

*NASA Conference Publication 2299*

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# NASA Space Biology Program

*Annual Symposium  
Program and Abstracts*

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*Program and abstracts of the  
Annual Symposium held in  
Arlington, Virginia  
October 12-14, 1983*

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*Annual Symposium  
Program and Abstracts*

**Thora W. Halstead, Editor**  
*NASA Office of Space Science and Applications  
Washington, DC*

**Program and abstracts of the  
Annual Symposium held in  
Arlington, Virginia  
October 12-14, 1983**

**NASA**

National Aeronautics  
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FOREWORD

The "8th Annual Symposium of the NASA Space Biology Program" was held in Arlington, Virginia on October 12-14, 1983. The activities included five-half days of presentations by Space Biology principal investigators, an evening of poster session presentations by Research Associates, and an afternoon session devoted to the Flight Experiments Program. This symposium continues to be the key U.S. annual symposium in gravitational physiology. Over 100 scientists attended this year's meeting.

I want to thank Donald R. Beam and Patricia L. Russell for their help in coordinating the symposium and Chris Bolcik and Judy Young for their valuable assistance.

Thora Halstead  
November 1983



## NASA SPACE BIOLOGY PROGRAM

## ANNUAL SYMPOSIUM

12-14 October 1983

Arlington, Virginia

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SESSION I: GRAVITY RECEPTOR MECHANISMS



A. PLANT GRAVITY RECEPTORS AND TRANSDUCTION





AMYLOPLAST MOVEMENT IN LIVING STATOCYTES

A. Carl Leopold and Fred Sack, Cornell University  
NASA Grant NSG-NAG-W3

Much evidence implicates amyloplast movement in plant graviperception, but it is not known how this signal is transduced into a differential growth response. Based on sedimentation rates derived from fixed tissues (1  $\mu\text{m}/\text{min.}$ ) it has been argued that amyloplasts cannot reach the lower wall during the presentation time (10-40+ sec.). Studies using fixed tissue are useful for deriving mean sedimentation rates, but cannot yield data on: (1) the movement of individual amyloplasts (2) the role of cytoplasmic streaming (3) the initial dynamic events occurring during the presentation time.

These limitations have been overcome by examining living tissue sections with a horizontally mounted microscope connected to a video camera and recorder. The use of Nomarski optics, an oil immersion objective and video contrast enhancement enable visualization of streaming and cell structure with high resolution. Gravistimulation is achieved by stage rotation. The cells studied were from the inner mesophyll, 1-3 mm from the tip of corn coleoptiles.

Amyloplast behavior is quite heterogeneous. Sedimentation may begin at different times and proceed at different rates (1-150  $\mu\text{m}/\text{min.}$ ). Amyloplasts often move through different pathways despite a similar starting position. They move singly or in clumps. While most amyloplasts sediment, some move laterally or upward. Similarly, once sedimented, amyloplasts often rise up and resediment. Statistically, amyloplasts spend more time near the lower wall, but amyloplasts were found in other parts of cell in vertically oriented, living and fixed tissue.

This heterogeneity in amyloplast movement largely results from an interaction between sedimentation and streaming. Amyloplast position with respect to a stream as well as the vigor, direction and size of the stream all affect plastid movement. Thus, lateral and upward movements occur when the strength of the stream overcomes the tendency towards sedimentation. Sometimes the amyloplasts are suspended in a network of fine cytoplasmic strands which appear remarkably narrow compared to the diameter of the amyloplast. Corn rootcap statocytes also contain fine cytoplasmic strands and show marked particle saltations.

A small but significant fraction of the amyloplasts reaches the lower wall of the coleoptile statocytes within the presentation time, calculated to be less than 40 seconds. This fraction includes rapidly sedimenting amyloplasts (e.g. those located in vigorous, downward streams) or those previously risen up from a sedimented position. Examination of cell sections from coleoptiles fixed at various times after gravistimulation confirms the conclusion based on videomicroscopy that some sedimentation is detectable by 30 seconds.

The kinetics of the early response to reorientation are consistent with the hypothesis that amyloplasts act by contact with a sensitive surface near the new lower wall and indicate that cytoplasmic streaming may provide an important vector in amyloplast redistribution in response to gravity.

INHIBITION OF GRAVITROPISM IN OAT COLEOPTILES BY CALCIUM CHELATION

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NASA Grant NSG 7480

Introduction When growing stems of sunflower and coleoptiles of corn and oats are moved from a vertical to a horizontal position, there is a rapid asymmetric redistribution of calcium across these tissues, resulting in significantly more calcium in cells on the upper side. In oat coleoptiles, much of this calcium, we have found, accumulates preferentially in the walls of cells on the upper side. Gravitropic curvature in coleoptiles and dicot stems occurs mainly as a result of the diminished growth of cells on the upper side of these organs. Since raising the calcium concentration of plant cell walls can inhibit cell wall loosening, and since the gravitropically stimulated calcium redistribution occurs before any visible bending, we have postulated that the calcium redistribution may be a necessary step in the events leading to gravitropism.

Plasma membrane located CaATPases are thought to be involved in pumping calcium out of cells into walls, and these pumps appear to be controlled by the calcium-activated regulatory protein, calmodulin. We found that the calmodulin antagonist, chlorpromazine, can block both gravitropism and the asymmetric redistribution of calcium in oat coleoptiles, without inhibiting their growth. These results called attention to the possibility that calmodulin, through its regulation of calcium pumps, may also participate in the transduction sequence which couples stimulus to response in gravitropism.

The calcium chelator ethylene glycol bis ( $\beta$ -amino ethyl ether) tetraacetic acid (EGTA) may be used as a probe to test whether calcium movements are important for gravitropism, since it can both chelate wall calcium and possibly interfere with the activation of calcium-dependent plasma membrane pumps. Here we report the effects of EGTA on the gravitropism of oat coleoptiles.

Methodology To improve reagent penetration, the coleoptiles to be treated were either excised at the base or had their apical tip (1 mm) removed before being perfused with the test solution. Plants were perfused with water or EGTA (10, 1, or 0.1 mM) while being rotated at 2 rpm on clinostats which allowed either continuous tumbling to negate any net gravitropic stimulus, or maintenance of one orientation of the coleoptiles to gravity during infiltration. Plants were kept in total darkness during clinostat perfusion and the post-perfusion measurements of length and bending height were quickly performed under dim green lights to the nearest 0.5 mm.

Results to Date A treatment period as brief as 2 hours in 1 mM EGTA completely blocks gravitropism in 50-60% of the treated coleoptiles without inhibiting growth. Only about 10% of the plants perfused in water failed to exhibit gravitropism. Subsequent perfusion of EGTA-treated plants with calcium completely restores gravitropism; post-perfusion with water does not. After perfusing in water for 10 hours, oat coleoptile segments show the same asymmetry of  $^{45}\text{Ca}$  distribution as reported earlier for non-perfused coleoptiles and sunflower hypocotyls. The degree of this asymmetry is reduced in those coleoptiles partially inhibited by perfusion in EGTA and is essentially absent in those coleoptiles completely inhibited by EGTA.

Discussion The results indicate that some cellular event necessary for gravitropism can be inhibited by EGTA without interfering with overall growth. The fact that calcium relieves this inhibition demonstrates both that the inhibition is reversible and that the inhibition was probably due to a reduction in the availability of free calcium required for one or more of the transduction steps of gravitropism. At the near neutral pH used in this study, EGTA is charged and would not be expected to readily cross membranes. One of its primary effects, then, is probably the binding of free calcium in the apoplastic space exterior to the cell membranes of coleoptile cells. This space would then have a low free calcium concentration and be buffered against changes. If a change in calcium concentration in the walls of some coleoptile cells were required for gravitropic growth to occur, the primary site of the inhibitory effects of EGTA on gravitropism would be in those walls. To date, all inhibitors known to block the asymmetric distribution of calcium in gravistimulated coleoptiles also block gravitropism.

## PROTEIN CHANGES IN LEAF-SHEATH PULVINI OF BARLEY (HORDEUM) INDUCED BY GRAVISTIMULATION

Name: Peter B. Kaufman and Il Song  
Affiliation: The University of Michigan  
NASA Grant NAGW-34

Some proteins from gravistimulated pulvini were analyzed by SDS-PAGE and a microdensitometer. The levels of salt-soluble proteins in the bottom halves of 24-hrs. gravistimulated leaf-sheath pulvini of barley plants increased amounts of some proteins (probably cellulase and invertase) as compared to the top halves.

### MATERIALS:

Barley plants (Hordeum) were grown at The University of Michigan Botanical Gardens for about 45 days. Shoots of those plants were gravistimulated for 2, 4, 8, 12, 24, and 48 hrs. in dark at 30° C. The graviresponding pulvini were excised and separated into 2 fractions: top halves and bottom halves. These tissues were used for SDS-PAGE analysis of the protein banding patterns.

### EXTRACTION OF PROTEINS:

The tissues were ground in a mortar with 0.02M Na<sub>2</sub>HPO<sub>4</sub>- citric acid buffer (pH 7.0) in 0.025M NaCl for 30 min. at 4° C. The extract was centrifuged at 2,000 x g for 15 min. at 4° C. A 5% final concentration of TCA was added to the supernatant. This fraction was then centrifuged at 12,000 x g for 30 min. to precipitate salt-soluble proteins.

A 0.05M borate buffer (pH 10.0) was added to the pellet residue from the salt-soluble protein extraction and was shaken overnight at 300 rpm with a gyrorotary shaker. This mixture was centrifuged at 2,000 x g for 15 min. A 5% final concentration of TCA was added to the supernatant. The supernatant was then centrifuged at 12,000 x g for 30 min. to precipitate alkali-soluble proteins.

### SDS-PAGE:

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical slab gel instrument (The Protein Double Slab Electrophoresis Cell, Bio-Rad) by a procedure adapted from Laemmli (1970). The gel dimensions were 1.5 by 180 by 160 mm, and the acrylamide concentration was 10%. The standard proteins used were phosphorylase B (Mol. Wts., 92,500), bovine serum albumin (Mol. Wts., 66,200), ovalbumin (Mol. Wts., 45,000), carbonic anhydrase (Mol. Wts., 31,000), soybean trypsin inhibitor (Mol. Wts., 21,500), and lysozyme (Mol. Wts., 14,400). Electrophoresis was conducted for 2 hrs. at 76 mA and stained with coomassie blue, silver stain, or silver stain followed by coomassie. The gels were photographed and scanned with the microdensitometer (Joyce, Loebel).

### RESULTS:

The SDS-PAGE patterns of salt-soluble proteins elicited by gravistimulation were shown in the top and the bottom halves of the gravistimulated pulvini as follows; at

least five proteins were increased in tissues derived from bottom halves of the pulvini in the approximate molecular weight ranges of 91, 57, 50, 22, and 17 kilodaltons. SDS densitometric scans indicated that two of them are probably cellulase and invertase.

Currently work is being performed to confirm the identification of these proteins.

I acknowledge the help of Ming Lin Hsieh, Paul Thompson and Dennis Irman who participated in this study and thank Thora Halstead for her suggestion that we undertake this type of analysis in our pulvinus system.

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

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NASA Grant NSG-NAGW-97, ORD 31479

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Resume: Gravistimulation causes an asymmetric distribution of the plant growth hormone, indole-3-acetic acid (IAA). We intend to determine in what tissue of the plant the IAA asymmetry arises so as to better localize the gravity sensing device.

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Recent studies from this laboratory have contributed three new facts to our knowledge of the response of plants to a gravitational stimulus: First, we confirmed by an unambiguous gas chromatographic-mass spectrometric method that the distribution of the growth hormone IAA, was affected by gravity (1); second, we showed that the hormone asymmetry occurred in which the cortical tissue of the stem - tissues which do not contain a vascular stele (2); and thirdly we showed that the hormone asymmetry was always 60:40, that is, 60% of the free IAA on the lower side of the horizontal stem and 40% on the upper side (2,3).

Now, by rather indirect reasoning, and owing to the peculiarly high concentration of free IAA in the stele and the relative paucity of free IAA in the cortical cells (4) we reasoned and postulated that the asymmetry arose in the vascular stele (2,5). Briefly stated the reasoning was that since so much of the free IAA occurred in the stele, and so little in the cortex, that a 60:40 asymmetry could not arise unless the asymmetry prevailed in the stele - just as it does in the cortex.

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This conclusion is important because it suggests that the early response to gravity may occur in the vascular stele and only secondarily be expressed in the cortex with the ensuing growth.

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The problem now is to test this hypothesis, and again we must return to methods - with an even greater demand for both precision and sensitivity. Mrs. Schulze has developed a method that we think will provide the sensitivity to directly test for IAA asymmetric distribution in the stele. The tissues - about one gram - are collected, frozen over solid CO<sub>2</sub>, thawed with acetone to which has been added [<sup>3</sup>H]-5-IAA, as internal standard (6), homogenized, filtered, concentrated, and chromatographed over a DEAE column (5). The IAA containing fractions are then pooled and the IAA converted to the pentafluorobenzyl ester (7) (PFB-IAA). This is chromatographed on a 5 micron C<sub>18</sub> HPLC column and the IAA containing fractions collected and pooled. The PFB-IAA is chromatographed by direct on-column capillary gc using a Varian cryogenic injector. The specific activity of the PFB-IAA is determined and then the quantity of IAA in the plant can be calculated. The method has been validated both for free IAA and IAA liberated by alkaline hydrolysis by a GC-SIM-MS method (1).

A second approach is being utilized by Dr. Yoshie Momonoki involving use of [<sup>3</sup>H]-5-indole-3-acetyl-myoinositol synthesized in our laboratory (6). IAA-myoinositol is a major IAA ester in both endosperm and shoot of corn (Chisnell, In Press). Dr. Momonoki has applied the [<sup>3</sup>H]IAInos to the endosperm of kernels of young corn shoots and is now studying the effects of gravistimulation upon the distribution of this conjugate of IAA.

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In summary, our approaches are leading us to the tissue in which the gravitationally-induced IAA asymmetry first occurs.

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THE ROLE OF CALCIUM IN THE GRAVITROPIC RESPONSE OF ROOTS

Michael L. Evans  
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NASA Grant NAGW-297

Previous research has indicated that gravity-induced calcium redistribution may play an important role in the gravitropic response of shoots. Calcium has been shown to move toward the upper side of gravistimulated shoots (1), and inhibitors of the calcium-activated regulator protein, calmodulin, have been shown to interfere with the gravitropic response of coleoptiles (2). We have examined the potential involvement of calcium redistribution in the gravitropic response of roots by testing: 1) the effect on gravitropism of calcium chelators applied to the root cap, 2) the ability of calcium gradients applied across the root cap to induce gravitropic-like curvature, and 3) the influence of gravity on the movement of  $^{45}\text{Ca}^{2+}$  across the root cap.

Calcium chelating agents (EDTA, EGTA) applied to the root cap of maize roots caused a loss of gravitropic sensitivity by the root. Gravitropic sensitivity could be restored by replacing the chelator with calcium chloride. These results indicate that a supply of mobile calcium in the root cap may be important in linking gravistimulation to the gravitropic growth response. This idea was further supported by the finding that asymmetric application of calcium chloride to the tips of vertically oriented maize roots caused gravitropic-like curvature toward the calcium source.

Roots which had been made insensitive to gravity by surgical removal of the root cap could not be made gravitropically sensitive by symmetric application of calcium to the decapped tip. However, decapped roots curved strongly toward a source of calcium applied asymmetrically to the decapped tip.

The movement of calcium laterally across intact roots was measured by applying  $^{45}\text{Ca}^{2+}$  in agar donor blocks to one side of the root and measuring the accumulation of radioactivity in agar receiver blocks on the opposite side. Using this method, it was found that strong lateral movement of calcium occurred across the root cap, with little lateral movement across the elongation zone or across the tips of decapped roots. When roots were oriented horizontally, the lateral movement of  $^{45}\text{Ca}^{2+}$  across the root cap became strongly (about three fold) polarized in the downward direction. These data indicate that gravistimulation causes polar movement of calcium toward the bottom of the root cap and that the calcium gradient may be a key factor in triggering the asymmetric growth which causes gravitropic curvature.

Further evidence that gravity-induced calcium redistribution may be important to the gravitropic growth response was obtained



in experiments testing the effect of light on the development of gravitropic sensitivity and on the occurrence of gravity-induced polar calcium movement in roots of maize. Roots of several cultivars of maize are known to lack gravitropic sensitivity when the seedlings are germinated and raised in the dark (3). In such cultivars, gravitropic sensitivity can be restored by exposure of the roots to light. When  $^{45}\text{Ca}^{2+}$  movement was measured in gravistimulated dark-grown seedlings of a light-requiring cultivar of maize, no polar calcium movement across the root cap was detected, and no gravitropic curvature occurred. When the dark grown seedlings were illuminated, the development of gravitropic sensitivity was paralleled by the establishment of polar calcium movement across the caps of gravistimulated roots. These results strengthen the possibility that gravity-induced polar calcium movement across root caps plays a role in the gravitropic growth response.

The relationship of calcium redistribution in the root cap to the establishment of asymmetric growth in the elongation zone remains unknown. Earlier work (4) has shown that cell to cell auxin transport depends upon simultaneous counter transport of calcium. We have found that treatment of the root tips of intact maize seedlings with inhibitors of auxin transport (naphthylphthalamic acid, triiodobenzoic acid, morphactins) prevents both gravitropism and gravity-induced polar transport of calcium across the cap. These findings indicate that calcium movement and auxin movement are closely linked in roots, and they raise the possibility that gravity-induced calcium gradients at the root tip lead to modified growth patterns in the elongation zone through calcium controlled movement of auxin in the root.

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CALCIUM ELICITED ASYMMETRIC AUXIN TRANSPORT IN GRAVITY  
INFLUENCED ROOT SEGEMENTS

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NASA Grant NAGW-368 and NSF Grant PCM-8207147

Auxin is a prime candidate for regulating and modulating the differential growth response of primary corn roots to gravity. Auxin, indole-3-acetic acid (IAA), can both promote and inhibit root elongation rapidly within a narrow concentration range. Thus growth regulation would require only small changes in auxin flux and cellular auxin concentration which in turn could be rendered in the short lag period for initiation of gravitropism. Since auxin is transported to/through the zone of elongation toward the meristem, it may serve as a direct communication link between the zone of elongation, site of gravitropic response, and the root cap (RC), site of gravity perception. When auxin transport is inhibited, gravitropism is also inhibited. Naphthylphthalamic acid (NPA) is one such inhibitor. It inhibits gravitropism only when applied to the apical growing and dividing region of the root. Application at the basal end of the root does not influence gravitropic curvature. NPA causes upward curvature when applied to the upper surface of horizontal, two day-old, intact corn roots. This effect is countered by application of IAA to the opposite side. NPA effects auxin accumulation in 1mm slices of apical root tissue but does not affect abscissic acid (ABA, another possible mediating hormone) net uptake. Such evidence strongly implicates IAA and its transport in the molecular control of root gravitropism.

In light of the accumulating evidence from Mike Evans' laboratory that calcium in the RC is important in determining the direction of gravitropic curvature in roots we have been investigating the relationships between IAA transport and apical calcium. In order to establish a mechanism for communication/regulation between the elongation zone and the RC of graviresponding roots we are researching the effects of calcium on lateral transport, acropetal/basipetal transport, and asymmetric distribution of IAA.

Corn seedlings were germinated vertically at 30°C for 2 days in light. Seedlings with straight roots 1-3cm were selected. Since both removal of the RC and EDTA (a calcium chelator) applied to the RC can prevent gravitropic curvature, it has been suggested that the source of calcium which elicits an effect on gravitropism is in the RC (Lee and Evans, personal communication). To test the effect of calcium and EDTA on acropetal IAA transport, the RC was gently teased off the meristem and replaced with an agar receiver block containing calcium, EDTA, or buffer alone. An apical 6mm segment was excised from the decapped root and held vertically in a plexiglas holder, the apical end on the receiver block. Donor blocks containing 3H-IAA were applied at the base. After 1.5 hr transport period the receivers were collected. 1mM calcium in the receiver did not effect efflux of label into the receiver. However, EDTA significantly reduced the label in the receivers whereas ABA enhanced the label content. EDTA was only inhibitory of label effluxing from the meristem. When acropetal transport was investigated in 6mm segments taken 3mm behind the root cap EDTA was not effective. The effect of EDTA at the meristem can be reversed by replacing EDTA with a calcium block. Calcium in the RC may regulate IAA translocation from the meristem to the RC.

Does asymmetrical calcium applied at the tip influence asymmetric distribution of acropetally transported IAA? To test this decapped, excised apical 6mm root segments were placed horizontally. Donor blocks were applied at the basal ends. Calcium and buffer agar blocks were placed opposite on the upper and lower surfaces of the root tip. After 1.5 hrs there was no significant cpm difference between the upper/lower receiver blocks at the tip. Asymmetrical calcium at the tip did not affect acropetal transport and asymmetrical distribution of label at the tip.

But calcium does strongly affect transport asymmetric distribution of basipetally translocated IAA. Donor blocks were applied at the meristematic apex, a calcium block placed on upper or lower surfaces of the tip, and receivers positioned on the upper/lower surfaces of the basal end of the segment. Asymmetric calcium at the tip resulted in greater accumulation of label in the basal receiver block on the same side as the apical calcium block. The opposite receiver block contained less label than that of controls. We are currently determining the identity of the label collected in the receiver blocks. Preliminary results will be presented.

In summary, calcium has been found to affect auxin acropetal efflux from the meristem and to elicit asymmetric transport of auxin travelling basipetally from the meristem. Calcium research has suggested that graviperception results in asymmetric calcium accumulation on the lower side of the root tip. We wish to suggest here that this calcium redistribution and asymmetry effects a reciprocal asymmetric distribution of auxin in the elongation zone by affecting a greater basipetal transport of auxin towards the site of gravitropic response. We will be testing this hypothesis further in intact roots with and without the root cap.

MECHANISM OF SHOOT GRAVITROPISM

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The objective of this research is to achieve a better understanding of the cellular basis of plant shoot gravitropism. Part of my recent work has centered on a critical evaluation of the role of auxin gravitropism. We have also begun work to evaluate an alternative hypothesis which links  $\text{Ca}^{+2}$  fluxes to the asymmetric growth that leads to gravicurvature.

Data will be presented showing the speed at which  $^3\text{H}$ -auxin is redistributed to the lower most tissues of gravistimulated Helianthus hypocotyls is rapid enough to be a causal agent for asymmetric growth. It will be shown that a proton gradient across the tissue is not necessary for auxin redistribution. This in turn suggests the lateral transport of auxin is the cause rather than the result of asymmetric acid efflux. Lastly, I will show that the magnitude of auxin redistribution (as determined by the movement of  $^3\text{H}$ -IAA) is sufficient to produce a substantial amount of shoot curvature. Collectively, these data suggest auxin plays a key role in shoot gravitropism.

