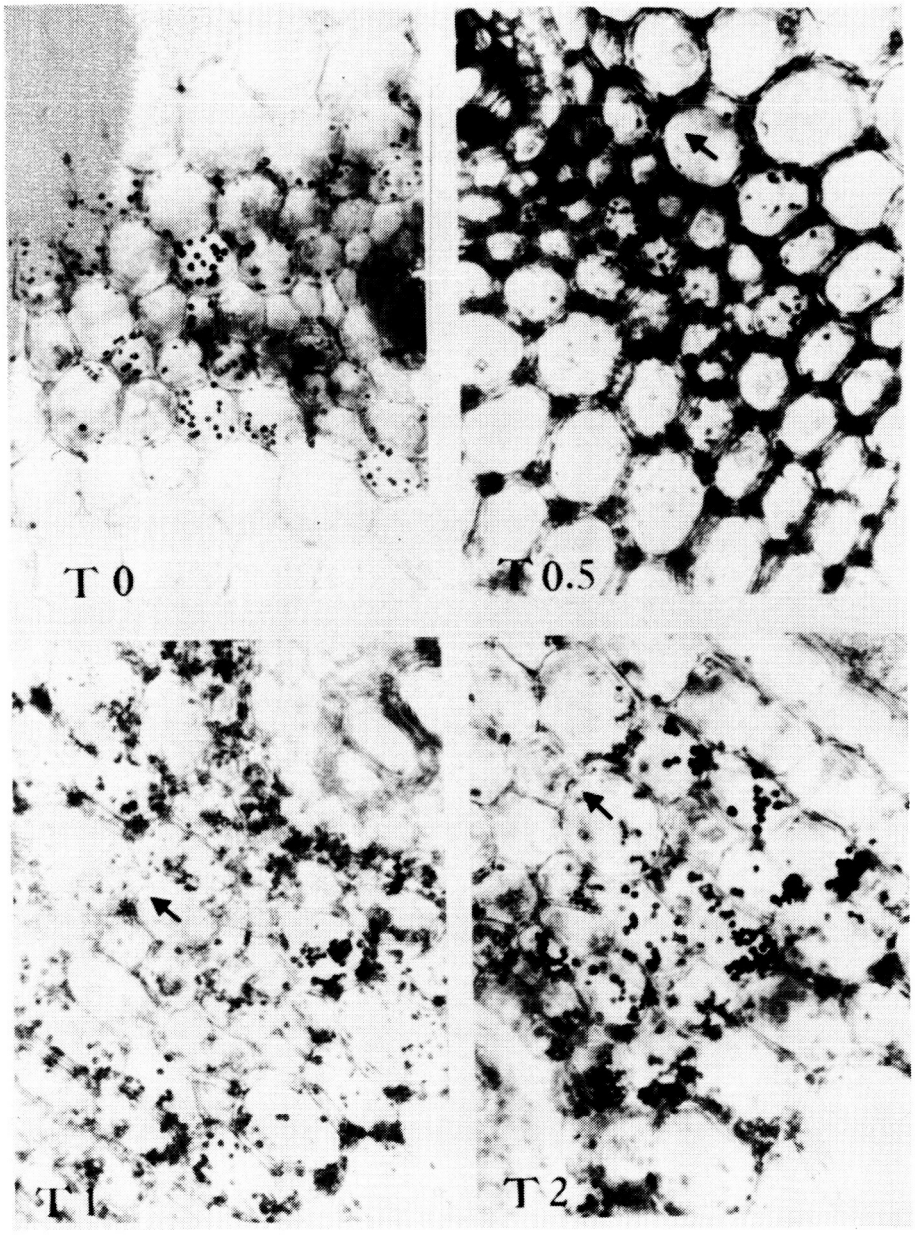

Figure 1. Barley shoot pulvinus cells showing time-course fall of I_2KI-stained starch grains in gravistimulated pulvini: T_0=zero minutes for gravistimulation (upright control); $T_{0.5}$= 1/2 minute; T_1=1 minute; T_2=2 minutes. At T_0, starch is located throughout statenchyma cells; at $T_{0.5}$, approximately one-third of starch grains are at bottoms of cells; at T_1, approximately two-fifths of starch grains are at bottoms of cells; at T_2, all starch grains are at bottoms of cells.

(2) Transduction Process in Cereal Grass Pulvini

(a) In lazy (prostate) corn plants, no asymmetry develops in free gibberellins or the gibberellin conjugates, in spite of the fact that pulvini of the shoots contain starch grains which can and do descend in response to gravistimulation. In contrast, in normal, upright corn plants, when the shoots are gravistimulated, the free, active gibberellins increase in levels in the lower halves (compared with the upper halves), whereas the inactive gibberellin conjugates accumulate in the upper halves of gravistimulated pulvini. This means that in lazy corn mutants, which do not respond to gravistimulation, there is a lesion in the transduction process that results in a failure for hormones such as gibberellins to become asymmetrically distributed and synthesized.

(b) Ethylene levels increase greatly in lower halves of gravistimulated oat shoot pulvini, but only 5.5 hours after upward bending has been initiated do we see the first evidence of this increase in ethylene. Thus, the increased ethylene in the lower halves appears to be the result of gravistimulation, and it probably develops as a consequence of the greatly increased free IAA levels we have measured in the lower halves of gravistimulated oat shoot pulvini. Exogenously applied ethylene does not stimulate the upward-bending response in gravistimulated pulvini, whereas both IAA and gibberellin (GA_4/GA_7) do.

(3) Asymmetric Growth Process in Gravistimulated Cereal Grass Pulvini

(a) Gravistimulated leaf-sheath pulvini of both oats and barley can reverse curvature repeatedly, i.e., up to 10 times, when shoots are rotated 180° after each upward-bending response. As a result, the pulvinus elongates up to five to six times its original length. This means that both graviperception and transduction mechanisms can be stimulated to recur repeatedly in a given graviresponsive pulvinus.

(b) A given graviresponsive leaf-sheath pulvinus in oats can lift a 20-gm weight to an angle of 34° after 24 hours of gravistimulation. This is 2,000 times its own weight (0.02 gm). This means that the pulvinus is a very efficient and sturdy system for elevating heavy shoot portions in prostrated cereal grass shoots. It also suggests that the wall-strengthening system (e.g., cell wall synthesis) must be at work during the upward-bending response.

(c) During the course of upward bending in a gravistimulated pulvinus, at least five proteins increase in amounts in the lower halves as compared with upper halves and with proteins in vertical control pulvini. One of these is invertase, whose activity increases 28-fold after 24 hours of gravistimulation. Another is beta-glucosidase, whose activity is enhanced twofold after 12 hours of gravistimulation. The increased invertase activity in the lower halves of the pulvini would provide hexose (glucose and fructose) for cell wall synthesis that is enhanced in the elongating cells in this half. The increased beta-glucosidase activity is one manifestation of cell wall-loosening that also occurs in these cells in the lower half. Cellulase activity increases early in both lower and upper

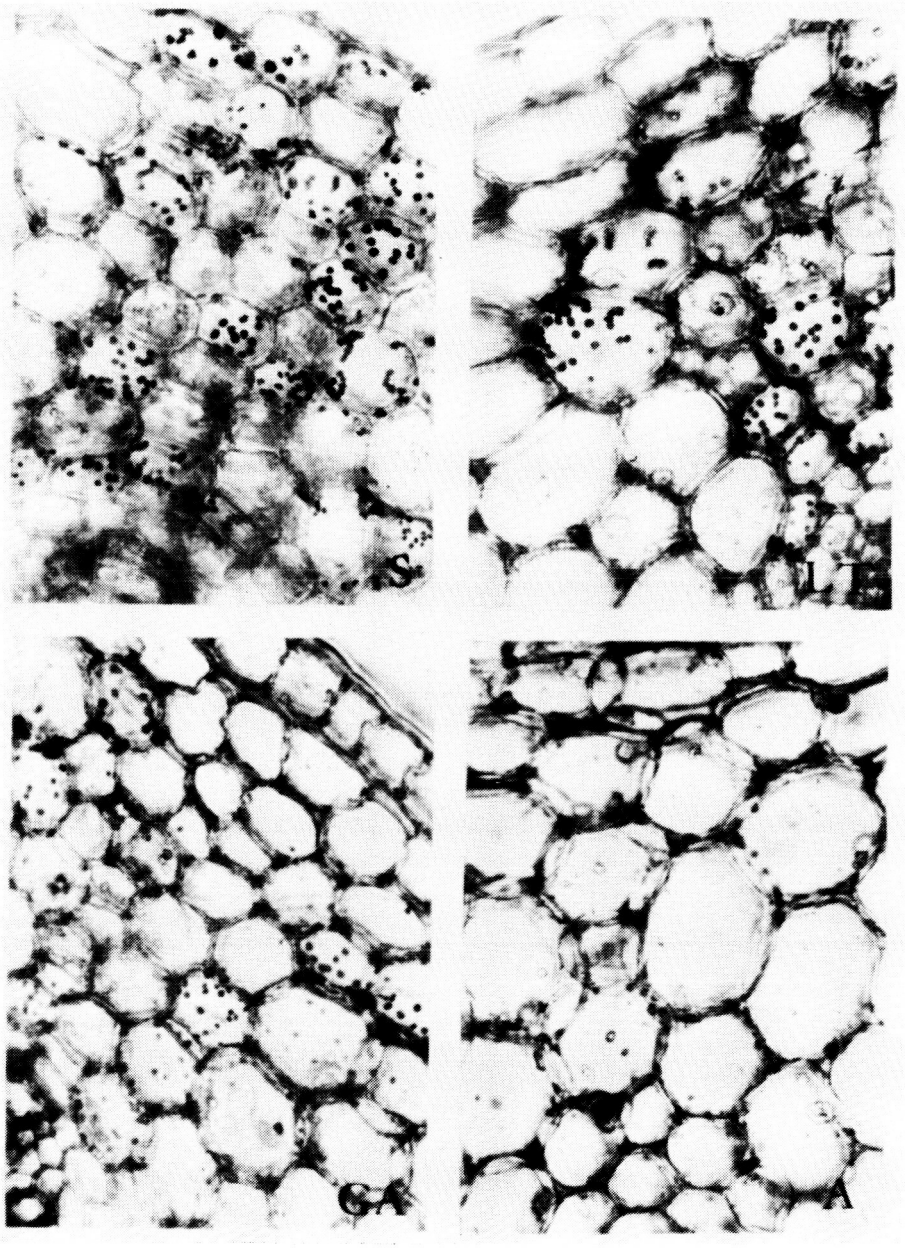

Figure 2. Barley shoot pulvinus cells, showing effects of various treatments on starch statoliths (graviperceptive organelles) in the pulvinus: S=0.1 M sucrose (many starch grains); LT=low temperature (4°C.) (many starch grains); GA=gibberellic acid (some decrease in starch grains); A=alpha-amylase (no starch grains).

halves; in the lower halves, its increase reflects another component of the cell wall-loosening process at work in the upward-bending pulvinus. The increased cellulase activity in the upper half would allow for cell wall folding or corrugation to occur in the upward-bending gravistimulated pulvinus.

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CELLS, EMBRYOS, AND DEVELOPMENT IN SPACE/MORPHOLOGY OF PLANT CELLS IN SPACE

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Description of Research

The ultimate objective of our overall research plan has been to ascertain whether flowering plants can carry out their full growth, development, and reproduction in a near-zero or hypogravity environment. Highly responsive experimental systems are being developed at different developmental or organizational levels--that is, free protoplasts from higher plant cells which can deposit new cellulose walls, divide, multiply, embark upon organized development, and ultimately give rise to organized plantlets; free somatic cells which by division under aseptic and heterotrophic (and even autotrophic) conditions may express morphogenetic competence and form somatic embryos which can, in turn, develop into plantlets; and finally at the level of plants as they develop from seeds or predetermined growing points. Recent emphasis has been placed on improving further the technology to effect regeneration of whole plants from daylily, carrot cells, and other cell, tissue, and organ cultures. The specific impetus to do this derives not only from the desire to understand better the developmental controls and plasticity of the systems, but to identify the means to minimize "hands on" operations at critical points of the regenerative cycles. This will enable us to establish the protocols which will be needed for automation of plant cell and tissue culture experiments in space. Also, since existing protocols to generate embryos have relied upon manipulation of exogenous hormone levels requiring "man-tended" operations, we paid considerable attention to selecting cells that do not require hormonal changes to organize. We have also sought to ascertain whether floral growing points can be caused to revert to vegetative growth modes.

Accomplishments

(1) (a) We completed analysis of roots of oats (Avena sativa var. 'Garry') grown by Joe R. Cowles, et al. of the University of Houston as part of their experiment "Gravity-influenced Lignification in Higher Plants" flown on Spacelab-2 in August of 1985.

(b) We have shown that the number of cell divisions in roots of space-generated seedlings are much reduced in number.

(c) The number of cells in division were about 10 times greater in ground controls than in flight samples.

(d) Chromosomal disturbances, such as chromosome

bridges and fractures, were also seen.

(e) Light and scanning electron microscope studies confirmed the karyological observations.

(2) (a) We have generated a high frequency somatic embryogenic carrot line autotrophic for exogenous auxin and cytokinin.

(b) The growth characteristics of the line have been studied showing that the stemline is light-dependent for its growth.

(c) The number of steps needed to yield plantlets consists of a single one--namely, to place a stemline of competent cells on a simple agar medium and wait for plantlets to form.

(d) Protocol variations have thus been introduced to the growing of carrot cells in this laboratory.

(3) Daylily cells have been systematically selected and challenged with protocol variations to test and control responsiveness.

(4) We can identify cell populations which can consistently do different things from a morphogenetic point of view.

Significance of the Accomplishments

The observations on the chromosomal disturbances and the reduced number of cells in division in the roots of space-grown oat seedlings raises important questions. It should be a high priority to ascertain whether the spaceflight conditions per se or microgravity was responsible for the poor root performance. Hopefully, this will be achieved as soon as possible. If it turns out to be indicative of reduced g conditions, appropriate countermeasures will have to be devised to enable long-term experiments on plants in space to be performed.

We know of no reports of cultures being generated that are capable of forming somatic embryos without use of exogenous growth hormones. Biotechnologists are very interested in the ability of angiosperm cells to grow in free cell suspensions and to develop into whole plants since it is the basis of cloning and genetic engineering operations. The evidence that cell multiplication and growth depends on the initial presence of exogenous growth factors is quite strong; more striking is the evidence that the type of growth, whether by cell division or enlargement, organized or unorganized, is dependent upon the interactive effects of various growth regulatory systems and their interaction with the external environments. One of the aims of our work has been to study those factors -- physiological, chemical, and environmental, especially microgravity and spaceflight conditions -- that have a bearing on the induction of totipotency and the orderly sequence of embryogenesis and organized growth in culture. In an attempt to seek answers here on Earth as to why some cells seem to be "more totipotent" than others, questions arise as to what conditions are necessary to cause certain cells to continue development. Do these conditions vary depending on the stage of development and

the portion of the plant from which the initial explants are taken? Is there a critical "switching point" at which certain signals must be delivered in order to bring about expression of competence? How is gravity involved? In carrot, the procedures have been thought by many to be now cut-and-dry--not so. The isolation of a totipotent line that is completely autotrophic as to exogenous growth regulators provides us with unique experimental material that now gives new insights as to growth factor modulation during expression of competence, since it permits perturbation from "outside" and "inside." Significance for space biology experiments is that we do not have to complicate matters by external hormone levels. From the perspective of developmental botany, it is noteworthy that the selection medium used by us at the initial point in the culture process is not as crucial as one might have supposed from the published literature. This emphasizes that alternative strategies exist to achieve the same result.

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THE AMYLOPLAST AS A GRAVITY-SENSING DEVICE IN PLANTS

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Description of Research

Research in this laboratory has been directed at elucidating the earliest processes of gravity sensing. Recent work has described in detail the kinetics of amyloplast sedimentation in corn roots and coleoptiles. In both cases, sedimentation is sufficient within the presentation time for the amyloplast to be acting as a statolith. To exploit this observation, dynamic gravistimulation-induced changes in the current pattern around the root tip were observed to assess the role of ion movement in early events of gravistimulation. These changes were also used as a diagnostic for evaluating the role of other metabolic processes in sensing, as distinct from growth and curvature.

We have been pursuing several other approaches to gain more information about the mechanism of sensing. We have been characterizing and attempting to identify the process in the sequence leading to gravicurvature that is blocked in nonphotoinduced roots. To begin to understand the process, individual components must be identified and placed in sequence. Understanding this component can also be a valuable tool for identifying earlier steps. We are in the process of producing paired homozygous lines of corn differing only in their need for red light to become graviresponsive. Finally, using cryofixation followed by freeze-substitution, we have attempted to measure changes in calcium concentrations within sensing cells following gravistimulation to identify the source of asymmetrically distributed calcium and to identify the tissue localization of redistributed calcium.

Accomplishments

(1) In corn roots, the endogenous current pattern is changed by gravistimulation only in the region adjacent to the columella and only after a lag equal to the presentation time.
(2) The change in the current pattern is indicative of a net upward current in the columella tissue coincident with initial sensing of gravity.
(3) Nonuncoupling inhibitors of calmodulin (Compound 48/80 and Calmidazolium) reversibly prevent gravicurvature and also the sensing-associated current shift.
(4) Triiodobenzoic acid, an auxin and calcium transport inhibitor, reversibly prevents gravicurvature, but does not reduce the sensing-associated current shift.
(5) Nocodazole, which blocks microtubule formation, does not inhibit gravicurvature.
(6) Treatment with the Ca-H antiporter A23187 will induce

graviresponsiveness in roots which have not been photoinduced. The effect is enhanced if endogenous calcium is supplemented.

(7) Abscisic acid will also induce graviresponsiveness in dark-grown roots.

(8) Cryofixation of whole corn-root caps produces good preservation of the three peripheral cell layers when a liquid nitrogen-cooled block is used, with characteristic ice-induced cytoplasmic aggregation in cells deeper in the tissue, including the columella. Freezing in stirred liquid propane results in severe ice crystal damage throughout the tissue.

(9) Three New York State breeding lines of corn have been found with a light requirement for gravisensitivity. One of these has been crossed with a normal line and the F_1 generation selfed. Backcrossing will follow to produce a pair of lines differing only in the one trait.

Significance of the Accomplishments

The shift in the electrical current pattern is closely tied to initial events in gravity sensing and therefore provides the first tool available for investigating the sensing process independently from growth and curvature. This approach has led to the conclusion that calmodulin is involved in the sensing process, but that sensing occurs without the transport of auxin.

The step which is not present until the root is exposed to red light can be induced by changing the concentration of intracellular calcium or of ABA. The production of paired genetic lines for this trait will be of great utility in discovering the blocked step.

Cryofixation of tissues for ion microprobe analysis is useful only for the superficial cell layers. The heat exchange properties of the tissue prevent good freezing of deeper tissues. The resulting damage allows diffusion of ions, which makes measured concentrations unrepresentative of those in the intact tissue.

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MECHANICAL STRESS REGULATION OF PLANT GROWTH AND DEVELOPMENT

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Description of Research

This project investigates the physiological mechanisms by which the growth and development of higher plants is affected by dynamic physical disturbances in the growth environment. At 1 x g, the increase in size (i.e., growth) and mass (i.e., photosynthetic productivity) of plants is retarded by seismic forces such as wind, shaking, or vibration, as well as thigmic forces such as the impact of precipitation, contact rubbing, or flexing of plant parts. Mechanical stresses capable of significantly inhibiting plant growth often are not accompanied by macroscopic signs of physical injury. Like drought or cold temperature, dynamic physical forces can create a condition of physiological stress within the plants they affect.

The physical signals received by plants growing in a spacecraft are atypical of the growth environment in which land plants originally evolved. If exposed to the acute seismic shock of launch for the first 8 minutes until main-engine cutoff, vegetative plant growth likely would be inhibited for several days relative to that of ground controls. Once in orbit, plants suddenly will be exposed only to a microgravity environment, or will they? Whether normal machine operation, spacecraft maneuver, or astronaut activity will create background noise or force pulses with sufficient gravity equivalents to alter growth or orientation remains to be determined. Will a plant developing in an environment largely devoid of a static physical force (i.e., gravity) be structurally weaker than a plant grown at 1 x g, and will slight vibration therefore cause even more effect on plant growth in microgravity than at 1 x g? Can controlled mechanical stimuli applied prior to launch or in orbit be used to condition or harden plants against unwanted tropisms or straight growth responses to perturbation in orbit?

Therefore, the objective of our research program (thus far Earth-bound) has been to characterize the nature and range of plant growth responses to periodic mechanical stresses and to reveal their physiological basis. Recent emphasis has focused on the potential for controlled mechanical stresses to acclimatize tender plants to better withstand the rigors of more stressful environments.

Accomplishments

Significant findings resulting from these studies are as follows:
(1) Seismic stress applied for 20 minutes twice daily at

160 or 190 rpm in a greenhouse inhibited growth in leaf area and leaf and plant dry weights of young soybean plants to the same extent as merely placing the plants outdoors under summer conditions. The same shaking treatments applied to outdoor-exposed plants had no further effect on growth. Thus, brief, periodic seismic stress effectively mimicked at least wind action in the outdoor environment in terms of equivalent reduction in certain growth parameters.

(2) Periodic seismic stress applied to young tomato plants in a protective environment inhibited internode elongation and decreased node and internode diameters (by the same proportion as the decrease in axial growth), but it increased ultimate shear strength, modulus of rupture, and modulus of elasticity for stems and petioles. The cellulose component of tomato stem fiber also increased. Thus, mechanical stress strengthens stem and petiole tissues and might be used as a structural toughening pretreatment before transfer of plants to a more stressful environment (outdoor environment, spacecraft).

(3) Tomato and soybean seedlings growing in a greenhouse were much less responsive to seismic treatment (agitation) in terms of growth inhibition if treatment was applied in the summer rather than in the winter. Experiments in which shade cloth of different densities was draped over benches indicated that plants generally are more responsive to mechanical stresses when grown and treated at low light intensities than at high light intensities. Since the light levels currently available in the present configuration of the Space Shuttle's Plant Growth Unit (PGU) are less than 5% of sunlight level, plant sensitivity to mechanical stresses should be high.

(4) Exogenous abscisic acid (ABA), a natural growth inhibitor, or seismic or thigmic stresses applied as a pretreatment in the greenhouse for several days before transfer of tender young eggplants outside to a more rigorous environment failed to acclimate the plants to outdoor exposure in terms of early recovery of relative growth rate or stem elongation. Thus, mechanical stresses or ABA did not condition eggplants to grow more vigorously than nonpretreated plants when subsequently placed in a stressful environment. Neither thigmic nor seismic pretreatments applied in a protective environment significantly affected endogenous leaf ABA content of eggplant after several days of pretreatment. However, thigmic pretreatment did prevent a brief rise in leaf ABA content one day after transferring plants outdoors, so mechanical pretreatments may have some value in desensitizing plants to stressful environments in terms of ABA fluctuations.

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GRAVIPERCEPTIVE MECHANISMS IN PLANT ROOTS

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Description of Research

The long-range goal of this research is to elucidate the mechanisms underlying how roots perceive and respond to gravity. We are interested in determining how plants translate an environmental signal (i.e., gravity) into a predictable growth response.

The specific objectives include:

(1) Determining what effectors mediate gravitropism. We attempted to attain this objective by engineering plants deficient in abscisic acid (ABA) and gibberellic acid (GA), two growth regulators that have been suggested to be effectors for root gravicurvature. We determined the gravireactivity of these plants' roots to determine if GA and/or ABA is necessary for root gravicurvature.

(2) Determining the influence of gravity on the movement of calcium, a putative effector of root gravitropism. We attempted to attain this objective by localizing calcium in cells of the root cap, which is the putative graviperceptive organ of roots.

(3) Determining the means and pathway of movement of gravitropic effectors in roots. We attempted to attain this objective by (a) studying the effects of electrical asymmetries on root curvature, and (b) investigating systems in which portions of the putative pathway for movement of gravitropic effectors is disrupted.

(4) Determining how root gravireactivity is altered by chemical, developmental, and genetic constraints. We attempted to attain this objective by systematically investigating roots whose nongraviresponsiveness results from genetic (i.e., agravitropic mutants), developmental (i.e., lateral roots), and chemical (i.e., treatments with morphactins) constraints.

(5) Determining if putative gravitropic effectors can induce curvature of normally nongraviresponsive roots. We attempted to attain this objective by determining if electrical and ionic (e.g., calcium) asymmetries across root tips would result in gravitropiclike curvature of agravitropic roots.

Accomplishments

(1) Our cytochemical data suggest that calcium moves acropetally (from base to apex) in the columella (i.e., putative graviperceptive) tissue of caps of horizontally oriented roots. We could find no evidence for lateral transport of calcium through the columella tissue.

(2) Large amounts of calcium are secreted into the mucilage

by peripheral cells of the root cap.

(3) Electrical and ionic asymmetries induce gravitropiclike curvature of graviresponsive and nongraviresponsive roots.

(4) Roots having undetectable levels of ABA and GA are strongly graviresponsive.

(5) Roots whose nongraviresponsiveness is due to chemical, developmental, and genetic constraints apparently perceive gravity and produce gravitropic effectors, but do not establish gradients of these effectors.

Significance of the Accomplishments

(1) The apoplast may be an important part of the pathway of movement of calcium (and other effectors) to the lower side of the root tip. We believe that gravity-induced electrical asymmetries may be important for moving calcium to the lower side of the root tip.

(2) ABA and GA are not necessary for root gravitropism.

(3) Nongraviresponsive roots respond to effectors believed responsible for curvature of graviresponsive roots. That is, roots whose nongraviresponsiveness results from chemical, developmental, and genetic constraints may all share a common mechanism for nongraviresponsiveness, namely, the inability to establish asymmetries of effectors across their tips.

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ROLE OF AUXIN IN NORMAL AND ABERRANT (WRONG-WAY) GRAVITROPISM OF TOMATO SEEDLINGS

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Description of Research

The overall goal of this research is to understand the entire process of gravitropism at a biophysical and biochemical level, that is, to dissect the mechanisms by which plants detect and orient with respect to gravity.

During the past year we have fulfilled our explicit goals for the study of auxin transport (signal transmission), and we have also made progress in understanding gravity reception/transduction and the ultimate curvature response.

Accomplishments

Signal Transduction: Primary Step. The most significant single activity of our laboratory, which has been joined by Professor Kathryn Edwards of Kenyon College for a sabbatical and leave, was to propose and provide preliminary evidence that mechanoreceptive ion channels of the type described in 1984 by Guharay and Sachs are responsible in plants for the reception of a variety of mechanical stimuli including friction, flexure, and gravity.

Our major goal in developing the idea was to elucidate the biophysics and biochemistry of the first step in gravity reception. However, for two important reasons we sought to achieve the first demonstration in plants of a representative mechanotransductive ion channel in a cell which does not respond to gravity: a suspension-cultured tobacco cell. First, we suspect that the gravitropic channel passes Ca^{2+}, has a low permeance for that ion, and is part of a macromolecular complex that tends to trap the ion before it reaches the bulk phase of cytosol. These properties would make the channel a poor one on which to perform preliminary patch clamp experiments. Second, we believe that root tip protoplasts from identified layers of cells in the columella are the best object for gravitropic patch clamping. Unfortunately, the process of protoplasting root tip cells and identifying them was much more tedious to work out and to perform than the process of obtaining suitable cells from many other tissues. Moreover, the small diameter of the root cap protoplasts makes them a little more difficult for preliminary studies than those of many other cell types.

Kathryn Edwards and I enlisted the efforts of Stanly Misler and Lee Falke of the Washington University Medical School in our patch clamping studies. Briefly, what we have found is a channel

which opens when pressures as low as 2.5 kPa (kiloPascals) are applied to the cytoplasmic face of the plasmamembrane, but not to the apoplastic face. The channel passes both anions and cations; the ratio of selectivity for Cl^- to K^+ is about 10 (Goldmann-Hodkin-Katz calculation). The conductance of the channel is high: for example, when there is 200 mM KCl and 10 mM CaCl in the patch pipette, conductance in the vicinity of the reversal potential is about 100 pS. We have inferred that the channel is a turgor sensor and osmoregulator.

Signal Transduction: Secondary Steps. Space Biology Program Research Associate J. Henry Slone has been studying auxin accumulation by plasmalemmal vesicles derived from pea and zucchini and the sensitivity of this system to the phytotropin family of gravitropic inhibitors. We believe that study of this *in vitro* system will aid in elucidating the proteins involved in gravitropism. We have amassed a great deal of descriptive information to use as a baseline for future experiments.

In this work, we have joined efforts with Professor Dabney Dixon, an organic chemist, and Terry Riehle, a postdoctoral associate, to isolate a protein already known to participate in controlling gravitropic lateral transport of auxin. This protein, which binds phytotropins, has been sought by many workers with as yet incomplete success. We have now succeeded in synthesizing a particular phytotropin with the desired special properties, and are now constructing affinity beads and testing whether they bind the modulatory protein which we are seeking. Obtaining this protein in pure, active form would be a breakthrough in the study of gravitropism, phototropism, and auxin transport, and possibly in more general studies of auxin effects.

Signal Transmission and Curvature. Experiments on gravitropic auxin transport, begun by Marcia Harrison when she was a Space Biology Program Research Associate, have now been completed. Results dealing with the putative influence of ethylene during gravitropism have been reported. Experiments dealing with the time-course, direction, and magnitude of auxin transport during the early transient positive ("wrong-way") phase of gravitropic curvature as well as during the major negative phase have been completed. These experiments also evaluated auxin transport in plants which, owing to sudden provision of relatively large amounts of auxin, carry out prolonged positive curvature as strongly as normal plants bend negatively. The major conclusions are that in light-grown tomato hypocotyls, auxin transport is evidenced early, certainly by the time curvature begins; that the ratio of auxin in the lower tissue to that in the upper is about 3:1 during the main phase of curvature; and that wrong-way responses are due not to reversal of auxin asymmetries but rather to wrong-way responses of the tissue to standard asymmetries. Moreover, the direction of gravitropic auxin asymmetry is unchanged when the system is flooded with auxin, although the magnitude of asymmetry is diminished: again, the wrong-way curvature is due to the special state of sensitivity of the

tissue. We believe that this information is important in sorting out the relationship of growth asymmetries to auxin asymmetries. Data on differential growth during the wrong-way responses are being collected.

M. Harrison has completed experiments elucidating how irradiation pretreatments can drastically shift the kinetics of gravitropism, with particular attention to how curving is distributed along the stem.

Statoliths. Because it is of relevance to our overall goal of understanding the mechanism of gravitropism, our collaboration with Tim Caspar and Chris Somerville on a phosphoglucomutase mutant of Arabidopsis thaliana which cannot make starch but which is nonetheless vigorously gravitropic is continuing. The experiments, though yet completed, appear to rule out the participation of amyloplast statoliths in the gravitropic curvatures of the roots and shoots of seedlings of this species.

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CALCIUM MESSENGER SYSTEM IN GRAVITROPIC RESPONSE IN PLANTS

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Description of Research

For plants to function and adapt efficiently to the changing environment, their cells must communicate with one another. All higher plants have built-in regulatory mechanisms which can be controlled in response to external and internal stimuli such as hormones, gravity, and light. It is becoming increasingly evident that calcium ions are important intracellular messengers in plants. Recent studies indicate that there are changes in the calcium localization in response to gravity. The changes in calcium localization occur soon after the stimulus is perceived and before any macroscopic response is detected. These results suggest that the stimulus perception in plants might involve the role of calcium as a second messenger. Investigations in our laboratory have indicated that calcium acts as a second messenger by coupling stimulus to response by regulating phosphorylation of proteins, a major mechanism in cellular regulation. We have shown that the calcium-promoted phosphorylation of several of these proteins is mediated by calmodulin, a calcium-binding protein of ubiquitous occurrence.

Protein phosphorylation/dephosphorylation is recognized as an important mechanism by means of which enzyme activities are modulated reversibly by second messengers such as calcium in response to various external stimuli. Protein kinases and phosphatases are involved in phosphorylation and dephosphorylation, respectively. The promotion of protein kinases by calcium and calmodulin provides a mechanism by which calcium can regulate diverse biochemical processes and physiological actions in plants. The occurrence of calcium and calmodulin-dependent protein kinases in a wide variety of plant tissues suggests that such a phosphorylation system could be of general importance in calcium action. These observations offer a promising approach for studying the role of calcium as a second messenger in the biochemical changes leading to tropic movement in plants.

Our primary objective is to study *in vitro* and *in vivo* protein phosphorylation and to establish the role of calcium ions as second messengers in gravitropic response in plants. Further research on calcium-regulated changes such as *in vitro* and *in vivo* protein phosphorylation should lead to a greater understanding of the role of calcium in plants.

Accomplishments

In vivo protein phosphorylation studies were performed using root tips of corn plants (Zea mays L.) grown in the dark. Phosphoproteins were analyzed by two dimensional gel electrophoresis. Distinct changes in phosphorylation were observed following altered calcium levels. Calcium depletion in root tips with EGTA and calcium ionophore (A23187) markedly decreased protein phosphorylation. However, replenishment of calcium following EGTA and ionophore pretreatment enhanced phosphorylation of proteins. Furthermore, phosphorylation of some of the polypeptides was markedly increased. Preloading of the root tips with ^{32}P in the presence of EGTA and calcium ionophore followed by a 10-minute calcium treatment resulted in increased phosphorylation indicating the involvement of calcium and/or calmodulin-dependent protein kinases. The calmodulin antagonist W-7 was effective in inhibiting calcium-promoted phosphorylation and also auxin-induced growth. These studies suggest a physiological role for calcium-dependent protein phosphorylation in calcium-mediated processes in plants.

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MECHANISM OF SHOOT GRAVITROPISM

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Description of Research

A basic question in plant physiology is the mechanism by which plants transduce information about the direction of gravity into an oriented direction of growth. Information gained about how plants respond to gravitropic stimulation will enhance our knowledge of a basic adaptive mechanism and allow us to better understand and control the orientation of plant organs in the space environment.

Briefly, we know that when a shoot is oriented in a horizontal position it begins to curve upward in a smooth arc after about 20-30 minutes. Reorientation is usually complete within 2-4 hours. This curvature response ultimately derives from enhanced growth of those cells comprising the lower portion of a horizontal shoot and a retardation of cell growth near the upper surface. Our goal is to understand the factor or factors responsible for this asymmetric growth. Toward this end our efforts have centered on the physiological properties of auxin and calcium as potential mediators of gravitropism.

During the current year we completed part of an ongoing effort to determine the role of calcium (Ca^{++}) in cell elongation, gravitropism, and auxin transport. Our major findings are listed below.

Accomplishments

(1) EGTA, a chelator of calcium ions, stimulates rapid cell elongation when applied in weakly buffered solutions but fails to initiate extension when applied in strongly buffered solutions. Quin 2, another chelator of calcium, fails to stimulate cell extension at all buffer molarities. Collectively these data indicate that Ca^{++} chelation per se does not result in cell wall loosening and thus cell extension growth.

(2) Polar auxin transport is enhanced by calcium application. As this effect can be mimicked by the addition of Ca^{++} to receiver blocks, the effect of calcium may be to regulate or to increase basal auxin secretion.

(3) Lateral auxin transport across gravistimulated shoots is also influenced by calcium. Data indicate that in vivo there may be an interaction between auxin and Ca^{++} lateral transport.

(4) We have discovered that young plant roots exhibit interesting patterns of fluorescence when treated with the calcium probe, chlorotetracycline (CTC). We hope to use CTC to directly visualize the patterns of calcium movement during

graviperception and response.

Significance of the Accomplishments

These data contribute to our overall understanding of plant gravitropism and asymmetric cell elongation by focusing on the interaction between calcium and the plant growth hormone auxin. In plant shoots it would appear that calcium levels do not directly regulate the rate of plant cell extension by influencing the physical properties of the cell wall. Rather, it seems more likely that gravity-induced calcium fluxes may influence auxin redistribution which in turn controls the rate of cell expansion and thus gravitropism.

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ROLE OF CALCIUM AND CALMODULIN IN CONTROLLING LIGHT-MEDIATED GRAVITROPISM

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Description of Research

Light greatly accelerates the gravitropic response of roots, coleoptiles, and stems in certain plants. This is taken as strong evidence that some cellular response initiated by light is the same as, or affects, one of the gravity-induced cellular responses necessary for gravitropism. The objective of this research is to elucidate the specific cellular processes that are altered by gravity and light during the induction of gravitropic growth in plants.

One of the key photoreceptors for light-regulated gravitropism is the pigment, phytochrome. The photoactivated form of this pigment, called Pfr, accelerates the gravitropic curvature of some shoots and roots. One important cellular function that is activated by both Pfr and gravistimulation and that would be expected to have an effect on gravitropism is the transport of calcium ions (Ca^{2+}) from inside cells out into cell walls. Increasing the concentration of Ca^{2+} in cell walls inhibits their extensibility. Both Pfr-regulated growth and gravitropic growth are characterized by the decreased growth rate of certain cells, possibly due in part to increased Ca^{2+} transport into the walls of these cells. The effects of Ca^{2+} on cell wall extensibility are thought to be mediated by Ca^{2+}-sensitive enzymes that regulate the loosening of load-bearing bonds and thus control how "stretchable" the wall is.

To learn more about Ca^{2+}-regulated activities both within the cell and in cell walls, we have used immunocytochemical methods to investigate the tissue and cellular locale of two calcium-binding regulatory proteins in gravitropically responding organs of plants. These two proteins are calmodulin and calcimedin. Last year we reported the tissue-level localization of calmodulin; this year, we localized calmodulin on the ultrastructural level, using immunogold methods, and we have localized calcimedin on the tissue level, using immunofluorescence methods. We have also raised antibodies against cell wall enzymes in order to learn more about their role in regulating cell wall loosening and gravitropic growth. Finally, we have tested whether light, calcium, and calmodulin influence nuclear functions, since rapidly modulated gene expression could play a role in the gravitropic response.

Accomplishments

The major findings from these studies are:

(1) Using immunogold methods, we have confirmed that calmodulin is associated with vacuoles, nuclei, and plastids, but is not associated with cell walls.

(2) Using immunofluorescence methods, we have found that calcimedin has a distribution in pea tissues different from calmodulin. In pea plumules, regions close to the apex show the clearest staining response. Most of this staining is in the cytoplasm where it has a distinctly punctate appearance, with little or no staining in plastids or nuclei. Immunogold localization studies will be needed to identify what structures are represented in this punctate staining.

(3) Mice inoculated with a protein preparation enriched for cell wall enzymes contain serum antibodies that will block at least three different wall enzyme activities: peroxidase, malate dehydrogenase, and glutamic oxaloacetic transaminase.

(4) From inoculated mice we have raised a library of monoclonal antibodies that recognize at least six different wall proteins.

(5) Pfr, calcium, and calmodulin modulate the phosphorylation of nuclear proteins and the activity of a chromatin-associated nucleoside triphosphatase.

Significance of the Accomplishments

Finding #1 extends and improves last year's findings by providing an independent confirmation, with greater structural resolution, that calmodulin is localized in plastids, vacuoles, and nuclei. All three of these organelles have been implicated as participants in the sensing or response phase of gravitropism. The lack of an immunogold signal for calmodulin in cell walls indicates that this calcium-binding protein may not be the target of calcium's action in plant cell walls.

Finding #2 is the first report of calcimedin localization in plant tissue. Many data implicate a role of calcium in the control of gravitropism, so it is critical to know what are the calcium-binding regulatory proteins in plant cells and where they function. Because calcimedin has a distinctly different tissue and subcellular distribution from calmodulin, it may regulate distinctly different Ca^{2+}-sensitive processes.

Finding #3 indicates that at least some plant cell wall enzymes are strongly antigenic, and that some of the antibodies raised against wall enzymes can block their activity. The antibody-producing spleen cells of mice containing antibodies against wall enzymes were used to make hybridoma cells that produce abundant monoclonal antibodies against these wall proteins, as described in Finding #4.

Finding #4 is likely the first successful attempt to raise a "library" of monoclonal antibodies against wall proteins. Since

this collection of antibodies was derived from mice which were making antibodies that blocked the activity of wall enzymes, it should include these antibodies, too. Each monoclonal antibody can be inexpensively produced in large quantities and used as a highly specific reagent to probe which enzymes mediate the wall extensibility changes that are so crucial for gravitropism.

Finding #5 calls attention to the possibility that gravitropically induced changes in the transport of Ca^{2+} could affect nuclear enzyme activities. Recent publications show that both Pfr and hormones promote rapid changes in gene expression that are important for the growth changes induced by these stimuli, so it now becomes pertinent to ask whether nuclear activity changes help mediate the rapid growth responses stimulated by light and gravity.

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CELLULAR POLARITY AND INTERACTIONS IN PLANT GRAVIPERCEPTION

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Description of Research

We do not know how a plant "senses" the direction of gravity. Many botanists suspect that heavy, starch-containing organelles--amyloplasts--play an essential role in this process, but it is unclear where amyloplasts act within the cell. The ultimate goal of our research is to contribute to the identification of the site of gravity reception in higher plants. Initial goals are to determine whether interactions occur between amyloplasts and two cellular components, the cytoskeleton and auxin transport carriers.

The cytoskeleton is composed of different systems of filaments in turn consisting of different proteins. Our previous observations of living statocytes (cells probably involved in gravity perception) indicate that gravity-induced amyloplast sedimentation is not straightforward but is influenced by cytoplasmic streaming in roots and shoots. Cytoplasmic streaming is thought to be mediated by microfilaments. If such a cytoskeletal system were present in statocytes, amyloplasts could perhaps trigger perception by "tugging" on these filaments or by repositioning membrane components with microfilaments as intermediaries. It is not known whether a microfilamentous cytoskeletal system is present in statocytes. Our initial work has attempted to demonstrate the presence of microfilaments in these cells.

Dr. Mark Jacobs has developed monoclonal antibodies to presumptive auxin transport carriers in pea stems. Using immunofluorescence, he has found that these hormone carriers are located in the cell membrane along the bottom of cells. Because auxin may be involved in inducing gravicurvature, it is important to understand the distribution of these carriers in vertical and horizontal organs. Work on this project has begun with a study of the structure of the pea stem as background for the immunofluorescence experiments.

Accomplishments

(1) Actin-containing microfilaments have been visualized in epidermal cells (nonstatocytes used as a favorable test system) using fluorescence microscopy. Microfilaments exist in these cells as either large cables, which are more or less straight, or as smaller and finer cables found around the periphery of the cells.

(2) Techniques were developed to fix amyloplasts in place and to serially section large organs using a Vibratome.

(3) Dark-grown pea stems contain a hook close to their apex. A starch sheath exists in the hook, but only some amyloplasts sediment close to the lower walls of cells in this region; sedimentation only occurs consistently when the cells become upright (just below the hook), about 1 cm above the zone where curvature takes place upon gravistimulation.

(4) Although the cells of the starch sheath eventually develop a casparian strip (which restricts solute movement in the cell walls), no such blockages are present in these cells at the stage when gravity perception seems to take place.

(5) Amyloplasts were often found sedimented upon, and in apparent contact with, endoplasmic reticulum located in the cytoplasm close to the lower cell wall.

Significance of the Accomplishments

Finding #1 suggests that it is technically feasible to determine whether microfilaments are found in statocytes from different organs and genera. The successful application of this technique to plants is significant because processing for electron microscopy destroys most plant microfilaments; a light fixation combined with enzymatic digestion preserves more microfilaments, which can then be visualized using fluorescence light microscopy.

The techniques mentioned in Finding #2 are particularly valuable for determining amyloplast distribution and position in large plant organs such as stems and coleoptiles. Chemical fixation maintains the amyloplasts in place and allows serial sectioning with the Vibratome. This method is rapid and convenient and preserves more cellular components and prevents major tissue shrinkage compared with classical methods of fixation (e.g., FAA) and embedding (paraffin).

Finding #3 provides structural background for studies of the distribution of the auxin transport carriers by immunofluorescence. It suggests that carrier distribution should be studied especially in the stem hook and in the 1-cm part of the stem below the hook, that is, in the region where amyloplast sedimentation occurs but above the zone where curvature takes place. Dr. Jacobs has only examined the internode below the zone capable of curvature. Because auxin is transported tip to base, if this hormone or hormone carriers are involved in the graviresponse, then clearly the upper part of the internode needs to be studied as well.

Finding #4 suggests that during the stage when the starch sheath cells are likely to be involved in gravity perception, there are no blockages between these cells and other cells. Thus, auxin flow in the apoplast is probably not restricted radially, and differences in hormone concentration, if present, must be actively maintained by the cell membranes.

Finding #5 is consistent with the hypothesis that amyloplasts can interact with endoplasmic reticulum. In fact, this is one of the few reports of amyloplasts actually contacting endoplasmic reticulum. It should be noted, however, that the effect of chemical fixation on these large, fluid-filled cells is uncertain.

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GRAVITROPISM IN LEAFY DICOT STEMS

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Description of Research

The goal of this research is to understand the mechanism of response of dicot stems to gravity; specifically, to understand (a) the mechanics of stem bending (that is, identifying the cells that grow by absorbing water, and elucidating what happens to the elasticity and plasticity of cell walls), and (b) the manner in which auxin controls or contributes to this growth.

The mechanics of stem bending. Perhaps the most striking observation about the mechanics of gravitropic bending in plant stems is the almost immediate halting of growth of top cells when a stem is turned to the horizontal. Bottom cells may continue to grow at about the same rate as when vertical, or their growth may speed up or slow down somewhat. This observation could be understood if cell walls of top cells quickly became less extensible upon gravistimulation, and this could occur in response to a reorientation of the direction of cellulose microfibril deposition or some other tightening of the wall. We have investigated microfibril orientations with transmission electron microscopy and by birefringence in polarized light.

The role of auxin. What brings about the changes in growth rates? The dominant theory (Cholodny-Went) suggests that the growth hormone, auxin, becomes more concentrated in bottom tissues than top tissues, accelerating growth of bottom cells. An alternative hypothesis suggests that changes in cell responsiveness to auxin might account for differential growth. Thus, the observed mechanics could occur as auxin drops to low levels in top cells or as top cells become less responsive to auxin. Researchers for many years have looked for auxin concentration gradients in stem tissues. Gradients and differences in growth rates between top and bottom tissues have been observed, but measured auxin may not be equivalent to active auxin, e.g., there may be bound auxin sequestered in inactive compartments. The sensitivity hypothesis has not been widely tested, so our experiments have been designed to do so.

Sensitivity can best be discussed in the context of Michaelis-Menton enzyme kinetics. As substrate concentration increases, reaction rate also is called V_{max}, and the substrate concentration at half of V_{max} is called K_m. A lower V_{max} means less enzyme; higher K_m means less tenacious binding of enzyme to substrate--the most interesting form of decreased sensitivity.

This approach can be applied to stem response to auxin by

plotting growth rate (analogous to reaction rate) as a function of auxin (analogous to substrate) concentration. We have marked soybean seedling hypocotyls, turned them to the horizontal, and immersed them in buffered auxin solutions. The hypocotyls were photographed at intervals, and bending as well as top and bottom growth were measured from the photographs. Auxin (indoleacetic acid) labeled with ^{14}C can be used to see how much auxin penetrates the tissues.

Accomplishments

(1) We found that mean microfibril orientations do not change significantly during gravitropic bending. Wall thicknesses remain fairly constant during the rapid elongation of bottom cells, indicating that wall deposition is continuing and compensating for the stretching of walls that occurs during growth. We saw a slight thickening of walls in top tissues, indicating that wall material continues to be deposited although walls are not stretching.

(2) Cell wall extensibility was measured for us by Sarb Viert in Robert E. Cleland's laboratory at the University of Washington, Seattle. A difference in extensibility between lower and upper tissue developed within 10 minutes so that extensibility of lower walls was greater than that of upper walls. During autotropic straightening, in which the curvature of the stem caused reorientation of tissue, there were corresponding changes in wall extensibility. Preventing stems from bending up caused the difference in extensibility between lower and upper tissue to increase throughout the experiment, leading to a much greater difference than in free-bending stems. This suggests that there are active processes causing this decrease in extensibility in upper tissue.