Recent data obtained by Stanley Roux, Michael Evans and others, suggests that  $\text{Ca}^{+2}$  fluxes may also be involved in gravitropic responses. According to this hypothesis, graviperception in shoots initiates a redistribution of  $\text{Ca}^{+2}$  toward the upper most tissues (i.e. opposite to the lateral transport of auxin). This asymmetry then might cause or amplify the differential growth response that results in shoot curvature.

- Central to the  $\text{Ca}^{+2}$  flux hypothesis are three assumptions:
- 1 ) Increased levels of cell wall  $\text{Ca}^{+2}$  inhibit cell extension.
  - 2 ) Decreased levels of cell wall  $\text{Ca}^{+2}$  accelerate growth.
  - 3 ) A  $\text{Ca}^{+2}$  asymmetry develops across shoots rapidly enough to be involved in asymmetric growth.

Data will be presented showing exogenous  $\text{Ca}^{+2}$  at 5 mM or above does inhibit both straight growth and gravitropism in Helianthus hypocotyls. However, lower levels either have no effect or stimulate curvature. Further, I will show that growth promotions caused by EGTA (usually taken to indicate  $\text{Ca}^{+2}$  chelation enhances growth) may be artifacts which derive from the protons released when EGTA chelates  $\text{Ca}^{+2}$ . Lastly, I will present data showing that in our hands gravistimulation does not result in a  $^{45}\text{Ca}^{+2}$  asymmetry. Together, these data indicate that we are far from being able to say with any certainty that  $\text{Ca}^{+2}$  fluxes mediate shoot gravitropism.

PROTEIN AND CAROTENOID SYNTHESIS AND TURNOVER IN GRAVISTIMULATED ROOT CAPS

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In certain cultivars of corn gravitropic bending occurs only after the root cap, the site of gravity perception, is exposed to light. Light appears to trigger or to remove some block in the gravity translation process. Using light-sensitive cultivars of corn we have shown that light affects various processes in the cap. Our current research is directed at elucidating the roles of these light-induced processes in gravitropic bending in roots.

Earlier we showed that protein synthesis in the root cap is required for gravitropic root bending. We now report that light preferentially enhances the synthesis of specific proteins. Using one- and two-dimensional gel electrophoresis we have analyzed the protein patterns from caps maintained continuously in the dark and have compared these patterns with proteins obtained from caps provided light. From our one-dimensional gels we have shown that within one-half hour of the light treatment the level of a low molecular weight protein(s) is enhanced markedly. Moreover, the turnover of these light-induced proteins is rapid, usually within an hour of formation. More refined analysis of these proteins was accomplished by using a double label ( $^{35}\text{S}$  and  $^3\text{H}$ ) coupled with one- and two-dimensional gel electrophoresis. The double label work confirmed the rapid light-induced synthesis and subsequent turnover of these proteins. We also were able to show that auxin was required for this synthesis. In caps maintained in tissue culture on medium lacking auxin, enhanced protein synthesis was not observed when the caps were illuminated.

Using two-dimensional gel electrophoresis coupled with the double label we had hoped to show whether the effects of light on protein synthesis were qualitative or quantitative. We thus far have been unable to demonstrate any qualitative differences in protein profiles between light versus dark or between plus versus minus auxin treated caps. We therefore conclude that light in combination with auxin leads to an enhancement in the levels of specific pre-existing proteins, rather than preferentially stimulating the de novo formation of selected proteins.

Carotenoid levels in root caps are markedly stimulated by light, with a 1.6-fold increase compared to roots maintained continuously in the dark. If roots are provided light, returned to the dark and after varying periods of time extracted for carotenoids we find that these pigments reach their maximum levels  $\frac{1}{2}$ -1 hour after the light treatment. After 3 hours in the dark the levels of carotenoids in the root caps diminish to values recorded for caps maintained continuously in the dark. This turnover of carotenoids corresponds temporally with the previously reported loss in the ability of light-treated roots to respond to gravity. From spectrophotometric data we have identified one carotenoid in particular, violaxanthin. This pigment has been suggested to serve as a precursor for substances with growth inhibitory properties. In earlier work the asymmetrical redistribution of an inhibitory growth substance in the root cap has been associated with gravitropic bending in roots.

Finally we report that we have had some success in the isolation of intact amyloplasts from root caps. This organelle is very fragile and successful isolation depends first on preparing protoplasts from the cells of the root cap.

SMALL GRAVITATIONALLY ELICITED VOLTAGE TRANSIENTS IN PEA STEMS

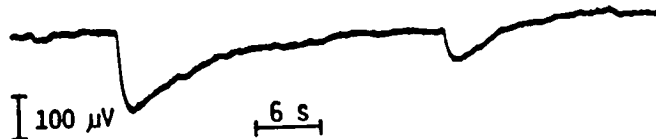
Barbara G. Pickard

Washington University in Saint Louis

NASA Grant NAGW-420

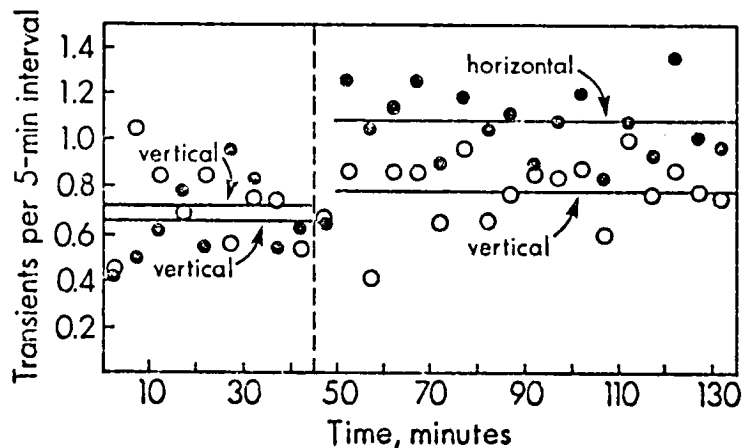
Experimental procedures. Seedlings of garden pea, *Pisum sativum* L., were grown on moist paper towels in the dark. Some were depleted of auxin by removing the plumule and hook 15 h prior to experimentation, according to the methods of Scott and Briggs (Amer. J. Bot. 50: 652, 1963). Excised shoots with or without the plumule and hook were mounted individually in vials filled with 2 mM KCl solidified with 1% agar. The stem was centered in a 3-mm-wide slot milled in a 6-mm-thick sheet of lucite so that the apical cut surface or the hook extended just above the slot. A 60  $\mu$ l drop of 50 mM KCl was placed in the slot so that it wrapped 3/4 way around the stem and maintained its position because of surface tension. A low-noise electrode made contact with the drop via a KCl and agar-filled tunnel drilled in the lucite. The assembly was mounted on a vibration-damped steel plate. The signal from the electrode was coupled to a Tektronix 3A3 amplifier by way of a band pass filter with -3 dB points of approximately 13 mHz and 2.8 Hz. This screened out the dc drift which occurred e.g. during plant response to auxin or to rubbing. The signal was displayed in the storage mode at amplification of 10  $\mu$ V mm<sup>-1</sup> and a sweep speed of 2 mm s<sup>-1</sup> on a Tektronix 564B oscilloscope. The operations were carried out at 24°C and 80% relative humidity in the dark, with minimal use of a dim green safelight.

Characteristics of the voltage transients. Typical extracellularly observed transients are illustrated below. The risetimes (10-90%) are approximately 200 ms, and the amplitudes range up to 600  $\mu$ V.



Gravitropic elicitation. Decapitated vertical stem segments were observed for 45 min, noting the number of transients  $>20$   $\mu$ V for each 5-min interval. Results for two sets of stem segments are plotted below; the average frequencies were  $0.72 \pm 0.05$  and  $0.66 \pm 0.04$  per 5 min.

One set of segments was swiveled gently to the horizontal position; then, recording continued for another 90 min. Transients continued in the vertical controls at a closely similar average rate  $0.78 \pm 0.04$  per 5 min. However, after the first 5-min interval the horizontally placed plants exhibited transients at an increased average rate of  $1.08 \pm 0.04$  per 5 min.



$n_{\text{expt}} = 28$

$n_{\text{cont}} = 22$

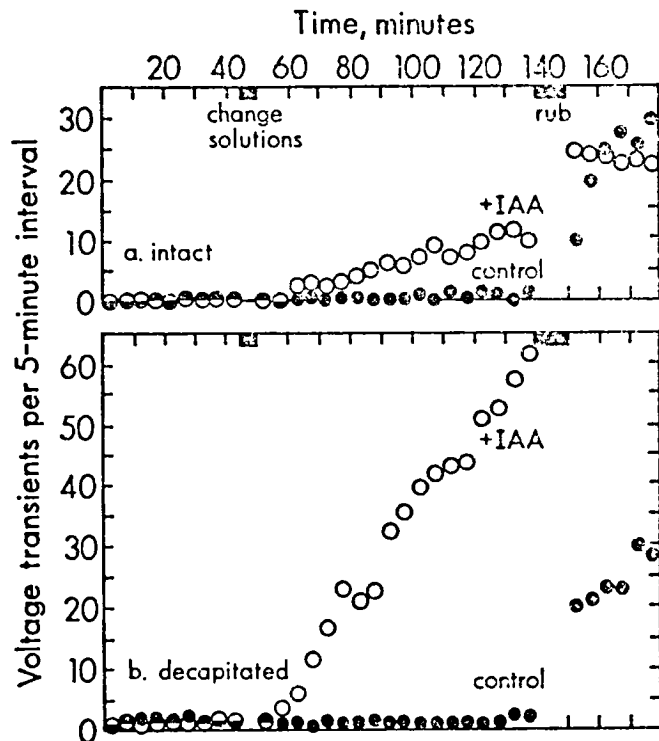
t-tests showed that the ~1.6-fold increase over background was statistically significant, with  $p < 0.1$  for each of the three possible comparisons.

The frequency of transients also increased following horizontal placement of intact shoots. However, it appears that this increase had two components: one due to gravity reception, and one due to the redistribution of indoleacetic acid (IAA) believed to mediate gravitropic curvature (or, more specifically, to the increase of IAA in the lower tissue). This interpretation accords with the following experiment.

Elicitation by IAA. After transients were recorded from two sets of intact shoots for 45 min, the contact drops of one set were replaced with 500  $\mu\text{M}$  IAA and recording was continued for another 90 min. As can be seen in Part a of the figure below, the frequency of the transients underwent increase. A very small increase was also measured for 100  $\mu\text{M}$  IAA.

The concentration of 500  $\mu\text{M}$  was chosen because, when applied to stems essentially as described above, it stimulates ethylene production as measured by gas chromatography. It appears that the abilities of a given treatment with IAA to stimulate ethylene production and to elicit transients may be closely correlated. There is considerable evidence that IAA on the lower side of a pea stem does reach concentrations high enough to enhance ethylene formation.

Elicitation of transients by application of IAA to IAA-depleted tissue is particularly dramatic: in Part b of the figure, the frequency rises fifty fold within the experimental period.



The frequency of transients also rises following both friction and flexure, which (like high concentrations of IAA) lead to enhanced ethylene production. Several other agents which stimulate ethylene production have additionally been found to elicit transients.

Remarks. The experiments were performed in partial test of a certain model for gravity reception. I suggest that the demonstrated small but significant gravitropic increase in voltage transients has only a secondary import for gravitropic curvature, and that transients are crosstalk which results because the sequence of receptive and mediational events has a step sharing certain properties with a step in friction, flexure and IAA elicited ethylene production.

MECHANISMS OF GRAVIPERCEPTION AND RESPONSE IN  
PEA SEEDLINGS

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NASA Grant NSG - 7290

During the past year, we have conducted research in two areas of interest to NASA: gravitropism and stress physiology. We reported at a previous Space Biology Symposium on a new method for the mass isolation and purification of multigranular amyloplasts from the bundle sheath parenchyma of etiolated pea epicotyls. These bodies, which displace within 2-3 minutes of exposure to 1 x g, are probably the gravity receptors (statoliths) in this plant. We have characterized these amyloplasts as having a double-membrane with a surface-localized ATPase, a high calcium content, and their own genomic DNA. We published a manuscript on this subject during 1983 which probably describes for the first time a repeatable technique for the isolation of statolithic amyloplasts from any plant (1). We are continuing to investigate these amyloplasts as to (a) the reasons for their especially high density, probably related to their starch content, (b) the possible identity of their DNA with the DNA of chloroplasts and unigranular amyloplasts, and (c) possible importance of their high calcium content.

Believing that "plants in space are plants under stress", we have enlarged the ongoing research on polyamines in our laboratory to encompass the remarkable stress-induced rises in the levels of a diamine, putrescine, because of increases in the synthesis and/or activity of an enzyme, arginine decarboxylase, which produces putrescine from arginine via agmatine. We have shown that this enzyme rises in response to water stress (2), pH stress (3) and nutritional stress (4). We are currently seeking to find a possible connection between polyamine metabolism with stresses induced by mechanical stimuli, including those induced by centrifugation and shaking.

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## B. ANIMAL GRAVITY RECEPTORS AND TRANSDUCTION



MAMMALIAN GRAVITY RECEPTORS: STRUCTURE AND METABOLISM

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NASA Grant NSC - Grant #NSG 9047

We are using Kratos high performance liquid chromatography (HPLC) instrumentation for amino acid analysis of rat otoconial complexes. Because we are using post column reaction of the separated amino acids with O-phthaldialdehyde (OPA), to make them fluorescent, and a fluorescence detector theoretically capable of detection in the 10 pmole range, we have been able to analyze the amino acids of otoconial complexes pooled by origin from only 10 rats. Our experimental findings indicate further that it should be possible to analyze complexes from only 3 rats, and perhaps fewer. This means that the method should be applicable to material from Space-flown rats even though only six animals might be available for study. The actual findings suggest that the organic otoconial phase is comparable in its complement of acidic amino acids to other calcium carbonate containing materials such as fish otoliths and certain mollusc shells. The organic material is high in acidic amino acids; and the relative proportions of aspartate, glutamate, threonine and serine appear to be similar to those found in neogastropod shells. This has significance in terms of the evolution of biomineralization processes occurring in the animal kingdom.

Another finding of evolutionary significance is that calcitic-type otoconia (rounded bodies and pointed ends) appeared in the vertebrate series prior to the evolution of birds and mammals, in which only calcitic otoconia are present. Aragonitic otoconia predominate in non-bird, non-mammal vertebrate species. Nevertheless, we found that calcitic-shaped otoconia occur in the turtle utricle and in all three receptor areas of the alligator vestibular system (utricle, saccule and lagena). The alligator saccule, however, contains a mixture of otoconial configurations, so that it is lagging behind the utricle in terms of the evolution of calcitic-type otoconia. The reptiles used in this evolutionary study were selected because of their positions on the phylogenetic tree. Turtles are considered to be close to the stem-line giving rise to mammals while alligators, which are more highly evolved than turtles, share a common ancestry with birds.

Aside from establishing that the calcitic otoconial type is phylogenetically old, the finding of different otoconial configurations in the saccule and utricle of reptiles provides one more indication that these vestibular areas are not confluent and that their ionic environments may differ. (It should be noted that we have not yet established whether the calcitic type of otoconium in alligators and turtles contains aragonite or calcite).

We have additional, ultrastructural findings of great interest in terms of the functioning of gravity receptors. These results are emerging from studies supported by the Flight Program and by this grant. In brief, we are demonstrating that the afferents supplying calyceal nerve endings to the Type I hair cells also supply Type II hair cells in specific patterns. These appear to form cell clusters that are interrelated functionally. Of equal importance, in physiological terms, the afferents give origin to at least one kind of vesiculated ("efferent") terminal ending within the macular neuroepithelium. Some of these vesiculated terminals can be shown to synapse back upon the parent "afferent" nerve fiber and thus correspond to autapses. They may function to boost signal transmission along the long, unmyelinated afferent nerve fiber. We have also been able to show that there are numerous interactions between "afferent" and "efferent" nerve fibers near the base of the neuroepithelium. The findings mean that integration of sensory information is taking place peripherally, before signals are transmitted centripetally.

AMPLITUDE DISTRIBUTIONS OF THE SPIDER  
HEARTPULSE IN RESPONSE TO GRAVITATIONAL STIMULI

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NASA Grant NAGW-242

The arachnid Nuctenea sclopetaria (Clerck), formerly A. sericatus, possesses a neurogenic heart. The heartbeat is under efferent control through a dorsal nerve arising from a brain center. Our previous reports have shown that the heartrate of this spider is also modulated by an afferent input associated with small increments of gravity. A compressive force on the order of 40 microN is sufficient to elicit a threshold change in heart-rate for a typical (100mg) spider. This obtains in a hyper-Gz field less than 1.001. The functional relationship between gravity and heartrate is logarithmic between the absolute threshold and at least 1.5 Gz. Based upon these findings and other observations of spider behavior a model has been proposed in which equilibrium and movement are maintained by changes in blood pressure. Thus, we have come to view the arachnid equilibrium system as a "weight detector" which employs a hydraulic compensatory mechanism.

During the course of the studies on gravity reception changes in the amplitude of the heartpulse became evident. Unlike the pulse of animals with myogenic hearts (i.e. the vertebrates) both rate and amplitude changes are remarkably variable, i.e. non-periodic in the spider. Peak to peak measurement cannot adequately describe these amplitude changes. (There are other examples among physiological response measures that also display non-periodic or pseudo-random waveforms: EEG, EMG.) The most important single measure with which to describe the amplitude characteristics of a complex wave is the root-mean-square (RMS) value. The RMS statistic ( the standard deviation of the waveform ) is related to the energy content of the alternating phenomena according to a square law and this could provide insights on the energetics of the arachnid cardiovascular system.

During the year we programmed a computer system which, either from magnetic tape or on-line, samples the cardiac response under different gravity conditions, digitizes and sorts the pulse amplitudes, displays the amplitude distribution, and calculates the RMS value. The distribution is the amplitude density function (ADF). The normalized ADF is the probability density function. The ADF gives insight into the statistical nature of the cardiac signal. The ADF calculated for the spider heart, whether taken at Gz=1 or in hyper-Gz appears "bell shaped". Statistical tests for normality indicate that the pulse amplitudes are distributed according to a Gaussian model. This confirms that the RMS measure is appropriate for a description of the changes in cardiac amplitude.

Our centrifuge has been modified; it now contains a laser diode and photodetector within the spider cradle. Consequently we can now measure the cardiac activity during continuous or in response to brief changes in radial acceleration. The ADF's taken from individual animals suggest that pulse amplitude may also be logarithmically related to Gz. This implies that as the functional weight of the animal is increased larger pulse amplitudes are interposed on the ongoing heartbeat. We can't be

sure at present whether these more vigorous beats appear randomly or in some time pattern.

We now have good evidence that the absolute threshold to  $G_z$  is exquisite in the orb-weaver. One might expect that the spider can make fine discriminations between  $G_z$  changes. The size of the JND (just noticeable difference) would expose this. It is, however, not necessarily the case that a low absolute threshold also predicts the fine sensitivity to stimulus differences. An example which comes to mind is electric shock in which threshold sensitivity is low but the discrimination (in human beings) of various levels of shock is quite poor. Delta  $G_z$  is a stimulus comparable to that produced by Coriolis. A brief change in the angular velocity of the centrifuge with the spider located at a fixed distance along the radius is a stimulus similar to an animal ambulating with a particular velocity in a rotating frame to a new radial location.

This year we improved our tilt table. An animal can be exposed to a tilt velocity of 0.03 degrees/sec or greater. Preliminary data indicate that the cardiac reflexes appear when tilt reaches about 2 degrees off the horizontal. Substantial individual differences have been noted. Electrophysiological recordings from the dorsal cardiac nerve are technically easy to take during tilt and we have developed a stable preparation for recording single units within the brain. Consequently we plan to examine the correlation between changes in the gravity vector and central nervous activity in the spider.

The observer of spider behavior should be impressed by the observation that in spite of her marvelous mechanical sensitivity she can literally "turn-off" unwanted stimuli. Thus, inordinately high amplitude/ low frequency vibrations, or strong winds blowing across the web may elicit little or no behavioral activity. Here we have an organism which appears to have evolved to the limits of mechanical sensitivity yet massive supra-threshold stimuli leave her imperturbed. Perhaps the CNS of the spider has evolved specific feature detectors or mechanisms of sensory inhibition which get rid of information not pertinent to the needs of the animal?



## SESSION II: PHYSIOLOGICAL EFFECTS OF GRAVITY





## A. STRUCTURAL MASS

STRUCTURAL DEVELOPMENT AND GRAVITY  
Emily Morey-Holton, Ph.D. NASA-Ames Research Center

BACKGROUND & OBJECTIVES

To validate and refine our rat model, used to simulate some of the physiological effects of spaceflight, we are continuing to define variables known to affect bone histomorphometry and physiology in this model. The variables studied during this grant period were: 1) changes in bone formation with age in two commonly used rat strains, 2) effects of cold stress on bone formation and apposition rates relative to changes in the same parameter in rats on the model.

In a 17-month study, body mass, growth rates, and tibial radiographic and histomorphometric measurements were compared in Sprague-Dawley (S/D) and Fischer 344 (F) rats aged six to sixty-eight weeks. The purpose of this study was to provide histomorphometric measurements of rat tibia from the juvenile to the adult period, and to compare tibial growth rates relative to both age and weight. The data obtained will be used to project bone formation rates in rats of these ages and strains and for planning tetracycline regimes for making these measurements.

A comparison of the physiological effects of unweighting the hind-limbs with those of cold stress was conducted in rats studied after one or three weeks of exposure. Adrenal weights, thymus weights, and corticosteroid levels were used as indicators of environmental stress. Changes in bone formation and apposition rates were measured using tetracycline labelling techniques. The objective of this study was to establish whether changes in cortical bone formation found in rats on the model are due to hind-limb unloading or involve a "stress" response.

RESULTS AND CONCLUSIONS

Growth-rate studies indicate that bone formation is most rapid at six weeks of age (S/D = 0.0575 mm<sup>2</sup>/day, F = 0.0475 mm<sup>2</sup>/day) and decreases sharply until approximately eighteen weeks of age (S/D = 0.090 mm<sup>2</sup>/day, F = 0.0110 mm<sup>2</sup>/day) when it plateaus. When plotted against age, bone formation rates for the two strains were, essentially, superimposed; whereas plotting formation versus weight and weight gain yielded parallel, but not superimposed curves. Further analyses are currently in progress.