(3) In our auxin immersion experiments, we found that increasing auxin concentrations inhibit gravitropic bending, and this occurs because top tissues are promoted in their growth, while bottom tissues are less affected or even inhibited. Plotting top and bottom growth separately as functions of auxin concentration gives the expected saturation curves with V_{max} approximately the same for both but a much higher K_m (an order of magnitude or two) for top tissues than for bottom tissues. This suggests that top tissues greatly decrease in sensitivity to auxin upon gravistimulation. We also found that the concentration of labeled auxin in the tissues, though it is proportional to external auxin concentration, was essentially equivalent in top and bottom tissues, contrary to predictions of Cholodny-Went.

We have also repeated experiments published in 1958 by Brauner and Hager. Seedlings (they used sunflower) were decapitated and left for some time in an upright position until they became depleted in or less sensitive to auxin. Then they were gravistimulated, returned to the vertical, and supplied with auxin, leading to bending in the expected direction. That is, they "remembered" the gravistimulation but could only express it

when given auxin. In our experiments with soybean hypocotyls immersed in various concentrations of labeled auxin, V_{max} was slightly decreased on the concave side (the side that had been uppermost during gravistimulation) and K_m was apparently unchanged. We need better data to be sure of this. It is clear, however, that labeled auxin concentrations were equal in both sides, again contrary to Cholodny-Went, which must suggest that the "memory" is some adjustment in the auxin-transport system so that more of the supplied auxin is moved to the convex side.

Significance of the Accomplishments

(1) The demonstration that the changes occurring on top and bottom cells of stems during gravitropic bending are not readily explainable on the basis of changes in cellulose-microfibril orientations indicates that we can now safely ignore the microfibril hypothesis.

(2) The demonstration that cell wall extensibility does change during gravitropic bending, with top cell walls becoming less extensible than lower walls, points out future directions of research on how auxin acts to change wall extensibility.

(3) (a) We confirmed earlier observations that increasing auxin concentrations in an external medium progressively inhibit gravitropic bending, and that this occurs primarily because the increasing auxin causes growth of top tissues. This was the first observation that allowed us to seriously consider a sensitivity hypothesis as a key to understanding gravitropic bending.

(b) We developed saturation curves showing growth rates of upper and lower hypocotyl surfaces as a function of external auxin concentrations and discussed these curves in terms of Michaelis-Menton enzyme kinetics; the curve for the lower surface shows little difference from that of a vertical stem surface in the same region, but the curve for the upper surface shows a sharp increase in K_m with little or no change in V_{max}, strongly suggesting that sensitivity of upper tissues to auxin greatly decreases upon gravistimulation. We also demonstrated that labeled tissue auxin is proportional to external auxin but similar in upper and lower tissues at all external auxin levels (true in the growth experiments as well as in the gravitropic "memory" experiment). The saturation curves and the "memory" experiments provide strong evidence for a sensitivity hypothesis and are difficult to reconcile with an auxin-transport idea. If this evidence is fortified by future work, researchers can finally break away from the Cholodny-Went idea that has held its grip on gravitropism research for 60 years. It would become highly desirable to understand the nature of tissue sensitivity to auxin and how it might change in response to gravistimulation. Present studies on calcium effects and related topics provide an excellent beginning for research on sensitivity.

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ANIMAL PROJECTS

EFFECT OF SKELETAL UNLOADING ON BONE FORMATION

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Description of Research

The long-range goal of our research program is to understand the effects of gravity on skeletal development and bone metabolism. Our present objectives are to define the effects of gravity or mechanical stress on bone formation and resorption, and to determine the mechanisms by which mechanical stress is coupled to bone cell activity.

Bone is a dynamic, living tissue. It is continually undergoing change or remodeling. This remodeling involves a delicate balance between bone formation and bone resorption. The balance is influenced by endocrine factors (e.g., vitamin D, parathyroid hormone (PTH), and the adrenal steroids), neuromuscular activity, and gravity. The effects of gravity on skeletal metabolism can be direct (e.g., through mechanical loading on bone) or indirect (e.g., through endocrine changes or changes in neuromuscular activity). Our objectives during the past year were to (a) determine if bone mineral maturation is influenced by skeletal unloading, (b) determine if PTH or adrenal steroids are involved in the bone loss induced by unloading, and (c) begin in vivo and in vitro studies to assess the role of electric fields in the coupling of mechanical stress to bone cell activity.

To accomplish these objectives we used the Holton rat model to produce skeletal unloading. Bone mineral maturation was studied using a density gradient fractionation technique. The influences of PTH and adrenal steroids were studied using parathyroidectomized and adrenalectomized animals, respectively. The role of electric fields is being studied using isolated bone cells in vitro and electrical stimulation of unloaded limbs in vivo.

Accomplishments

(1) Demonstrated that skeletal unloading reduces the accumulation of dense, highly mineralized mature bone, in addition to reducing bone formation.

(2) Demonstrated that adrenalectomy does not prevent the inhibition of bone formation induced by skeletal unloading. Studies in parathyroidectomized animals are ongoing and results are not yet available.

(3) Established an in vitro primary bone cell culture capable of producing collagen and which shows evidence of intercellular electrical and chemical communication.

(4) Established an animal model in which a portion of the skeleton can be unloaded and simultaneously stimulated with a capacitively coupled electric field.

Significance of the Accomplishments

One of our primary objectives has been, and continues to be, to define the effects of gravity or mechanical stress on bone. We have shown that not only does mechanical unloading produce a transitory inhibition of bone formation, but it also impairs the maturation of bone mineral. The maturation effect may be related to the abnormality in bone formation or it may represent a second, entirely independent response to mechanical unloading. The fact that adrenalectomy does not prevent the decrease in bone formation induced by unloading indicates that neither changes in the circulating concentrations nor changes in bone sensitivity to the adrenal steroids are likely to be responsible for the inhibition of bone formation. This is consistent with our previous observation that the abnormalities in bone formation in unloaded limbs are not due to changes in either the circulating concentration or bone sensitivity to the active vitamin D metabolites. Studies in parathyroidectomized animals are underway. To date, our cumulative data suggest that the bone changes induced by mechanical loading are not due primarily to either endocrine changes or changes in bone sensitivity to endocrine factors. Instead, they suggest that unloading directly influences bone cell activity. We hypothesize that the mechanism coupling mechanical load to cellular activity involves changes in electrical potential. To examine this hypothesis we are establishing *in vitro* and *in vivo* models to study the effects of externally applied electric fields on bone cell metabolism.

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WEIGHTLESSNESS SIMULATION: PHYSIOLOGICAL CHANGES IN FAST AND SLOW MUSCLE

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Description of Research

Functional load is an important regulatory factor for maintaining the normal physiological status of skeletal muscle. The lack of production of regular muscle force in zero gravity is a contributing factor leading to atrophy and changes in metabolic activity and other muscle fiber characteristics. This research is directed toward an understanding of the influence of load-bearing on muscle, in particular the mechanisms by which muscle controls fiber type, acetylcholinesterase, acetylcholine receptors, and metabolic enzymes such as superoxide dismutases (SOD), glutathione (GSH) peroxidase, cytochrome c oxidase, and fumarase.

During 1985, research has focused on the effect of the absence of load-bearing ("non-load-bearing") on (a) high energy compounds such as phosphocreatine and the adenine nucleotides, such as ATP, ADP, and AMP; and (b) enzymes involved in the metabolism of free radicals such as superoxide and hydrogen peroxide. Disuse was induced by denervation and hindlimb unloading.

Accomplishments

(1) Changes in high energy nucleotides with disuse.

Following denervation or hindlimb unloading-induced muscle disuse in rat, qualitative changes in high energy phosphate compounds in the slow-twitch soleus and the fast-twitch extensor digitorum longus (EDL) muscle were determined using reversed phase HPLC. A dramatic decline of phosphocreatine (PC) levels occurred 3 days after denervation in both muscles (53%). Unloading-induced muscle disuse caused a maximum reduction within 7 days in soleus (50%) and in EDL (30%). This effect was maintained up to 2 weeks with a parallel rise of creatine (34%) in suspended soleus. Detailed analysis of adenine nucleotides indicated that following 3 days of denervation, a marked decrease in the ratios of ATP/AMP (>88%), compared with ATP/ADP (>42%), occurred as a result of a decrease in ATP and a more than threefold rise in AMP level. Unloading-induced muscle disuse greatly reduced the ratio of ATP/AMP in soleus (50%), while in EDL no significant change in any of the adenine nucleotides studied was seen.

(2) Disuse and changes in superoxide dismutases.

The effects of denervation and non-load-bearing were determined on the free radical scavenging systems in relation to

the mitochondrial oxidative metabolism in the slow-twitch soleus and fast-twitch EDL muscles of rats. The muscle tissue levels of five enzymes were studied 2 and 5 weeks following denervation and hindlimb unloading. Recently developed radioimmunoassays were utilized for the selective measurement of cuprozinc (cytosolic) and mangano (mitochondrial) superoxide dismutases. Total tissue content of cuprozinc superoxide dismutase (CuZnSOD) showed a slight decrease after denervation in slow but not in fast muscle. Mangano superoxide dismutase (MnSOD) and fumarase decreased markedly at 2 weeks and returned toward control levels by 5 weeks, the changes appearing to be greater in slow than in fast muscle. At 2 weeks, cytochrome c oxidase decreased significantly in slow but not in fast muscle. Glutathione (GSH) peroxidase markedly decreased at 2 weeks in slow muscle, and returned toward control levels at 5 weeks, whereas the total enzyme activity in fast muscle did not change through 5 weeks. Baseline activity was 10-fold higher in slow than in fast muscle.

Total tissue content of CuZnSOD (cytosolic) showed no significant change in both muscles following 2 weeks of hindlimb unloading. MnSOD (mitochondrial) decreased markedly after 2 weeks in soleus (50%) with no change in EDL.

GSH peroxidase, cytochrome c oxidase, and fumarase were markedly reduced in both muscles. This is in contrast to the denervation changes which induced a specific reduction of these enzymes in soleus, while in EDL no significant changes were seen.

Significance of the Accomplishments

(1) While muscle disuse-associated effects such as atrophy, changes in morphology and fiber type, increase in acetylcholinesterase, and increase in acetylcholine receptors are well documented, the findings of a decrease in high energy phosphate compounds are new. Excitation-contraction coupling, the process by which the action potential triggers muscle contraction, is energetically linked to phosphocreatine and ATP. The decrease in these compounds seen with hindlimb unloading is probably responsible for some of the changes observed with disuse, such as synthesis, contractility, and Ca^{++} uptake mechanisms. The greater deficit and the loss of selectivity seen with denervation is due to loss of trophic regulation of the enzymes involved in the synthesis of phosphocreatine and ATP.

(2) The present study has demonstrated that disuse results in a marked decrease in MnSOD concentrations in both slow and fast muscle, but only a marginal change in CuZnSOD levels. To our knowledge, this is the first report indicating a selective modification of CuZnSOD and MnSOD levels in muscles in response to depression of cell function, including oxidative metabolism.

The decrease in MnSOD concentrations was associated with the anticipated decrease in fumarase activity, indicative of a decrease in mitochondrial activity. These data collectively suggest that MnSOD concentration reflects mitochondrial free-

radical production and that this is in turn a function of mitochondrial metabolic activity.

The 10-fold higher GSH peroxidase activity in slow muscle, when compared with that of fast muscle, may have important practical implications. Histochemical classification of muscle fiber types is based on differences in activity of enzymes distributed in these muscles. Since GSH peroxidase activity is significantly higher in slow muscle, the histochemical identification of this enzyme may be a useful tool in studies of muscle fiber type identification.

Of interest is the fact that denervation induced significant changes of all enzymes measured in soleus but not in EDL. This is in contrast to the effect of non-load-bearing which induces similar changes in both muscles. The underlying mechanisms for this difference in response to disuse induced by denervation and non-load-bearing remains to be investigated. Only an exact knowledge of these processes will allow us to develop a rational approach to establish proper techniques that will prevent disuse-induced changes.

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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 objective of this research is to determine the influence of gravity on connective tissue development and maintenance. Previous studies have shown that hypogravity results in reduced new bone formation. The function of other connective tissues, whether they normally calcify or not, also is influenced by alterations in the mechanical forces exerted upon them. The importance of gravity and its role in these tissue responses is a fundamental question and remains largely unknown.

Two sources of material were available to us during this past year: (a) In Spacelab-3 (SL-3), 12 rats were subjected to 7 days of spaceflight and hypogravity conditions. An additional 12 rats were "flight-simulated" but did not experience hypogravity. The Spacelab experiment involved many investigators, and skeletal samples from the animals were received postflight. (b) The Earth-based model of Morey-Holton provided skeletal samples from rats whose posterior limbs were released from weight-bearing. This condition mimics skeletal responses similar to those found in hypogravity or spaceflight environments.

Accomplishments

(1) Results from the SL-3 experiment were difficult to determine because there was a significant delay between landing and analysis following spaceflight, thus the animals had begun to recover from their hypogravity experience. The bone-forming cells showed a slight reduction in cell size, compared with ground-based controls, but no apparent change in bone-forming ability.

(2) In animals with non-weight-bearing limbs, there is a temporary reduction in new bone formation, which can be related to several changes in the cells of bone:

(a) The cytoplasmic vesicles that transport bone matrix proteins to the cell surface show a shift in their size, which indicates a reduction in the rate of new protein movement through the cell cytoplasm.

(b) A protein associated with the cell surface (an enzyme called alkaline phosphatase) appears to be reduced in amount in those cells demonstrating reduced bone formation.

(c) A carbohydrate which can be used as a marker for noncollagen synthesis, has been found in the bone-forming cells, and it provides a good indicator of matrix synthesis by these cells. This carbohydrate is not found in other types of

connective tissue cells.

(d) Granules within the cytoplasm of bone-forming cells show a distribution along the cell membrane adjacent to the matrix and are not found along other cell surfaces (Figure 1). Thus these granules may be a sensitive indicator of cell polarity and can be used to indicate whether gravity influences organization of the cell relative to adjacent cells or matrix.

(e) The cell responsible for remodeling bone and regulating bone destruction (i.e., the osteoclast) contains a carbohydrate which is found only in the acidic compartment of the cell. This carbohydrate may be a specific indicator for resorptive activity for each osteoclast.

(f) A specific cytoplasmic enzyme (called dipeptidyl peptidase II) has been found in bone-forming cells but not in bone-resorbing cells. This enzyme could be used by the bone-forming cells to degrade specific proteins within these cells and thereby control the amount of protein being released to form new bone matrix.

(g) An enzyme has been found which can be stimulated by

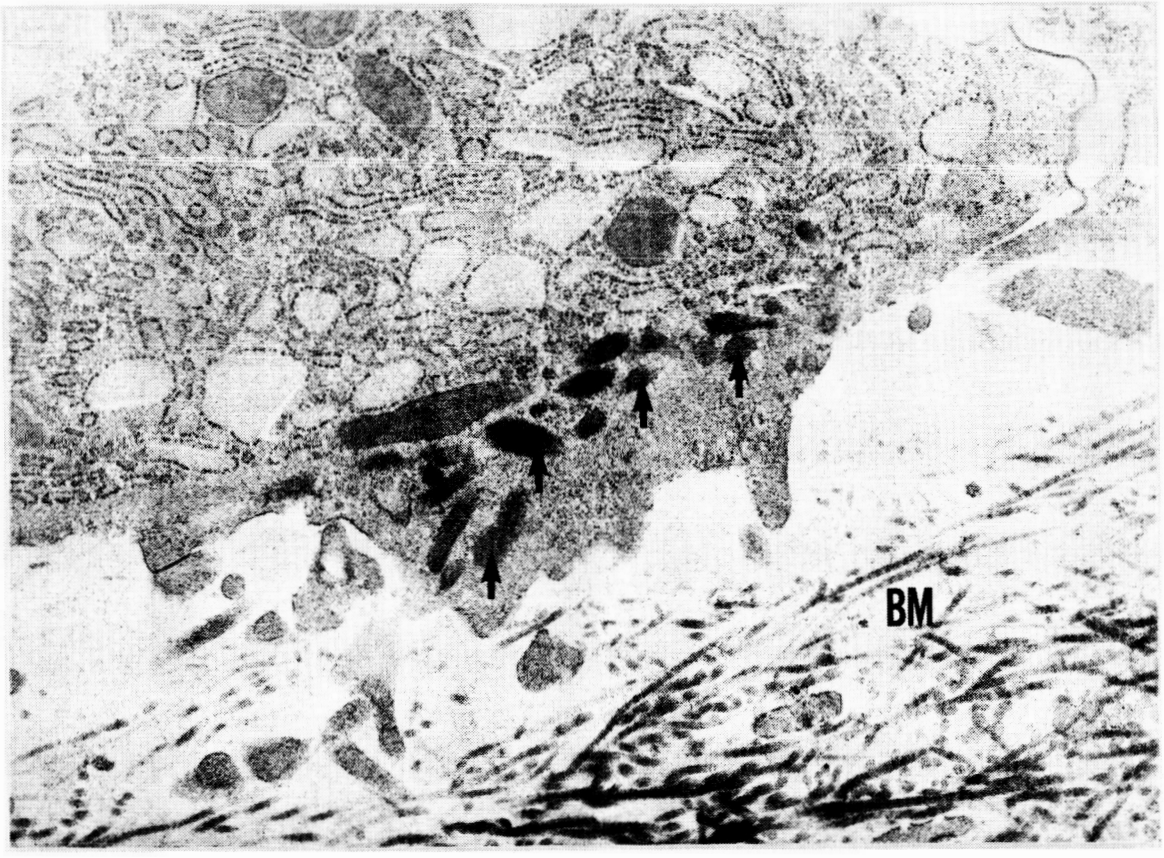

Figure 1. An electron micrograph (magnification 32,000 X) which shows secretory granules (arrows) oriented along the cell surface adjacent to bone matrix (BM). These granules are directed to one side of the cell prior to their release, suggesting that their accumulation within the cell may be a gravity-dependent process.

the level of ionic calcium in the extracellular fluids. This enzyme is localized along blood vessels, but not along the bone-forming cells.

Significance of the Accomplishments

The mechanism whereby hypogravity or non-weight-bearing can influence cellular activities is unknown. However, from our studies, a hypothesis can be presented which brings together the various accomplishments. The hypothesis would suggest that the system of supporting structures within each cell would be affected by the presence or absence of gravity. This supporting structure (called a cytoskeleton) is arranged like an internal skeleton to provide mechanical support for each cell and to support the cell's internal structures. If this internal support is inhibited from functioning properly, proteins synthesized by the cell would be carried to the cell surface in reduced amounts (see accomplishments 2a and 2b), overall cell size might be reduced (accomplishment 1), cell orientation or attachment to other surfaces might be affected (accomplishment 2d), and degradation of proteins within the cell cytoplasm might be altered (accomplishments 2e and 2f). The internal skeleton of cells may be influenced directly by the presence or absence of mechanical force, so perhaps the reduction of gravity will directly express itself on the skeletal system of each cell. For this to happen, we might also have to assume some sort of attachment between the connective tissue cell and other cells or between cells and the matrix which normally transmits force to the cell surface. This direct transfer of force between cell and matrix remains to be proven. Based on this hypothesis, we will now begin to study the skeletal systems of these cells by more direct methods. In addition, in other tissues, we know that cellular skeletal activities can be regulated by alterations in extracellular calcium levels (accomplishment 2g) and, in these instances, mechanical forces may not play a role. Therefore, we have to consider a change in cell calcium as another potential regulator of the cytoskeletal system which, for some cells, would be the most critical change due to hypogravity.

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GROWTH AND DIFFERENTIATION OF MAMMALIAN EMBRYONIC TISSUES EXPOSED TO HYPERGRAVITY *IN VIVO* AND *IN VITRO*

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Description of Research

Gravitational changes have been shown to produce corresponding changes in development. Our laboratory has been studying the effects of altered gravity on the development of the mammalian skeletal system, which is known to be responsive to gravitational changes. Because of limited opportunities for experimentation in weightlessness, data from ground-based systems is required for planning and prioritization of flight experiments. Two such systems are the clinostat (for simulated null g studies) and the centrifuge (for excess g studies). The experiments reported here are studies of the effects of excess g on mammalian skeletal development *in vivo* and *in vitro*.

Exposure to excess gravity enables us to identify aspects of skeletal development which are sensitive to gravitational changes, and which are likely to be affected by exposure to microgravity. The use of *in vivo* and *in vitro* systems allows us to distinguish any direct effects on the embryo from indirect effects resulting from changes occuring in the mother. At present, and for some time to come, *in vitro* experiments, such as those conducted on the 1985 Space Shuttle D-1 mission, may be our only source of information about effects of weightlessness on mammalian development.

In our *in vitro* studies, embryonic mouse tissues were excised and exposed to excess g on a culture centrifuge. For *in vivo* studies, adult mice were placed on a small animal centrifuge and were mated after they had adapted to the excess g force. The prenatal development of the embryos, particularly of the skeletons, was then assessed.

Accomplishments

(1) Frequency analyses of developmental stages of serial sections of embryonic mouse limbs centrifuged *in vitro* showed significantly greater percentages of cartilage and precartilage regions in centrifuged limbs.

(2) Premature cell death occurred in the medial epithelial edge of embryonic palatal shelves cultured singly, showing that this result (seen in previous studies of paired shelves) was not the result of increased contact between shelves.

(3) Estrus induction was delayed in about 24% of centrifuged female mice, as determined by vaginal smears.

(4) Interactive image analysis of long bones of alizarin-stained 18-day fetuses showed reduced areas of ossification in fetuses centrifuged during development.

(5) Form factor analysis established that the major effect of in vitro and in utero centrifugation was a shortening of the developing elements within the limb.

Significance of the Accomplishments

(1) Finding #1 is significant because it confirms the validity of the ground-based centrifuge as a predictor of microgravity effects. Differentiation was accelerated by exposure to excess g; results from the Spacelab (SL)-3 and D-1 flights show that microgravity delays differentiation of chondrocytes in epiphyseal growth plates, and of Drosophila.

(2) Fusion of embryonic palatal shelves cannot occur unless cells of the intervening epithelia undergo a programmed cell death. Acceleration of this differentiative step was seen in paired palatal shelves exposed to excess g, but could have been due to increased cell contact. Acceleration of cell death in shelves cultured singly shows that exposure to excess g accelerated differentiation, and that gravity has an effect at the organ level.

(3) In spite of considerable evidence to the contrary, mice are sometimes said to be unaffected by gravitational changes. Results of finding #3 show that even after adaptation (this study was begun after 6 months of centrifugation), differences between centrifuged and control mice exist.

(4) Not only mice, but mouse fetuses as well, are affected by chronic centrifugation. The only other investigation of effects of gravitational changes on prenatal skeletal development was carried out by Soviet scientists, prior to exposure of pregnant rats to microgravity on Cosmos 1129.

(5) The mouse limb system was chosen for these studies because previous teratological studies had shown in vitro results to be comparable to in utero results. We have previously shown that exposure to excess g in vitro or in utero suppresses limb morphogenesis; the shortening of limb elements in both systems is yet another point of identity.

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OTOCONIA CALCIFICATION PROCESS - A CHICK EMBRYO MODEL

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Description of Research

This research is investigating subcellular details of processes leading to calcification of organic matrix of statoconia in the developing chick embryo (Gallus domesticus). Studying the subcellular details of the organic matrix before and after calcification of the statoconia is important because earlier work in mammals and in birds has shown that secretion of an organic matrix precedes the formation and the calcification of the statoconia. In the chick, these processes occur at a very rapid rate.

Consequently, critical periods of development exist during formation of the gravity-sensing system in the developing chick. A microgravity environment may very well affect the formation of statoconia during critical periods of development. Therefore, visualization of the manner in which the initial associations of the organic matrix and calcium, integration of calcium into the matrix, and spatial distributions of calcium component takes place should be carefully investigated in order to establish normative data and values that will be useful for interpreting changes that may occur during exposure to the microgravity environment.

Species with genetically defective otoliths have demonstrated the importance of statoconia for detection of gravity and maintenance of balance. Thus, any effect to these organs will produce significant changes in the ability of the organism to orient to gravity and the environment. For this reason, studying statoconia formation is an important aspect in relation to the solution of Earth and space problems.

Accomplishments

Two main hypotheses underlie ongoing investigations in our laboratory in regard to statoconia formation in the chick.