As shown in table 1, there is a statistically significant ( $p < 0.025$ ) increase in adrenal weight between the one-week cold stressed and the one-week unweighted groups, as well as between the three-week cold stressed and three-week unweighted groups. A statistically significant ( $p < 0.001$ ) decrease in thymus weight between these same two sets of groups is also evident from table 1. Muscle weights were obtained on the gastrocnemius, extensor digitorum longus, and soleus. Only data for the soleus is listed in table 1 since it was the one muscle in which true atrophy was evident.

Bone formation rates, expressed as percent change from control for consecutive weeks of experimentation for both cold stressed (cs) groups and unweighted (uw) groups, showed dramatic drops during the first week of experimentation (-28% for both groups). By the second week, rates in cs were beginning to return to control levels (-16%), whereas uw were still suppressed. By the third week, cs animals were only slightly different from controls (-10%) while uw animals were -20%. These data suggest that only the initial changes in bone formation in uw rats could be related to "stress"; however, any influence of glucocorticoids on bone formation in uw rats at this time period is questionable because of adrenal and thymus weight differences.

PARAMETERS MEASURED IN  
COLD STRESS/MODEL STUDY

	ADRENAL (mg)	THYMUS(mg)	BOOY MASS(g)	SOLEUS(mg)
<b>1 Week Cold Stressed</b>				
cold	52.5000 +/- 7.9779	426 +/- 78.5	184.00 +/- 17.11	131.171 +/- 10.609
control	40.9857 +/- 3.9519	685 +/- 67.8	227.43 +/- 20.40	161.886 +/- 14.671
<b>1 Week Unloading</b>				
unloaded	40.9143 +/- 6.1080	600 +/- 150	185.29 +/- 16.51	83.400 +/- 7.447
control	42.1143 +/- 7.3222	564 +/- 54.8	179.29 +/- 10.31	144.071 +/- 14.126
<b>3 Week Cold Stressed</b>				
cold	62.6143 +/- 7.8575	425.1 +/- 84.9	255.86 +/- 14.69	148.757 +/- 11.954
control	46.7143 +/- 4.6770	600.5 +/- 99.6	293.57 +/- 9.47	201.057 +/- 15.763
<b>3 Week Unloading</b>				
unloaded	47.3429 +/- 3.3723	576 +/- 96.9	235.57 +/- 14.34	86.533 +/- 13.669
control	48.2143 +/- 6.2213	622 +/- 115.8	258.00 +/- 11.65	187.143 +/- 8.323
Basal	37.25 +/- 4.19	602 +/- 32.5	151.60 +/- 2.07	109.72 +/- 11.87

Basal = animals terminated at the beginning of the experiment  
n = 7 for all groups except Basal for which n = 5  
values expressed as mean +/- standard deviation

## BONE LOSS IN TAIL-SUSPENDED RATS IS RESTRICTED TO THE UNWEIGHTED LIMBS

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We have developed a model using rats suspended by their tails to simulate weightlessness. This model permits us to study the effect on bone of unweighting the hind limbs and lower spine in comparison to the continued weighting of the forelimbs and upper spine. Furthermore, the more generalized metabolic effects of weightlessness can be studied.

Intestinal calcium transport measured by several different techniques at different calcium concentrations was found not to be altered in this model of simulated weightlessness. This indicates that weightlessness does not have a striking generalized metabolic effect on calcium homeostasis in this model. However, suspended rats had a significantly lower calcium content in the unweighted bones, such as the tibia and lumbar vertebra, compared to pair fed controls. The normally weighted bones, such as the humerus, cervical vertebra, and mandible, did not show differences between suspended and control rats. The differences in bone calcium content between suspended and control rats increased with time of suspension; no significant differences were seen after 5 days, whereas the differences were greatest at 15 days (the longest time evaluated). Despite the progressive disparity in bone calcium content in the tibia and lumbar vertebra of suspended rats compared to that in control rats, radio calcium uptake by such bones showed a biphasic pattern. Initially, calcium uptake by the unweighted bones was lower (2-5 days); by 15 days calcium uptake had rebounded to higher than control values. These data suggest an initial inhibition of bone formation followed by an increase in bone formation, possibly secondary to increased bone resorption when the rats were initially suspended. Normally weighted bone did not show these changes. Appositional rates at the tibia-fibula junction were depressed in the suspended rats when measured from 2-15 days, however, suggesting that bone formation does not "catch up" during this interval. When dietary calcium content was altered, the calcium content of bone was directly proportional to the calcium content of the diet in both suspended and pair fed controls. Rats fed a very low calcium diet had fragile bones regardless of whether they were suspended. In contrast, rats fed a high calcium diet had much more calcium in their bones. These data suggest that a high calcium diet may reduce bone loss during space flight, providing at least one means of countering the bone loss of weightlessness.

We conclude that the predominant mediator of bone loss during simulated weightlessness is localized to the unweighted bone. Such bone loss is not due to a change in intestinal calcium transport or a generalized effect on bone, as might be expected by alterations in PTH,  $1,25(\text{OH})_2\text{D}$ , or corticosteroid levels.

The role of PTH and  $1,25(\text{OH})_2\text{D}$  in the bone changes induced by simulated weightlessness.

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Space flight results in certain characteristic changes in the skeleton. It has been hypothesized that these abnormalities are a direct result of the weightless state. To determine the role of PTH and  $1,25(\text{OH})_2\text{D}$  in the bone changes associated with weightlessness we studied bone metabolism under various dietary conditions using an earth based rat model system which simulates weightlessness. In this model, rats are suspended by their tails such that their rear limbs are completely unloaded while their fore limbs are normally loaded. Unlike earlier models (Morey, E.R., Bioscience 29:168, 1979) in which rats were suspended from their backs, tail suspension allows nearly normal growth and weight gain.

Rats weighing approximately 180g were equilibrated on one of 4 diets and then suspended for 2 weeks. Nonsuspended, age matched pair fed animals acted as controls. The four diets used contained the following concentrations of Ca/P; 0.1%/0.3%, 0.4%/0.3%, 1.2%/0.8% and 2.4%/1.2%. At the end of 2 weeks all animals were sacrificed.

Tibial dry weight and ash weight were consistently lower in suspended animals than pair fed controls except in the very low Ca/P diet group. No differences were detected in the humerus between suspended and control animals in any diet group. Limited histological examination of tibia from each dietary group tended to support these biochemical findings. These results confirm that skeletal unloading diminishes bone mineralization. They also demonstrate that in the tail suspension model only those portions of the skeleton actually unloaded are affected. The serum concentration of Ca was virtually identical in all groups. Serum Pi decreased from  $6.3 \pm 0.5$  mg/dl to  $4.8 \pm 0.6$  mg/dl in the control animals as dietary Ca/P increased but did not change in the suspended animals. The concentrations of PTH and  $1,25(\text{OH})_2\text{D}$  in suspended and pair fed animals within each diet group were nearly identical. Diet had little effect on serum PTH concentrations (range = 0.53 ng-Eq/ml, high Ca/P diet to 0.72 ng-Eq/ml, low Ca/P diet) but produced striking changes in  $1,25(\text{OH})_2\text{D}$  concentration (range = 74 pg/ml, high Ca/P diet to 230 pg/ml, low Ca/P diet). The similarity in circulating PTH and  $1,25(\text{OH})_2\text{D}$  concentrations between suspended and control animals within a given diet group suggests that the bone changes induced by simulated weightlessness are not due to changes in the serum concentrations of these hormones. This hypothesis

is consistent with the fact that the bone changes are localized and do not occur over the entire skeleton. If unloading caused a shift in the serum concentration of a given hormone it would be expected to elicit an equal response from both loaded and unloaded bones.

In summary, our findings suggest that skeletal unloading induces a localized defect in the unloaded bone which results in abnormal growth and mineralization. Clearly this defect is not the result of a change in the circulating concentrations of PTH or  $1,25(\text{OH})_2\text{D}$ . However it may involve a change in bone sensitivity to these hormones. In other words, skeletal unloading may make the unloaded bone more or less sensitive to a systemic factor which in turn could account for a change in bone metabolism.

SIMULATING CERTAIN ASPECTS OF HYPOGRAVITY: EFFECTS ON THE MANDIBULAR  
INCISORS OF SUSPENDED RATS (PULEH MODEL)

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We have studied the effect of a hypogravity simulating model on the rates of mandibular incisor formation, dentinogenesis and amelogenesis in laboratory rats. The model is the Partial Unloading by Elevating the Hindquarters (PULEH) of Morey (The Physiologist 24:S45, 1981). In this system, rat hindquarters are elevated 30-40° from the cage floors so as to completely unload the hindlimbs, but the animals are free to move about using their forelimbs. This Model replicates the fluid shift changes which occur during the weightlessness of spaceflight and produces an osteopenia in the weight bearing skeletons (Metab. Bone Dis. 4:69, 1982). This study chronicles the histogenesis and/or mineralization rates (tetracycline method) of the mandibular incisor during the first 19d of PULEH in young growing rats (37-56d of age).

Method: During 19d PULEH, the suspended animals and their controls were injected with tetracycline (10mg/kg) at T<sub>0</sub>, T+7d, T+14d, and T+19d to provide for the incorporation of fluorescent time markers in the bone and teeth (visible by UV microscopy). At autopsy, the jaws were embedded undecalcified in methyl methacrylate. The blocks were mounted in a special jig to assure that cuts through the bone would be made radially through the center of curvature of the rodent incisor, i.e., coincident with radii of curvatures of both the incisor and the axis of rotation of the jig. In the right mandible, for example, sections were cut at 10° intervals from the incisal tip toward the basal formative end, changing to 5° intervals in the last 20-30° of arc due to the requirement to obtain sufficient samples through the rather narrow zone of enamel formation [about 20° of arc]. Each section was about 100um thick. They were mounted serially for measurements of the distances (um) between successive tetracycline lines (dentinogenesis, UV microscopy) and accumulated enamel thickness (amelogenesis). Linear regression analysis was used to calculate both the slopes of the individual dentin markers within each tetracycline labeling interval, and to calculate the rate of enamel formation. The rate of dentinogenesis was calculated from an average of the labeled intervals in the distal, mesial, labial, mesial-labial, distal-labial and lingual directions. Amelogenesis (rate, duration of formation, & completed thickness) was calculated from measurements of the tissue in the mesiolabial-labial and labial directions. For any particular interval of tetracycline labeling, the rate of incisor elongation (=incisor formation rate) was calculated as the average of the (6) values for the rates of dentin formation (um/d) divided by their corresponding slopes (um/degree). Values for the radius of curvature of the mandibular incisor were then utilized to convert formation rates from degrees/day to mm/day.

Results: In control rats (37-56d of age), the rate of incisor formation decreased from 18.6 um/d to 17.9 um/d, while the rate of dentinogenesis decreased (as an average of all surfaces) from 19.2 um/d to 17.5 um/d. Within this interval of time, the average thickness of enamel increased (ex. labial surface) from 115.0 um to 136.6 um, and the average rate of enamel formation (over the last 7-9 days) was 16.2 um/d. Nineteen days- PULEH failed to alter the normal rates of incisor formation, dentin mineralization, enamel thickness and enamel formation.

Conclusion: We conclude that 19d PULEH does not alter the normal growth patterns in the rat mandibular incisor. Parallel investigations have also shown that 10- 14d PULEH does not affect the chemical status of the mandibular bone per se, and that such a time span is insufficient to elicit the maturational deficit observed in the mandibles of rats flown for 18.5d in the Soviet Biosatellite Cosmos-1129. Unless the duration of PULEH is critical, the cephalad fluid shift common to PULEH and spaceflight animals cannot be solely responsible for the Spaceflight-induced bone and tooth maturational deficit. Because the mandibles of PULEH rats remain antigravity postured, the absence of bone and tooth changes in the suspended animals emphasize the importance of gravity unloading to the onset of abnormal changes in the non-weight bearing skeletal elements. Decreased gravity and, hence, gravity unloading, cannot be mimicked in ground-based models of hypokinesia.



THE EFFECTS OF SPACEFLIGHT ON THE MINERALIZATION OF RAT INCISOR DENTIN

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The lower incisors of male rats flown for 18.5d on the Soviet Cosmos-1129 Biosatellite were sectioned and chemically analyzed with an electron microprobe in order to determine whether there were specific effects of spaceflight on dentin formation. Control tissues were obtained from rats housed under identical conditions in a land-based mock-up of the Biosatellite. Calcium (Ca), phosphorus (Pi) and sulfur (S) concentrations (=glycosaminoglycan index)s in the dentin were measured in continuous traverses across growth increments defined as "recent" since they were (a) formed 0-5d (80um band adjacent to the pulp) before recovery of the spacecraft, and (b) had not been much affected by secondary mineralization processes. The data were Fourier analyzed (cosine waves of various frequencies) were fitted to the data) to determine the spectra of chemical growth rhythms.

Calcium and P were found to be more concentrated in the newly forming dentin of the Flight rats than in comparable regions of control tissues. There was no significant difference in the mean S-concentration between the two groups, but the pattern of S distribution in the recently formed dentin from the Flight rats was different from that in the Synchronous Control group. Sulfur fluctuations in Flight animals periodically peak above the irregular background fluctuations, but there are no comparable sulfur peaks across the dentin in the Controls. These observations indicate that spaceflight has measureable effects on dentinogenesis, and they may also bear on the problem of the regulatory role of proteoglycans in mineralization and in the maturation of mineral and matrix moieties in skeletal tissue.

INFLUENCE OF STRESS, WEIGHTLESSNESS, AND SIMULATED  
WEIGHTLESSNESS ON DIFFERENTIATION OF PREOSTEOBLASTS

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The periodontal ligament (PDL) nuclear volume assay for osteoblast progenitors (Am. J. Anat. 165: 373-384, 1982) was utilized to study the effects of: 1) 18.5 days of weightlessness aboard Cosmos 1129, 2) stress of restricted feeding (Cosmos vivarium controls), 3) stress of noise and vibration to simulate space flight (Cosmos flight controls), and 4) 21 days of head-down suspension via the Morey-Holton model for simulated weightlessness. Nuclear size of fibroblast-like cells in PDL on the anterior surface of maxillary first molars was classified as: 1) A cells, self-perpetuating precursors with a nuclear volume  $< 80\mu\text{m}^3$ , 2) B cells, non-osteogenic fibroblasts with a nuclear volume of  $80-119\mu\text{m}^3$ , 3) C cells, preosteoblasts that are in G<sub>1</sub> stage of the cell cycle with a nuclear size of  $120-170\mu\text{m}^3$ , and 4) D cells, preosteoblasts that are in G<sub>2</sub> stage of the cell cycle with a nuclear size  $> 170\mu\text{m}^3$ .

Percentage of PDL Cell Types According to Nuclear Size (mean  $\pm$  S.E., n=5)

Nuc. Size	Cosmos 1129		18.5 days of space flight	Simulated Weightlessness	
	Vivarium Control (restricted food)	Flight Control (noise, vibration)		Suspended 21 days	Control Ad Lib.Fed
A	22.0 $\pm$ 2.9	15.8 $\pm$ 3.0*	33.8 $\pm$ 4.3*	46.1 $\pm$ 4.2*	28.9 $\pm$ 3.5
B	27.4 $\pm$ 1.5	24.2 $\pm$ 1.5*	33.0 $\pm$ 2.9*	33.0 $\pm$ 1.8	33.1 $\pm$ 2.0
C	32.2 $\pm$ 2.0*	31.0 $\pm$ 2.9*	22.8 $\pm$ 1.7*	15.0 $\pm$ 2.3	20.2 $\pm$ 2.8
D	18.4 $\pm$ 1.4	28.1 $\pm$ 1.6*	11.0 $\pm$ 2.1*	5.5 $\pm$ 1.1*	17.8 $\pm$ 1.8
	*p<.05 vs. Ad Lib.Fed	*p<.05 vs. Ad Lib.Fed	*p<.05 vs. Flight Ctl.	*p<.05 vs. Ad Lib.Fed	

Compared to the ad lib. fed control, the vivarium control had greater numbers of C type cells, indicating the stress of restricted feeding increased the numbers of preosteoblasts. Cosmos flight simulation (noise and vibration) produced a pronounced shift from A and B to C and D cells compared to the ad lib. fed control, suggesting a higher rate of preosteoblast differentiation and/or greater incidence of G<sub>2</sub> block of preosteoblasts. Relative to the flight control, 18.5 days of weightlessness increased the numbers of A and B cells and decreased C and D cells which is consistent with a block in preosteoblast differentiation. Compared to the ad lib. fed controls, rats suspended for 21 days to simulate weightlessness had more A cells and fewer D cells, which is similar to the effect of weightlessness. Since physiological stress tends to enhance preosteoblast differentiation, while weightlessness and simulated weightlessness inhibit it, their mechanisms of action in depressing bone formation are different. Both weightlessness and simulated weightlessness (head-down suspension) produce a suppression of preosteoblast differentiation which is independent of physiological stress.

Title: Morphological and Histochemical Studies of Bone and Cartilage During Periods of Simulated Weightlessness.

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Introduction: Rats which have been subjected to spaceflight for 2-4 weeks have shown considerable loss in ability to form new bone (Morey and Baylink, 1978). Animals which are placed into non-weight bearing positions, as a model to simulate the absence of gravity here on the earth's surface, can be made to show a similar decline in new bone formation (Morey, 1979). The mechanisms underlying these changes are poorly understood but are apparently the result of reduced transmission of gravitational force to the skeletal cells.

Methodology: Adult rats (300-700 grams) are suspended with their hind legs in a non-weight bearing position and the head in a downward tilt. This model has been described previously by Dr. E. M. Holton. The animals are free to move about on their fore limbs and can eat or drink ad libitum. Controls are pair fed. Experimental periods vary from seven to twenty-one days.

Results: Previous studies have shown that the bone forming cells (osteoblasts) are normally in cell-cell communication with other similar cells. Thus, lamellae of bone matrix are deposited as a combined effort by hundreds of these cells working in concert. When the animals are placed into a non-weight bearing position, the cells demonstrate reduced communication, their cell membrane-associated enzyme activity decreases, and overall new matrix production is decreased. This result, in the long term, may be explained by the reduced cell-cell communication and some sort of feed back mechanism which directs the cell to reduce its synthetic activity as a result of reduced mechanical stress applied to the skeleton. However, more recently we have documented two new histochemical findings in these same bone-forming cells:  
(1) The Golgi activity of these cells seems to be consistently

unchanged, regardless whether the cells are from suspended animals or not. (2) The lysosomal degradation of collagenous material within the cytoplasm of osteoblasts seems to be greatly increased in cells from suspended animals.

We interpret all of the above findings to mean that collagen synthesis and its packaging via the Golgi system remains normal in both the suspended and control animals. However, the increase in lysosomal and degradative activity in the bone forming cells from suspended animals, suggests that increased intracellular degradation of procollagen is occurring. Thus the genetically directed function of these cells still continues; ie, to synthesize procollagen and prepare it for export from the cell to form new bone matrix. However, this synthesized product is then internally degraded because an environmentally derived signal appears which directed the cells to not release their synthesized product but to degrade it. This control mechanism which overrides the signal for collagen release is apparently sensitive to environmental factors and a change in gravitational forces is one change to which the cells are sensitive.

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## METABOLIC ALTERATIONS CAUSED BY SUSPENSION

### HYPOKINESIA IN LEG MUSCLES OF RATS

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Albino rats (130-170 g) were suspended by a cast around the base of their tails consisting of medical elastomer and orthopedic gauze. By use of a clip, the rats were attached to a pulley which allowed them complete access to food and water and the freedom to groom themselves. This study used two groups of animals: 1) weight bearing control which were tail casted but allowed to walk on all four limbs, and 2) hypokinetic with no load bearing of the hindlimbs. Data were collected for 5 to 15 animals per group.

The control and hypokinetic rats gained weight at a steady and similar rate over the six days of the experiment. Hypokinesia for 6 days led to significantly lower relative weights of the soleus (-26%), gastrocnemius (-10%) and plantaris (-9%) muscles. Hypokinesia did not affect the relative mass of the anterior tibialis or extensor digitorum longus (EDL) muscles.

To account for the changes in muscle mass, protein metabolism was measured in soleus and EDL. Protein synthesis was 36% lower and protein degradation was 36% greater in the atrophied soleus. In the EDL, hypokinesia had no effect on degradation but did decrease synthesis slightly.

Enhanced turnover of protein in atrophying muscle requires the increased export of nitrogen derived from the degradation of certain amino acids by muscle. Both alanine (ala) and glutamine (gln) serve as vehicles for removing this excess nitrogen. These amino acids were studied in terms of non-protein production (synthesis + tissue losses) and net synthesis. The release of non-protein derived ala and its synthesis were unaffected by hypokinesia. The release of non-protein derived gln was 35% slower in the hypokinetic soleus and the incubated tissue level of gln was 43% lower. Because the tissue levels of glutamate were unaffected, the ratio of gln/glu fell significantly.

These data suggested that the capacity of hypokinetic soleus to synthesize gln might be diminished. However, assays of glutamine synthetase activity in muscle showed 2-fold more activity than in the control muscles. Since this activity was higher, it was possible that ammonia may have limited the release of gln in hypokinesia. Addition of ammonium chloride had a similar effect on synthesis of gln in control and hypokinetic soleus and the basal rates were also similar. This result is surprising since the greater activity of glutamine synthetase in hypokinesia should have produced a larger effect of ammonia addition.

Since glutamate levels of soleus are not affected by hypokinesia, we considered whether ATP might have been limiting for gln synthesis. However, hypokinetic soleus had a 53% greater content of ATP than control incubated muscles. The EDL showed no changes. Clearly availability of ATP and glutamate are not limiting factors for synthesis of gln in hypokinetic soleus muscle. The higher levels of adenine nucleotides suggest less AMP deamination and therefore a diminished supply of  $\text{NH}_3$ .

Biochemical and histochemical  
adaptations of skeletal muscle  
to rat suspension

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The influence of rat suspension on soleus disuse and atrophy was investigated to measure changes in fiber area and number and to determine if suspension elicited changes in lysosomal protease activity and rate of calcium uptake by the sarcoplasmic reticulum. Other current research goals are to determine the influence of rat suspension on myosin light chain phosphorylation and succinate dehydrogenase activity.

Since Jaspers and Tischler (4) reported soleus muscles from suspended rats increased rates of protein degradation, and since muscle protein degradation may involve lysosomal (acid) proteases, the activities of these proteases were tested in suspended rats. Compared to control, acid protease activity in soleus muscles were significantly increased by 32 and 35% after rat suspension intervals of four days and two weeks, respectively.

An inverse relationship exists between isometric contraction duration and rate of  $Ca^{++}$  uptake by the sarcoplasmic reticulum. Since rat suspension evokes soleus speeding, the influence of rat suspension on SR  $Ca^{++}$  uptake was investigated using the method of Fitts et al. (2) to determine if SR regulated the duration of soleus contraction. In soleus muscles from rats suspended two and four weeks,  $^{45}Ca^{++}$  uptake was statistically unchanged when compared to control.

While the concepts that training evokes hypertrophy and disuse elicits atrophy have been demonstrated in several skeletal muscles, a current controversy exists as to whether a change in fiber number occurs with training (3). Consequently, a current goal of our research is to determine whether a change in fiber number of the soleus muscle is evoked by either rat suspension or recovery from suspension. Documenting a change in fiber number requires establishing an equality between muscle weight and the product of density, average fiber area, fiber length and fiber number. To date we have measured average fiber area. In response to rat suspension for two and four weeks, the average area of Type I fibers significantly decreased by 38 and 50%, respectively. After one week recovery from a two week suspension, both Type I and II fiber areas increased significantly above non-suspension control values (14 and 47%, respectively).

Myosin light-chain phosphorylation has been suggested as a possible control of tension development in skeletal muscle (6). To determine whether light-chain phosphorylation is involved in the change in muscle function evoked by rat suspension, the responses of treppe and post-tetanic potentiation, which are indicators of changes in myosin light-chain kinase activity, are initially being investigated. Subsequently, if these two functional parameters indicate an involvement of light-chain phosphorylation, myosin light-chain kinase activity will be measured. At present, depressed

treppé responses have been shown by gastrocnemius muscles from suspended rats.

Succinate dehydrogenase (SDH) activity has been reported to be reduced in skeletal muscles of immobilized rats (5) and to be increased in both heart and skeletal muscle after endurance training (5). In rats suspended for four weeks, SDH activity was found to be unchanged in either the gastrocnemius or soleus muscles or in the heart.

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PHYSIOLOGICAL CHANGES IN FAST AND SLOW MUSCLE WITH  
SIMULATED WEIGHTLESSNESS

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A rat hindlimb suspension model of simulated weightlessness was used to examine the physiological characteristics of skeletal muscle. The physiological sequelae of hindlimb suspension were compared to those of spinal cord section, denervation by sciatic nerve crush, and control. Muscles examined were the predominantly slow (Type I) soleus (SOL) and the predominantly fast (Type II) extensor digitorum longus (EDL).

(1) Effects on biochemical characteristics.

Animals were sacrificed at intervals of 1-5 weeks after operation and the SOL and EDL muscles assayed for total acetylcholinesterase (AChE) activity. The control EDL-AChE activity was about twice that of the control SOL. With hindlimb suspension EDL-AChE activity did not significantly change, however, SOL-AChE activity increased to over twice control when expressed as activity per gram muscle wet weight. Spinal cord section also resulted in an increase in SOL-AChE activity, but produced a small decrease in EDL-AChE activity. Individual molecular forms of AChE were assayed after hindlimb suspension. In the SOL, the increase was of all four major forms: 16S, 12S, 10S, and 4S. In the EDL, the 4S and 10S decreased and the 16S increased slightly.

(2) Effects on histochemical characteristics.

After intervals of 1-5 weeks, animals were sacrificed and the SOL and EDL muscle sections reacted for actomyosin ATPase activity. Sections were analyzed morphometrically by computer. The control SOL was composed of 79% Type I fibers. With hindlimb suspension the proportion of Type I fibers decreased to 55% with the concomitant increase being predominantly of Type IIa but also of Type IIb fibers. The control EDL was composed of 4% Type I fibers. With hindlimb suspension the proportion of Type I fibers decreased to 1.4% with the associated increase being of Type IIa fibers. Spinal cord section produced qualitatively similar results with the proportion of Type I fibers in the EDL being less than 1%.

(3) Effect on reinnervation.

The effect of altered motor unit activity on reinnervation after sciatic nerve crush was examined. In control rats, denervation resulted in a decrease in EDL-AChE activity which gradually recovered towards control as reinnervation was established at 4-5 weeks post-op. SOL-AChE also initially decreased but as reinnervation began, at 2-3 weeks post-op, AChE activity increased to more than twice control activity. Subsequently the SOL-AChE activity returned to control over the following 2-3 weeks. With hindlimb suspension and denervation (both operations on the same day), there was a delay in the recovery of EDL-AChE activity toward control levels. Also, the SOL-AChE activity overshoot was delayed and truncated. Qualitatively similar results were seen with denervation after spinal cord section.

Conclusion.

Two procedures which alter motor unit activity, hindlimb suspension and spinal cord section, produce changes in characteristics of skeletal muscles that are dependent upon fiber type. The SOL develops characteristics more representative of a fast muscle, including smaller Type I fiber proportion and higher AChE activity. The EDL, which is already predominantly fast, loses most of its few Type I fibers, thus also becoming

"faster". These data are in agreement with the studies in which rats experienced actual weightlessness.

The reason for this pattern of changes with simulated weightlessness may be found in an understanding of the physiological factors which influence fiber type. Under normal gravity the soleus of animals and man is a postural muscle tonically firing at a moderate rate to maintain the leg and/or body somewhat perpendicular to the surface of the earth. The gastrocnemius may also serve this same function, but it is usually relatively silent during standing, becoming more active during ambulation for foot extension or the thrust of "pushing off". The EDL, on the other hand, usually has only the inertia of the foot and compliance of antagonist muscles as its load during foot flexion. During weightlessness there are two major changes in these relationships. The first is that the soleus no longer has to fulfill a weight bearing function, and therefore takes up a task more resembling that of the EDL. The second is that the effort required for locomotion is much decreased for the powerful muscles of the legs. Therefore, the degree of motor unit activation and recruitment required to make a patterned movement for both muscles is much less than normal. This results in decreased activation of the large number of muscle fibers that are not among the lowest in recruitment order. Therefore, under the influence of a pattern of motor unit activity which resembles that of the EDL, the slow SOL converts, within its physiological constraints, to having characteristics of a "faster" muscle.

The reinnervation data show that there is clearly a delay in reinnervation with both hindlimb suspension and spinal cord section. Further studies are necessary to determine the characteristics of motor unit activation associated with each of these treatments. Nevertheless, it is apparent that the speed of reinnervation is at least partly dependent upon the pattern of motor unit activity. This has implications not only for weightlessness, but also for basic theories of nerve-muscle interaction.

## Appropriateness of the small-cage-reared rat as a model for the study of altered-activity effects

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Within genetically imposed limits, the fatigue-resistance capability of muscle varies according to the chronic demands of usage imposed on the muscle. Given the fiber-type distribution within a muscle, its fatigue-resistance can be utilized as an indicant of its physiological status. We have employed such a strategy and have previously suggested (Stuart and Enoka, 1982) that the hindlimb musculature of rats raised in cages constructed to minimum FDA specifications (46-49 x 25-28 x 20 cm; 4-7/cage) are physiologically inappropriate for the study of altered-activity effects. This proposition was based upon two observations from the medial gastrocnemius muscle ( $n = 7$ ) of Sprague-Dawley rats (500 g, 100 d); first, a substantial disparity ( $\sim 277\%$ ) in the peak forces (twitch and tetanic) elicited by neural and direct-muscle stimulation, and second, a reduction in force during the fatigue test (2 min of 1 Hz trains with each train lasting 330 ms and including 13 stimuli; Burke et al., 1971) that was greater (79%) than theoretically expected (62%).

Among the variables which could account for the disparity between the forces associated with nerve and direct-muscle stimulation are current spread to adjacent musculature, multiple stimulation caused by an inappropriate stimulus pulse width and impairment of the neuromuscular junction. The first possibility was addressed by examining the force produced by direct-muscle stimulation of the test muscle (soleus or medial gastrocnemius) once all the other muscles in the compartment had been either extirpated or tenotomized and on other occasions by stimulating the test muscle once it was ischemic. The disparity was not affected by extirpation-tenotomization and similarly, pursuant to ischemia the force elicited by direct-muscle stimulation of the test muscle was negligible ( $<1\%$ ). These observations suggested that the adjacent musculature did not contribute significantly to the difference in force.

Subsequently, variable combinations of stimulus strength (0.5-110 V), pulse width (0.1-10.0 ms), and electrode numbers (1-4 pairs) were used for direct stimulation of the soleus and medial gastrocnemius muscles. For pulse widths of less than 1.0 ms, the peak twitch force increased as a sigmoidal function of stimulus strength to some common plateau value. Furthermore, the minimal stimulus strength (threshold) necessary to elicit a twitch response was inversely related to pulse width. Threshold was also affected by the number of intramuscular stimulating electrodes (stainless steel,  $\sim 120 \mu\text{m}$  diameter), being least in medial gastrocnemius with four pairs of electrodes but of comparable magnitude in soleus with 2-6 pairs. However, for pulse widths of 1.0 ms and greater the peak twitch force-stimulus strength relationship did not plateau but rather force continued to increase in a direct relationship with stimulus strength. These data indicated that the longer pulse widths ( $\geq 1.0$  ms) resulted in multiple activations of the muscle in response to a single direct-muscle stimulus (see also, Close and Hoh, 1968; Desmedt and Hainaut, 1968). Since the original observations (Stuart and Enoka, 1982) were based upon a 2.0 ms pulse width for direct-muscle stimulation, the measurements were repeated using pulse widths of 0.1 and 0.2 ms for neural and direct-muscle activation, respectively. In contrast to the previous observations, there was no difference in the mean peak twitch force obtained by the stimulus protocols; the nerve-elicited values were  $97.9 \pm 5.9\%$  of those produced by direct-muscle stimulation. Thus it appears that the nerve-direct muscle-disparity objection to the small-cage-reared rat was due to an artefactual observation.

The second objection, however, that of undue muscle fatigability during the fatigue test, has not been so readily dismissed. Medial gastrocnemius has continued to display greater fatigability (peak tetanic force =  $-83 \pm 12\%$ ; EMG amplitude =  $-90 \pm 6\%$ ) than would be expected (force =  $-62\%$ ; EMG =  $-23\%$ ) based on its fiber-type distribution. More significantly, however, a substantial variability in force decline during a six-minute fatigue test has been exhibited by both the soleus ( $n = 5$ ) and medial gastrocnemius ( $n = 3$ ) muscles. Figure 1 illustrates the force decline profile of four muscles, selected to represent the least and greatest force reduction seen for the two muscles. As indicated above, this variability in fatigue resistance within a muscle (i.e., soleus vs medial gastrocnemius) is interpreted as reflecting wide variations in the physiological status of the two muscles. In a similar vein, the failure site(s) associated with the force reduction appeared to vary among the muscles. The nerve-elicited peak twitch forces were  $97.9 \pm 5.9\%$  and  $77.9 \pm 19.9\%$  of those obtained by direct-muscle stimulation before and after the fatigue test, respectively. This post-fatigue difference was correlated significantly ( $r^2 = 0.89$ ,  $p < .05$ ) with the amount of fatigue (i.e., force reduction) exhibited during the test, indicating a variable neural contribution to whole-muscle fatigue (Joyner et al., in press).

To further address these issues, we are currently raising two populations of Sprague-Dawley rats (125-150 d), one group in the conventional minimum-specification cages and the other group in a large cage (320 x 183 x 99 cm; 15/cage) which includes "exercise" wheels and climbing structures. We anticipate that one of the environment-size effects will be to decrease this variability in fatigue resistance. At this juncture, however, the issue of the appropriateness of the small-cage-reared rat remains unresolved.

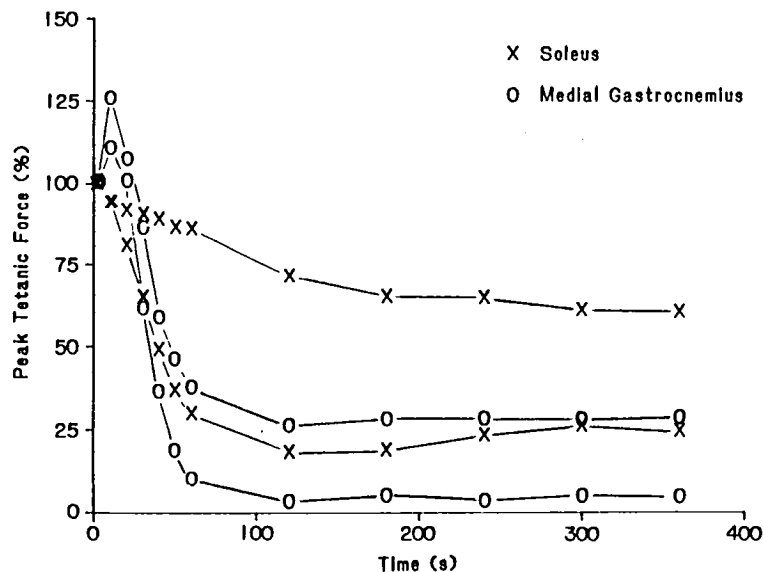


Figure 1. Peak tetanic force elicited during the six-minute fatigue test. The graph illustrates the variability (least and greatest observations) in fatigue resistance seen for the two muscles.

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## B. FLUID DYNAMICS AND METABOLISM



## HOMEOSTASIS IN PRIMATES IN HYPERACCELERATION FIELDS

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The homeostatic capabilities of animals are sensitive to changes in the ambient acceleration environment. Such changes in centrifuged rats and dogs include depressed body temperatures, alterations in circadian timekeeping and changes in body composition. To date, however, little work has been done examining these changes in man or any other primate. Over the last year, we have examined various homeostatic responses of a non-human primate, the squirrel monkey (*Saimiri sciureus*) to acute changes in the acceleration environment. When these animals were exposed to a hyperdynamic field the body temperature was consistently depressed and the animals showed behavioral indications of increased drowsiness. Further, time of day played a significant role in influencing these responses.

Initially, loosely restrained squirrel monkeys were exposed to hyperdynamic fields. The centrifugation consisted of a 60 minute step change in the acceleration environment from 1G to 2G in the z axis (head-to-toe). The animals were allowed to acclimate to the centrifuge at 1G for as much time as necessary for the body temperature to stabilize prior to centrifugation (60 to 120 minutes). Body temperature was measured via a thermistor inserted six centimeters past the anus and taped to the base of the tail. Temperature information was recorded continuously on a strip chart recorder. The data was subsequently digitized at one minute intervals, and average responses were computed. Videotapes were also made of the animals' behavioral response throughout the centrifugation, including control and recovery periods before and after centrifugation.

All animals demonstrated significant depressions of body temperature while in the hyperdynamic environment. After the centrifuge was started, there was generally a small increase in body temperature. Within 5 to 10 minutes, however, a continuous decline in temperature became apparent. The average decrease in body temperature was 1.4°C and the body temperature depression was maintained for the duration of the step change. Approximately five minutes after the animal was returned to 1G the body temperature began to rise.

The primate sleep responses to 70 minute exposures to the 2G<sub>z</sub> hyperdynamic fields were also examined. We initially noted that centrifuged animals, when observed visually, periodically demonstrated a sleeping behavior when in the hyperdynamic field. We further examined this phenomenon with electrophysiological recording of the EEG in a group of chronically implanted primates. During the precentrifugation phase, the animals showed various amounts of "napping" behavior in which slow wave sleep occurred on a periodic basis. (Rapid eye movement sleep was not observed during this daytime study.) Upon centrifugation, however, sleep was inhibited for several minutes after which the slow wave sleep pattern began to recur. The amount of sleep never reached the baseline level of the precentrifugation phase. Postcentrifugation, the amount of sleep increased significantly and was maintained above baseline levels for the entire 70-minute postcentrifugation phase. This response was proportional to field intensity, in the animals studied at both 2 and 3 G<sub>z</sub>. At 3 G<sub>z</sub>, the animals showed an increased response in that there was a greater rebound in slow wave sleep during the postcentrifugation phase. It is interesting to note that slow wave sleep has always been inversely correlated with body temperature. That is, lower body temperatures are usually associated with increased

sleep. In this experiment, the correlation did not hold. During centrifugation, the amount of sleep observed during the fall in body temperature was less than that seen in the control phase where body temperature was higher.

Finally, we compared the thermoregulatory responses of squirrel monkeys to 70-min  $2 G_z$  exposures during the day and night. Again, the response of the animal during the day was, on average, a  $1.5^{\circ}\text{C}$  fall in colonic temperature, mediated at least in part by an increase in vasomotor heat loss. This response was highly reproducible and seen in all animals. In contrast, at night the temperature response was completely eliminated. Individual animals showed small deviations in temperature either in an upward or downward direction, but on the average there was no change in colonic temperature. Similarly, the skin temperatures did not show any major deviations in vasomotor heat loss. These results demonstrate that although the centrifuge induced changes in body temperature are very prominent and reproducible during the day, these animals can regulate their body temperature independently of the gravitational field during the night.

We then investigated this day-night difference in body temperature response in a series of rats. Historically, all rats exposed to hypergravitational fields have been examined during the day, which is the rest phase of these nocturnal animals. We wished to determine whether the light versus the dark had a significant influence on the response or whether the magnitude of the response was a function of the activity level of the animal. We observed the previously reported  $2^{\circ}\text{C}$  response when centrifuging the animals during the day. However, upon exposure to  $2 G_z$  at night, which is the animal's active phase, the body temperature fall was even larger. This indicates that although the monkeys were able to regulate body temperature more precisely than the rats, both species showed an increased ability to maintain body temperature during their rest phase when their circadian body temperatures were at a minimum.

Current efforts are proceeding to examine the influence of chronic acceleration on these and other homeostatic systems. We are especially interested in examining the new steady-state levels of these homeostatic variables, and their 24-hr rhythmicity. Finally, we are developing protocols to further describe the mechanisms by which these responses are produced.



SET-POINT CHANGES IN HIERARCHICALLY-ARRANGED THERMOGENIC SYSTEMS

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NSG-2234

INTRODUCTION: The use of hypergravic fields to test 1 G models of thermoregulatory mechanisms builds on several studies of the thermal responses of animals in gravitational fields of 1-4 G. Early work by Oyama and his colleagues demonstrated a marked fall in core temperature ( $T_c$ ) followed by a period of relatively unchanged body temperature. However, if the rats were exposed for several days to a constant hypergravic field,  $T_c$  slowly increased toward normal values. Similar measurements on dogs and monkeys have shown that a decreased  $T_c$  in hypergravic fields is not unique to the rat. Thus, as manifested by a fall in  $T_c$ , thermoregulatory mechanisms in several species of mammals appear to be altered in hypergravic fields.

In the present study, rats acclimated to either 23 or 5°C were concurrently exposed to cold and hypergravic fields. The experiments were designed to test the proposal that mammals have parallel controllers for thermoregulation. That is, Satinoff has proposed that thermoregulatory mechanisms are independently controlled by distinct neurocontrollers arranged in parallel. In the present study, each neurocontroller is considered to include components within the hypothalamus, as well as those additional components of the central nervous system involved in signal processing. For example, the controller for shivering would be composed of a hypothalamic component and of neural pathways to and including the spinal cord. [In the normal functioning of the controller at low ambient temperature, an oscillatory pattern for shivering appears to be generated in the spinal cord in response to a descending tonic signal.] The afferent limb to this shivering controller includes cold receptors in the periphery, and the efferent limb includes axons of spinal motor neurons that synapse on skeletal muscle cells.

METHODS: Techniques for oxygen consumption measurements. Closed-circuit procedures for measuring oxygen consumption at 1 G environments were adapted for use on a centrifuge (2.1 m radius). The oxygen consumed by a rat in a closed chamber was replaced via Krogh-type spirometer. The amount of oxygen consumed was determined by counting the number of times the spirometer delivered a fixed amount (8.36 ml) of 100% oxygen to the chamber that was initially filled with room air. To absorb CO<sub>2</sub>, a layer of soda lime was placed on the bottom of the respiratory chamber beneath a wire screen.

Experimental procedures. Thirty-two Long-Evans hooded male rats were maintained on a 12 h light:dark cycle and provided with food (Simonsen's white diet) and water ad libitum. These rats were divided into two groups. One group, RT, was acclimated to 23°C; the other, CA, was cold acclimated by exposure to 5°C for at least 6 wk. Rats from both groups were subjected to the same experimental protocol. RT and CA rats of comparable size ( $441 \pm 24$  and  $444 \pm 20$  g, respectively) were used for comparisons of oxygen consumption measurements. The RT rats were  $128 \pm 17$  days old and the CA rats were  $168 \pm 12$  days old at the time of the experiment.

Two types of experiments were conducted. In the first, RT and CA rats were transferred to the centrifuge from the rooms in which they were acclimated. After 30 min they were placed for 1 h at  $23.0 \pm 2.6^{\circ}\text{C}$  (mean  $\pm$  SE) in the respiratory chamber described above. They were then exposed to cold ( $7.6 \pm 1.9^{\circ}\text{C}$ ) by surrounding the chamber with ice for the next 3 h. During the 2 h of this cold exposure, the rat was concurrently exposed to a hypergravic field. Oxygen consumption was measured and recorded simultaneously with chamber temperature on a Varian chart recorder (using Veco 32/A thermistors and Houston chart recorders). In the second series of experiments, the RT and CA rats were restrained in plastic holders within the respiratory chamber.  $T_c$  and tail temperatures ( $T_t$ ) were measured with thermistors. In addition to temperature, oxygen consumption was measured throughout exposure to 1 and 3 G at  $22.9 \pm 1.8$  or at  $10.0 \pm 1.5^{\circ}\text{C}$ .

DISCUSSION: In our experiments, RT and CA rats were used to evaluate the different relative contributions of shivering and nonshivering thermogenesis to the increased oxygen consumption of the cold-exposed rats in hypergravic fields. The lower magnitude of the cold-induced oxygen consumption observed when cold-exposed rats are moved from 1 G to hypergravic fields is probably due to an inactivation of shivering rather than nonshivering thermogenesis. Our observation that shivering, but not nonshivering thermogenesis, appears to be impaired by hypergravic fields is consistent with the representation of central thermoregulation by multiple controllers. Current work centers on a further characterization of potential shifts in set-points of controllers for the effectors for heat production and heat conservation, and build on several studies on temperature regulation in hypergravic studies (e.g., 1,2,3).

CURRENT EFFORTS: In addition to studies on shifts in set-points of each controller and adaptation of thermoregulatory systems to hypergravic fields, we have also been developing mechanical and electronic systems for the measurement of averaged vestibular responses on rats.

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GRAVITY, BODY MASS AND COMPOSITION, AND METABOLIC RATE

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Investigation of the scale effects of increased gravitational loading by chronic centrifugation on metabolic rate and body composition in metabolically mature mammals has progressed well. Individual oxygen consumption rates in groups of 12 each, 8-month-old, hamsters, rats, guinea pigs, and rabbits have been measured at weekly intervals at 1.0 *g*, then at 2.0 *g* for 6 weeks. Six of the animals have been sacrificed immediately after removal from the centrifuge for body composition analysis, and oxygen consumption rate measurements for estimation of metabolic rate have been continued on the remaining 6 at 1.0 *g* for an additional 4 weeks. The biochemical body composition analyses are in process, and preliminary results on the metabolic rate changes were reported during the 5th Annual Meeting of the IUPS Commission on Gravitational Physiology in Moscow on 28 July 1983, as follows.