First, the segregation of cell type, cytodifferentiation of vestibulosensory epithelia, and the synaptogenesis of afferent and efferent nerve fibers onto hair cells follow well-defined gradients, and the events are coordinated in time and space (stage and location).

Second, statoconia exist first as unmineralized primitive statoconia that originate from segmentation of immature otolithic

membrane, and at least part of the process of calcification occurs by the incorporation of calcic granules into the organic matrix.

During the past year, attention was directed to the second hypothesis. It was determined that, in the chick, statoconia emerged as units from a segmenting membrane of fibrillar organic material. The amorphous mass of fibrillar material is secreted progressively by the supporting cells of the saccular and utricular macula, in a fashion that resembles the manner in which the supporting cells of the auditory portion of the ear secrete the tectorial membrane (Figures 1,4).

This new notion of segmenting membrane provides an important step toward understanding the formation of the otolith, since most work conducted in other species has dealt with statoconia that already have characteristic geometric shapes. Consequently, until results similar to the ones obtained here with the chick are also shown for other species, one cannot be certain whether or not segmentation of the membrane is a process characteristic only of the chick embryo. In any event, this observation has allowed us to determine that while calcification of the organic matrix may start very early (e.g., membrane is segmenting), it seems that there is a critical period during which calcium is incorporated at a very fast rate. In the chick, this period falls between 8 and 11 days of incubation (stages 33-37). It is intriguing, though, that if statoconia form by seeding of a nucleus (e.g., central core), the center of each statoconia remains relatively empty in relation to the periphery (Figure 5), indicating that if calcium exists in the core it is not tightly bound to the organic matrix. One may ask then, why is calcium selectively extracted from the area that is supposed to be the seeding nucleus (center core)?

Figure 1 (facing page). The organic matrix over the maculae appears to aggregate and form an immature otolithic membrane through a process that resembles the secretion of the tectorial membrane in the cochlea. Segmentation of the immature, uncalcified, otolithic membrane in the saccule (S) gives origin to statoconia.

Figure 2 (facing page). Higher magnification of area boxed in Figure 1. Fibrous material secreted by the supporting cells of the saccule (S) thickens at its outer edge (arrows) and individual statoconia begin to form. This process continues and statoconia are produced by segmentation of the membrane. The fibrous substance remains in contact with the microvilli of the supporting cells. The saccular macula might serve as a locus for statoconia initiation and might also provide the necessary material for the assembly of utricular statoconia. In the utricle (U), statoconia were not seen attached to the statoconial mass while segmentation is apparent in the saccule (S).

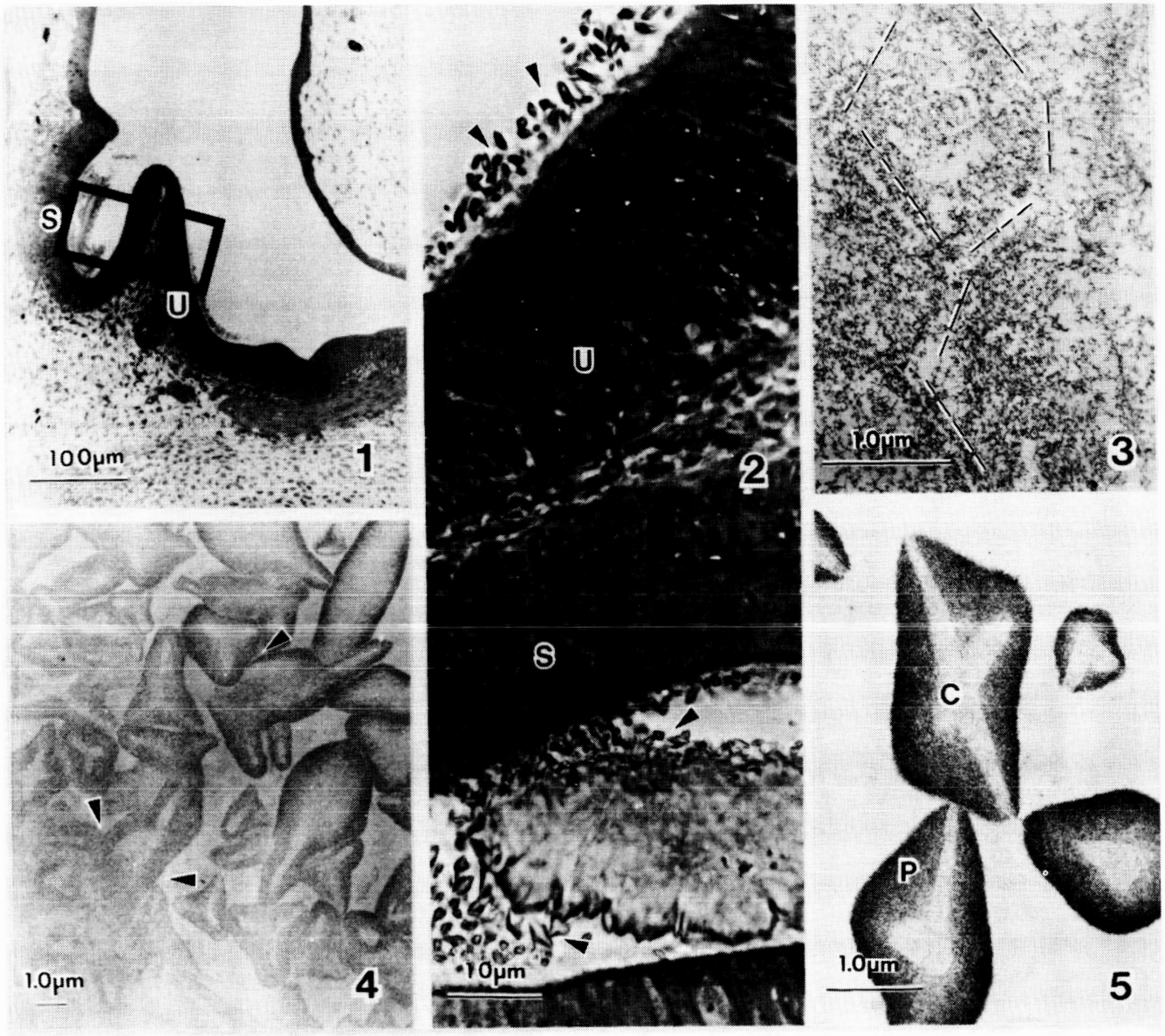

Figure 3. TEM micrograph showing part of the segmentation process (dotted lines). Separation lines appear in the lower part of the membrane and statoconia appear.

Figure 4. The upper portion of the otolithic membrane contains individual statoconia, some seen here still attached to the segmenting part of the membrane (arrows).

Figure 5. By the time statoconia have separated from the statoconial mass, their periphery (P) has thickened, but the central core (C) is relatively empty.

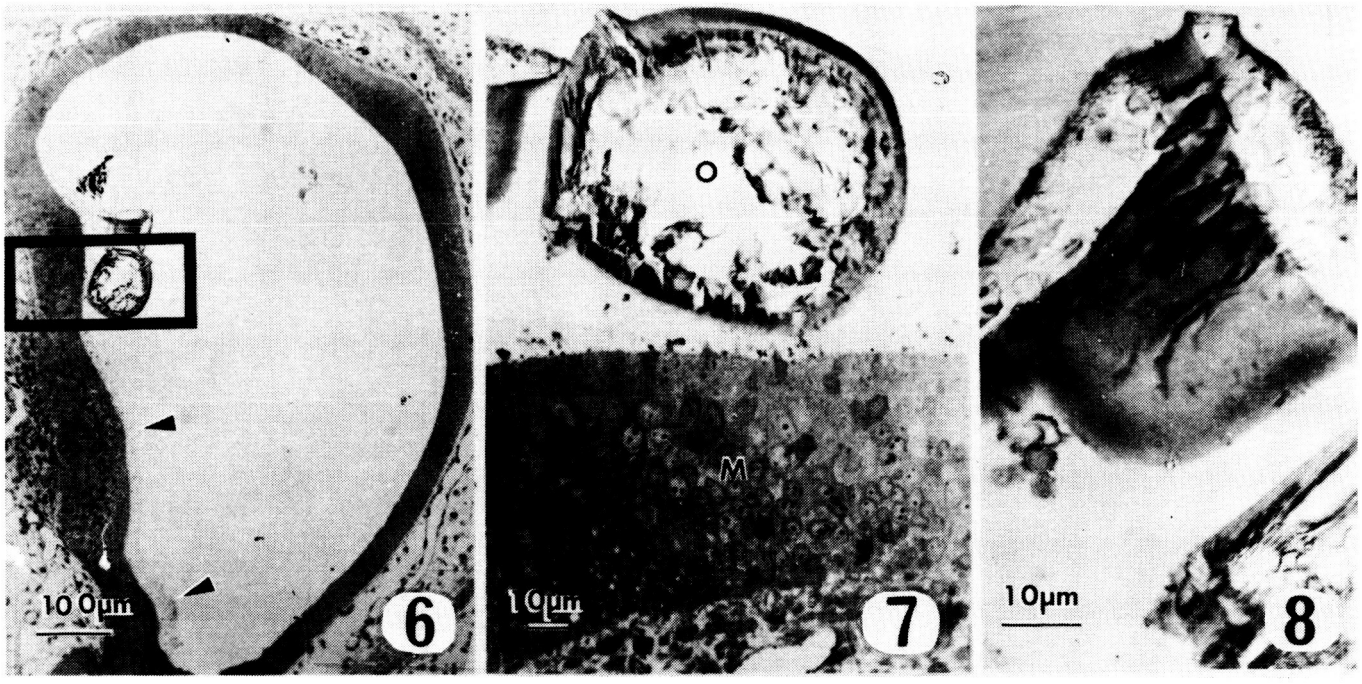

Figure 6. Saccule from a 10 day-old embryo injected on the 3rd day of incubation with the carbonic anhydrase inhibitor Diamox. Normal development of statoconia has been modified, and the organic matrix is scarce (arrows). Available calcium and the organic matrix produced may have fused and form two giant statoconia in this ear. No statoconia were seen in the utricle of this embryo.

Figure 7. High magnification of area outlined in Figure 6. The giant statoconia have numerous small crystals enclosed by a ring of amorphous material.

Figure 8. The giant statoconium formed in this ear lacked the organization and geometric shape that characterizes individual statoconia in normal ears.

We are very confident that the segmenting process is not an artifact of fixation. The process has been arrested in at least four different embryos and the same was observed in the left and the right ear. Between stages 26 and 30, a statoconial mass begins to segment in the saccule of the chick embryo and statoconia are formed. In the utricle of the same embryos, similar masses were not observed to the same degree (Figures 1,2). Thus, there is an abundance of material available for statoconia formation in the saccule that is not matched by that found in the utricle. Similarly, segmentation of the primitive otolithic membrane is more advanced in the saccular macula than the utricular macula. At the same time, there are more statoconia in the saccule than the utricle of the same age. This may indicate that the saccule could serve as an initiating locus for statoconia formation in the inner ear. At first, the organic

matrix is not tightly packed, but as the embryos mature, the fibrils of organic matrix inside statoconia are packed more tightly than the fibrils of organic matrix (substance) holding the statoconia together.

Diamox is a diuretic that is also used in treatment of glaucoma. Diamox inhibits the enzyme carbonic anhydrase. We have taken advantage of Diamox's ability to produce partial inhibition of statoconia formation to examine the fate of calcium following interference of metabolic pathways of the enzyme carbonic anhydrase. In embryos injected with this substance, we observed ultrastructural changes similar to those observed during the initial stages in the formation of the membrane of normal embryos. In other embryos, statoconia were absent and in their place were giant statoconia masses surrounded by a ring of organic matrix substance, inside of which was inorganic calcium not associated with the organic matrix (Figures 6,8). Histochemical staining of this preparation allows the visualization of organic calcium within the epithelia and as it is associated with statoconia. We observed positive stained organic calcium within the apical cytoplasm of the cells of the macula as well as in the endolymphatic space immediately above the macula. We are continuing this investigation to determine the origin and fate of the organic calcium that we can see in granular form (20-150 nm).

Since formation of the statoconia takes place in two separate processes, that is, segregation of the membrane and calcification of the statoconia, one should be able to elucidate the biochemical composition of the organic matrix by performing certain assays on the membrane prior to calcification of the statoconia. We have recently begun doing so and have obtained preliminary results indicating that there are at least five major proteins in the otolithic membrane. We are also beginning to use high pressure liquid chromatography in order to elucidate the nature of these proteins.

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HOMEOSTASIS IN PRIMATES IN HYPERACCELERATION FIELDS

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Description of Research

The ultimate goal of this research program is to understand the role of gravity in influencing the physiology of living organisms. Of particular interest are the physiological mechanisms leading to adaptation of an organism to an altered gravitational environment. Included in these responses are 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 gravitational loading are poorly understood. Such changes in centrifuged animals include depressed body temperature, alterations in the circadian timekeeping system, 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 provided both a demonstration of these systems' responsiveness to gravity as well as efforts to elucidate the underlying mechanisms of the responses. Further, this program is focused on the responses of the whole organism (primates and rodents) to further understand the interaction between the various physiological systems of interest. This research has 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 acute and chronic g fields ranging from 1 to 20 g. The research accomplished in the past year has examined the responses of primates to altered fields (2 g) for up to 10 days exposure duration. Additionally, control studies at 1 g have begun to examine the neural control mechanisms integrating the circadian timekeeping system with those of body temperature and sleep-wake cycles. Finally, an opportunity to examine both rodents and primates in the microgravity environment was utilized with the advent of Spacelab-3 during this reporting period.

Accomplishments

(1) The presence of a hyperdynamic environment depresses primate body temperature for an extended period (more than 48 hours). The magnitude of this response is dependent upon both time of day (body temperature falls more during the animal's day than its night) and the circadian phase at which the acceleration

field is initiated (subsequent fall in body temperature is lower when the acceleration field is initiated during the animal's subjective night).

(2) Exposure to prolonged (7 days) 1.5-g field leads to a reduction in the average amplitude and 24-hour mean of the circadian feeding rhythm, which persists for up to 96 hours prior to the animals' recovery to baseline.

(3) The circadian timekeeping system has a significant influence on body temperature, sleeping, and drinking. The suprachiasmatic nucleus (SCN) of the hypothalamus plays a major role in the generation of circadian timekeeping in the squirrel monkey. However, although the 24-hour distribution of these variables is no longer rhythmic in animals with the SCN removed, the 24-hour level is still at the homeostatic level.

(4) The microgravity of spaceflight has a significant influence on the circadian timekeeping system of rodents. The 24-hour mean heart rate rhythm was depressed in rats in microgravity; however, the rhythm remained synchronized at 24-hours light-dark cycle. In contrast, the body temperature rhythm did not appear to retain the synchronization to the 24-hour light-dark cycle that was seen in the heart rate rhythm.

(5) The functional metabolism of the paraventricular nuclei was shown to be depressed in the rats following 7-day exposure to microgravity. Further, animals that were marginally dehydrated in the flight were noted to have elevated levels of neuronal metabolism.

(6) Squirrel monkeys that flew on Spacelab-3 showed on average the same depression of circadian rhythmicity of feeding behavior and recovery time of feeding behavior as seen in the 1.5-g centrifuged animals cited above (2). This physiological adaptation resulted from exposure to altered tonic levels of the dynamic environment (hyper- and hypodynamic environments produced similar responses).

Significance of the Accomplishments

In general, findings #1, #2, and #4-6 demonstrate the response of mammals (primates and rodents) to altered levels of gravitational loading (increased and decreased). The response of both species underscores the significant delay (i.e., days) in the adaptation of these organisms to changes in the dynamic environment. For example, finding #1 demonstrates that body temperature is depressed for an extended period of time as a result of exposure to a hyperdynamic environment. Further, this response is dependent upon the circadian phase at which the change in the dynamic field is initiated. Similarly, exposure to hypergravitational fields (accomplishment #2) or hypodynamic fields (accomplishment #6) also leads to multiple-day recovery periods for the feeding patterns of these animals.

Accomplishment #4 demonstrates an apparent change of the utilization of photic information by animals in a microgravity environment. That rodent body temperature rhythms may not synchronize to the 24-hour light-dark cycle is a unique finding.

Historically, such observations have only been made in primates exposed to the microgravity environment. That such a change in photic response only occurs in the temperature rhythm further underscores our understanding of the circadian timekeeping system and photic entrainment.

Finding #5 demonstrates the persistence of neural control mechanisms in the hypodynamic environment. That the reduction in neuronal activity occurred in the microgravity environment is consistent with the perception that there is a redistribution of body fluids in the microgravity environment leading to a diuresis as a result of a perceived excess level of hydration. The animals which were marginally dehydrated in the flight also had higher levels of activity, as well as cell size increases, in these control nuclei. These observations would indicate a functioning neural control mechanism for fluid balance in the central nervous system.

Finding #3 provides us with continuing information regarding the underlying mechanisms of the organization of these various physiological systems. That the suprachiasmatic nucleus plays a major role in the regulation of the circadian timekeeping system in primates is crucial to our understanding of gravitational influences. With such information, we hope to begin to define how control systems perceive information regarding changes in gravity and how they modify the regulated levels to new steady states.

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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 goal of this research is delineation of the neural pathways and transmitters that mediate the changes in the secretion of renin and other hormones that affect salt and water metabolism. Special emphasis is placed on changes in the secretion of these hormones produced by gravitational and stressful stimuli. Evidence from this laboratory indicates that stimulation of serotonergic neurons in the dorsal raphe nucleus of the midbrain increases renin secretion, and that these neurons project to the mediobasal hypothalamus (Karteszi, et al., Neuroendocrinology 34: 323-326, 1982). The initial goal of the project was to determine how the message got from the hypothalamus to the renin-secreting cells in the kidneys. We have good evidence that the final common pathway is sympathetic, with stimulation of renin secretion by way of beta-adrenergic receptors in the kidneys. The present goal of the research is to determine by the production of discrete lesions the exact parts of the hypothalamus and brainstem that are involved in regulating renin secretion. For this purpose, our initial stimulus to renin secretion, administration of the serotonin-releasing para-chloroamphetamine (PCA), has been supplemented by the psychological stress of immobilization and the postural stress of tilting to the upright position. In addition, we have begun to study the transmitters involved in the afferent pathways from the hypothalamus to the sympathetic neurons that innervate the kidneys.

Accomplishments

(1) Additional research of the type begun in 1985 has made it clear that increases in plasma renin activity produced by head-up tilt and immobilization, as well as administration of the serotonin-releasing PCA, are reduced or abolished by lesions of the paraventricular nuclei of the hypothalamus and cuts made behind these nuclei. Sham lesions and lesions in several other parts of the hypothalamus do not have this effect. However, only the response to PCA is abolished by lesions of the serotonin-secreting neurons in the dorsal raphe nucleus. Head-up tilt and immobilization responses are unaffected or even potentiated.

(2) We have demonstrated that the increase in plasma renin activity produced by a low sodium diet is also prevented by lesions of the paraventricular nuclei.

(3) However, at least in preliminary experiments, we have

found that lesions of the paraventricular nuclei reduce the plasma concentration of renin substrate. This finding is significant because it indicates that the changes in plasma renin activity produced by paraventricular lesions could be due to deficient substrate secretion rather than decreased renin secretion. Research currently being pursued should permit us to choose between these alternatives. We will measure the total concentration of renin in plasma as well as its activity in response to the stimuli studied before, and we will also measure the concentration of substrate.

(4) Like the plasma renin activity response to PCA, we have found that the plasma renin activity response to immobilization is mediated by a final common pathway that is sympathetic, since the response is prevented by the beta-adrenergic blocking drug propranolol. The response to head-up tilt also seems to be blocked by propranolol, but the data on this point are not yet complete.

(5) We have obtained evidence that oxytocin- and vasopressin-secreting neurons may be part of the pathway from the hypothalamus to the sympathetic outflow that is involved in the renin responses. Brattleboro rats that congenitally lack vasopressin in their brains have exaggerated plasma renin activity response to PCA, immobilization, and head-up tilt. Conversely, in preliminary experiments, it appears that intraventricular injection of an oxytocin antagonist increases plasma renin activity.

(6) We are now measuring blood pressure and heart rate as well as plasma renin activity during head-up tilt. With these measurements, it will be possible to determine whether paraventricular lesions exert a depressive effect on all baroreceptor responses, or whether they exert a selective effect.

(7) In other experiments conducted in collaboration with Dr. Lanny Keil at NASA Ames Research Center we have collected additional data on the long-term endocrine effects of head-down tilt in rats. Plasma renin activity, ACTH, and corticosterone have been measured 1, 2, 3, 5, and 7 days after the start of hindquarter unloading. The procedure does not produce any increase in ACTH or corticosterone at these time intervals, and plasma renin activity is also normal. Additional experiments measuring these variables at times shorter than 1 day and intervals longer than 7 days are underway.

Significance of the Accomplishments

The preliminary finding that lesions of the paraventricular nuclei reduce plasma angiotensinogen is significant because it suggests that the previously observed alterations in plasma renin activity could be due to alterations in the rate of secretion of substrate rather than renin. Plasma renin activity is determined by measuring the amount of angiotensin I generated when plasma is incubated, and this generation is limited by the amount of substrate in the sample. There is considerable evidence that the secretion of substrate from the liver is under endocrine control, and it may be that paraventricular lesions disrupt anterior

pituitary function with a consequent decrease in hormonal stimulation of substrate secretion. As noted, measurement of plasma renin concentration and substrate, as well as plasma renin activity in the plasma samples will permit rapid resolution of the question. In the meantime, research during 1985 has made it clear that the paraventricular nuclei occupy a central position in the regulation of plasma renin activity. Serotonergic neurons from the dorsal raphe nuclei converge on the paraventricular nuclei, but the responses to immobilization and head-up tilt are mediated by impulses that reach the paraventricular nuclei by other pathways.

Our preliminary data to date suggest that oxytocin-secreting neurons in the brain mediate increases in plasma renin activity whereas vasopressin-secreting neurons mediate inhibition. Further experiments are being carried out using specific oxytocin and vasopressin agonists and antagonists. Conceivably, these experiments could lead to practical manipulation of the secretion of renin and hence aldosterone by oxytocin or vasopressin antagonists. The experiments in which we will measure blood pressure and heart rate as well as plasma renin activity with head-up tilt and other stimuli are important because they will settle the question of whether the effects of lesions of the paraventricular nuclei are selective or general.

The results of the study of hormone levels in unloaded rats are significant to date because they demonstrate that at least from the 1st to the 7th day, hindquarter unloading, if performed correctly, is not a stress. This is important in terms of its use as a model of the weightlessness of spaceflight.

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EFFECTS OF HYPOGRAVITY ON SYNAPTOGENESIS IN CELL CULTURE

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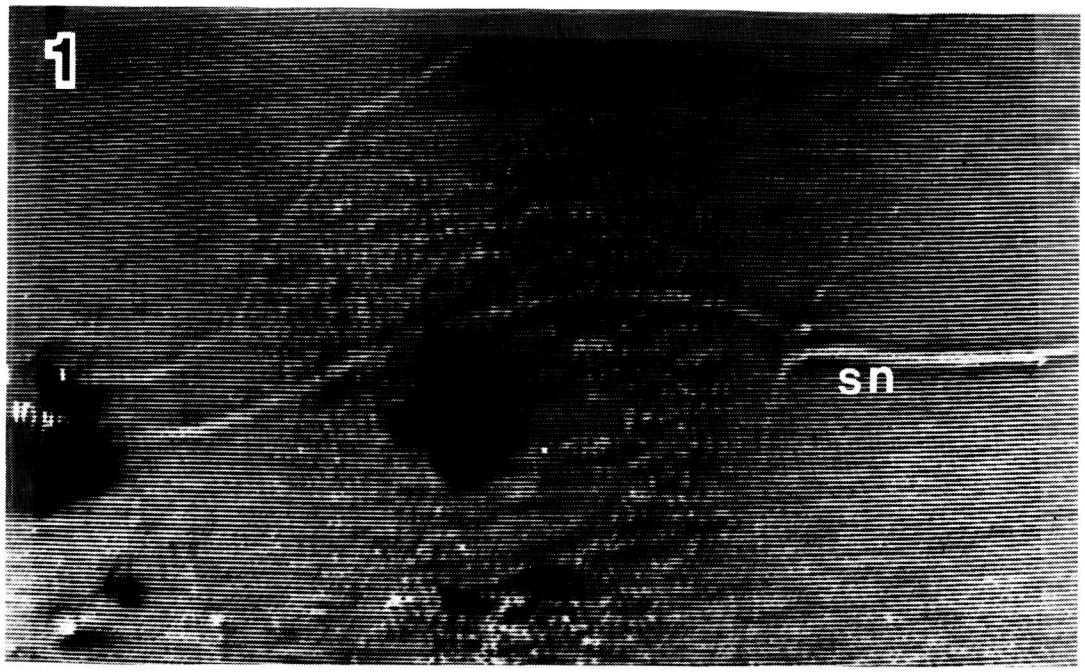
Description of Research

Multigenerational survival in space will depend on the ability of organisms to progress successfully through embryonic development in the relative absence of the heretofore most constant of all environmental factors--gravity. Therefore, to insure the well-being of adult biosystems during prolonged stays in microgravity, and to anticipate possible problems during development of humans and animals under these new conditions, we must understand how cells behave and communicate with each other when growing and developing under microgravity conditions.