Metabolic rate was increased significantly in all species, and stabilized after 2 weeks at 2.0 *g*. Statistical analysis of the data revealed that the larger the animal the greater was the increase in mass-specific metabolic rate, or metabolic intensity, over the 1.0 *g* value for the same animal, with the result that the interspecies allometric scaling relationship between metabolic rate (MR) and total body mass (TBM) is different at 2.0 *g* compared to 1.0 *g*. At 1.0 *g* the classic Kleiber allometric relationship is expressed as

$$MR_{1.0g}, \text{kcal} \cdot \text{hr}^{-1} = 2.82(\text{TBM}, \text{kg})^{0.756}; r = 0.998$$

whereas at 2.0 *g* from our data the allometric relationship becomes

$$MR_{2.0g}, \text{kcal} \cdot \text{hr}^{-1} = 3.30(\text{TBM}, \text{kg})^{0.813}; r = 0.993.$$

Analysis of covariance has shown that the positioning constant at 2.0 *g* is increased by 17% at 2.0 *g* at the  $P < .001$  level, and the exponent is increased by 8% at the  $P = 0.008$  level. Thus, our initial hypothesis that augmented gravitational loading should shift the allometric relationship between metabolic rate and body size by an increase in both parameters is supported. The results also support the broader hypothesis that gravitational loading is an important contributor to mammalian energy requirements. Finally, we continue to predict that abatement of gravitational loading in spaceflight will result in a lowering of both allometric parameters in the Kleiber equation.

Definition of the thermoneutral zone for mature animals of the 4 mammalian species, hamster, rat, guinea pig and rabbit, has been completed, and the effect of departure from the thermoneutral zone on the allometric scaling of metabolic rate on total body mass has been examined. These results were also reported during the Moscow meeting, as follows.

Direct body heat loss rates were measured in a gradient layer calorimeter on 6 animals 8 months of age for each of the 4 species during exposure to different ambient air temperatures from 20°C to 36°C at 50% relative humidity for 4 hours. The results showed that the minimum metabolic rate for hamsters occurred at 29°C, with a bandwidth of  $\pm 1^\circ\text{C}$  within which the metabolic rate did not change by more than 5%. For rats the minimum occurred at 27°C with a bandwidth of  $\pm 2^\circ\text{C}$ , and for guinea pigs and rabbits it occurred at 29°C with a bandwidth of  $\pm 2^\circ\text{C}$ . It was also found that if all 4 species were maintained at  $29^\circ\text{C} \pm 1^\circ\text{C}$ , the metabolic rate in all 4 would be within 10% of the minimum value for each. Furthermore, the relationship of metabolic rate (MR) to total body mass (TBM) for the animals at 29°C could be expressed by the allometric equation

$$\text{MR}_{29^\circ\text{C}}, \text{kcal}\cdot\text{hr}^{-1} = 2.91(\text{TBM}, \text{kg})^{0.766}; r = 0.979,$$

and the equation parameters are not significantly different from the classic Kleiber relationship.

However, the metabolic rate data for 24°C ambient temperature yielded the allometric relationship

$$\text{MR}_{24^\circ\text{C}}, \text{kcal}\cdot\text{hr}^{-1} = 3.51(\text{TBM}, \text{kg})^{0.676}; r = 0.987,$$

in which the equation parameters are significantly different from both our 29°C relationship and the Kleiber relationship. The positioning constant is elevated because of the higher metabolic rates at the lower ambient temperature, and the exponent is lessened because the metabolic rate of the smaller animals was increased proportionately more at the lower temperature than was that of the larger animals.

Our results confirm that the condition of thermoneutrality is important for metabolic scale effect studies. Furthermore, our results show that the thermoneutral zone for the species considered here is a narrow one. Our measurements also permitted partitioning of total body heat output into sensible heat loss by radiation, conduction and convection, and into latent heat loss by evaporation of water from the body surface, as a function of ambient temperature at 50% relative humidity. It was found that significant species differences exist with respect to partitioning of heat loss.

A year ago we reported on a method for estimating total body skeletal muscle mass from measurements of total body creatine content in mature individuals of the 4 species, hamster, rat, guinea pig and rabbit. The method involves the use of conversion factors for each species derived from measured values of the creatine content of fat-free skeletal muscle. Because it appears that age differences as well as species differences may exist in muscle creatine content, we are now in process of measuring creatine content of skeletal muscle from animals of various ages from 1 month to 5 months for each of the 4 species of interest to supplement our earlier measurements on animals of 8 months and more of age. The results will provide a basis for deciding on the feasibility of estimating total body skeletal muscle mass in young animals as well as mature individuals.

EXTENSIONS OF SUSPENSION SYSTEMS TO MEASURE EFFECTS OF  
HYPOKINESIA/HYPODYNAMIA AND ANTIORTHOSTASIS IN RATS

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NASA Grant NSG- 2325

Suspension systems are used to simulate hypokinetic/hypodynamic (H/H) and anitorthostatic (AO) responses seen under conditions of weightlessness. Growing rats (male, Sprague Dawley, 180-200gm) in H/H suspension with unloaded hindlimbs for one and two weeks respond with muscle atrophy (soleus>gastrocnemius=plantaris>EDL) and increased excretion of nitrogenous end products such as urea, NH<sub>3</sub> and 3 methyl histidine (JAP, 48:479-486, 1980, Aviat. Space, Environ. Med., in press, 1983). Since muscle is in a dynamic state of synthesis and breakdown of protein, relationships between protein, RNA and DNA contents in the four muscles which reflect weight bearing and non-weight bearing functions were assessed. Protein and RNA progressively decreased over a one and two week period of H/H suspension: soleus>gastrocnemius=plantaris>EDL. Concomitant analysis of DNA contents showed there were no changes. Our interpretation was that protein synthesis was slowed during H/H. As with muscle mass, protein and RNA levels recovered rapidly after removal from H/H.

"Scaling effects" were considered with a series of comparable experiments using adult rats. Older rats (525 gm) lose body weight (20%) during the first week of H/H, whereas, growing rats (180-200gm) fail to gain weight. The decrease in muscle mass in adult rats was comparable with that in growing rats: soleus>gastrocnemius=plantaris>EDL; increased levels of urea and NH<sub>3</sub> excretion were also evident. Recovery is also rapid, within a few days, upon removal from H/H suspension. Initial observation suggests that much of the body weight loss is a decrease in body fat.

AO rats (which are also H/H) respond with diuresis, natriuresis and kaliuresis in a manner comparable to responses seen when thoracic blood vessels are volume loaded (JAP, 49:576-582, 1980). Orthostatic rats (O) have no comparable responses. Since cardiovascular measures could be used to further characterize these differences (i.e., AO vs. O), a series of blood pressure (BP) studies were done. With fluid and electrolyte changes occurring during the first days of AO, we used one and three day suspended subjects with carotid cannulations to measure systolic, diastolic, mean arterial and pulse pressures. Also, AO and O suspended rats were rapidly tilted 70° head-up. One and three day (AO) rats respond with increased BPs. Rapid head-up tilting showed an immediate increase in BPs except in three day AO rats. This functional cardiovascular response provides another differentiation of the two types of positioning during suspension and another source of data arguing for care in utilization of suspension models in mimicking weightlessness.

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Title of research Project:

Circulation in the rat (NASA grant NAg-2-87)

Hypothesis and objectives. Antiorthostatic hypokinesia mimics circulatory effects of weightlessness. Using an animal model that was developed (by Holton and modified by Musacchia), we are studying circulatory mechanisms that occur during exposure to head-down (20°) hypokinesia as well as during readaptation of the rats to control conditions. The cardiovascular measurements are done in control experiments on unrestrained unanesthetized rats and in the same animals in hypokinetic conditions or during readaptation to free activity.

Animals and techniques. Because surgery and anesthesia drastically decrease cardiac output and other circulatory parameters in rats (Popovic, V. P. and Kent, K. M. Am. J. Physiol. 207, 767-770, 1964; Popovic, Pava, Popovic, V. P., Schaffer, R. and McKinney, A. S. Proc. Soc. Exp. Biol. 154, 391-396, 1977; Popovic, Pava, Schaffer, R. and Popovic, V. J. Neurosurgery 48, 962-969, 1978). Unanesthetized, unrestrained, resting Sprague-Dawley rats (200 ± 10g) are used in all experiments. Aorta and right ventricle of the animals are permanently cannulated ten to fifteen days before the experiment. Arterial and central venous blood pressure, intraventricular ECG, cardiac output, and other cardiovascular parameters are measured with adequate techniques already developed and routinely used in our laboratory.

The research accomplished during the last 12 months is summarized here:

1. "Stress hormones" in antiorthostatic hypokinesia. We have continued to study "stress hormones" in the blood of head-down hypokinetic rats. Determination of the levels of these hormones represents a way to measure stress imposed on the rats by placement of the harness and by the head-down position. We have already shown that ACTH, corticosterone, and prolactin (determined by radioimmunoassays) were elevated in the early exposure to antiorthostatic hypokinesia, but that after six-seven days of exposure the animals adapted to the unusual position, as judged on the basis of return of these three hormones to the normal level. In the continuation of this study we measured plasma growth hormone and plasma catecholamine levels in rats before, during and after seven days of antiorthostatic hypokinesia. The sampling was done through chronic aortic cannula three times before, three times during (on the first, third and seventh day) and three times after release of the animals from the harness (second, fifth and tenth day) and antiorthostasis. Only 0.3 ml of blood was withdrawn each time for hormone determination. Selection of growth hormones and of catecholamines was based on their common link to the existence of exogenous stressors. Plasma catecholamines were

elevated on day 1 and day 3 of the antiorthostatic exposure. The plasma growth hormone level was decreased only on day 1. On day 7 of the exposure, the plasma levels of both hormones were only slightly above the control levels. After release from the harness (and after return to their own cages) the hormone levels were again elevated though not as much as during the antiorthostatic exposure. The elevation lasted 2-3 days.

2. Harness versus negative tilt, stress factors in antiorthostatic hypokinesia. Similar study as described above has been done using antiorthostatic hypokinetic rats; that is hypokinetic exposure was the same as before but without head-down position (orthostatic animals). The increase in the plasma level of stress hormones was very small and it lasted only a few hours (the first day). The level of hormones was at or near control values during the next 6 days. Removal of harnesses did not induce any increase of studied hormones.

3. Immobilization and hypokinesia, physiological differences. Some of the investigators that are studying similar problems as the NASA's small animal consortium are using other animal models than the Holton. Especially the scientists from the Eastern block are using often complete or almost complete immobilization of the animals (rats or bigger domestic animals). We have collected as much information about immobilization and will review comparatively physiology of immobilization and physiology of hypokinesia. We have studied in our laboratory hormonal stress levels during 30 min immobilization in rats repeated seventeen times during a period of seventeen days. The main difference was that during repeated immobilization adaption processes were completely absent while during exposure to antiorthostatic hypokinesia the animals adapt after 3-4 days and in the case of orthostatic hypokinesia this process is only a few hours long.

4. Determination of ADH and angiotensin II in the blood of antiorthostatic animals. Though this study is still in progress we have determined that the ADH level was decreased the first day of exposure to antiorthostatic hypokinesia but the angiotensin II level was only slightly elevated. Aldosterone levels are being presently determined in antiorthostatic rats.

5. Intravascular administration of glycerol and cardiac output in antiorthostatic rats. We have shown earlier that administration of glycerol expands plasma volume to such a degree that total interstitial space is decreased by approximately 2 percent and that the effect is observed even after 8-12 hours of glycerol administration. In this study we have administered glycerol to antiorthostatic rats 1, 3 and 7 days after exposure to hypokinesia.

6. Circadian rhythm and cardiac output in antiorthostatic rats. Several years ago we concluded on the basis of colonic temperature in rats that in early beginning of antiorthostatic exposure circadian variations are absent. An abnormal temperature regulation and often hypothermia were observed that might disturb the circadian regulations. Recently we measured cardiac output in antiorthostatic rats 4-7 days after initiation of hypokinesia and we found that it follows circadian patterns.

REGULATION OF HEMATOPOIESIS IN THE SUSPENDED RAT  
AS A MODEL FOR SPACE FLIGHT.

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NASA Grant NAGW-308.

During the first year's effort on this project, a series of studies was completed in which a variety of routine hematological and other parameters were obtained from sequential sampling of control and suspended rats. These data showed that, during suspension, the rats failed to gain weight at the same rate as the controls, ate and drank significantly less, demonstrated a transient increase in peripheral hematocrit and RBC count, a transient decrease in MCH (with the overall result that the hemoglobin concentration did not change significantly), suppressed reticulocyte counts and a progressive decrease in MCV but no change in RBC shape. The hemoglobin P50 was shifted to the right. Leukocyte counts were variably decreased but no significant changes in platelet numbers were noted. Post-suspension, evidence of anemia was present from a reduced RBC count, hemoglobin, hematocrit, and MCV. A leukocytosis was also noted. These changes were largely independent of the site from which the blood was obtained, the size of the rat or the mechanism of suspension. However, indwelling arterial catheters produced a significant increase in RBC production (presumably as a consequence of a response to hemolysis). As a result non-catheterized animals are now used. These are suspended in a harness thus leaving the tail in good condition for injections and blood sampling.

The past year's efforts have been directed at the collection of data aimed at understanding changes in blood volume during suspension. As part of these studies the following parameters were investigated; RBC survival, in vitro leukocyte reactivity to PHA, bone marrow and spleen cellularity and morphology, ferrokinetics, and the hematopoietic inductive microenvironment. Blood volume (red cell mass and plasma volume) appears to be decreased as early as the second day of suspension. This is associated with a decrease in bone marrow cellularity (particularly of the erythroid elements) and reduced radioiron incorporation. Leukocyte reactivity remains unchanged although there is considerable variability from rat to rat. <sup>51</sup>Chromium-labeled RBC are cleared more slowly in suspended rats than in control animals. However, when the animals are returned to the usual vivarium environment or if their RBC are transfused into normal animals, the cells are cleared much more rapidly than are cells from control rats. These phenomena are most clearly observed towards the end of seven days of suspension but are also apparent in the first one or two days. The rapid clearance of these RBC may be contributing to the



post-suspension decrease in RBC count and hematocrit which we had previously considered were due only to plasma volume repletion. Another mechanism possibly contributing to the anemia, is the weight loss and/or reduced nutritional intake. Rats, fed the same quantities of food and water as those consumed by rats during suspension, show similar, but less marked, decreases in body weight, blood volume, radioiron incorporation and reticulocytes, than their suspended counterparts.

In summary; we have shown that, during antiorthostatic hypokinesia, a variety of hematological effects are apparent in the peripheral blood of rats. These are also manifest at a whole body level in a reduced red cell mass and plasma volume. To date, we have identified two factors which appear to contribute to these changes - a reduced food and/or water intake, and a change in RBC survival. The relative importance of these and other factors are currently under investigation.



## C. MECHANISMS OF PLANT RESPONSE

## GRAVITROPISM IN LEAFY DICOT STEMS

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NASA Grant NSG-7567

During much of the period covered by this report, the principal investigator was on sabbatical in Austria and Israel. His research plus that of 3 students in Utah is encompassed by the following three topics (underlined):

Plant Responses to Clinostating and Mechanical Stress (Chauncy S. Harris). In an attempt to separate plant responses to mechanical stresses from responses to gravity compensation, we have now automated six treatments: 1) upright stationary controls, 2) horizontal clinostat, 3) intermittent clinostat (plants upright 3.3 minutes out of every 4 minutes, horizontal and rotated once in the remaining time), 4) inversion every ten minutes (plants upside down half the time), 5) inversion and immediate return to the vertical, and 6) vertical rotation. Plant height and diameter changes were measured at ink dots with a vertical-translator microscope (50X) with dial indicators, allowing measurement without touching the plants. Leaf epinasty was measured as in our previous studies (reciprocal of radius of circle matching leaf curvature). Plants used were Xanthium strumarium (cocklebur) and Lycopersicon esculentum (tomato). Flowering response of cockleburs was also studied.

As in past studies, epinasty appeared only on clinostated and on inverted plants, both subjected to gravity compensation. Effects on stem elongation and thickening were much more ambiguous, however. Horizontal rotation promoted the elongation of partially mature stems, for example, but inhibited the youngest portions. There was no increase in stem diameter. Mechanical stress (clinostating, intermittent clinostat, vertical rotation) slows the transport of the flowering stimulus. Induction of flowering is attenuated by prolonged gravity compensation (clinostating), and ethylene probably plays a role.

The Mechanics of Gravitropic Stem Bending (Wesley J. Mueller and Frank B. Salisbury). Cocklebur stems were subjected to four treatments: 1) horizontal restrained stems (stem placed between two stiff wires and wrapped with thread), 2) horizontal free bending, 3) vertical controls, and 4) stems restrained for up to 48 hours and then released by cutting the threads. Stems bent immediately, typically to about 135°. Measurements used techniques of stereo photogrammetry with two 10.2 X 12.7 cm (4 X 5) cameras, mounted perpendicular to the horizontal base, 21 cm apart, and 100 cm above the base. Both tops and bottoms of horizontal stems were photographed. Length measurements were taken between dots of india ink on stems by projecting the negatives onto a digitizer interfaced with a microcomputer.

Growth nearly stops on the top of horizontal free-bending stems and is accelerated on the bottom compared to controls. In certain regions on the top, growth not only stops but surface dimensions actually decrease slightly indicating shrinkage. Horizontal restrained plants grow during restraint, on both top and bottom. Upon release, the top of the stem shrinks considerably (sometimes to the dimensions before the stem was laid on its side), and the bottom elongates. These results emphasize the cessation of growth in the upper stem tissues as soon as stems are turned to the horizontal.

When a vertical stem is sliced longitudinally from the tip toward the

base, the two halves bend away from each other indicating that internal stem tissues are under pressure compared to surface tissues. Tension and pressure in castor bean stems (*Ricinus communis*) was studied by making horizontal cuts at 2.5-mm intervals halfway around one side of the stem. Depth of the cuts was controlled by the extent to which a razor blade protruded from between two pieces of a metal holder. Depth of the cuts was measured on free-hand sections after the degree of bending (always away from the side of the cuts) had been measured. Plotting degree of stem bending as a function of depth of the cuts showed that maximum bending was achieved when the cuts were a little less than two millimeters deep, extending through the epidermis, collenchyma, cortex, and at least into the phloem of the vascular bundles. Thus, pressure seems to be localized in the pith (even in these hollow stems), and tensions apparently exist in all the remaining tissues.

A device was built to cut longitudinal sections of measured thickness from the center of plant stems. When this is done with castor bean stems, outward bending of the two halves is greater and usually equal for the two halves of a vertical stem. When horizontal stems are bending upward, however, the upper half of the longitudinal slice always bends about 20° more than the lower half, emphasizing the importance of tissue tensions of the upper half of free-bending stems. Split, longitudinal, central sections were placed in sorbitol solutions of varying concentrations. Stems placed in solutions or pure water hypotonic to the cell sap continued to bend as pith cells took up water; in pure water, bending reached about one thousand degrees. Hypertonic solutions caused an inward bending (a straightening) of the two stem halves. Further development of these approaches combined with the digitizer should provide further insights into the mechanics of stem bending.

Effects of a Unilateral Application of Ethephon on Gravitropic Bending (Rosemary White). Solutions of ethephon (as Florel--3.9%: (2-chloroethyl) phosphonic acid) were painted on one side of stems and hypocotyls of young tomato, cocklebur, castor bean, and sunflower (*Helianthus annuus*) plants. Stems of vertical plants bent away from the side of application within 2-3 h; the response was most marked in the hypocotyls of tomatoes, but cockleburs showed no response other than an injury of the shoot apex. When ethephon was neutralized to pH 6, vertical stems did not bend away from the side of application, and there was no stem damage, but leaves developed epinasty, a typical ethylene response. When solutions of buffered acid or even unbuffered HCl were applied to one side of vertical stems, bending occurred away from the side of application, epinasty did not develop, but stems were damaged in a manner similar to that produced by unbuffered ethephon. There was no plant response to application of NaCl or KCl.

Buffered ethephon solutions (pH 6) or acid solutions applied to one side of clinostated plants produced highly inconsistent results. There was no consistent bending away from or toward the side of application. Ethephon solutions (pH 6) applied unilaterally increasingly inhibited total stem elongation with increasing ethephon concentrations--but no bending was observed. When ethephon (pH 6) was applied to the bottom of stems just turned to the horizontal, gravitropic bending was delayed. Acid solutions applied to the bottom of horizontal stems greatly delayed bending--an unexpected result in light of the experiments just described. Ethephon (pH 6) applied to the upper surface of horizontal stems caused a small delay in gravitropic bending, but acid solutions applied to the top of horizontal stems caused downward bending.

MECHANICAL REGULATION OF PLANT GROWTH & DEVELOPMENT

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Gravity is a static physical force which has obvious significance for plant growth and development. Mechanical disturbance in the form of wind and precipitation are dynamic physical forces which have a significant growth-limiting impact on plant growth in the outdoor environment. In the wind-protected environment of a growth chamber or greenhouse, natural mechanical disturbance may be mimicked by periodic application of seismic (shaking) or thigmic (contact) treatment. Although mechanical stresses generally limit plant growth, yield, and productivity, they also strengthen stems and have potential to substitute for some aspects of gravity in an orbiting spacecraft.

Soybean (Glycine max Merr. Wells II) and Eggplant (Solanum melongena cv. Black Beauty) grown and shaken in a greenhouse exhibited decreased internode length, internode diameter, leaf area, and fresh and dry weight of roots and shoots in much the same way as outdoor-exposed plants. Perhaps more important than decreased dimensions of plant parts resulting from periodic seismic treatment is the inhibition of photosynthetic productivity that accompanies this stress. Soybean plants briefly shaken or rubbed twice daily experienced a decrease in relative as well as absolute growth rate compared to that of undisturbed controls. Growth dynamics analysis revealed that virtually all of the decline in relative growth rate (RGR) was due to a decline in net assimilation rate (NAR), but not in leaf area ratio (LAR). These growth dynamics parameters are related to each other according to

$$\text{RGR} = \text{NAR} \times \text{LAR}.$$

Lower NAR suggests that the stress-induced decrease in dry weight gain is due to a decline in photosynthetic efficiency. Possible effects on stomatal aperture were investigated by measuring rates of whole plant transpiration as a function of seismo-stress, and a transitory decrease followed by a gradual, partial recovery was detected. Leaf water potential of soybean was significantly higher 30 minutes after 5 minutes of shaking at 240 rpm, relative to that of undisturbed controls. Both water status effects are consistent with a transitory, stress-induced reduction in stomatal aperture.

Seismic stress caused an immediate drooping of soybean leaves (at the pulvini), which did not recover to their equilibrium position for as much as 2 hours, depending on their mechanical stress pre-history. Reorientation of drooped leaves to a horizontal position resulted in elevation of attached leaf photosynthetic rate by shaken plants above that of control plants, an apparent artifact resulting from stress and/or leaf reorientation, since

overall plant photosynthetic productivity was retarded by shaking. Continuous photosynthetic rate measurements of whole plants, including the shake period, revealed an immediate decline in carbon dioxide assimilation for at least 40 minutes following a 5-minute seismic stress episode, followed by a gradual recovery which is not yet complete 1 hour after shaking. Long-term measurements of the photosynthetic stress response, as well as resistance component analysis of leaves with or without reorientation from the droop position, are in progress.

Measurement of Thigmomorphogenesis and Gravitropism by  
Non-intrusive Computerized Video Image Processing.

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NASA grant NAGW 96

Until now, auxonometry has always intruded on the plant in some way. These intrusions have included touching the plant, tying a string to the plant and illuminating the plant with unidirectional light. We have developed a video image processing instrument: "DARWIN" (Digital Analyser of Resolvable Whole-pictures by Image Numeration) This instrumentation includes both video image enhancement and image analyses and can be used to count and measure any images that the computer can "see". We have programmed DARWIN to measure stem or root growth and bending, and coupled it to a specially mounted video camera to be able to automatically generate growth and bending curves during gravitropism.

In practice, the plant holder is fixed in relation to the camera, so that however they are reoriented, the image on the CRT remains the same. The camera controls are used to enhance the image, so that the contrast between the object and its background are maximized. The resolution of the instrument is limited only by the magnification used with the camera. The reproducibility error is less than 0.5%. The growth of the plant is recorded on a video cassette recorder with a specially modified time lapse function. At the end of the experiment, DARWIN analyses the growth or movement and prints out bending and growth curves.

We have used this system to measure thigmomorphogenesis in light grown corn plants. If the plant is rubbed with an applied force load of 0.38 N., it grows faster than the unrubbed control, whereas 1.14 N. retards its growth. Image analysis shows that most of the change in the rate of growth is caused in the first hour after rubbing.

When DARWIN is used to measure gravitropism in dark grown oat seedlings, we find that the top side of the shoot contracts during the first hour of gravitational stimulus, whereas the bottom side begins to elongate after 10 to 15 minutes. The rate of elongation of the bottom side is somewhat faster than the elongation of both sides during the previous vertical growth.

These experiments show that the growth of plants can be measured in a completely non-intrusive manner, and that



high-resolution growth and bending curves can be obtained  
thereby

The Roles of Callose, Elicitors and Ethylene in Thigmomorphogenesis and Gravitropism.

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NASA grant NAGW 96

We have demonstrated a correlation (both temporal and through the inhibitor, 2-deoxy-D-glucose) of callose deposition and ethylene evolution in mechanically perturbed (MP) bean or pine stems or in gravitationally stimulated corn shoots. In the proposal, it was suggested that the callose, which is deposited on the inside of the cell wall, and adjacent to the plasma membrane causes, in some way, the ethylene production. There are two possible hypotheses which might explain the mechanism. In the first, it is possible that the mechanical pressure of the callose might press against the membrane, distort and perturb it locally, and that this perturbation might activate the enzymatic system which converts ACC to ethylene, and which is thought to reside in the plasma membrane. Although such an hypothesis may be involved in the MP induced ethylene production, we have tested a second hypothesis: That there is a chemical activation of the enzyme system by the callose which is being deposited in apposition with it.

We now have generated data that suggests that the latter supposition may indeed be true. Callose, which is believed to be mostly a B-(1,3) glucan with some b-(1,4) linkages is made by vascular plants. Other B-(1,3) glucans are made by other types of plants. For example, laminarin, a B-(1,3) glucan with some B-(1,6) linkages is of algal origin and is available commercially. We have treated bean stem sections with laminarin and found that it will cause an increase in ethylene evolution. The increase is greatest at pH 5.8, the calculated pH of plant cell walls. In our hands, 0.5% laminarin produces the best effect. In another experiment, carboxymethyl cellulose (a soluble form of cellulose, B-(1,4)glucan) caused a 2% increase and starch (a B-1,6)glucan) caused a 7% increase in ethylene production, while laminarin caused a 36% increase. Laminarin may not be the best model for callose since it has some B-(1,6) linkages instead of some B-(1,4) linkages. UDP-glucose is believed to be the natural precursor for natural callose biosynthesis. We have tried adding UDP-glucose to stem sections and find that it causes large amounts of callose to be deposited at the cut ends. Quantifying aniline blue staining with computer-assisted video image analysis, we find that UDP-glucose treated stems produce 3.3X their amount of callose

as non-treated controls. Accordingly, we compared the effect of exogenous UDP-glucose to that of exogenous laminarin on ethylene production. Ten mM UDP-glucose caused a 35% increase in ethylene production over the control, in an experiment where 0.5% laminarin or MP caused a 74% increase.

All of these experiments support the hypothesis that there is something special about the B-(1,3)glucan configuration, so that when it is deposited next to, and comes in contact with the plasma membrane, it can activate the enzymatic conversion of ACC to ethylene. At present, there is considerable variation in the ability of the laminarin to induce ethylene production (e.g. 30-74% greater than controls). We plan to standardize our methods better when we start to use extracted callose instead of laminarin. However, in all cases, laminarin did induce significantly more ethylene evolution than did the controls.

We have also shown that elicitor-like activity is caused by MP, and that this elicitor activity can cause the same morphological effects as do MP. This activity peaks at 1 h after MP and therefore may also be involved in the early events of thigmomorphogenesis. If callose is broken down by a B-(1,3) glucanase, later in the progress of the syndrome, a B-(1,3) glucan oligosaccharide might result. This possibility is currently under investigation.



BIOPHYSICAL MECHANISM OF DIFFERENTIAL GROWTH DURING GRAVITROPISM

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NASA Grant NSG-NAGW-480

The goal of this research is to determine the mechanism of gravitropic curvature in plant stems at the biophysical and the cellular level. The reorientation of plant organs under the influence of gravity is due to differential growth of the upper and lower sides of the organ. In the case of stems, the lower side grows at a faster rate than the upper side. The rate of plant cell enlargement is governed by four biophysical parameters:

- (1) the extensibility of the cell wall,
- (2) the minimum stress in the cell wall required for wall expansion (the "yield threshold"),
- (3) the osmotic pressure difference between the cell contents and the water source, and
- (4) the hydraulic conductivity of the pathway for water uptake.

Gravitropic response must involve differential alteration of one or more of these four parameters on the two sides of the growing organ. Each of these factors will be examined to assess the role it plays in gravitropism.

Pea stem internodes will be studied, since much background information concerning their growth physiology is available and they are well suited for many of the experimental procedures requiring turgor pressure measurements. The first step in this program will be to define quantitatively the growth response of pea stems to gravistimulation using a high-resolution marking technique. This information will indicate where and when to look for changes in the biophysical parameters governing growth.

A modified pressure probe will be used obtain data on the osmotic characteristics of the growing tissue. Turgor pressure of cells on the upper and lower sides of the gravitropically responding stem will be directly measured with this apparatus under conditions of zero transpiration. The change in turgor pressure, compared with controls, will indicate whether the change in growth rate is due to a change in the water uptake characteristics of the tissue. For example, a decrease in the hydraulic conductivity or the osmotic pressure gradient should induce a decrease in cell turgor pressure. In contrast, a decrease in the yielding characteristics of the cell wall will result in no change in turgor or an increase in turgor (i.e., under conditions where water uptake is at least partially limiting growth).

Hydraulic conductivity of cell membranes will be measured directly with the pressure probe. At the same time, the volumetric elastic modulus of the cell, which governs reversible changes in cell size, will be measured. Two other methods will be employed for evaluating hydraulic conductivity. One method measures only differential changes in the hydraulic conductivity of the two sides of the stem. The other calculates hydraulic conductivity as the ratio of the growth rate over the water potential gradient between the growing tissue and the water source. The water potential of the growing cells will be calculated from measurements of turgor pressure and osmotic pressure of the cell sap.

The osmotic pressure of the cell sap on the two sides of the stimulated stem will be measured by a bulk method and a microtechnique. The bulk method involves measuring the osmotic potential of cell sap expressed from bisected halves of pea stems. The microtechnique requires that the vacuolar contents from single cells be removed using the microcapillary of the pressure probe and measured with a freezing point osmometer capable of measuring volumes as low as 100 pl (ca. the size of a cell). This microtechnique will permit detection of osmotic pressure changes which are localized to the epidermal cell layers. There is evidence that the epidermal layers control the growth of stems.

The yield threshold and the extensibility of the cell wall will be measured by a modification of the conventional procedure which involves incubation of segments in solutions of different osmotic pressures. A second, novel method for estimating the yield threshold will be to remove the water supply from the growing tissue and measure the consequent decrease in turgor pressure. In theory, continued loosening of the cell wall should decrease the turgor pressure to the yield threshold. From the combined data on turgor pressure, yield threshold and growth rate, it will be possible to calculate wall extensibility.

The cell wall free space solution of growing stem tissues has been found to contain a significant concentration of solutes (osmotic pressure ca. 2 bars). The presence of these solutes must be taken into consideration when calculating hydraulic conductivity from the water potential data. By use of a perfusion method, the cell wall solution will be sampled from the two sides of gravistimulated internode segments to examine the possibility of differential alteration of the external osmotic pressure. The pH and calcium concentration of the solution will also be assayed.

From the results of these experiments, it will be possible to construct a quantitatively accurate model of the biophysical basis of differential growth induced by gravistimulation.

## SESSION III: ROLE OF GRAVITY IN DEVELOPMENT





## A. PLANT STRUCTURE, GROWTH AND DEVELOPMENT



CELLS, EMBRYOS AND DEVELOPMENT IN SPACE

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Work in this laboratory continues to focus on the demonstrable totipotency of cultured somatic cells of various higher plants and has examined the conditions which regulate this propensity to be controllably released. This has been done with special reference to cells obtained from cultured explants of daylily and carrot. For purposes of identifying the variables in question, work has been carried out almost exclusively in liquid media. The events that intervene between the aseptic isolation of tissue explants, the culture of small derived units and free cells and the propagation in large numbers of adventive or somatic embryos to plantlets have been traced and certain definitive stages at which control may be exercised have been identified. In daylily, morphologically competent units can now be propagated with a high degree of precision in rotated liquid cultures in bulk, and under the conditions of continuous neutralized gravity, the development is restricted. After plating in/on semi-solid media, development progresses so that embryo-plantlets are obtained. The feasibility of using protoplasts from morphogenetically - competent cells, especially those of daylily, has been examined in further detail. Cytological examination of cells, plantlets and fully grown plants has, in the past, permitted characterization of various chromosomal events and conditions, thus permitting better use of the systems in probing problems of space plant biology experimentation. But the efficiency of our systems has posed several problems for they are labor-intensive and require concentrated effort. Methods have now been improved which permit quicker analysis. Access to material which can be accurately karyotyped should permit effective use of these highly sensitive and defined systems to distinguish between problems of radiation damage, exposure to microgravity or conditions of space flight in a much more convenient fashion.

The detailed karyology of the developmental pathways and specifics of growth and differentiation exhibited by aseptically cultured daylily cells and protoplasts as they yield callus which undergoes transition to discrete plantlets has been analyzed exhaustively using the new procedures. The techniques which now have now been worked out permit conclusive and definitive establishment of karyotype of cells and protoplasts with a high degree of confidence. A special feature of the system is that the techniques minimize the number of cells in each unit necessary for analysis thus reducing potential of erroneously interpreting the production of chimeras. Some of our past protoplast work in particular has suggested that polyploids or chimeras may be induced as a result of experimental procedures or possibly from spontaneous chromosomal abnormalities since leaves of some control plants occasionally produced small white islands of cells (variegation).

Field observations and measurements of bloom characteristics of large field plantings of daylily (4 plots of over 100,000 blooms!) cultured in

different ways and for different periods has been particularly instructive. Data comprised of number of scapes, number of blooms, floral morphology, color etc. have permitted us to establish the mode and means of culture best suited to a specific set of needed experimental protocols.

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IN VITRO SEED TO SEED GROWTH ON CLINOSTATS

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The effect of a long term micro-gravity environment on the life cycle of plants is unknown. Whether higher plants have evolved to a stage where removal or reduction of gravity is detrimental to plant life cycle and thus fatal to the plant species, is an unanswered question. In space plants have been successfully grown through the various stages of their life cycle. Attempts to grow plants as a continuous integral process from seed to seed through one generation have not been successful until recently.

Culture of plants through multiple generations has not been accomplished in space nor in ground based studies. We are investigating the effect of long term simulated weightlessness by growing consecutive generations of plants continuously on clinostats using the cruciferous plants Arabidopsis thaliana (L.) Heyhn. and Cardamine oligosperma Nutt.

We have successfully grown Arabidopsis from seed to seed of the next generation on clinostats. All of these plants were healthy and produced more seeds than their upright stationary controls. The methods and techniques to accomplish this goal were developed during the past year. From our investigations, we conclude that the failure of previous investigators to grow vigorous seed producing Arabidopsis plants under space and clinostat conditions in vitro was due to the suboptimal cultural conditions they used. We have overcome these shortcomings.

Three important factors in culturing plants on a clinostat have emerged from the first year's work. These are container size, gas exchange, and moisture level.

The size-of-container experiment has shown that the larger the container, the faster the plant grew and flowered. Conversely, the smallest size used, 20 x 150 mm, severely restricted growth and the plants did not flower by the end of the experiment. The containers in this experiment were covered by Saran Wrap, which effectively blocks the passage of CO<sub>2</sub> necessary for photosynthesis. The plant growth rate, seed development, and final plant size observed in this experiment more likely reflects the original amount of CO<sub>2</sub> in the containers. On the other hand, the accumulation of metabolites in the container atmosphere or the size of the container itself may have had a role here. The initiation of bolting and rate of bolting were respectively delayed and suppressed.

The development of Arabidopsis and Cardamine was affected by the gas exchange capacity of the container. Transmission of various gases such as CO<sub>2</sub> and water vapor is blocked most effectively by Saran Wrap, followed by Mylar film, then by cotton plug and least by polyurethane foam plug. In cultures covered by Saran Wrap, the plants readily formed flowers but were strongly inhibited in seed pod development; likewise, but less so, for cultures covered by Mylar film. Cultures with open top, cotton plug and polyurethane foam plug readily produced seeds. Thus, in order for Arabidopsis or Cardamine to form seeds in vitro, free gas exchange with the ambient atmosphere appears to be

necessary. It is not known what changes occur in the gas ratio of the atmosphere within the culture tubes nor what levels of the various gases are required for seed development.

The amount of moisture contained in the culture was found to affect the general morphology of Arabidopsis. Agar medium cultures planted soon after cooling and sealed with Saran Wrap or Mylar film retained a high amount of water which then condensed on the walls, drained down, and covered the emerging seedling. These seedlings, growing in a wet environment, developed more slowly, produced larger, more numerous leaves, and more stems than cultures with cotton plugs, polyurethane plugs, or opened to air, which usually did not have free standing water on the surface of the agar. Furthermore, under clinostat treatment, more roots were formed in cultures having high moisture content and part of these roots grew "above" the agar medium along the wet walls obscuring the stems.

The cause of the enhanced root growth "above" the agar was not determined, but may be due to the preferential growth of roots in solutions having a low osmoticum. The free water, part of which may have originated as transpired water, would probably be lower in its osmotic potential than that found in the agar medium and could thus be the mitigating condition for the enhanced root growth "above" the agar medium. These are just conjectures and need to be substantiated with direct evidence. For this reason, the effects of various osmotic potentials on Arabidopsis and Cardamine root growth need to be determined and correlated to the osmoticum of the free water and that of the agar medium. The basis for the enhanced root growth may possibly be determined in this manner.

From the findings of the past year, we conclude that gas exchange with the atmosphere is necessary and appears to ameliorate the problems of small container size and excess moisture. Therefore, we selected for our studies the polyurethane foam plugged test tube as our culture container. It is not known at this time if lack of CO<sub>2</sub>, the accumulation of metabolic products such as ethylene or excess moisture are singly or in combination responsible for the lack of seed set and poor growth seen on the limited gas exchange cultures.

This year's work has thus produced information that for the first time makes possible the task of growing Arabidopsis for multiple generations on clinostats. Since no information is available on the effect of continuous simulated or true weightlessness on the growth, flowering and seed production in sequential generations of plants, we plan in the following years to carry out studies using Arabidopsis thaliana (L.) Henyh. A second species, Cardamine oligosperma Nutt., will be used to corroborate the Arabidopsis findings.

IMPORTANCE OF GRAVITY FOR PLANT  
GROWTH AND BEHAVIOR

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NASA Grants:

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RESEARCH — Accomplishments during the past fiscal year consisted of (a) completion of research on a study of the kinetics of damping out of circumnutation when the axially directed *g*-force has been abruptly eliminated (Research Task NULYRL). The methodology took advantage of simulated hypogravity to test a currently popular model for circumnutation. (b) further experiments were accomplished on a comprehensive study, underway for several years, to validate (or invalidate) the use of clinostat rotation as a hypogravity simulation device. A few more experiments remain to be done on this study before it can be written up for publication (Research Task SIMCOM). (c) Some of our earlier (unpublished) observations on plant seedlings' resistance to *g*-loading were evaluated, interpreted, and a paper was submitted for publication (Research Task HYGEFF). (d) In what has been called "Shuttle middeck locker ecology" an attempt to acquire reliable empirical information on the thermal profile experienced by test packages housed in middeck lockers (MDDL) during shuttle flights at long last seems to be yielding some results. Since the first such data were obtained incidental to the HEFLEX Bioengineering Tests (HBT) on STS-2 and STS-3 requests from the SL-1 IWG and from other sources including NASA HQ to obtain similar data on all missions prior to STS-9 were uniformly unavailing. However on STS-8, four temperature recorders were placed in different MDDL locations and the data they acquired are now available. Since the means for stabilizing and maintaining any chosen temperature in the Shuttle middeck within the limits most biological experiments would require is patently not within the design capabilities of the Shuttle, potential MDDL users will be interested in any and all empirical measurements that may help to establish MDDL environment prospects.

SCIENTIFIC MEETINGS — Three meetings were supported: (a) A workshop on space biology at DFVLR-Institute of Aerospace Medicine, Cologne, Germany (Sponsored by ELGRA, ESA, and DFVLR). An introductory Paper has been published in the workshop proceedings. (b) A meeting of the International Union of Physiological Sciences Commission on Gravitational Physiology took place in Moscow. A research paper was presented and the manuscript is now in press. (c) At a meeting of the American Society of Plant Physiologists in Ft. Collins, CO, a paper was presented at a symposium on plant gravitational physiology. A manuscript is in preparation.

FUTURE RESEARCH PLANS — In order of urgency the following tasks are projected: (a) To complete task NULYRL (see above) by writing a manuscript for publication; (b) To finish experimental work on task SIMCOM (see above) and prepare a manuscript for publication; (c) To measure the hyponastic response attributable to an axially directed  $g$ -force, in this case a controlled variable centripetal force. This task relates to a proposed model to account for hyponasty as a clinostatting artifact; (d) To measure for small transverse  $g$ -forces the shoot tip displacement associated with such  $g$ -loading. Preliminary tests have been performed to validate the methodology. This task relates to the "noise level" of zero  $g$  simulation by the horizontal clinostat. (e) To provide indirect support to accepted or candidate flight experiments, HEFLEX, GTHRES, FOTRAN.

PUBLICATION EFFORTS, 1983 — The following papers are published, in press, or in preparation:

- Brown, A. H. The significance of gravity on biological systems. Space Biology with Emphasis on Cell and Developmental Biology, Proceedings of a Workshop at DFVLR - Institute of Aerospace Medicine, Cologne, 9-11 Mar. 1983; ESA SP-206 May 1983. pp3-9
- Brown, A. H. Resistance of mature Arabidopsis plants to mechanical deformation in relation to  $g$ -force during development. Paper presented at Fifth Annual Meeting IUPS commission on Gravitational Physiology. Manuscript in press in The Physiologist.
- Brown, A. H. and Chapman, D. K. Use of simulated hypogravity to test a model for circumnutation. Paper presented at Amer. Soc. Plant Physiol. symposium on gravitational physiology, Aug. 1983. Manuscript in preparation.
- Brown, A. H. and Chapman, D. K. Experiments on plants grown in space: A test to verify the biocompatibility of a method for plant culture in a microgravity environment. Manuscript in press in Annals of Botany.



THE ROLE OF GRAVITY IN LEAF BLADE CURVATURES

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NASA Grant NAGW-131

In the past year we have gained useful information on several aspects of leaf blade growth. The most important observations, discussed in more detail below, are as follows:

1. We have observed the  $^{14}\text{C}$ -IAA moves preferentially in a gravipositive dorsiventral direction through the blade. This movement is inhibited by inversion of the blade.
2. We have verified by direct measurement that the responding cells in leaf blade hyponasty are in the lower epidermis and bundle sheath cells.
3. We have characterized two additional responses in the leaf. In addition to blade curvature, the leaf shows petiole curvature and changes in the liminal angle subtended by the pulvinus.
4. We have studied ethylene production under a number of conditions and note that the blade, rather than the petiole or pulvinus, is the principal site of auxin-promoted ethylene synthesis.
5. We have reviewed the effects of a variety of agents on the blade, including gibberellic acid, abscisic acid, vanadate, low pH buffers, and blue light.

I. Auxin Transport Studies:

We have long been interested in the hypothesis that auxin applied to the upper surface of the leaf moves to the lower side in a gravipositive auxin transport system. Indirect evidence supports this hypothesis: 1) auxin transport inhibitors applied to the lower (but not the upper) side of the leaf inhibit hyponasty and produce epinasty 2) ethylene and ethylenegenic agents, which act as auxin transport inhibitors in some systems, inhibit hyponasty and promote epinasty; 3) inversion and clinostat rotation inhibit hyponasty; 4) temperatures at which transport is inhibited in other systems inhibit hyponasty; 5) the time course of curvature is comparable to that of other responses in which transport has been demonstrated. However, alternative hypotheses (differential dorsiventral sensitivity to auxin; direct growth effects of ethylene; differential ethylene synthesis, etc.) could also explain some of the phenomena observed.