It is already known that exposure to microgravity, even during short orbital flights, alters systemic functions in flight crews in at least four systems: the vestibular apparatus, skeletal muscle, bone, and cardiovascular systems. It is therefore reasonable to anticipate that prolonged exposure to microgravity during much longer flights, especially if such exposures were to take place during the early stages of biological development, might result in significant alterations in cell and tissue organization and function. This is especially true for developmental processes, since they are particularly labile to environmental perturbations even under normal conditions.

The primary objective of this research is to formulate, on the basis of experimental simulation, a working hypothesis which would define the site and mechanism of gravity perception by nerve and muscle cells. This formulation will serve to understand how cell development and intercellular communication under conditions of microgravity might be affected. The fundamental premise of this objective is that developing organisms must be able to perceive gravitational forces even as they evolve from a single to a multicellular system. Taken to its logical conclusion, this means that a gravity "sensor" or "receptor" must exist in individual cells. This premise is based on experimental results obtained by exposing nerve and muscle cells, growing in culture, to simulated microgravitational conditions. Since experimentation in actual microgravity is



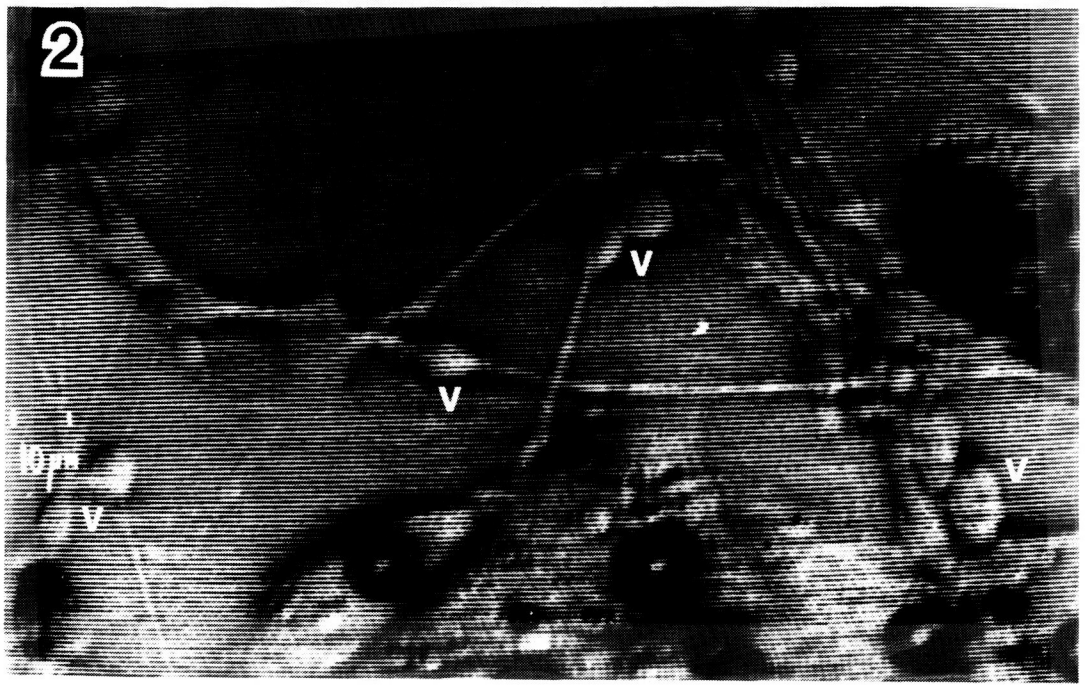


Figure 1. Neuritic projections growing from an explant of a nerve cord grown under control, stationary conditions. Note the absence of varicosities and the smooth neurite (sn) profiles.

Figure 2. Neuritic projections growing from a nerve cord explant grown under constant clinorotation of 10 rpm for 36 hrs. Very large and frequent varicosities (v) are present along the shafts of these neurites. These indicate possible damming (accumulation) of axoplasmic material due to problems with cytoskeletal structures within the neurites.

presently limited by the duration and availability of spaceflights, this question has not yet been fully addressed.

The simulation of microgravitational conditions is accomplished by placing culture dishes containing embryonic nerve and muscle cells on a horizontal clinostat rotating at speeds of 1-50 rpm for periods of up to 72 hours. During this time, the muscle and nerve cells (isolated from embryos of Xenopus laevis, the South African toad) normally mature to express typical phenotypic expression of nerve and muscle. These expressions include: formation of contractile apparatus, production of neuritic extension, and contact formation between nerve and muscle (expressed in the formation of the neuromuscular junction) complete with transmitter release, accumulation of cholinergic receptors, and trophic interactions between the two cell types. Such interactions constitute the normal development of a typical synapse, which is the essential communication gateway among nerve cells in the brain. Any deviation from this normal pattern under a clinorotation regime indicates the likelihood that nervous system function of, at least developing, systems might be seriously compromised when exposed to microgravity for extended periods of time.

To assess changes induced by clinorotation in cultured nerve and muscle cells, the following measurements were made: a) micromorphologic observations of cell perimeter, area, and shape; b) size of nucleus and nucleolus; c) distribution patterns of acetylcholine receptors in innervated and noninnervated muscle cells; and d) morphology, number, length, and diameter of neurites produced by nerve cells. These extensive observations permit the formulation of hypothetical mechanisms underlying the observed changes. For example, changes in nuclear parameters may indicate altered genetic capabilities; altered receptor distribution in innervated muscle cells may indicate a breakdown of neurotrophic controls; and changes in neurite morphology may reflect abnormal synthesis of cytoskeletal elements responsible for the production and shape of these essential neuronal extensions.

Accomplishments

(1) Clinorotated nerve and muscle cells showed typical phenotypic expressions: muscle cells produced striations (the expression of contractile proteins) and nerve cells produced neurites which grew and made contact with muscle.

However:

(2) Muscle cell striations appeared later in clinorotated cultures, and yolk platelets (the energy supply of the cell) disappeared more slowly.

(3) Myocyte nuclei were enlarged (by as much as 200%), as were nucleoli (Figures 3-6). The number of nucleoli often exceeded the normal complement of two per cell.

(4) The number and distribution of acetylcholine receptors on the surface of muscle cells was reduced in number and

organization.

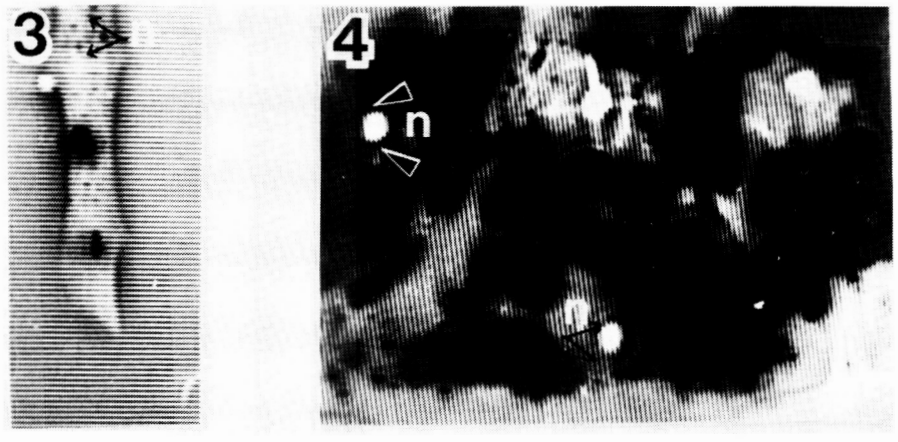
(5) The number and length of neurites produced by nerve cell bodies was reduced. A large number of prominent varicosities (enlargements) appeared along the shaft of the neurites (Figures 1,2).

(6) The number of neurites produced in the presence of trophic agents (embryo extract, laminin) was reduced by as much as 70% in comparison with stationary controls.

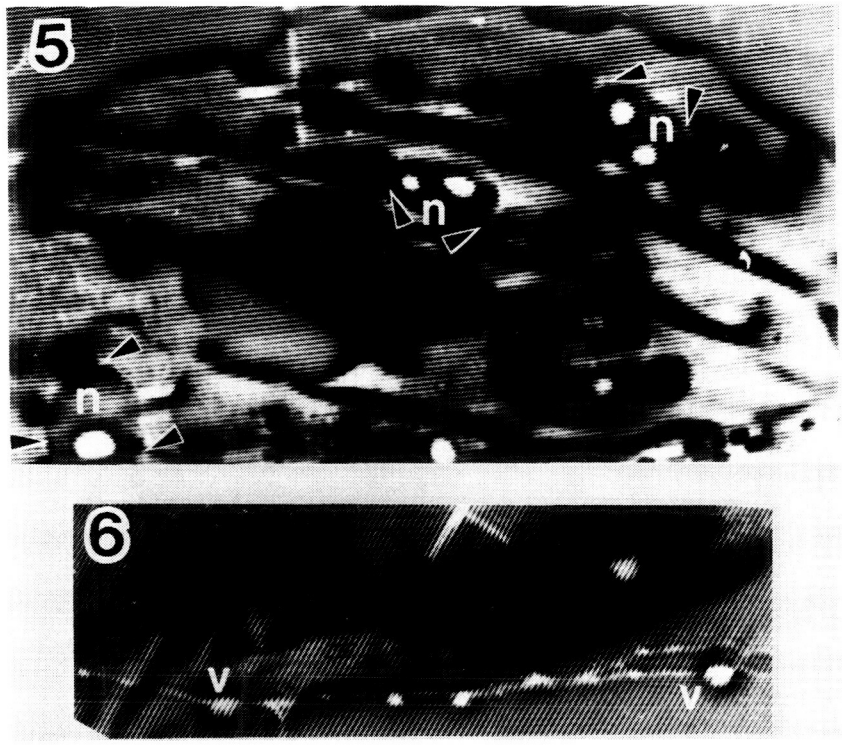
Significance of the Accomplishments

The profound morphologic changes discovered subsequent to clinorotation indicate that the embryonic nerve and muscle cells can survive simulated microgravity, but that their development is altered in significant ways. Taken together, the findings suggest that cell maturation is delayed after slow clinorotation. Thus, the late appearance of striations, the retarded consumption of yolk platelets, and the production of fewer and thinner neurites indicate subnormal expression of cell characteristics. In addition, these cells do not respond normally to environmental cues such as trophic substances or surface contact.

These findings suggest that normal embryonic development may be severely affected if it were to occur in space. Therefore, ways must be found to (a) understand the mechanisms responsible for these microgravity-induced changes, and (b) minimize these effects in consideration of normal progeny development during long-term colonization of space. As a first step, a working hypothesis has been formulated, on the basis of the findings listed above, to further test the effects of simulated microgravity on cell development and communication.



Figures 3 & 4. Examples of normal, stationary muscle cells after 36 hrs in culture. Note the relatively small size (compare with Figures 5,6) of the nuclei (n) and also the relatively small size of the muscle cell (Figure 3). Arrows delineate borders of nuclei.



Figures 5 & 6. Examples of muscle cells rotated for 36 hrs at 1 and 10 rpm, respectively. Note the especially prominent nuclei and their nucleoli (n). Arrows delineate borders of nuclei. Note also the varicosities (v) in the single neurite in Figure 6 (cf. the explant in Figure 2).

Working Hypothesis

All eukaryotic cells, except plant cells and some unicellular organisms, contain a subcellular organelle which has been linked to two major functions--cell division and the enucleation of the cytoskeleton of the cell. This organelle, the centriole, is adjacent to the nucleus and appears to be attached to it. The centriole also communicates morphologically with the cell surface via the cytoskeleton. Although the molecular structure of the centriole is not yet fully understood, it appears to consist of two orthogonal protein cylinders, one of which is parallel to the large surface of cells as they grow in culture (Bornens et al., 1977). On the basis of electron microscopic evidence, Bornens suggested that the centriolar cylinders rotate slowly, about 1 Hz. He further proposed that the centriole acts as the gyroscope of the cell, so as to provide the cell with a kinetic reference point, thus controlling cytokinesis, cell shape, and size. (These roles would be of particular importance in nerve and muscle cells where cell shape and motility are essential expressions of their function). He also suggested that the centriole would ensure coherence in cell metabolism and that this regulation might be coordinated through the cytoskeleton.

Bornens (Biol. Cell. 35: 115-132, 1979) concluded that "gravity influences the equilibrium position of the centriole." Clearly, due to the dynamic nature of the centriole, alterations in the intensity and directionality of gravity may be expected to directly alter cell function.

This hypothesis must, of course, be tested directly. The evidence presented here and in other studies, while consistent with the hypothesis, is still circumstantial. Certain findings, however, bear specific mention as they appear to be crucial. First, the alterations in nuclear and nucleolar size reported here, can be predicted to occur if the integrity of the centriole has been affected by clinorotation. Second, the relative disorganization of membrane-bound receptors is similarly expected if, secondary to alterations in the centriole, the structure of the cytoskeleton is also compromised. This is so because many membrane-bound proteins, including the acetylcholine receptor, have been shown to be stabilized somehow by the cytoskeleton (Bloch, J. Neurosci. 3: 2670-2683, 1983). Third, results reported in this study from clinorotated cells should be consistent with data reported from direct interference with the integrity of cytoskeletal elements. Indeed, it is of interest to note that Letourneau and Ressler (J. Cell Biol. 98: 1355-1362, 1984) showed that exposure to taxol, which promotes microtubular polymerization, produces microtubular tangling and looping which appear as large varicosities at neuritic terminals. The neuritic varicosities described here (Figures 2,6) may have a similar origin. Similarly, Domnina et al. (J. Cell. Sci. 74: 267-282, 1985) reported that epithelial cells exposed to cytochalasin and colcemid, agents which destroy microtubules, produce thin cytoplasmic extensions which appear to contain prominent varicosities.

In summary, I have shown that clinorotated cells display morphologic alterations that are consistent with the hypothesis that altered gravitational forces may act via the cell's centriole. The observations reported here bear strong, albeit circumstantial, resemblance to those reported to occur subsequent to interfering with centriolar and cytoskeletal integrity. Such a resemblance may be ascertained by direct and pharmacologically independent testing of the proposed hypothesis.

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THE EFFECTS OF HYPERGRAVIC FIELDS ON NEURAL SIGNALING IN THERMOREGULATORY AND VESTIBULAR SYSTEMS OF THE RAT

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Description of Research

Studies have continued on the neural processing of sensory information of rats in hypergravic fields. The specific neural systems considered were mammalian temperature regulation and vestibular function. Previous studies in a variety of laboratories have shown that the rat, dog, and monkey have an impaired ability to regulate their body temperature when exposed to hypergravic fields. Thus, one set of experiments using the rat was directed toward clarifying mechanisms underlying this impairment. A second set of experiments was directed toward describing the response of the vestibular system to angular accelerations as the temperature of the central nervous system was controlled. The rat was chosen as an experimental animal in both sets of experiments because there are studies at Earth gravity, 1 g, that provide basic background for further studies both at zero-g and in hypergravic fields from 1.5 to 4 g.

A new series of studies recently started is based on the striking findings from Spacelab-3 that there is an elevation of serotonin binding in the hippocampus of rats exposed to microgravity for 7 days. Thus, at the cellular level, a neural change associated with microgravity has been identified, and this observation allows one to focus on a particular neurotransmitter in a particular region of the brain.

Accomplishments

(1) Rats acclimated to a gravitational field of 2.1 g are able to regulate their core temperature better when cold stressed at 2.1 g than are rats acclimated at Earth gravity (1 g).

(2) Rats acclimated to 2.1 g showed an increase in tail temperature (T_t) and a fall in core temperature during exposure to 5.8 g. Thus, rats acclimated to 2.1 g were not able to regulate their temperature when exposed to higher acceleration fields.

(3) Acclimation did not result in a change in thermoregulatory ability at 1 g.

(4) The interpeak latencies of brainstem responses evoked by vestibular stimuli were increased as brain temperature was decreased.

Significance of the Accomplishments

Finding #1. Groups of rats were acclimated to gravitational fields of 1 or 2.1 g. That is, one group was born and raised at 2.1 g and belonged to the 12th generation of rats living continuously on a centrifuge in a 2.1-g field, except for brief periods of routine care at 1 g. The finding that rats acclimated at 2.1 g could thermoregulate better when cold stressed at 2.1 g is important because it shows that acclimation to a hypergravic environment can modify the activity of a neural control system in mammals.

Finding #2. The significance of the observation that rats acclimated to 2.1 g were not able to regulate their core temperature when first exposed to 5.8 g is that acclimation at one level of a hypergravic field does not improve the ability of the animal to thermoregulate at higher field levels.

Finding #3. This experiment completes the series of experiments described above by testing the ability of a rat acclimated to 2.1 g to regulate core temperature over a range of 1 to 5.8 g. In addition, experiments were conducted on the mechanisms underlying control of temperature regulation in rats acclimated to 2.1 g. (We would like to thank Dr. Jiro Oyama at NASA Ames Research Center who provided the acclimated animals).

Finding #4. The interpeak latency of responses was precisely measured and shown to increase as brain temperature decreased in a continuation of an earlier experiment (Physiologist 27: S87-S88, 1984). The significance of these experiments is that they describe in more detail the response of the brainstem responses of the vestibular system in rats. The noninvasive experiments provide the basis for experiments which could be performed in a microgravity environment.

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MICROGRAVITY-INDUCED EFFECTS ON PITUITARY GROWTH HORMONE CELL FUNCTION

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Description of Research

The long-range goal of the research is to determine the extent to which mammalian pituitary growth hormone (GH) cell function may be affected in spaceflight.

Specific major objectives are:

(1) Analysis of GH cell function in pituitary glands of four large (400 gm) and six small (200 gm) male rats flown on the Spacelab-3 (SL-3) mission in April-May 1985.

(2) Begin ground-based research ultimately leading to a pituitary cell culture experiment in microgravity.

Mammalian GH regulates a number of key biological processes. For example, GH (a) increases skeletal growth, (b) increases protein synthesis, (c) decreases carbohydrate uptake into cells, (d) increases breakdown of fat, and (e) is a mitogen. Since GH probably controls these metabolic activities in muscle and bone, tissues known to be affected by spaceflight, it is important to determine if the mechanisms governing synthesis and secretion of GH are modified in microgravity. The problem is complicated by the fact that a number of GH variant forms are known to exist in the pituitary gland. The biological activities of these variants also differ. Preliminary evidence from our laboratory suggests that intracellular processing of GH variants may be unique in different subpopulations of GH cells. Finally, it is likely that the nature of the GH molecules being released from different GH cells is under physiological control (e.g., brain peptides/amines). The overall thrust of our research is therefore to: (a) examine mechanisms of intracellular processing and secretion of GH variants, (b) improve our ability to detect biologically active GH forms secreted from GH cells in culture, and (c) identify molecules coming from the brain which may regulate these activities.

The experiments that were done in 1985 to meet the two major objectives can be conveniently described by the following methodological approaches:

(1) For glands from SL-3 rats:
  (a) Determination of GH cell ultrastructure.
  (b) Determination of GH cell number.
  (c) Determination of GH content/GH cell.
  (d) Determination of GH release from cells in culture.
  (e) Determination of GH release from cells reimplanted

into rats on Earth.

(f) Determination of immunoactive GH variants secreted in vitro.

(g) Determination of bioactive GH variants secreted in vitro.

(2) For ground-based cell culture experiments preparatory to a flight experiment: establishment and validation of a glass vial pituitary cell culture system for flight.

Accomplishments

(1) Spacelab-3

(a) GH cell ultrastructure was not affected by flight.

(b) The percentage of GH cells in glands of the large flight animals was 44%, whereas controls contained 37%. This difference is considered significant since a laser flow cytometric procedure, which permitted objective counting of 200,000 cells/group, was used.

(c) The percentage of GH cells in glands of small flight animals was not different from controls.

(d) Flight cells contained more intracellular hormone than controls.

(e) Flight cells released less GH into culture medium than controls. Differences were most dramatic in cells from the small rats.

(f) The ability of GH cells from small flight animals to synthesize GH in vitro was significantly less than controls. This was also true in cells from large animals, although the differences were less marked.

(g) Immunoblotting techniques indicated that the different GH forms secreted into the culture medium were not modified as a result of flight.

(h) Fractionation of culture media by high pressure liquid chromatography indicated that flight cells did not release a high molecular weight GH (>60 kD) that is rich in bioactivity.

(i) After reimplantation into hypophysectomized rats, flight cells from both large and small rats released only ~50% as much GH into the host as cells from corresponding controls.

(2) Establishment and validation of a glass vial pituitary cell culture system for flight.

(a) The flight hardware, which has been made, is designed to accommodate 164 4 ml glass culture vials in a zero box which can be placed in a 37°C incubator on the Shuttle middeck.

(b) Several culture media have been tested for use in maintaining rat pituitary cells in a secretory mode for 9 days. To date, the optimal medium formulation is αMEM + 5% calf serum + 0.2% $NaHCO_3$ + 25 mM HEPES + antibiotics, pH 7.4 at 37°C.

(c) The amount of immunoactive GH released from cells in vials over 9 days is proportional to the number of cells put into the vial.

(d) The amount of immunoactive GH released from cells in sealed 4 ml glass vials is virtually identical to the amount released from cells maintained on plastic surfaces, which are

used in more conventional culture systems. This establishes the validity of the glass vial culture system for spaceflight.

(e) The total amount of immunoactive GH synthesized by 200,000 cells in closed vials over 9 days ranges from 10 to 30 μg.

(f) Viable, ultrastructurally intact GH-containing cells can be recovered from the vial after 9 days.

(g) The 3T3 mouse fibroblast GH bioassay, which is sensitive to 10 ng, has been established in this laboratory.

(h) Bioactive GH, released into the medium, can be detected by the 3T3 assay.

Significance of the Accomplishments

The results from our analyses of pituitary GH cell function in rats flown in microgravity indicated that several important changes occurred relative to cells prepared from animals kept on the ground. In sum, we believe that the changes reflect the existence of a zero-g "secretory lesion" in at least some of the GH cells. It is emphasized that the "shutdown" is not a total one; that is, some GH was released from cells of flight animals. Little is known regarding the mechanism of this effect. It may reflect adaptation to a new environment. (The fact that the flight cells were not as effective in reinitiating animal growth on Earth is consistent with the hypothesis that the flight cells may not be as responsive to stimulatory agents from the brain). In adapting to the new environment, is it possible that microgravity signals are "sensed" by tissues (such as muscle, bone, brain) which, in turn, "inform" the pituitary of the need for an adaptive change? Or, on the other hand, is it possible that microgravity has direct effects (i.e., is the lack of gravity sensed directly) on the pituitary GH cell itself? The results of a preliminary cell culture experiment done on the STS-9 mission suggested that this latter possibility might indeed be correct. In this respect, our findings relating to the development of a valid cell culture system designed to accommodate large numbers of samples (to ensure statistical validity) are crucial.

Finally, while the cellular/subcellular mechanism(s) responsible for microgravity-directed modifications of GH cell function remain obscure, the observation that GH cells prepared from flight rats were unable to release a high molecular weight form offers the first solid "clue." Future work can be expected to shed light on this interesting response.