We therefore decided to measure auxin movement through the leaf directly. Agar discs containing a specified concentration ( $10^{-3}$  to  $10^{-6}\text{M}$ ) of  $^{14}\text{C}$ -IAA were layered over 10mm discs which were layered over agar receiver discs. Receiver discs were removed for counting at specified intervals. The amount of IAA in donors, blades, and receivers was quantitated with a Tri-Carb liquid scintillation counter. In other experiments, the donor disc was applied to the lower (ventral side and the receiver disc was placed on the upper side. In further experiments, the blade disc was inverted and movement in both directions was measured. Movement of auxin showed a strong preferential gravipositive direction.

II. Cytological Studies:

SEM photomicrographs of whole leaves and sectioned leaves taken from various angles confirm the earlier observation that the greatest growth during hyponastic curvature is the expansion of cells in the lower epidermis and in the bundle sheath cells.



## The Role of Gravity in Leaf Blade Curvatures

### III. Petiole and Pulvinar Response:

The auxin treatment that produces blade hyponasty subsequently results in a reduction of the liminal angle (pulvinar epinasty) and petiolar epinasty. Excised pulvini and petioles do not show a surge in ethylene production when immersed in auxin, but leaf blade discs in auxin solution show a substantive increase in ethylene production. When treated directly with auxin, both pulvini and petioles respond on the treated side - i.e., auxin to the upper side produces epinasty and to the lower side produces hyponasty. However, when it is the blade that is treated with auxin, both pulvini and petioles show epinasty. Rotation promotes the pulvinus and petiole effects, but inhibits hyponasty. Studies with ethylene inhibitors show that ethylene is required for the pulvinar response.

### IV. Ethylene Production:

We had previously observed that ethylene was produced in response to IAA treatment and in response to clinostat rotation. However, ethylene does not have a dramatic effect on leaf blade morphology. Ethylene is moderately epinastic but is not as effective in producing blade epinasty as are auxin transport inhibitors like TIBA or NPA. It does, however, have an epinastic effect on the petiole and pulvinus, resulting in a change in the position of the blade from its "day" diageotropic position towards the vertical "night" position.

The main site of ethylene production in the leaf is in the blade, not the petiole or pulvinus, and ethylene production in auxin treated leaves does not begin until after leaf hyponasty has begun. This is important in helping us understand the adaptive mechanism of the leaf. The change in position of the blade is a response in the pulvinus after ethylene is produced in the leaf in response to IAA. The blade, via auxin distribution, may be the sensor; the pulvinus, the responder. We do not yet know how ethylene operates within the pulvinus to produce its effect, but we do know that no leaf angle change occurs in the absence of ethylene (as in leaves treated with the ethylene inhibitor AVG) or if ethylene action is inhibited (as in leaves treated with silver nitrate). Blade epinasty, however, can occur without promotion of ethylene production in leaves treated with auxin transport inhibitors.

### V. Nastic Curvature:

Early investigations showed that organic acids and buffers at low pH sporadically produced hyponasty in the absence of auxin. To eliminate these irregular responses, we used auxin in pH 7 phosphate buffer for our bioassay. In effect, we designed our system to avoid the acid growth effect because it was irregular and inconsistent. However, because of its importance in other systems, we have done some investigations. We find that low pH buffer does not promote hyponasty in statistically significant amounts. Sodium vanadate, an inhibitor of ATP driven proton secretion, did not produce significant epinasty. However, when applied to the lower surface of the leaf opposite IAA upper, it did inhibit hyponasty. In these experiments, sodium vanadate also inhibited leaf expansion and so we cannot conclude that its effect indicates the importance of proton secretion in this system.

We had earlier noted that dark treatment promotes hyponasty and bright light treatment inhibits curvature. We are now looking at curvature response to red, far red, blue, green, and white light. Preliminary studies show that blue light inhibits hyponasty and far red light promotes it.

AROMATIC BIOSYNTHESIS IN PINE TISSUES

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NASA Grant NSG-9042

Pinus elliotii is a woody plant species responsive to gravity and capable of synthesizing large quantities of lignin. Lignification begins very quickly after germination; lignin can be detected in the vascular region within 4 days after germination and rapidly progresses up the hypocotyl. Young pine seedlings will bend in response to geostimulation for about 10 days after germination, with the most rapid response time occurring in 4- to 5-day-old seedlings. Various chemicals have been used to establish their effects on the geotropic response in this gymnosperm species. IAA will completely arrest the geotropic response for 18 to 24 hr. Afterward the seedlings will respond to geostimulation as if they had not been treated. The same pattern of response will occur with a second IAA treatment. If the synthetic auxin, 2-4,D, is used, the georesponse is permanently blocked. The method of application does not appear to be critical; addition of auxin to only one side of the seedling gave results similar to those obtained by treating the entire seedling. The geostimulated bending response in young pine seedlings also is inhibited by cycloheximide. Interestingly, cycloheximide ( $10^{-5}$  to  $10^{-3}$  M) does not inhibit the photoresponse in young pine seedlings. Rifampicin ( $10^{-3}$  M) and actinomycin D ( $10^{-4}$  M) did not affect the georesponse in pine seedlings.

Proteins have been isolated from the upper and lower sides of the bend region and examined by gel electrophoresis. The protein bands from the two bend regions appear to be similar. We have sent tissue to Dr. Peter Kaufman, who is having another look at the electrophoresis patterns.

Under our most optimum conditions we are able to demonstrate a 15 to 20% reduction in lignification in pine seedlings using clinostats. The condition that causes the greatest reduction in lignification, however, is the absence of light. We have previously shown that if seedlings develop in the absence of light very little lignin is synthesized, and after they are exposed to light, lignin synthesis rapidly increases. This study is being expanded to include cellulose synthesis, lignin, lignin-synthesizing enzymes, and phenolic compounds related to the lignin biochemical pathway in an effort to understand the requirements for, and regulation of, lignin synthesis.

Cinnamyl alcohols are the intermediate monomeric precursors for lignin synthesis. These substrates, however, are not present in cells as free alcohols but as glucosides. Therefore, the enzyme,  $\beta$ -glucosidase, which hydrolyzes the cinnamyl alcohol glucoside to make the free alcohols available to peroxidase, is a critical enzyme in the lignin biosynthetic pathway. Research with histochemical stains have shown this enzyme to precede lignin synthesis. We have looked for this enzyme in young pine seedlings using synthetic precursors.

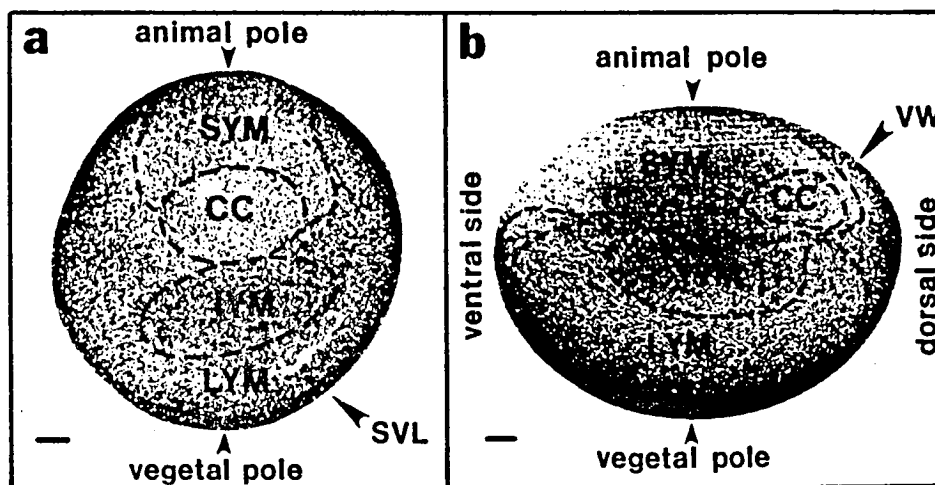
## B. ANIMAL DEVELOPMENT



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Cytoplasmic Rearrangements Associated  
With Amphibian Egg Symmetrization

Cytoplasmic rearrangements which follow fertilization were monitored in normal and inverted eggs. A set of yolk compartments was resolved by cytological analyses of both normally oriented and inverted eggs. Those compartments were characterized by their yolk platelet compositions and movement during egg inversion. In addition to the major yolk masses which contain either small, intermediate or large platelets, minor cytoplasmic compartments which line the egg cortex were also identified:



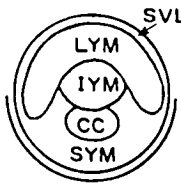
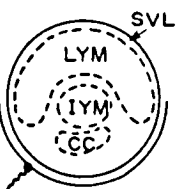
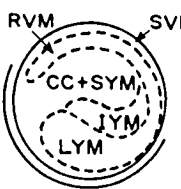
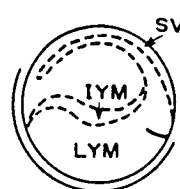
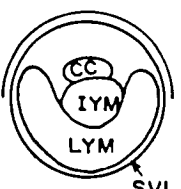
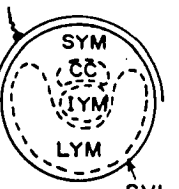
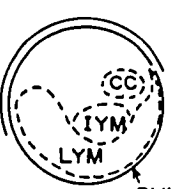
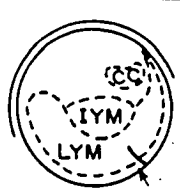
(a) = fertile Xenopus egg; (b) = 1st cleavage egg.

Abbreviations: CC = central cytoplasm; LYM = large yolk mass; IYM = intermediate yolk mass; SYM = small yolk mass; SVL = subvitelline layer; VW = vitelline wall. Magnification bars = 100  $\mu$ m.

During egg inversion the yolk compartments shift. Those yolk mass shifts occurred only after the inverted egg was activated (by sperm, electrical or cold shock). The direction of shift of the major yolk components, rather than the sperm entrance site (as in normal orientation eggs), determines

the dorsal/ventral polarity of the inverted egg. Among different spawnings the rate of shift varied. Eggs that displayed the fastest rate of shift exhibited the highest frequency of developmental abnormalities during organogenesis.

Interpretation of novel observations on cytoplasmic organization provided criticisms of some earlier models. A new "Density Compartment Model" was developed and presented as a coherent way to view the organization of the egg cytoplasm and the development of bilateral symmetry.

DENSITY COMPARTMENT MODEL				
	Preloading (radial symmetry)	Release (activation)	Symmetry Breaking	D/V Polarity
process in inverted eggs				
supporting data	description of major yolk zones Fig. 1,2,3,7	ability of yolk masses to move following ferti- lization and artificial acti- vation - Fig. 1,6, 8,11,14	shift of yolk masses in direction of "tilt" Fig. 4,6,11,12, 14	involution site determined by egg "tilt" Fig. 4,5
postulated process in normal orientation eggs				
	density com- partments established during oogenesis and maturation	release of compartments by fertilization and artificial activation	assymmetric shift of compartments in response to sperm aster growth, "tilt", cortical contrac- tion, etc.	involution site determined by new compart- ment associa- tions and/or assymetries
selected support- ing literature references	Nieuwkoop, 1956; Ubbels, 1977.	Herkovitz and Ubbels, 1979; Radice et al., 1981.	Born, 1885; Pasteels, 1964; Ubbels et al., 1983; Slack, 1982.	Ancel and Vintemberger 1948; Scharf and Gerhart, 1980

The data which generated the above model will be discussed.



Effects of Simulated Weightlessness on Mammalian Development  
I. Development of Clinostat for Mammalian Tissue Culture and  
Use in Studies on Meiotic Maturation of Mouse Oocytes

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NASA Grant NAGW-346

The long-range goal of our research is to examine the effects of weightlessness on three aspects of mammalian reproduction: oocyte development, fertilization, and early embryogenesis. Our first objective was to simulate zero-gravity conditions within the laboratory by construction of a clinostat designed to support in vitro tissue culture. The initial studies then centered on examining the effects of simulated weightlessness on meiotic maturation in mammalian oocytes using mouse as the model system. Specific endpoints have included assessing the timing and frequency of germinal vesicle breakdown and polar body extrusion, as well as the structural and numerical properties of meiotic chromosomes at Metaphase I and Metaphase II of meiosis.

The clinostat we constructed is based upon the design originally developed by Tremor and Souza (Space Life Sciences 3:179-191, 1972) for studies of the effects of simulated zero gravity on amphibian development. The major adaptations that were necessary were due to the unique in vitro culture requirements of mammalian oocytes and embryos, including (a) a temperature of 37°C, (b) a constant atmosphere of 5% CO<sub>2</sub> in air, and (c) constant humidity. In addition, a major technical consideration lay in the selection of an appropriate culture vessel made of tissue culture grade plastic or glass, of small diameter, and allowing for exchange of gases. Sterile 96-Well Micro Test III tissue culture plates (Falcon 3072) were chosen. The center well of each plate was filled with 400 µl of media and overlaid with paraffin oil. A non-air tight lid was placed over the plate and the plate was then mounted onto the metal support plate of the clinostat.

Oocytes were recovered from ovaries of CD-1 mice and placed into the culture system with care taken to minimize the interval between oocyte collection and start of rotation ( $\leq 10$  min.). After 16 hours of incubation under clinostat rotation or control static conditions, oocytes were removed, observed under the dissecting microscope for polar body formation and gross morphology, and processed for cytogenetic analysis. Initial experiments involved assessing the extent to which our static controls, using the culture system modified for the clinostat, compared with the rate of meiotic maturation under standard conditions. We then began experiments in which oocytes were rotated on the clinostat at various RPM. The cytogenetic endpoints examined included the rate of germinal vesicle breakdown and progression through to Metaphase I or II. In addition, the oocytes were examined under a dissecting microscope immediately upon removal from the clinostat for any obvious cellular abnormalities such as a granular or necrotic appearance of the cytoplasm, fragmentation, rupture of the zona pellucida, clumping of cumulus cells, etc. The analysis is now being extended to include detailed examination of the chromosomes obtained.

A total of 37 experiments have been analyzed, representing rotations at 0,  $\frac{1}{4}$ , 1, 10, and 30 RPM. Qualitative evaluation of the condition of the cells was made

immediately following rotation. There was clearly no obvious production of fragmented or otherwise damaged cells at any rotation rate. In some of the experiments, some granularization of the oocyte cytoplasm was noted. This kind of observation is very imprecise at the light microscopic level, however, and we hope to extend these observations to include ultrastructural analysis at the electron microscopic level.

There were no differences between static and clinostat rotated oocytes in the percentage of germinal vesicle breakdown. However, it appears that a lower percentage of oocytes reached the second meiotic division (MII) during clinostat rotation. This preliminary observation will be tested with additional rotation rates and the preparations will be further examined for numerical or structural abnormalities in the chromosomes.

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

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NASA Grant NAGW-438

In about 10 years or so, men and women from Earth will be long-term inhabitants of a space station aboard which plants and animals will be growing and developing in gravities other than that of Earth. How these gravitational changes will affect development is indicated by a number of experiments involving a variety of organisms. In general, results from these experiments indicate that differentiation is speeded up under excess G and slowed in low or null G.

Previous studies in this laboratory have shown that excess gravity (2.6 G) is able to suppress morphogenesis in embryonic mouse limbs developing in vitro by promoting early differentiation. These earlier studies were carried out with BGJ<sub>b</sub> medium containing 25% fetal calf serum and been repeated with serum-free medium (BGJ<sub>b</sub> plus 25% salt solution) to insure that the changes seen in centrifuged limbs are not due to sedimentation of necessary serum components. Morphogenetic scoring of the various elements in fixed whole mounts of control limbs showed that the serum-free medium supported limb development just as well as did medium with serum. Also, centrifuged limbs attained scores similar to those of centrifuged limbs in the previous study, showing that the possible unavailability of serum components involved in growth regulation was not a factor in the lower morphogenetic scores seen in centrifuged limbs. Other observations seen in the previous study were repeated: there is a proximo-distal gradient of sensitivity to gravity's teratological effects with distal elements (i.e., less differentiated ones) being more susceptible and proximal ones less so; only certain stages in limb development exhibit geo-sensitivity and these stages are the same as for other (i.e., chemical) teratogens; centrifugation may cause limb elements present at explantation to resorb; and finally, the lack of morphogenesis seen in centrifuged limbs is due to early differentiation of cells.

Recently, the effects of exposure to excess gravity on fusion of the embryonic mouse secondary palate have been studied. During fusion, the palatal shelves first adhere by means of glycoproteins appearing along the medial epithelial edge (MEE). The contacting epithelia then reorganize and undergo programmed cell death, allowing the underlying mesenchymes to come in contact. The process of cell death occurs in vitro at about the same that it occurs in vivo.

Palatal shelves from 13- and 14-day embryos were excised and cultured in contiguous pairs. Experimental cultures were exposed to 2.6 G in a culture centrifuge; controls were placed in the same incubator. After 24 hours, palates were prepared for light or electron microscopy.

Palates examined by light microscopy were scored as to highest degree of fusion: no fusion (0), epithelial fusion (1), epithelial deterioration (2), or complete fusion (3). When palates were excised at 14 days, epithelial deterioration was seen in 75% of the controls and complete fusion in 25%. Centrifuged palates, however, showed 75% complete fusion and 25% epithelial deterioration. No fusion was seen in control palates excised at 13 days, but centrifuged palates had 50% with complete fusion, 25% with epithelial deterioration and 25% with epithelial fusion.

For electron microscopy studies, sections were taken from the middle region of the palatal medial epithelial edge (MEE), the last region of the palate to fuse in vitro. Ultrastructurally, cells of both palatal types had numerous mitochondria and ribosomes, short sections of slightly expanded RER and Golgi vesicles. Control MEE had tightly apposed cell membranes and numerous desmosomes; in centrifuged MEE, desmosomes had been removed and there was much intercellular space. Nuclear membranes were intact in control MEE, but showed marked deterioration in MEE of centrifuged palates. Few lysosomes and no necrosis were seen in control MEE; centrifuged MEE had numerous lysosomes as well as necrotic cells. Basal lamina were intact in controls, but interrupted in centrifuged palates.

This study shows that palatal cell death and fusion are accelerated under 2.6 G. The results are consistent with the theory that gravitational increases speed up the differentiation process. Excess gravity's effect is assumed to operate by compression induced changes in cell shape and intercellular contacts that mimic normal developmental events.

Studies on in vitro limb development under excess G are continuing using a number of biochemical and quantitative cytological techniques including automated image analysis. Problems being addressed include construction of a dose-response curve and duration of exposure needed to effect a change.

In addition, a small animal centrifuge for in vivo centrifugation studies has been constructed, and a vertical, slow-rotating clinostat has been developed for simulated 0 G studies.

Rodent CNS Neuron Development - Timing of Cell Birth and Death

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NASA Grant No. NAGW-83

The establishment of the complex central nervous system of mammals is an exercise in precision: precision timing of cellular birthdates, precision migratory movements of neuroblasts from the generative sources to their defined terminal structures, precision establishing proper connectivity with relating structures and tracts. Failure in such precision during any of these processes provides the groundwork for aberrant neural relationships resulting in death of the organism or unusual behavioral responsiveness to otherwise normal stimuli.

Each and every neuron is normally generated within a precise four-dimensional system in which its generative "stem" cell is defined in x, y, and z axes at a particular and highly specific embryonic time. While a certain degree of latitude is to be expected in each of these parameters, the 'normal' range of each variable appears to be quite restricted in terms of conferring specificity on new neurons. Prior studies in this area have utilized a variety of labelling paradigms with tritiated thymidine to determine the generative timing of certain selected neuronal structures in amphibian and avian species although studies on timing of mammalian neurogenesis have been relatively limited (see review by Schultze and Korr, 1981). Recently, detailed studies of a complete longitudinal series of staged Wistar rat embryos has been reported by Altman and Bayer in Wistar rats (summarized in Altman and Bayer, 1982) and by the current studies of Keefe (Keefe, 1982; Keefe, manuscript in preparation) in both Wistar rats and C57B16/j mice. As will be detailed later (Tables 1+2), these studies, utilizing different timing paradigms on the same species, have extended our understanding of the timing patterns of cellular generation of central neural structures into many of the more important regulatory nuclei of the brainstem and ganglia of the cranial nerves. The fact that differences in observed times of neuronal generation are minimal from different laboratories utilizing different techniques on Wistar rats derived from different parent stocks reflects the precision of the underlying embryonic process.

This report will center upon data obtained from a staged series of single paired injections of tritiated thymidine to pregnant Wistar rats or C57B16/j mice on selected embryonic days [rat series: every day from E9 => Birth; mouse series: days E9, 11, 13, 15, 17] and several postnatal times. All injected specimens were allowed to come to term, each litter culled to six pups (equilibrate nursing load) and specimens were sacrificed on PN28, with fixation and embedding for paraffin and plastic embedding. The results reported here are derived from serial paraffin sections of PN28 animals exposed to autoradiographic processing and plotted with respect to heavily-labelled cell nuclei present in the selected brain stem nuclei and sensory ganglia. Counts from each time sample/structure have been totalled and the percentage of cells in the total labelled population/structure represented by each injection time interval plotted (Table 1 and 2).

The generation of large neurons of the vestibular nuclear complex occurs early in development, roughly coincident in timing with the initial establishment of the first cells of the vestibular ganglia from the sensory epithelium of the involuted otocyst. Separate zones of the otocyst contribute the initial cells of the maculae of both

utricle and saccule over a much longer time span. Coincident with the onset of generation in this system, a "root cap" is established along the lateral neural tube (?neural crest origin?) which interfaces with the initial afferent projections into the medulla, shows labelling throughout embryogenesis (Schwann cells?) and becomes constricted and disappears by PN1.

Cell death among neurons is evident from earliest generation times (e.g., invaginating otocyst) but is most obvious in two prominent waves: (1) late embryonic period and (2) first postnatal week. The timing of the first wave appears to vary from system to system while the second wave appears coincident with the demise of the "root cap". Our concern with cell death timing is twofold: (1) Is cell death preferential with respect to time of neuron generation [Does early birth lead to longer life?]; (2) Can cell death parameters be utilized to mimic cell generation timing? Future plans for analysis of cell death parameters and equation with generation times in the vestibular and proprioceptive systems of both rats and mice correlating ground-based and flighted [COSMOS 83] specimens will be presented.