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STUDIES OF PRENATAL AND POSTNATAL DEVELOPMENT AND FUNCTIONAL DIFFERENTIATION OF THE VESTIBULAR SYSTEM IN NORMAL AND CENTRIFUGED WISTAR RATS

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Description of Research

The long-range goal of this research is to determine the effects of altered gravity loading upon the functional development of mammalian central vestibular and proprioceptive systems. Specifically, it is focused upon understanding the mechanisms that underlie the response plasticity that must be present within the developing mammalian central nervous system to facilitate adaptational responses of the system to a variety of novel environmental experiences presented during the perinatal [Embryonic (E)18 to postnatal (PN)21] period. The development of the vestibular and proprioceptive systems offers a series of unique "windows of opportunity" to study such responses to altered functional loading by varying gravity exposure during selected developmental intervals.

Developmental adaptations in the central nervous system in response to unique environmental exposures may result from (a) alterations in the number of neurons being generated in the impacted system, or (b) variations in the establishment of synaptic fields (system integration patterns) or pathways. Accumulating evidence suggests that while both mechanisms appear to be applicable, the choice of the response mechanism maximally expressed is determined by both the degree of environmental variation to be accommodated and the sensitivity of the affected system at the time of encountering the altered environment.

Two possible mechanisms exist to facilitate organismic responses to altered functional gravity loading by varying the numbers of neurons generated during the development of vestibular and proprioceptive systems:

(1) modification in the neuronal generative period by:
    (a) recruitment of additional generative sites;
    (b) extending the generative interval of sites with primary responsibility for the impacted system; or
(2) modulation of normal neuronal cell death patterns in either peripheral or central elements of the affected system.

During prior years of this project, we developed and analysed timed embryonic, fetal, and early postnatal specimen series, utilizing tritiated thymidine autoradiographic techniques, to fully document the normal generative sites and birth time intervals of primary neurons comprising the vestibular periphery

and central nuclei, as well as the brain stem nuclei bearing primary responsibility for processing general somatic proprioceptive input. These studies, conducted in standard laboratory housing at Earth-normal gravity, have established that all neurons comprising the vestibular nuclei arise during a single contiguous time interval from ependymal sites that are specific for each nucleus. Variation in the time of cell origination for any single nucleus across a number of samples is less than 24 hours, reflecting the degree of precision contained within this aspect of the developmental process.

The second potential manner of altering the number of responsive neurons in a specific system, thereby impacting the sensitivity of that specific modality, would be to modulate the extent of normal neuronal cell death in that system. Figure 1 presents a theoretical example of the potential impact of altered functional loading upon neuronal populations responsive to altered gravity levels during development. This concept of functional loading suggests that each neuronal system is capable of responding to its particular modality during development by altering the number of responsive neurons continuing through the postnatal maturation period into adult life. The central element of Figure 1 demonstrates the case in which, at Earth-normal gravity, an appropriate amount of nerve cell death occurs, resulting in the establishment of the normal (100%) complement of responsive neurons.

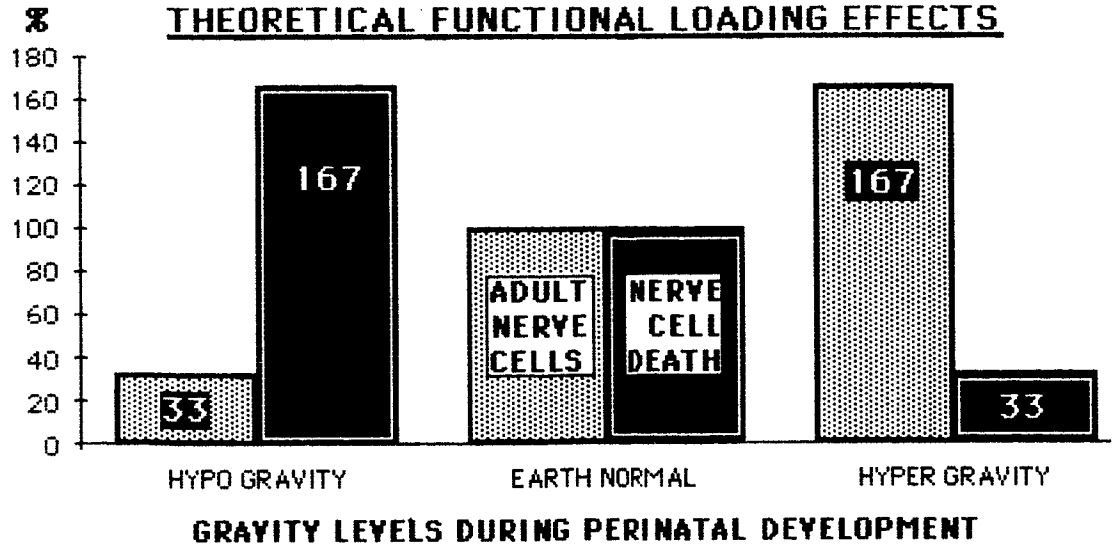

Figure 1. Representation of theoretical effect of altered functional loading on neural populations sensitive to altered gravity during development.

If the functional loading effects of an altered gravity level are expressed by altering the number of mature neurons through changes in the normal pattern of neuronal cell death, then: (a) in hypergravity exposures, the number of responsive neurons would be increased, with an accompanying reduction in cell deaths, and (b) in hypogravity exposures, the number of responsive neurons would be decreased with an elevation of neuronal cell deaths.

Of particular concern is the potential for marked reduction of responsive neurons during exposure to null-gravity throughout the perinatal period, with the resulting organism incapable of subsequent proper responses to Earth-normal gravity encounters.

During the past two years we have striven to establish the timing and extent of normal neuronal cell death in the mammalian vestibular and proprioceptive systems. We have shown that such neuronal cell death occurs in two discrete time periods during development at Earth-normal gravity: (a) Immediately following the initial cytodifferentiation of the neuroblast, at the time of coupling with the "guidance factor." This cell death period is very site specific and lasts for approximately 48 hours (begins ~48 hours after initial cell births and extends ~48 hours after time of last cell birth). The number of neuronal cell deaths during this period appears to represent 20 to 30% of the neurons being produced in the specific region. (b) During the postnatal maturation period, when large numbers of neurons, approximating 50% of the population of each nucleus, undergo cell death. This period begins around PN7 and extends into the third week of postnatal life (duration appears to be dependent upon the specific nucleus).

During the past year we have initiated a series of studies to test the functional loading hypothesis as it applies to vestibular and proprioceptive systems exposed to altered gravity levels during various stages of development in Wistar rats.

Accomplishments

(1) We concluded morphometric and timing analyses of thymidine-injected, staged (E11 thru PN7) normal Wistar SPF rats. The emphasis in this series has been upon the interval E10 through E18, the period of maximum generation for vestibular nuclei neurons. We have extended the thymidine-timing studies throughout the full perinatal (E18 - PN21) period in Wistar SPF rats.

(2) Analyses of cell death timing in vestibular and cochlear nuclei in available materials derived from normal gravity, Wistar SPF specimens were continued. Two cell death intervals were recognized: shortly after initial migration of neuroblasts from generative zones (therefore very time specific), and around PN7. The prenatal period of cell death is primarily associated with the initial differentiation coupling of the primitive neuroblast with the guiding radial glial fibers. The

additional specimens required to fully define the extent and duration of the second phase of normal neuronal cell death during the early postnatal period have been technically processed and are in computer-aided analysis.

(3) We concluded a comparative study of cell death parameters between normal gravity specimens and samples exposed to null-gravity during E13-E18 on Cosmos 1514. Large variability in Cosmos specimens and apparent slowing of development during spaceflight produced numerical differences (not statistically significant) in initial cell death period (E18). However, no apparent differences were observed in the cell death counts, which are normally at a low level during the PNO time frame, following five days of maternal readaptation to 1 g.

(4) We have continued three-stage testing of functional gravity loading during development, utilizing continous exposure to 1.71 g and 2.16 g (hypergravity) from conception through E16d (Group III), PNO (Group II), and PN28 (Group I). All dams have received a single IP injection of tritiated thymidine on a schedule such that two dams from each group have been injected on days E14, E16, and E18. The pups are analyzed at two time periods: on PN0, litter-reduction culls are utilized; all remaining pups are analyzed on PN28. All of Group III is being transferred from hypergravity to 1 g at E16, half of Group II litters are being cross-fostered to 1 g mothers at 1 g, while all of Group I is being retained in hypergravity to analysis on PN28. All groups are being sampled at birth and at PN28 for shifts in timing of neuron birth intervals and comparative cell death parameters. Analysis is in progress on the first test sample of animals exposed to hypergravity through day E16.

(5) Continuation of exposure of three critical developmental stages to 2.16 g. This portion of the study is designed to demonstrate the feasibility of flight experiments and to establish the ground-based data required for these experiments. Two sets of specimens from Group I, representing days E9, PN0 and PN28, have been processed and are awaiting analysis time. All dams receive a single IP injection of tritiated thymidine on exposure days E9, E14, and E20, respectively. Three dams of each group will be analyzed 3 hours post-injection, while the remaining three dams from each group have been returned to and maintained at Earth-normal gravity through birth. Cross-fostering of offspring will be arranged and uteri of the experimental dams will be analyzed.

Our research, focusing upon neuronal generation, has basic applications for both mammalian development and postnatal maturation during null-gravity spaceflight, as well as for increasing our understanding of fundamental developmental neurobiological mechanisms.

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GRAVITY PERTURBATION AS A PROBE FOR ANALYZING PATTERN SPECIFICATION IN EARLY AMPHIBIAN EMBRYOGENESIS AND HIGH RESOLUTION ANALYSIS OF GRAVITY ORIENTATION OF AMPHIBIAN EGG CYTOPLASM

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Description of Research

The fertile amphibian egg provides an exceptional model system to study the effects of gravity perturbation (microgravity of spaceflight to hypergravity by centrifugation) on early embryonic pattern formation. After fertilization, the activated amphibian egg orients with respect to the gravity vector. Between fertilization and the first cell division, the egg undergoes many changes at the cell surface as well as internal cytoplasmic rearrangements which are involved in the bilateral symmetrization necessary to establish a single normal primary embryonic axis. The bilateral symmetrization of activated amphibian eggs is sensitive to manipulations with respect to the gravity vector such as rotation and inversion. Therefore, by using gravity orientation (egg tilt, rotation, and inversion) and gravity perturbation (microgravity--spaceflight, microgravity simulation--horizontal clinostat rotation, and hypergravity--centrifugation), insight into fundamental embryonic events such as cytoplasmic organization, bilateral symmetrization, and morphogenetic pattern specification can be gained.

Gravity orientation experiments have shown that the cytoplasm is organized into discrete compartments based on an unequal and discontinuous distribution of the major organelle in amphibian eggs--yolk platelets. In this study, we analyze the organization and the function of the egg cytoplasm using nonyolk markers for cytoplasmic organization under normal and inverted egg conditions.

Accomplishments

(1) Monoclonal antibodies were prepared against a subset of nonyolk egg cytoplasmic proteins.

(2) The nonyolk cytoplasmic components (proteins) are localized within the fertile amphibian egg into a new "compartment" separate from the major yolk platelet compartments (Figure 1).

(3) In inverted eggs the nonyolk compartment moves with respect to the gravity vector as do some yolk platelet compartments.

(4) The nonyolk compartment is established independently from the yolk platelets during the growth and organization (oogenesis) of the amphibian.

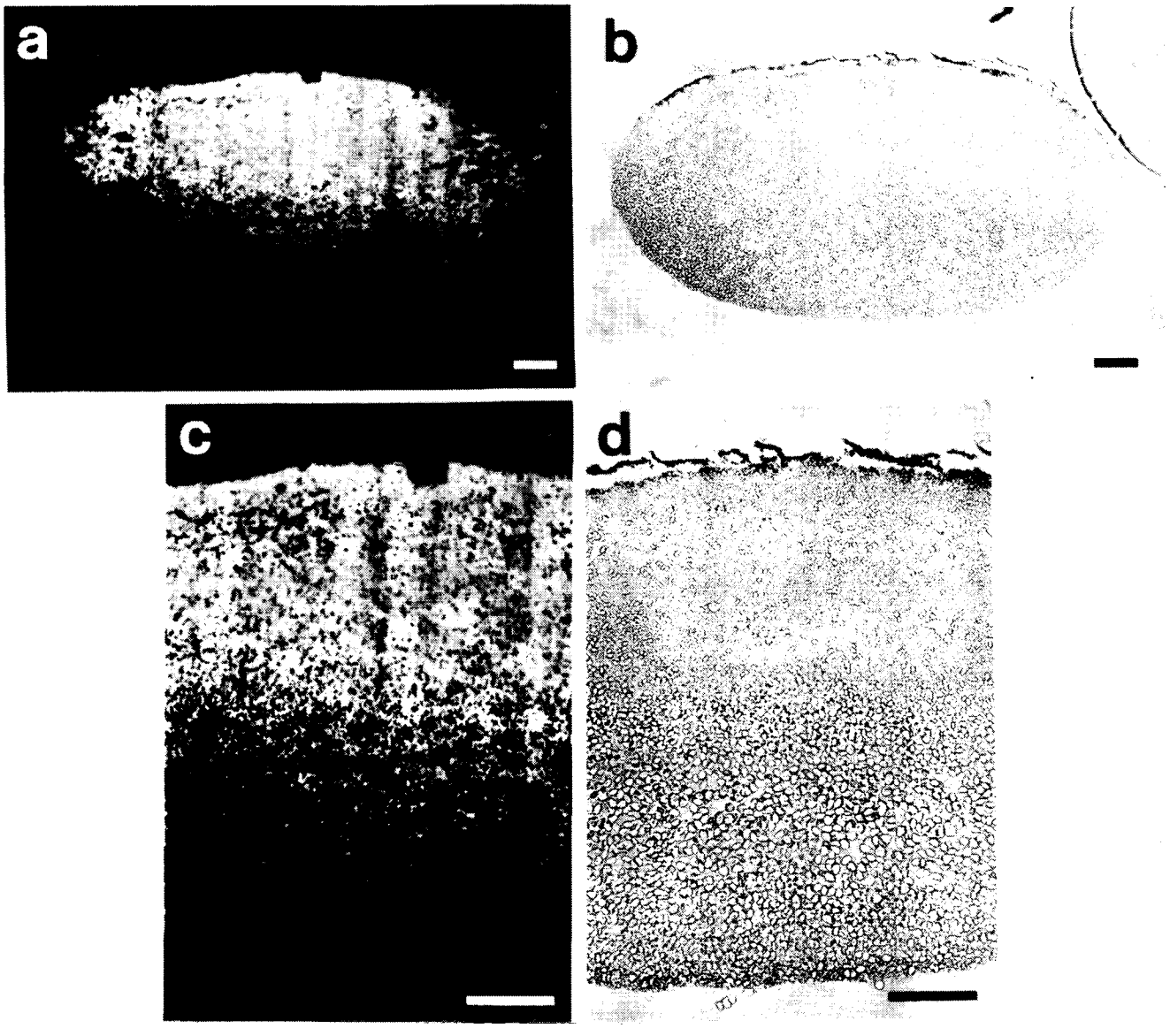

Figure 1. Indirect immunofluorescence labelling pattern of early first cleavage eggs by MoAb H8a. (a) and (c) are fluorescence micrographs and (b) and (d) are corresponding phase contrast micrographs. Note that the antigen recognized by the H8a MoAb, although enriched in the animal hemisphere, is not restricted to the cytoplasm containing primarily small yolk platelets. The central region of the egg containing intermediate and large yolk platelets also is enriched in H8a antigen. However, the more vegetal cytoplasm containing yolk platelets and vegetal subcortical region shows reduced antigen. A similar compartmentalization of antigen was also recognized by MoAb C7e. Bars = 100μ M.

(5) A new method was used to visualize a localized region of the amphibian egg cytoplasm: the germ plasm.

Significance of the Accomplishments

(1) The achievement of preparing monoclonal antibodies, accomplishment #1, provides nonyolk markers for amphibian egg organization at the molecular level. The method used to develop these monoclonal antibodies can be used to produce other markers to further dissect the compartmentalization of the amphibian egg cytoplasm.

(2) Finding #2 of localization of the nonyolk cytoplasmic components provides evidence for the compartmentalization of the amphibian egg at the nonyolk level. The nonyolk compartment is not congruent with any previously described yolk compartment. This observation provides new insight into cytoplasmic organization. This will provide a way to understand the function of the amphibian egg cytoplasm in bilateral symmetrization at a finer level than was previously possible by studying yolk platelets alone.

(3) The observation that the nonyolk compartment moves with respect to the growth vector shows that the nonyolk compartment is gravity sensitive, and supports the idea that cytoplasmic compartments move as units (yolk and nonyolk components).

(4) Finding #4 that the nonyolk compartment is established independently of the yolk platelets shows that the compartment has a unique origin and therefore is not an indirect consequence of yolk deposition.

(5) The development of the method mentioned in finding #5 provides for a new way to track the hypothesized germ cell determinant--germ plasm--in amphibian embryogenesis. Germ plasm appears as a localized "compartment" in the first cleavage amphibian egg and in contrast to most other compartments of amphibian egg cytoplasm is not significantly gravity sensitive in inverted embryos.

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STRUCTURAL DEVELOPMENT AND GRAVITY

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Description of Research

The ultimate goals of this research are to learn what turns bone cells on and off and if/how they communicate with each other and with their environment, and to understand how alterations in structural matrix might induce alterations in mineral crystal size or composition. To accomplish these goals, both ground-based and flight experiments are essential.

Gravity is a major factor determining the amount of structural support required by Earth-bound organisms. The hypothesis of this research effort is that skeletal support structures will change during spaceflight and that the degree of change will be dependent upon the growth rate of the bone and the length of exposure to flight; changes in both quality and quantity of bone will occur. Most ground-based research is done in rats exposed to simulated spaceflight; two flight experiments have been approved and will allow gathering of more information to support or negate the hypothesis.

Accomplishments

The major findings during 1985 were:

(1) Bone labels given to flight rats on Spacelab (SL)-3 did not interfere with other experiments on this flight, suggesting that these compounds did not adversely affect rats prior to or following launch.

(2) A significant suppression of periosteal bone formation rate at the tibiofibular junction was noted in the large flight animals on SL-3, suggesting that depressed bone growth occurs in a mission as short as 7 days.

(3) Rats with rear limbs unloaded using a back harness showed chronic (2-week) suppression of many parameters in unloaded bones and a suppressed gain in body mass compared with controls, whereas animals unloaded using tail-traction showed no difference in weight gain compared with controls, and most bone parameters were only transiently altered.

(4) The influence of substrate on enzyme productivity of cultured bone cells was initiated. Preliminary data suggest that alkaline phosphatase activity is significantly elevated in cells cultured on collagen, as compared with cells cultured on plastic or coverglass.

Significance of the Accomplishments

Finding #1, that bone labels do not interfere with other experiments, is a continuing study which must be done on any

flight experiment involving multiple investigators to assure that markers allowing measurement of bone growth do not affect other studies being done in flight or in ground control animals.

Finding #2 suggests that changes in bone growth can be significant as quickly as a week in space in growing animals. Also, these animals were older than preferred but still showed significant alterations.

Finding #3 suggests that in animals with impaired growth, bone changes may be more pronounced and chronic during unloading whereas in normally growing animals such changes may be transient, lasting only about 2 weeks. However, regardless of the type of unloading, addition of bone mass to the surface of the bone shaft was still suppressed, suggesting that this particular part of bone depends significantly upon loading for normal growth while growth in length may be more genetically driven.

Finding #4 is a preliminary observation that was collected while setting up bone cell culture work in the laboratory. The data show that the bone-forming cells are more active when allowed to grow on a normal substrate than when grown on surfaces without substrate.

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HYPERGRAVITATIONAL EFFECTS ON MAMMALIAN FETAL AND NEONATAL GROWTH AND DEVELOPMENT

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Description of Research

The purpose of the research is to increase our understanding of the fundamental role of Earth's gravity as well as abnormal gravitational fields on mammalian growth and development. Current studies are focused on the effects of hypergravity exposures using the technique of chronic centrifugation to: (a) determine the stages of development affected by hypergravity, (b) delineate those structural and functional elements that are affected, (c) establish mathematical relationships between changes in development and different field intensities, and (d) quantify the scaling effects of gravitational force on different sized animal species with respect to growth and development.

During the past year, postnatal growth and developmental studies on mice and rats were completed over the entire range of hyper-g intensities available using our 12-ft radius centrifuge. A series of prenatal studies on rats conceived and reared in hypergravity at different intensities ranging from 1.0 g up to 2.0 g were also completed. The first study on reproduction of guinea pigs in hypergravity (1.27 g and 1.71 g) along with postnatal growth and developmental measurements were also completed during the past year.

Accomplishments

(1) Femur and tibia/fibula mass measurements of rats conceived and reared in hypergravity were completed. Significantly higher tibia and fibula/body mass ratios were found in hypergravity animals compared with normal gravity controls.

(2) Growth rates of fetal rats in hypergravity were not markedly different from rates in normal gravity. Body masses of 22-day-old fetuses conceived and reared at 1.48 g were the same as controls and were slightly smaller (3-5%) at 1.71 g and 2.03 g.

(3) A housing system for mating, reproduction, and growth studies of guinea pigs on the 12-ft radius centrifuge was devised and successfully tested. Using the new system, it was found that guinea pigs can mate and successfully produce viable litters without any problems up to as high an intensity as 1.71 g.

(4) Growth rates of pregnant guinea pigs were found to be inversely related to the gravitational field intensity, and the mean birth mass of the newborn pups showed a similar response. Guinea pigs conceived and reared at 1.27 g were approximately 13%

smaller than normal gravity controls at birth, while at 1.71 g, they were 21% smaller than controls.

(5) Postnatal growth rates of hypergravity-conceived guinea pigs showed the continued slower rates exhibited during the gestational period.

Significance of the Accomplishments

Results from these and previous studies show that minimal effects of hypergravity exposure occur in mice and rats during the gestational period, which may be attributed mainly to their relatively small body size. In contrast, results from the guinea pig study have shown very significant changes occurring during the same period in terms of growth of the fetus. The marked suppression of growth of the fetal guinea pig in hypergravity lends further support to my hypothesis that a critical body size (mass) of approximately 40-50 grams must be attained before moderate hypergravity exposures will induce a significant effect on normal growth and developmental patterns of mammals. The guinea pig fetus well exceeds this critcal body size in utero. Our comparative studies of both prenatal and postnatal growth and development on mice, rats, and guinea pigs show that progressively larger and more significant effects are obtained as the size of the species increases. Accordingly, the preferred animal for developmental studies in microgravity or fractional gravity (space centrifuge) may be the guinea pig, which is most likely to be affected to the greatest extent in such abnormal gravitational environments.

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GRAVITY, BODY MASS AND COMPOSITION, AND METABOLIC RATE

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Description of Research

The long-range objective of this project has been to evaluate the influence of Earth's gravity on the metabolic energy requirements and load-bearing functions of the body in terrestrial mammals. Two approaches have been used: one is the allometric analysis of the relationship between body mass and various physiological characteristics among species of differing body size; the other is the examination of these same characteristics in animals subjected to modulation of gravitational loading as through the weightlessness of spaceflight or through the increase in loading produced by chronic centrifugation.

We have measured oxygen consumption rate and body composition of a large series of laboratory animals of different ages and both sexes, representing five species: mouse, hamster, rat, guinea pig, and rabbit. The series has provided a 200-fold range of body size from 25 grams to 5 kilograms, sufficient to establish allometric scale effects. We developed a standard procedure for examination of the elemental composition of the whole body, as well as measurement of body water, fat, and creatine content, which was used to study our series of animals. The procedure was also used to examine a series of rats after 18.5 days of weightlessness in the joint USA/USSR Cosmos 1129 Biosatellite flight, and another series of rats after 14 days of hindquarter unloading. Finally, we measured oxygen consumption rate during, and body composition after, 6 weeks of chronic centrifugation at twice normal gravity (2.0 g) in adult animals of four species: hamster, rat, guinea pig, and rabbit.

Accomplishments

Findings from these studies during 1985 are as follows:

(1) Body fat content in all four species examined after 6 weeks at 2.0 g was 25-50% less than that measured in control animals at 1.0 g.

(2) Water content of the fat-free body mass was unchanged at 2.0 g.

(3) The potassium and creatine contents of the fat-free body mass exhibited a small, but statistically significant reduction at 2.0 g.

(4) The calcium, phosphorus, magnesium, and sodium contents of the fat-free body mass were all elevated in the rat, guinea pig, and rabbit at 2.0 g.

(5) The calcium, phosphorus, magnesium, and sodium contents

of the fat-free body mass were unchanged in the hamster, both at 2.0 g and 3.0 g.

Significance of the Accomplishments

The reduction in body fat content by 25-50%, which occurred in all four species kept at 2.0 g for 6 weeks, has been noted by previous investigators. It appears to be a specific effect of chronic centrifugation on fat metabolism, the nature of which is not known. This effect is worthy of further investigation.

The water balance of the body does not appear to be affected by augmented gravitational loading at 2.0 g, as judged by the constancy of the water content of the fat-free body mass. However, the reduction in potassium content of the fat-free body suggests a perceptible reduction in body cell mass, and a corresponding reduction in creatine content implies that the reduction was largely of the skeletal muscle mass of the body.

The most striking effect of augmented gravitational loading was that observed on the calcium, phosphorus, magnesium, and sodium contents of the fat-free body mass. Practically all of the body calcium, most of the body phosphorus, and much of the body sodium are found in the bone mineral of the body. Since the body content of these elements was significantly elevated in the rat, guinea pig, and rabbit after 6 weeks at 2.0 g, it seems clear that the bone mineral content of the body was increased at 2.0 g in these three species. Conversely, the hamster did not display an increase in bone mineral mass, even at 3.0 g. Thus, it would appear that there may be a body mass threshold for skeletal responses to augmented loading somewhere between the hamster and the rat in body size. The existence of such a threshold would be an important consideration for future spaceflight experiments.

The results of our centrifuge study also permitted direct comparison of the 2.0-g rat data with the rat data from the Cosmos 1129 flight, inasmuch as the same analytical procedures were used in both studies. The Cosmos 1129 rats exhibited a 17% decrease in the body bone mineral fraction of their fat-free body mass after 18.5 days of weightlessness. In contrast, the 2.0 g rats exhibited an increase of 18% in body bone mineral after 6 weeks of chronic centrifugation. Thus, we have concluded that the bone mineral mass of the body is directly proportional to gravitational loading over the range of 0 g to 2.0 g, emphasizing the dynamic nature of the weight-bearing function of the skeletal system of the body.

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BONE CELL KINETICS OF SIMULATED WEIGHTLESSNESS

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Description of Research

The osteogenic (bone-forming) components of bone modeling (change in size and/or shape) and probably remodeling (turnover of preexisting bone) are inhibited during spaceflight. The ultimate goals of this research are to: (a) define the cellular mechanism of osteoblast (bone-forming cell) production and (b) determine how this process is suppressed in microgravity. Spaceflight is the only experimental means known for probing the gravity dependence of osteoblast production.

The present cell kinetics research utilizes DNA labeling (^{3}H-thymidine), mitotic activity, and nuclear size as indices of the proliferation and differentiation aspects of osteoblast histogenesis (production of bone-forming cells). The overall thrust of these studies is to determine the relative influence of gravity, mechanical loading, and physiological stress on osteoblast histogenesis in weight-bearing (tibia and ulna) and non-weight-bearing (maxilla) bones.

Previous research in periodontal ligament (PDL), the osteogenic interface between tooth and bone, revealed there are three kinetically and/or morphometrically (nuclear volume) distinguishable cell types involved in osteoblast (Ob) production: (a) self-perpetuating, less differentiated precursor cells (A); (b) committed osteoprogenitor cells (A'); and (c) pre-DNA synthesis preosteoblasts (C cells) and post-DNA synthesis preosteoblasts (D cells). The histogenesis sequence is A -> A'==> C -> D -> Ob. The rate limiting step in differentiation of an osteoblast is an increase in nuclear volume (A'==>C) to form a preosteoblast. This morphological manifestation of change in genomic expression (differentiation) has proven to be an effective tool for assessing inhibition of bone formation during spaceflight. Preosteoblast numbers are markedly suppressed in microgravity, suggesting a block in osteoblast differentiation. However, specific influences of factors, such as decreased skeletal loading, loss of extracellular fluid, and physiological stress, are not established.

Principal questions in the past year focused on: (a) definition of the cellular compartments and timing of the proliferation and differentiation steps in the histogenesis sequence for producing osteoblasts; (b) influence of 18.5 days of microgravity during Cosmos 1129 on the total number and tissue distribution of

osteogenic cells in the primary spongiosa (PS) (spongy bone immediately below the growth plate) of flight ulnae (minor weight-bearing long bones); (c) optimal ^{3}H-proline (protein label) dose and autoradiography (detection) conditions to assess bone matrix formation; and (d) determining whether a 7-day flight is sufficient to produce a suppression in preosteoblast formation.

Experiments were: (a) completion of the analysis for a control series of rats examined at hourly intervals over a complete 24-hour circadian cycle, (b) nuclear volume analysis of Cosmos 1129 ulnae, (c) autoradiographic analysis of maxillae and long bones of 6-8 week old rats injected with ^{3}H-proline and analyzed 1 hour or 6 days later, and (d) Spacelab-3 (SL-3) flight.

Accomplishments

(1) Mathematical modeling successfully simulated our previous studies of osteogenic induction in rat PDL. This indicates that the four cells (A, A', C, D) previously implicated in osteoblast histogenesis are the only major compartments in the osteoblast histogenesis sequence.

(2) Mathematical derivation of the relative contribution of fibroblast-like B cells (nuclear volume 80-119 μm^3) demonstrated that they are non-osteogenic fibroblasts. Furthermore, B cells are not induced to proliferate under conditions of short-term osteogenic stimulus.

(3) Differential distribution of osteogenic cells in Cosmos 1129 ulnae (PS) revealed that preosteoblast numbers are also inhibited in long bones by 18.5 days of spaceflight.

(4) In preparation for Spacelab-4 (SL-4), the optimal ^{3}H-proline dose was found to be 3.0 μCi/gm body weight and an autoradiographic exposure of 14 days was ideal.

(5) For all flight and control groups, the older (large) SL-3 rats had significantly fewer preosteoblasts than the younger (small) animals.

(6) Insignificant trends toward increased A/A' but decreased C/D cells are consistent with suppression of preosteoblast formation within the 7-day flight period and partial recovery during the 12-hour postflight period.

(7) A morphometric (image analysis) method has been developed for assessing osteogenic activity and/or potential of a broad range of skeletal tissues.

Significance of the Accomplishments

Finding #1. This research has resulted in the first report of the complete proliferation and differentiation sequence for osteoblast histogenesis under physiological conditions. These results may have important implications in understanding the mechanisms of skeletal adaptation and metabolic bone disease.

Finding #2. Morphometric distinction of nonosteogenic (B) from osteogenic (A, A', C, D), fibroblast-like cells is a major

milestone in bone cell kinetic methodology. It is now possible to directly study individual cellular elements in a mixed connective tissue population.

Finding #3. The spaceflight-related decrease in osteoblast numbers, previously noted in the non-weight-bearing maxilla, is probably a systemic effect. These results demonstrate that the nuclear volume method, developed in PDL, is also applicable to growing long bones.

Finding #4. This is a technical refinement to optimize the information which can be derived from the upcoming SL-4 experiment.

Finding #5. Since this is the first description of a method for directly assessing an age-related decrease in osteogenic potential, it may have important implications for understanding the pathophysiology of skeletal aging.

Finding #6. Nuclear volume analysis is a sensitive index of the gravitational dependence of normal osteoblast histogenesis.

Finding #7. Since nuclear volume analysis does not require any drugs or radioactive markers, it is an ideal, nontoxic method for investigation of the disturbance of osteoblast histogenesis during spaceflight.

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MAMMALIAN GRAVITY RECEPTORS: STRUCTURE AND METABOLISM

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Description of Research

The goals of this research are twofold: to understand the basic organization of mammalian macular end organs, and to shed light on the role calcium plays in their functioning. These receptors have been alluded to generally as gravity receptors, but my more recent work focuses on them as biological linear accelerometers. I believe that they cannot be understood except by examining them in this light. Thus, interactions with persons knowledgeable in physics and fluid dynamics will be essential as the work proceeds.

The otoconial masses of macular end organs are subjects of interest both as biomineralized objects and as test masses in a bioaccelerometer. Thus, some of our effort has been spent in analyzing the protein composition of otoconia. Work with Kenneth Pote, a graduate student, is showing that the internal organic material of otoconia can be separated from external material through suitable chemical and fixation procedures. He has transmission electron micrographs of samples treated similarly to those analyzed by gel electrophoresis to demonstrate exactly what has happened to the organic materials within and outside the otoconia by the various treatments applied. This research shows that the otoconia have a central core, a peripheral area of greater size, and a thin "skin" of organic material which covers even the terminal faces. The most prominent protein inside the otoconia has a molecular weight of ~85,000-90,000 daltons. Calcium binding does not appear to be associated with this material, however, because mobility changes (in the presence and absence of calcium) did not occur. However, mobility of a lower molecular weight material (~18,000 daltons) seems to have been affected by calcium. This work is being repeated. If it holds, it would fit a hypothesis presented in some of our first work with gel electrophoresis, that a protein of this weight might bind calcium. The work on otoconia is related to our general interest in the localization and utilization of calcium in rat linear accelerometers. We have been successful in localizing calcium to glycocalyx material around the stereocilia and kinocilia; to some of the cisterns of smooth endoplasmic reticulum that are present at the hair cell membrane and within the calyceal nerve endings; along the striated organelles; at nodes of Ranvier; and at synapses, in the synaptic membrane and in vesicles. This was done using a precipitation method involving the sodium salt of N,N-napthaloylhydroxylamine (NHA), considered to be highly specific for calcium (Zechmeister, Histochemistry 61: 223-232, 1979). The reaction product is

calcium-NHA. That calcium is present in the granules we precipitated has been proven by two different microprobe instruments and by electron energy loss spectra.

Accomplishments

(1) Reconstructions of rat saccular macula showed that both type 1 and type 2 hair cells are integrated into the same neural circuits leading to the brain (Figure 1).

(2) Proteins of rat otoconial complexes have been characterized according to molecular weight. A protein of ~85,000-90,000 MW is quantitatively the most significant within the otoconia.

(3) Glycocalyx (cell coat) has been demonstrated to bind stereocilia of the tuft together, and to bind the kinocilium to the sterocilia in specific ways.

(4) Glycocalyx has been shown to bind calcium, with a gradient along the sterocilia.

(5) Calcium has been demonstrated at specific sites within the hair cells and within nerve endings.

Significance of the Accomplishments

(1) Previously it was thought that each kind of hair cell had its own nerve connections to the brain. Our findings mean that integration of incoming information is occurring peripherally. We have also been able to demonstrate that the macula is organized on the basis of sensory fields, and resembles the retina in that summation, convergence, divergence, feedback, and feed-forward all occur. If we can model this system, it will be like modeling a microcosm of the brain.

(2) The major protein within the otoconia has been identified, but its functional significance remains obscure. It does not seem to be the material that binds calcium.

(3) Work with several histochemical methods has demonstrated that hair cells have much glycocalyx, and that glycocalyx (a special kind of cell coat) holds the stereocilia together. The kinocilium and stereocilium comprise a functional unit. This will be important when we try to model how the system works.

(4) It is this cell coat that binds calcium. Probably both high and low affinity sites are present. We are the first to show this, and to show that a gradient is present. However, this work must still be considered in its infancy. We may have only demonstrated the high affinity binding sites. Further work is required.

(5) This distribution of calcium at specific sites is meaningful in functional terms. It shows that calcium is important in the regulation of many activities carried out by the hair cells.

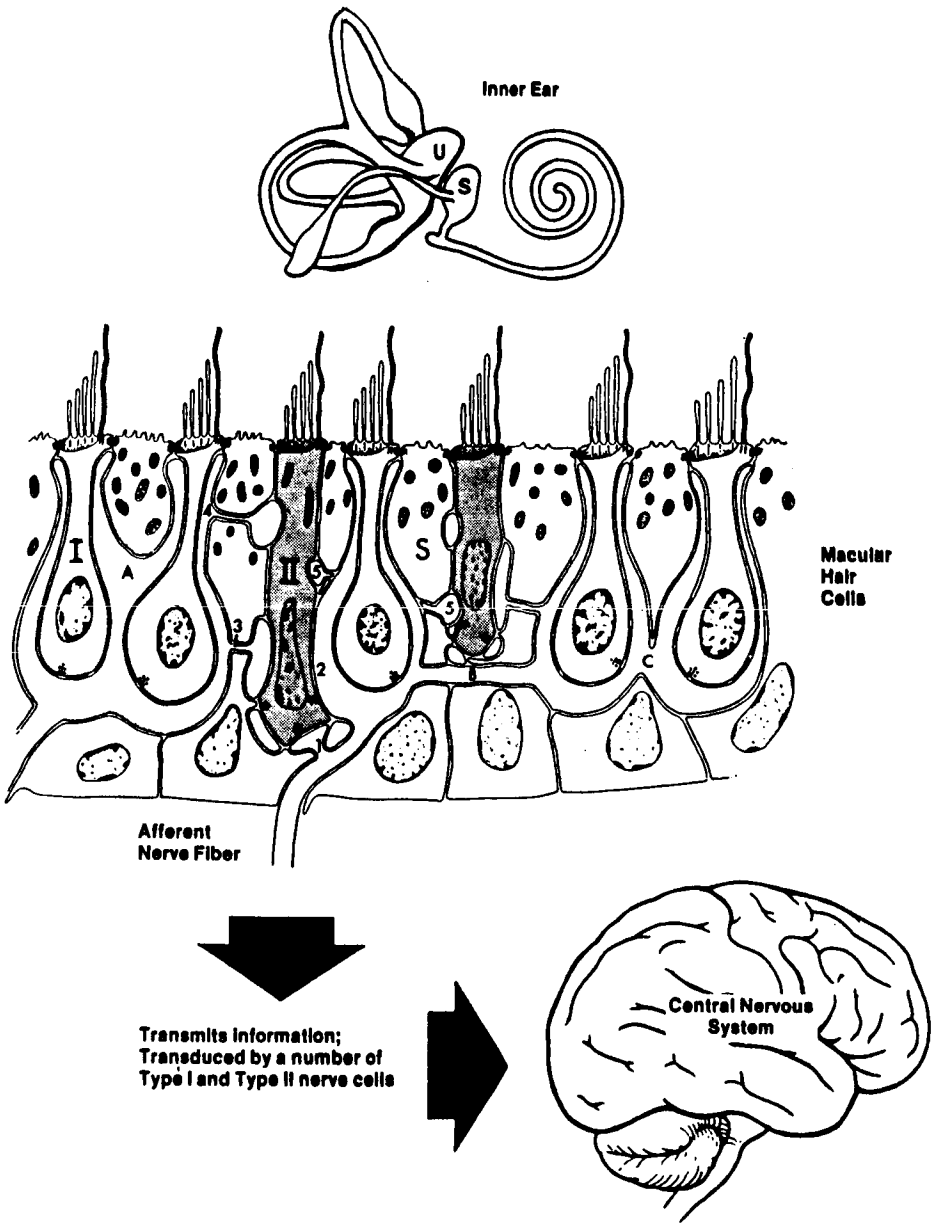

Figure 1. Representation of gravity perception and information transfer in mammalian vestibular system.

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EFFECTS OF MICROGRAVITY ON SEA URCHIN FERTILIZATION AND EARLY DEVELOPMENT

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Description of Research

Since gravity is a pervasive influence on all developing systems, there are reasons to envision that this force can affect fertilization and embryogenesis. Indeed, development in some systems is abnormal when the orientation of gravity is constantly changed in clinostat experiments. To determine the effect of microgravity and the universal influence it has on fertilization and early development, this series of experiments is aimed at understanding microgravity effects during fertilization, embryogensis, and spicule calcium deposition in sea urchins. Sea urchin development, a model system for exploring the cell and molecular basis of fertilization and embryogenesis, has several advantages for this investigation. Spaceflight experiments should be quite feasible since the gametes and embryos withstand manipulations. In addition, the normalcy of fertilization and early cleavage stages can be compared with the knowledge about gravitational influences on amphibian eggs. Furthermore, skeletal calcium is deposited into the embryonic spicules within a day of fertilization, permitting studies on the effects of gravity on bone calcium.

Accomplishments

(1) Suitable culture chambers and clinostats were constructed, permitting experiments on the effects of rotation at varying speeds.

(2) Cultures were rotated either perpendicular or parallel with the gravitational vector, and numerous parameters of normal fertilization, cleavages, and embryogenesis were monitored.

(3) At rotational speeds of 0.25, 5, and 60 rpm, normal fertilization and early development was observed in the experimental and control cultures. These results provide the framework on which to design spaceflight experiments.

Significance of the Accomplishments

The importance of these studies is in providing a baseline from which future research on the effects of microgravity can be assessed in this model system. This research demonstrates that, unlike other studied developing organisms, sea urchin fertilization and early development does not appear to be influenced by constantly changing gravitational vectors. These results can now be used to begin to determine the culture

conditions and holding chambers necessary for a flight experiment. In addition, since many of the basic processes of embryogenesis are shared by most animals, an understanding of the events in this system will help explain the effects of microgravity on gravity-sensitive developmental events.

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EFFECTS OF WEIGHTLESSNESS ON AURELIA EPHYRA DIFFERENTIATION AND STATOLITH SYNTHESIS

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This report on the use of jellyfish for microgravity-related research requires a special introduction because many people are not familiar with jellyfish life stages and structures. The most intriguing nature of jellyfish polyps is their ability to metamorphose, giving rise to tiny (2-3 mm) immature medusae called ephyrae (Figure 1, left). The ephyrae have a different form from polyps and can pulse and swim. Metamorphosis of the entire ephyra takes place in 5 days at 27°C. Ephyrae form simple gravity-sensing structures called rhopalia (Figure 1, right), which resemble, in a less-complicated way, the gravity receptor structures of higher organisms. Rhopalia are composed of statoliths in statocysts (Figure 2), mechanosensory cells (hair cells), neurons, and supporting cells, cell types which are found in inner ears of higher animals.

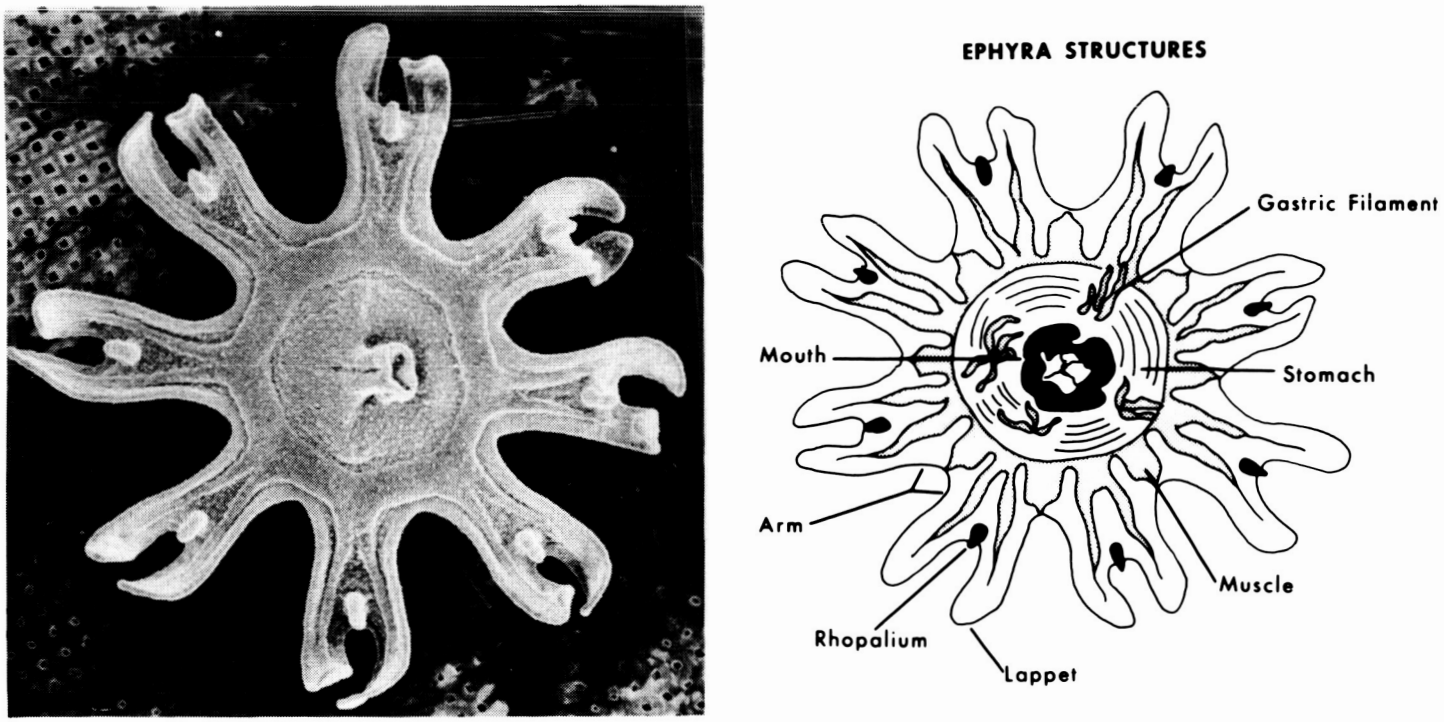

Figure 1. Left side. Photograph of an ephyra, an immature medusa stage of jellyfish. Right side. Drawing of an ephyra, showing the location of various structures.

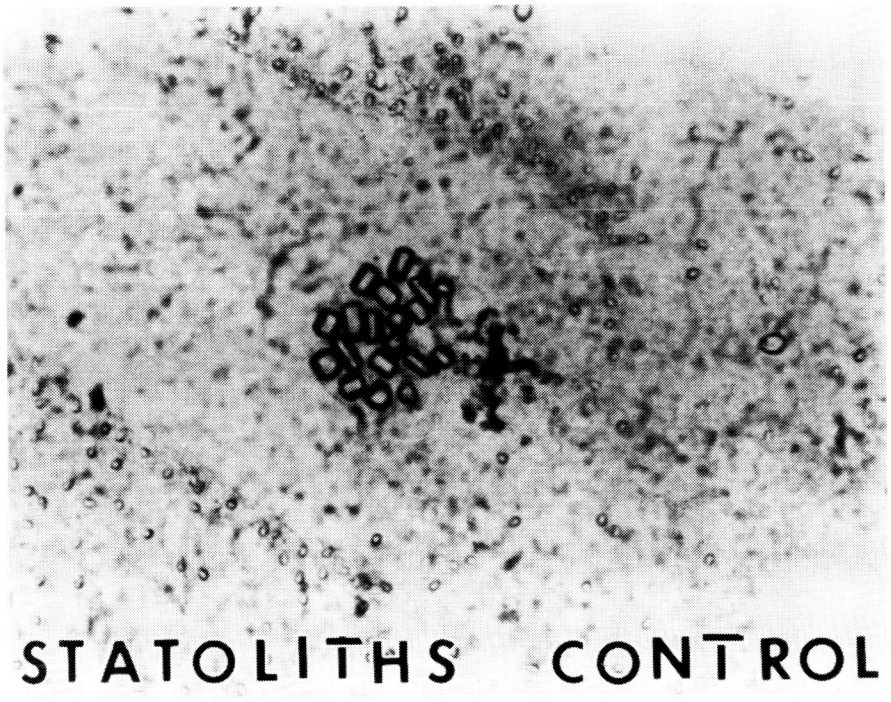

Figure 2. Statoliths, which are found in rhopalia. Rhopalia are the gravity-sensing structures of jellyfish ephyrae.

Description of Research

The long-range goals of the research are to discover the role(s) of gravity on the behavior and the development of Aurelia ephyrae and of their graviceptor structures and to discover the effects of microgravity on ephyra and rhopalia development after short-term (7-day Shuttle flight) and long-term (Space Station experiment) exposure to a microgravity environment.

Specific objectives include:

(1) Determination of whether the microgravity of outer space will modify: the development of ephyrae from polyps; the development of the graviceptors of ephyrae; the formation or demineralization of statoliths of rhopalia; and the swimming/pulsing behavior of ephyrae.

(2) Comparison of the features listed above in ephyrae which developed in space with those that developed on Earth, to discover the role that gravity plays in the development of ephyrae, their graviceptors, and their behavior.

Experiments conducted:

(1) Clinostat: The effects of clinostat rotation on the synthesis of statoliths was studied. For each experiment, groups of six metamorphosing polyps were rotated in the horizontal or vertical plane and controls were kept stationary in these planes.

Ten ephyrae from each group were collected after 5 or 6 days at 27°C and the numbers of statoliths per rhopalium were counted. Three experiments were done for each rotational speed of 1/15, 1/8, 1/4, 1/2, 1, and 24 rpm.

(2) High Voltage Electron Microscopy (HVEM) of Rhopalia: Several hundred micrographs of rhopalia of ephyrae were obtained using the HVEM. Comparison of mechanosensory cells of rhopalia on the exumbrellar (top) side of the organism with those on the subumbrellar (under) side revealed a similar structural pattern, which indicates that the ephyrae may not have a touchplate as has been reported for medusae. Of particular interest was the tracing of the ciliary filaments throughout the cell from the basal body of the cilium to the plexus of filaments resembling neurofilaments at the base of the cell. The hair cells are in very close association with the nerve cells and the mineralizing statocytes.

(3) Cloning: A series of experiments was done to establish the validity of cloning polyps for their ability to produce normal ephyrae. Clones were developed by inducing metamorphosis in polyps, checking their ephyrae for normality, and retaining the polyp bottom pieces from the strobilae for restoration to the polyp condition. These polyps were then cloned by separating them into a culture dish and retaining their asexual progeny. After several hundred polyps had developed from each bottom piece, 10 organisms were induced to metamorphose from each clone. Ephyrae from the metamorphosed polyps were checked microscopically for normality of form and pulsing ability. Over a period of approximately 9 months, the organisms from two of the clones have consistently given rise to a high level of normal ephyrae, as compared with organisms randomly chosen from the cultures.

(4) Parabolic flight experiment: Ephyrae with and without statoliths (created by a low sulfate environment during development) were provided for Dr. C. Oman of MIT for a parabolic flight last summer and for a recent ground-based experiment. The results of these experiments are being analyzed. Earlier studies by Dr. Oman during parabolic flight indicated that ephyra swimming/pulsing behavior is inhibited at 0 g.

(5) Statoliths and pulsing behavior: A student project was performed relating statolith number and pulsing behavior. Ephyrae which had developed in low sulfate sea water and controls were compared with regard to statolith numbers and pulsing numbers per minute. In general, ephyrae with no statoliths pulsed at lower rates than those with statoliths. Further studies need to be done, however.

Accomplishments

(1) Statistical analyses using an ANOVA and the New Duncan's Multiple Range Test revealed that statolith numbers were statistically significantly reduced in ephyrae which had developed while rotating at 1/4 and 1/2 rpm in the horizontal plane as compared with stationary and vertically rotated controls.

(2) Using methods we developed for HVEM studies, we obtained several hundred micrographs of rhopalia of ephyrae. While they are still under investigation, it was found that mechanosensory cells (hair cells) are present on the exumbrellar and subumbrellar areas of the rhopalia (and not restricted to a touchplate) and that the ciliary filaments of the hair cell cilia extend throughout the cell to the base of the cell and become integrated with filamentous materials at the base of the cell.

(3) We have developed clones of polyps which will give rise to a high percentage of normal ephyrae. These will be used for the planned flight experiment.

(4) We finished planning the jellyfish Shuttle flight experiment which had been manifested for flight in December 1986 prior to the Challenger accident.

Significance of the Accomplishments

(1) In Aurelia, the statolith-bearing graviceptor structures are utilized for positional orientation with respect to gravity during swimming. The finding that 1/4 or 1/2 rpm rotation of developing ephyrae in the horizontal plane reduced their statolith numbers suggests that statolith synthesis is sensitive to gravity and that the formation or mineralization of statocytes will be modified in the microgravity environment of space.

(2) An understanding of the cellular organization of the graviceptor structure is essential for understanding how it works on Earth and how it may be modified by microgravity. Significant progress was made toward this goal through the use of a very special microscope, the HVEM housed in Boulder, Colorado, which makes it possible to examine unusually thick sections of tissue and thereby to determine how cells are interrelated. Of special interest is the relationship between the sensory cells (hair cells) and the cells which make the statoliths and the relationship between the sensory cells and the nerve cells which interact with the muscular system causing the pulsing activity. Completion of these ongoing studies will achieve this goal and prepare the investigator for interpretation of the results to be obtained from the planned flight experiment.

(3) The cloning experiments described above are the first in which jellyfish have been cloned successfully for normality. This research guarantees that jellyfish polyps with the capability of producing normal ephyrae will be available for the flight experiments as well as for ground-based experiments.

In summary, current progress demonstrates that developing and mature ephyrae are especially suited for microgravity research. These organisms are tiny, require little or no care during spaceflight, and have the capability of forming gravity-sensing structures. If the graviceptors do not form in space, we will deduce that gravity was needed for normal development (having controlled for other factors), and that gravity plays an important role in the normal development of these structures on Earth. If graviceptors do form in space, we will study them in

detail using various types of microscopes, including the electron microscope, to determine whether they developed normally in space as compared with controls on Earth. An in-depth understanding of the mechanisms through which these organisms sense and respond to gravity is important in understanding how other organisms are affected by gravity and will illuminate the role(s) of gravity in the development and functioning of organisms on Earth as well as the effects of microgravity on biological organisms in space.

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MUSCULAR ACTIVITY, PHOSPHOLIPIDS, PROSTAGLANDINS, AND SIZE

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Description of Research

The primary goal of this research project has been to investigate the involvement of muscular prostaglandins in the established relationship between muscular activity and changes in muscular size. Increased muscular activity by exercise, passive stretching, direct electrical stimulation, and decreased activity with accompanying muscular atrophy have been reported to elevate levels of muscular prostaglandins. Possible control of muscular size by prostaglandins has been indicated in studies of isolated muscle by results which related prostaglandins E_2 and $F_2\alpha$ to muscular protein degradation and synthesis, respectively. Further support has been provided by studies of indomethacin, which blocks prostaglandin synthesis. In these studies, indomethacin reduced both muscular prostaglandin levels and protein turnover in isolated muscles and lowered insulin-induced increases in both muscular prostaglandin levels and protein synthesis in awake rats.

Specific research goals have included investigation of the influence of indomethacin on muscular size. Since arachidonate release from membrane phospholipids is the rate-limiting step in prostaglandin synthesis, the influences of muscular activity and inactivity on phospholipids and arachidonate content in the soleus muscle have been investigated. Intracellular factors which control arachidonate release have been investigated in chick muscle culture.

Accomplishments

(1) Hindlimb unloading, which reduced soleus activity and simulated microgravity, reduced selectively the concentration of phosphatidylethanolamine in the soleus muscle of rats.

(2) Indomethacin treatment of rats during recovery from hindlimb unloading enhanced the recovery of phosphatidylethanolamine.

(3) Indomethacin reduced the weight gain of the soleus muscle during recovery from hindlimb unloading; hypertrophy of both fast and slow fibers was reduced.

(4) In chick muscle culture, a calcium ionophore A23187, which increased cytosolic calcium, and melittin, which stimulated phospholipase activity, elicited release of arachidonate from muscular phospholipids.

Significance of the Accomplishments

Finding #1 supports a previous report (Fernandez et al., Muscle and Nerve 2: 118, 1979) that muscular phosphatidylethanolamine was selectively reduced during a reduction of muscular activity. This previous report also presented evidence that the muscular content of this phospholipid was under neural control.

Finding #2 suggests that the content of phosphatidylethanolamine in soleus phospholipids may be influenced by cellular mechanisms which are involved with arachidonate metabolism.

Finding #3 supports the hypothesis that muscular prostaglandins may mediate changes in muscular size during changes in muscular activity.

Finding #4 supports other data which indicate that the level of cytosolic calcium influences liberation of arachidonate from muscular phospholipids.

In summary, evidence supports the hypothesis that a change in muscular activity increases cytosolic calcium, calcium-dependent phospholipase activity, arachidonate liberation from phospholipids, prostaglandin synthesis, and protein turnover. Other evidence supports the hypothesis that soleus inactivity evokes a reduction in the concentration of phosphatidylethanolamine and that alteration in this phospholipid may be involved with changes in cytosolic calcium.

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Templeton, G.H., Padalino, M., and Wright, W. Control of Arachidonic Acid Release in Chick Muscle Cultures. Physiologist 28(6, Suppl.): S135-S136, 1985.

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SKELETAL MUSCLE METABOLISM IN HYPOKINETIC RATS

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Description of Research

Research in this laboratory is aimed at identifying the mechanisms which lead to the metabolic adaptations of muscle to the condition of not bearing weight, as may occur with bedrest or weightlessness. Ultimately, the sequence of these adaptations will be defined to determine which factors contribute most to the loss of muscle mass, and thus to the mechanics of muscle wasting.

In studying the metabolic adaptations of muscle to unloading (i.e., lack of bearing weight), a number of important areas require attention. The loss and/or the failure to accumulate protein by unloaded muscle requires extensive studies of the formation and breakdown of these proteins. It is important to know whether certain groups of proteins are affected specifically, and to identify the mechanisms which lead to the loss or growth failure of muscle. Hence, work has continued to further identify those factors that are instrumental in initiating or prolonging the response of muscle protein to unloading. A second focus has been on the fate of amino acids, which are the individual components of protein. Most amino acids are simply taken into or released from muscle and its protein. Certain key amino acids, however, play more vital roles, such as providing small amounts of energy or serving as a means of ridding the muscle of nitrogen waste. Of particular interest are leucine for energy and glutamine for waste removal. Extensive studies of their metabolism have been conducted. Finally, besides fats, glucose (or its storage form, glycogen) serves as an important source of energy and may be used in the formation of glutamine. Hence, alterations of glucose and/or glycogen metabolism could have important effects on muscle function. Because of its sensitivity to insulin, glucose metabolism can provide important indications of altered responses of muscle to this vital hormone. Some of insulin's many functions in muscle include: uptake of glucose, storage of glycogen, and maintenance of muscle protein mass.

Accomplishments

(1) Protein metabolism.

(a) Relative responses of protein mass were similar in muscles of rats subjected to unloading on Earth and in muscles of rats flown on Spacelab 3.

(b) Constant tension of the unloaded soleus muscle prevents loss of protein and the alterations of protein metabolism.

(c) Insulin is more effective in controlling protein metabolism in the unloaded soleus muscle.

(2) Amino acid metabolism.

(a) Tyrosine in the soleus muscle increases, whether the muscle is unloaded on Earth or in space.

(b) Aspartate in the unloaded soleus decreases, whether from flown rats or those studied on Earth.

(c) Constant tension of the unloaded soleus can prevent alterations in amino acid metabolism.

(d) The diminished formation of glutamine by the unloaded soleus results from the reduced use of energy by this muscle due to lowered activity.

(e) Metabolism of leucine is greater when the soleus muscle is unloaded due to increased activity of a key enzyme in its pathway for breakdown.

(3) Glucose metabolism.

(a) The increased metabolism of glucose by insulin in the unloaded soleus is due to a higher concentation of sites (receptors) to which insulin can bind on the muscle.

(b) The concentration of membrane proteins for transporting glucose appears to be greater in the unloaded soleus.

(c) Despite a higher concentration of glycogen in the unloaded soleus, the activity of the enzyme for its synthesis is lower, as is the activity of the enzyme for glycogen breakdown.

Significance of the Accomplishments

The combined findings listed as 1a, 2a and 2b above suggest that the model used in Earth-based experiments to unload rat hindlimb muscles must mimic, at least to a certain extent, the unloading which occurs in the microgravity of space. Thus, we have gained more confidence that the model will help us to develop the most important questions to be tested in future Shuttle and Space Station experiments. These experiments will concern the mechanisms of adaptation to a lack of gravity.

Findings 1b and 2c illustrate that the simple action of passive stretch, a routine procedure used by physical therapists, is a more potent factor than the lack of bearing weight. Such a possibility is also planned for testing in space.

Findings 1c, 3a and 3b point to a promising area of future work. The greater response to insulin resulted from a higher concentration of insulin receptors. This increase occurred apparently because this particular protein was not lost as the muscle underwent wasting. Hence, the concentration effectively rose because the muscle decreased in size. A similar conclusion may be drawn for the protein responsible for moving glucose into the muscle. Taken together, these facts show that these proteins in the membrane were not subject to the same deleterious effects of unloading. In contrast, these proteins are not protected when

muscle wasting is induced by severing the nerve supply. Extensive studies in these areas can help to better understand whether multiple mechanisms for muscle wasting exist.

Findings 2d and 2e point to the extensive metabolic alterations which occur as muscle adapts to unloading. These changes are most likely secondary responses to the lack of bearing weight rather than primary effects that contribute to muscle wasting.

Accomplishment 3c suggests that accumulation of glycogen by unloaded muscle is not due to increased storage but rather to marked reduction in glycogen use. The increased concentration of glycogen seems to shut down any further formation of glycogen.

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EFFECTS OF SIMULATED MICROGRAVITY ON MAMMALIAN DEVELOPMENT AND EXPRESSION OF CALCIUM BINDING PROTEINS

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Description of Research

The long-range goal of this research is to assess the effects of altered gravitational environments on mammalian development and differentiation.

This laboratory has previously shown that mouse oocytes rotated on a clinostat in an axis perpendicular to the gravity vector can exhibit anomalies of the meiotic maturation process. Rotation at 100 rpm produced an inhibition of the progression of oocytes into Metaphase II of meiosis. The use of clinostat rotation as a means with which to reorient cells relative to the gravity vector is now being extended to studies on fertilization and early embryogenesis. Ova were combined with capacitated sperm and rotated at 100 rpm, the rate at which effects were observed in the oocyte maturation experiments. No alterations in the efficiency of fertilization have been noted. By morphological criteria at the light microscope level, the zygotes appeared normal, although developmental potential of these zygotes has not been evaluated. In addition, a modification of the experimental system has greatly enhanced the validity of the clinostat model for examination of the effects of altered gravity. Cells (2-cell embryos) are embedded in low melting point agarose and placed in the well of a tissue culture chamber which is mounted on a clinostat. This provides the advantage of immobilizing the cells such that effects due to the movement of a free-falling body during rotation can be reduced.

Accomplishments

(1) Reorientation of mammalian oocytes relative to the gravity vector, through the use of a clinostat, can affect their meiotic maturation. Oocytes rotated at 100 rpm exhibited an inhibition in the number of cells which reached metaphase II of meiosis. This presumably represented an inhibition of chromosome movement.

(2) Ova which had matured meiotically in vivo under a normal gravitational environment were used in experiments in which the process of fertilization under clinostat rotation was examined. Ova were combined with fully capacitated spermatozoa, placed immediately into the clinostat rotation system, and cultured for 8 hours. The rotation speed of 100 rpm was chosen since this was the rate at which effects were observed on meiotic

maturation. No abnormalities in the appearance of the fertilized ova or differences in the efficiency of achieving normal fertilization were observed. In addition, no increase in the proportion of ova which underwent parthenogenetic activation was observed.

(3) A modification of the culture system has been developed and is being used for our current studies on early embryogenesis under clinostat conditions. In this new system, embryos are immobilized in the center of the axis of rotation. This is achieved by embedding the cells in low melting point agarose, which is then adhered to the culture dish. We have achieved development up to the compacted morula stage of early development and predict that we will be able to allow development to proceed to the blastocyst stage.

Significance of the Accomplishments

(1) The observation of an effect on a division process of cells under conditions of reorientation relative to the gravity vector is significant at several levels. First, an inhibition of chromosome separation and/or movement at this stage of meiosis would affect the ability of the egg to be fertilized. This would result in impaired fertility if the same event occurs *in vivo* under conditions of microgravity. Second, this inhibition is interesting in light of the fact that effects on mitotic divisions have been observed in cells which were flown in space and in astronauts.

(2) The experiments on fertilization under clinostat rotation suggest that formation of the male and female pronuclei is not sensitive to reorientation relative to the gravity vector. The fact that no increase in the proportion of parthenogenetic activation was seen is also important, since mammalian ova are exquisitely sensitive to a variety of chemical and physical agents which result in spontaneous or parthenogenetic activation. Finally, although the fertilized ova appeared normal, we do not yet know the developmental potential of such fertilized ova.

(3) For our initial studies on the effects of clinostat rotation on mouse embryogenesis, we modified a key feature of our clinostat--that of the position of the cells during rotation. We developed a means to immobilize the cells during clinostat rotation, permitting the cells to remain at the axis of rotation, yet not interfering with cellular development. This eliminates effects due to the movement of free-falling bodies in the experimental system.

PUBLICATIONS

Wolgemuth, D.J. and Grills, G.S. Early Mammalian Development Under Conditions of Reorientation Relative to the Gravity Vector. Physiologist 28(6, Suppl.): S75-S76, 1985.

Wolgemuth, D.J. and Grills, G.S. Early Mammalian Development Under Conditions of Reorientation Relative to the Gravity Vector (Abstract). Physiologist 28: 262, 1985.

SPECIAL ACTIVITIES

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Space Biology Research Associates' Program

NASA's Space Biology Program provides a unique opportunity to train individuals to conduct biological research in space and to continue relevant ground-based research. 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 program was developed to train this cadre of biologists by offering Research Associate Awards to young scientists. These grants provide opportunities to work on projects directly related to Space Biology in laboratories that provide the necessary facilities and a suitable research environment. It is anticipated that these scientists will develop research careers in 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. To date, 52 awards have been made. There have been 32 awardees, 20 of whom have received a second year of funding. These scientists come from different disciplines, including: zoology, developmental biology, botany, and physiology (animal and plant). They have worked in laboratories at: University of California at Berkeley, Irvine, and Davis; Stanford University; University of Texas at Austin and Dallas; University of Houston; Texas A&M; University of Michigan; Wayne State University; Washington University in St. Louis; University of Washington in Seattle; Duke University; Tufts University; Indiana University; University of Louisville; Cleveland Clinic; National Institutes of Health and National Institute of Mental Health; University of Pittsburgh; University of Pennsylvania; Rockefeller University; Princeton University; Yale University; and Dartmouth College. In June 1980 there were 19 laboratories participating. Presently (May 1986), there are 47 laboratories in the program.

The scientists who have completed this program have accepted positions in colleges and universities, with research laboratories, and with NASA. Dr. Jay Buckey is a co-investigator and project manager for Dr. Gunnar Blomqvist's Spacelab-4 project; Dr. John Garavelli is working in the Extraterrestrial Research Division at NASA-Ames; Dr. Steven Black is working with Dr. Kenneth Souza at NASA-Ames; and Dr. Mark Cooper is working with Dr. Emily Morey-Holton at NASA-Ames. In addition, many of the Research Associates have been asked to participate in NASA panels, national workshops, and national meetings. There have been 63 publications in refereed journals and as many abstracts of papers presented at national and international meetings. Several Research Associates have submitted proposals for direct funding from the NASA Life Sciences Program.

Each year, in the fall, the Research Associates attend and

present papers at the annual American Society for Gravitational and Space Biology (ASGSB) meeting, often in conjunction with the American Physiological Society/International Union of Physiological Sciences (APS/IUPS) Commission on Gravitational Physiology. The Research Associates are also encouraged to participate in other national meetings of their choice and are encouraged to publish in refereed journals. At the completion of their award period, they are required to submit a final report. These reports are on file in the Project Director and Scientific Advisor's office (Dr. X.J. Musacchia, University of Louisville).

Research Associate Awardees

As stated previously, this program has provided awards for 32 Research Associates. They are listed below, alphabetically: names, award terms (in parentheses after their name), host laboratory, and current location:

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

Dr. Thomas Bjorkman (7/1/86 - 6/30/87) will be working on "Mechanism of Gravity Sensing in Plants" in Dr. Robert Cleland's laboratory at the University of Washington, Seattle, Washington.

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

Dr. Harry Blair (7/1/84 - 6/30/86) is working on "Cellular Mechanisms of Bone Degradation" in Dr. Steven Teitelbaum's laboratory at the Jewish Hospital/Washington University Medical Center, St. Louis, Missouri.

Dr. Thomas Brock (7/1/86 - 6/30/87) will be working on "Comparison of Changes in Protein Synthesis Induced by Gravity and Auxin Treatment in Pulvini and Coleoptiles of Oat (Avena sativa L.)" with Dr. Peter Kaufman at the University of Michigan, Ann Arbor, Michigan.

Dr. Jay Buckey, Jr. (7/1/82 - 6/30/84) worked on "2-D Echocardiography as an Accurate Mean for Measuring Left Ventricular Volume and Central Venous Pressure During Zero-gravity" in Dr. C. Gunnar Blomqvist's laboratory at the University of Texas Health Sciences Center, Dallas. At the present time, he is the co-investigator and project manager for the Spacelab-4 project in Dr. Blomqvist's laboratory, Dallas, Texas.

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

Dr. Denis Clohisy (7/1/86 - 6/30/87) will be working on "Mechanisms of Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at the Jewish Hospital/Washington University Medical Center, St. Louis, Missouri.

Dr. Mark Cooper (1/1/85 - 12/30/86) is working 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 also doing some work with Dr. Emily Morey-Holton at NASA-Ames Research Center, Moffett Field, California.

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

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

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

Dr. Cheryl Gould (7/1/84 - 8/30/85) worked on "Effect of Weightlessness on Various Immunological Functions Using a Marine Simulated Space Flight Model" in Dr. Gerald Sonnenfeld's laboratory at the University of Louisville, Louisville, Kentucky. She is now developing a Tissue Banking Program for the Community Blood Center, Dayton, Ohio.

Dr. Martha Gray (7/1/86 - 6/30/87) will be working on "The Correlation of Applied Strain Distribution 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.

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 at Marshall University, Huntington, West Virginia.

Dr. Gary Jahns (1/1/83 - 4/30/84) worked on "Interactions of Light and Gravity on the Growth, Orientation, and Lignin Biosynthesis in Mung Beans" in Dr. Joe Cowles' laboratory at the University of Houston. He is continuing to work with Dr. Cowles, Houston, Texas.

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.

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' laboratory at the University of Michigan and the second year in Dr. Dennis Drescher's laboratory at Wayne State University. He is now an Assistant Professor at Wayne State University, Detroit, Michigan.

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

Dr. Konrad Kuzmanoff (7/1/83 - 7/30/85) is working on "Isolation and Identification of B-glucan Synthetase: A Potential Biochemical Regulator of Gravistimulated Differential Cell Wall Loosening" in Dr. Peter Ray's laboratory at Stanford University. He is continuing to work with Dr. Ray, Stanford, California.

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 working with the International Genetic Science Partnership in Talpiyot, Jerusalem, Israel.

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 the Science and Curriculum Coordinator in the Space Life Sciences Training Program at Kennedy Space Center, Florida.

Dr. Dean Murakami (1/1/85 - 12/30/86) is working 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, Davis, California.

Dr. Mary Musgrave (7/1/86 - 6/30/87) will be working on "Studies on the Respiratory Metabolism of Plants Under Space Flight

Conditions" in Dr. Boyd Strain's laboratory at Duke University, Durham, North Carolina.

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

Dr. Farrel R. Robinson, Jr. (7/1/84 - 6/30/86) is working 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.

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

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

Dr. J. Henry Slone (7/1/85 - 6/30/87) is working 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, St. Louis, Missouri.

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, Kentucky. He is now an Assistant Professor at Indiana University, Bloomington, Indiana.

Dr. Julianna Szilagyi (7/1/81 - 12/30/81) worked on "Progressive Hemodynamic Changes in Simulated Weightlessness" in Dr. Carlos Ferrario's laboratory at the Cleveland Clinic, Ohio. She is now an Assistant Professor at Baylor College of Medicine, Houston, Texas.

Dr. Yasuhiro Torigoe (1/1/84 - 12/30/85) is working 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.

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16. Abstract

This report consists of individual technical summaries of research projects of NASA's Space/Gravitational Biology Program. This Program is concerned with using the unique characteristics of the space environment, particularly microgravity, as a tool to advance knowledge in the biological sciences; understanding how gravity has shaped and affected life on Earth; and understanding how the space environment affects both plant and animal species. The summaries for each project include a description of the research, a listing of the accomplishments, an explanation of the significance of the accomplishments, and a list of publications.

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