TABLE 1 - WISTAR / 8PF RAT DATA

PER CENT NEURONAL POPULATION FORMED IN EACH STRUCTURE ON SELECTED EMBRYONIC DAYS  
(Pooled bilateral data from three non-littermates/time point)

DAY	SVN	LVN	MVN	IVN	NMT	NGR	NCU	NCE	SSC	HSC	PSC	MUT	MSA	VGA
10		*			*							10	*	
11	5	10	*	*	15	*	*		*		*	30	15	*
12	20	45	10	20	20	10	10		15	10	10	25	40	10
13	50	30	20	45	10	60	40	5	35	25	10	15	25	35
14	15	10	40	25	*	10	25	15	20	30	40	10	15	25
15	10	*	30	10	45	15	15	30	11	20	25	5	5	10
16	*	*	*	*	10	5	10	40	10	10	10	5	*	10
17					*	*	*	10	5	*	*	*	5	5
18							*	*	*	*	*	*	*	*
19									*	*	*	*	*	*
20									*	*	*	*	*	*
P+1									*	*	*	*	*	*
P+7									*	*	*	*	*	*

(\* - labelled cells comprise less than 3% of population)

HSC - horizontal canal crista  
 IVN - inferior vestibular nucleus  
 LVN - lateral vestibular nucleus  
 MSA - macula of the saccule  
 MUT - macula of the utricle  
 MVN - medial vestibular nucleus  
 NCE - external cuneate nucleus  
 NCU - medial cuneate nucleus  
 NGR - gracilis nucleus  
 NMT - medial/lateral trapezoid  
 PSC - posterior canal crista  
 SSC - superior canal crista  
 SVN - superior vestibular nucleus  
 VGA - vestibular ganglion

TABLE 2 - C57B16/j MOUSE DATA

PER CENT NEURONAL POPULATION FORMED ON SELECTED EMBRYONIC DAYS  
(Pooled bilateral data from two non-littermates/time point)

DAY	SVN	LVN	MVN	IVN	NMT	SSC	HSC	PSC	MUT	MSA	VGA
9	*	5			?	*	*		*	?	?
11#	10	40	20	15	25	20	10	15	20	10	?
13	60	50	40	45	30	50	45	10	60	70	50
15	30	*	30	35	35	25	30	50	10	10	30
17#			5	5	10	5	15	25	10	5	*
p-1								*	*	*	
p-7#								?	?	?	
p-21#											

(# - only single specimen analysed this time point)

(\* - labelled cells comprise less than 3% of population)

(? - incomplete analysis due to technical problem)

HYPER-GRAVITATIONAL EFFECTS ON METABOLISM AND THERMOREGULATION

Jiro Oyama

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Our animal hyper-gravitational research deals with two major problem areas: 1) initial and short-term exposure effects (stress effects) and 2) chronic, long-term exposure effects involving developmental and adaptational effects.

During the past year, our investigations have focused on: a) quantifying changes in thermoregulation with graded G-intensities in rats b) further delineating the effects of duration on gluconeogenesis, gluconeogenic hormones and substrates, and glucose homeostasis c) reproduction and neonatal survival rates under different G-intensities.

Thermoregulation. Series of hyper-G stress experiments were run in which 3 groups of rats (6 rats/group) were subjected to different G-intensities simultaneously on a 1.7 m radius centrifuge. Rectal and tail temperatures were recorded continuously from each rat before and during centrifugation using a 40 channel system. Graded G-intensities ranging from 1 to 5.2G were used for exposures up to 2 hrs. The rectal temperature decreases showed a graded response with incremental increases in G-intensity between 1 and 1.5G and then leveling off with higher G-intensities. The sensitivity of the rectal temperature response was such that as little as a 0.1G difference could be detected. The transient increase in tail temperature which accompanies the initial fall in rectal temperature was also a graded response with G-intensity up to approximately 1.8G. The smaller rise in the tail temperature response with G-intensities higher than 1.8G indicates the activation of sympathetic vasoconstriction at these higher intensities. Other studies in which rats were centrifuged under head-up and head-down (29°) positions showed no differences in their temperature responses from rats in the normal (horizontal) position. The temperature responses effected by centrifugation have been determined to be due to the G-intensity and independent of the rotational rate or radius of centrifugation. Forthcoming investigations will be on the effects of additional stressors (temperature) combined with hyper-G on the thermoregulatory system and will include heat loss and heat production studies.

Gluconeogenesis. Plasma concentrations of lactate and glycerol as well as hormones affecting gluconeogenesis (catecholamines, glucagon, insulin) have been determined in rats exposed to 3.1G for varying periods from 0.25 hr. up to 24 hrs. Lactate and catecholamines show a transient increase; insulin and glucagon show a sustained increase and glycerol a sustained decrease compared to control levels. Results from these studies indicate that the initial, rapid rise in blood glucose with hyper-G stress is due mainly to an increase in gluconeogenesis. A procedure for preparing viable hepatocytes from livers of hyper-G stressed rats for in vitro gluconeogenic measurements has been set-up and preliminary studies initiated with labelled alanine. These in vitro studies will be continued and extended along with the in vivo studies.

Reproduction & Survival. Reproduction and neonatal survival studies have been conducted at 2.1G. Survival rates of the newborn rats which averages around 50% is significantly higher than at 2.5G used in previous studies (ca 15%). Our current colony maintained at 2.1G includes rats from 11 successive generations. It is planned to use some of these rats for fetal and neonatal developmental studies dealing with gluconeogenesis and glucose metabolism in the near future.





ABSTRACTS - RESEARCH ASSOCIATES



Failure of Vincristine induced Twinning

Michael Binder MD  
Dartmouth Medical School  
Sponsor: Dr. Wm Layton Jr

Mammalian ova do not contain axes of symmetry from which are derived embryonic axes of symmetry. Mammalian axis determination is an early embryologic event occurring at about the time that monozygous twinning occurs. Vincristine Sulfate has been reported to cause monozygous twinning in mice (Kaufman MH & O'Shea KS, 1978, Nature 276:707) and I attempted to reproduce their work in several strains of mice. Over 3200 embryos were examined without any twins being found. To rule out the possibility that vincristine caused twinning plus some lethal malformation (with subsequent resorption of the embryo) the embryos were examined 36-60 hours after vincristine treatment.

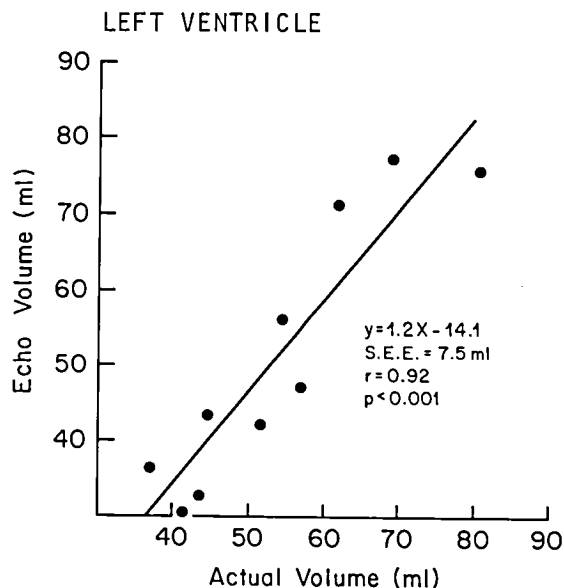
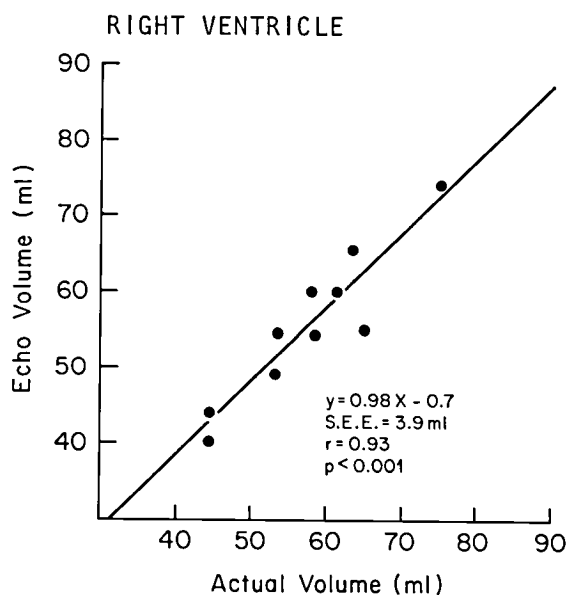
TWINNING OF AMPHIBIAN EMBRYOS BY CENTRIFUGATION

Steven D. Black  
University of California, Berkeley  
Sponsor: Raymond E. Keller

In the frog Xenopus laevis, the dorsal structures of the embryonic body axis normally derive from the side of the egg opposite the side of sperm entry. However, if the uncleaved egg is inclined at  $1g$  or centrifuged in an inclined position, this topographic relationship is overridden: the egg makes its dorsal axial structures according to its orientation in the gravitational/centrifugal field, irrespective of the position of sperm entry. Certain conditions of centrifugation cause eggs to develop into conjoined twins with two sets of axial structures. A detailed analysis of twinning has provided some insight into experimental axis orientation. First, as with single-axis embryos, both axes in twins are oriented according to the direction of centrifugation. One axis forms at the centripetal side of the egg and the other forms at the centrifugal side, even when the side of sperm entry is normal to the centrifugal force vector. Second, if eggs are centrifuged to give twins, but are inclined at  $1g$  to prevent post-centrifugation endoplasmic redistributions, only single-axis embryos develop. Thus, a second redistribution is required for high-frequency secondary axis formation. This can be accomplished by  $1g$  (as in the single centrifugations) or by a second centrifugation directed along the egg's animal-vegetal axis. Since only a brief centrifugation is required, the timing of experimental twin formation can be studied. Third, eggs irradiated with ultraviolet light to inhibit axis formation can be "twice rescued" by double centrifugation to yield twin embryos with their dorsal axes at the centripetal and centrifugal sides. Taken together, these observations suggest that centrifugal force achieves redistributions of endoplasmic materials which must in some way mimic a step in the egg's normal axialization process. Further research is directed toward understanding the nature of these apparently important rearrangements.

CARDIAC CHAMBER VOLUMES BY ECHOCARDIOGRAPHY USING A NEW MATHEMATICAL METHOD: A PROMISING TECHNIQUE FOR ZERO-G USE. Jay C. Buckey (Research Associate), James M. Beattie, F. Andrew Gaffney, J.V. Nixon, C. Gunnar Blomqvist (Sponsor). University of Texas Health Science Center, Southwestern Medical School, Dallas, Texas.

Accurate, reproducible, and non-invasive means for ventricular volume determination are needed for evaluating cardiovascular function in zero-G. Current echocardiographic methods, particularly for the right ventricle, suffer from a large standard error. We tested a new mathematical approach, recently described by Watanabe et al., on 10 normal formalin-fixed human hearts suspended in a mineral oil bath. Volumes are estimated from multiple two-dimensional echocardiographic views recorded from a single point at sequential angles. The product of sectional cavity area and center of mass for each view summed over the range of angles (using a trapezoidal rule) gives volume. Multiple (8-14) short axis RV and LV views at 5.0° intervals were videotaped. The images were digitized by two independent observers (leading-edge to leading-edge technique) and analyzed using a graphics tablet and microcomputer. Actual volumes (x) were determined by filling the chambers with water. These data were compared to the mean of the two echo measurements (y). The graphs are shown below; for either line neither the slope nor the intercept differed significantly from 1 and 0 respectively. Analysis of views obtained every 2.5° did not improve accuracy.



The individual volume (ml) data were:

Heart #	1	2	3	4	5	6	7	8	9	10	Mean
Actual LV	41.3	62.0	37.0	43.3	57.0	44.6	80.6	69.0	51.6	54.6	54.1
Echo LV	30.4	71.4	36.3	32.7	47.2	43.2	75.8	77.3	42.1	55.9	51.2
Actual RV	44.0	61.6	58.6	44.6	58.0	65.0	63.6	53.3	75.3	53.6	57.8
Echo RV	40.4	60.3	54.5	44.3	60.2	55.1	65.8	49.2	74.3	54.6	55.9

Interobserver variations, analyzed by linear regression, were small:  $r = .85$ , S.E.E. = 5.5 ml for RV,  $r = .95$ , S.E.E. = 5.7 ml for LV. The results show an excellent correlation and reasonable accuracy over a fairly narrow range of left and right ventricular volumes.

Future work is planned to study accuracy *in vivo* and the effect of using different echo views. Intra- and interobserver variation needs to be further assessed.

PRESYNAPTIC ELEMENTS INVOLVED IN THE MAINTENANCE  
OF THE NEUROMUSCULAR JUNCTION

Name: George Howard Burrows  
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Sponsor: Marshall Nirenberg

Alterations in the neuromuscular junction have been observed in rats preceding loss of muscle mass (Portugalov and Ilyina-Kakueva, 1973). In view of the possibility that these alterations involve changes in the secretion of myotrophic agents by presynaptic motor neurons, an investigation was undertaken to characterise a neuronal factor which is thought to be involved in the initiation and maintenance of cholinergic synapses. This factor, which is secreted into the incubation medium by NG108-15 neuroblastoma x glioma hybrid cells, induces the aggregation of nicotinic acetylcholine receptors on primary cultures of rat hindlimb myotubes. Previous attempts to purify this factor had not met with success. Extensive washing of the NG108-15 cells with hepes-buffered salt solution followed by short (4 hour) collection times resulted in the collection of incubation medium containing maximal aggregation activity with as little as 5 ug secreted protein per ml of fresh medium. A three-fold increase in specific activity was obtained after DEAE anion exchange chromatography. Previous studies by Bauer et al. (1981) have suggested that the factor is a protein of 400K daltons. If this is correct, maximal activity in the purest fraction is obtained at a concentration of less than 5 nM protein. Moving boundary gel electrophoresis demonstrated four bands of protein in this preparation. To date numerous efforts to recover activity from these gels have failed.

Synapse formation between NG108-15 cells and rat myotubes depends on differentiation of the neuroblastoma x glioma cells. NMR spectroscopy was employed in order to examine the metabolic effects of PgE1/theophylline-induced differentiation. Phosphorous NMR demonstrated that the cells retain high levels of ATP for 30 minutes in glucose-supplemented salt solution. Proton NMR revealed a striking increase in the glutamine content of the differentiated cells. The significance of this effect to the maintenance of the neuromuscular junction remains to be investigated.

Identification of a Volatile Phytotoxin from Algae

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Sponsor: Dr. Franklin Fong

ABSTRACT

One objective of space plant research is to develop closed ecosystems which can sustain human life for long duration space missions and in extraterrestrial habitats. Early attempts to construct model closed ecosystems revealed that algal cultures produced a gaseous effluent which was toxic to higher plants.

The objectives of this research project were to develop a trap system for isolating fractions of volatile algal phytotoxin and to characterize the major components of the isolated phytotoxin fractions. A bioassay using Phaseolus vulgaris seedlings was developed to aid in investigating the properties of the phytotoxin produced by cultures of Euglena gracilis var. bacillaris and Chlorella vulgaris. Two traps were found, 1.0 M hydrochloric acid and 0°C, which removed the phytotoxin from the algal effluent and which could be treated to release that phytotoxin as judged with the bioassay procedure. It was also determined that pretraps of 1.0 M sodium hydroxide and 1.0 M potassium bicarbonate could be used without lowering the phytotoxin effect. Ammonia was identified in trap solutions by ninhydrin reaction, indophenol reaction and derivatization with dansyl chloride and phenylisothiocyanate. Ammonia at the gaseous concentrations detected was found to have the same effects in the bioassay system as the volatile phytotoxin. It is possible that other basic, nitrogen containing compounds which augment the effects of ammonia were present at lower concentrations in the algal effluent, but no experiments to characterize such minor components are contemplated at this time.

This research was supported by a NASA Research Associate Award Grant, NAGW-70, to Dr. John S. Garavelli and by a NASA Controlled Ecological Life Support Systems Program Grant, NCC-2-102, to Drs. Franklin Fong and Edward A. Funkhouser. We also wish to acknowledge the Texas Air Control Board for their assistance.

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Pea Amyloplast DNA is Qualitatively Similar to Pea Chloroplast DNA

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To fully understand the role of statolithic amyloplasts in the graviperception mechanism of higher plants it is necessary to understand their development and regulation in gravisensitive tissue or statocytes. It has previously been shown (Gaynor & Galston, *Plant and Cell Physiol.* 24(3):411-421 1983.) that intact, gradient-purified multigranular amyloplasts contain significant amounts of DNA and RNA. Further analysis of the DNA content of amyloplasts has shown that this DNA, which is insensitive to DNaseI, is sensitive to nuclease digestion following osmotic lysis suggesting that it is contained within the organelle and not simply adsorbed to the envelope.

Amyloplast DNA (apDNA), when subjected to digestion with restriction endonucleases, yields patterns nearly identical to that of DNA from mature pea chloroplasts (ctDNA). Southern transfers of apDNA and ctDNA, probed with the large subunit (LS) gene of ribulose-1,5-bisphosphate carboxylase (Rubisco), shows hybridization to the expected restriction fragments for both apDNA and ctDNA. However, Northern transfers of total RNA from chloroplasts and amyloplasts, probed again with the LS gene of Rubisco, shows that no detectable LS message is found in amyloplasts although LS expression in mature chloroplasts is high. Likewise, two-dimensional polyacrylamide gel electrophoresis of etiolated gravisensitive pea tissue shows that both large and small subunits of Rubisco are conspicuously absent; however, in greening tissue these two constitute the major soluble proteins. These findings suggest that although the informational content of these two organelle types is equivalent, gene expression is quite different and is presumably under nuclear control.



PARTICIPATION OF ETHYLENE IN GRAVITROPISM

Marcia Harrison  
Washington University in Saint Louis  
Barbara G. Pickard

In shoots of many plants, of which tomato (*Lycopersicon esculentum* Mill.) is an example, ethylene production is substantially increased during gravitropism (Abeles, Ethylene in Plant Biology, Academic Press, 1973). Most of the evidence suggests that auxin and associated effectors mediate upward curvature and that ethylene retards it or participates in a counterreaction, but some recent publications suggest that ethylene may be a primary mediator of upward curvature. As a first step toward elucidating the role of ethylene in gravitropism, detailed time-courses of ethylene production in isolated hypocotyl segments and whole plants are being measured for gravistimulated and upright tomato seedlings.

In the first experiment, seedlings were set upright or laid horizontal and then, at 15-min intervals, sets of hypocotyls were excised and sealed into gas-tight vials. Air was sampled for ethylene 40 min later. A steady long-term rise in ethylene production begins after 15 min gravistimulation. It is possible that this increase is a consequence of the accumulation of indoleacetic acid (IAA) in the lower tissue of the hypocotyls.

However, because the lag for IAA-induced ethylene production is usually at least 15 min, it was desirable to resolve the observed lag more accurately. Therefore, a similar experiment was performed with hypocotyls excised after 5, 10, and 15 min. A 5-min stimulus consistently resulted in elevation of ethylene after 40 min, whereas 10-min and 15-min stimuli resulted in ethylene values relatively close to those of controls. Either the 5-min stimulus enhances the formation of wound ethylene (which begins after the plants have been in the vials about 30 min) or else the brief gravitational stimulus independently causes an increase in ethylene production which can be measured after 40 min incubation. This unanticipated early effect made a new method of measurement necessary. Therefore, in a second kind of experiment, whole seedlings were enclosed in sealed chambers and air samples were withdrawn at 5-min intervals. Stimulated seedlings produced more ethylene than controls during the first 5-min interval, but not appreciably more during the second. This suggests the possibility that the ethylene production induced during the first 5 min occurs immediately rather than after a lag, and thus much too soon to be controlled by redistribution of IAA.

Our major purpose is to examine the causes and effects of ethylene production during gravitropism. Therefore, we will next study whether the long-term rise in ethylene production depends on IAA redistribution and also how it influences curvature. Additionally, however, the new evidence suggesting the existence of an unanticipated early burst of ethylene requires exploration and, assuming confirmation, explanation. Tentatively, we propose evaluation of the hypothesis that it is due to release of 1-aminocyclopropane-1-carboxylic acid from the vacuoles where it accumulates (Guy and Kende, Plant Physiol. 72 suppl., 38, 1983); perhaps such release could result from bombardment of the vacuoles by shifting amyloplasts.

INTERACTIONS OF LIGHT AND GRAVITY ON GROWTH, ORIENTATION,  
AND LIGNIN BIOSYNTHESIS IN MUNG BEANS

Name: Gary C. Jahns  
Affiliation: University of Houston  
Sponsor: Dr. J. R. Cowles

Mung beans (*Vigna radiata* L.) seedlings grown on the third Space Transport Mission (STS-3) showed marked orientation problems (some of the stems elongated horizontally and many of the roots were growing upward) and had a lower lignin content than the ground-based controls. This research was initiated to determine if the atypical growth characteristics of mung beans grown in microgravity could be simulated using horizontal clinostats. To date most of the effort has focused on the design, construction and testing of the clinostats. In order to closely approximate the growth conditions of the plants grown in the plant growth unit on STS-3, cylindrical lexan "mini"-chambers were constructed. The clinostat drive mechanism was designed to permit gas sampling and/or gas flow through the chamber during rotation. In addition, the clinostat apparatus has provisions for mounting a light source on the rotational axis of the clinostat to investigate the phototropic response of plants grown in simulated zero  $g$ . Results thus far have shown that plants grown using these clinostats in the horizontal position exhibit similar growth characteristics to the plants grown on STS-3 (disorientation of both stems and roots), while the vertical stationary and vertical rotating controls exhibit normal growth. In the near future, lignin content as well as two enzymes in the lignin pathway are to be analyzed in both simulated zero  $g$  and 1  $g$  controls.

CELLULAR LOCALIZATION OF  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase IN THE MAMMALIAN VESTIBULAR SYSTEM

Thomas P. Kerr

University of Michigan

Sponsors: Dr. Muriel D. Ross, Dr. Stephen A. Ernst

The vestibular system of the inner ear, which mediates inertia-dependent sensation, possesses two types of hair-cell sensory organ: the maculae, or otolithic organs, and the ampullae. In each of these structures, the hairs of the receptor cells extend into a compartment filled with endolymph, a unique extracellular fluid distinguished by its high concentration of potassium ion and low concentration of sodium ion. Although the endolymphatic compartments associated with the individual sensory organs are interconnected, the endolymphatic space ultimately forms a closed system, bounded on all sides by epithelia comprising the "membranous labyrinth" (Figure 1). Electrochemical considerations indicate that the steep gradient of potassium concentration between endolymph and other extracellular fluids must result from active transepithelial transport of potassium ion.

The mechanisms regulating endolymphatic ion concentration are of physiological significance for two reasons. First, the sensory transduction process is thought to commence with a flow of ionic current from the endolymph into the hair cell. This current is carried by endolymphatic potassium ion, which is therefore essential to receptor cell function. Secondly, the ionic concentrations of the endolymph apparently control endolymphatic volume. Available evidence indicates that the osmotic pressure of the endolymph is determined primarily by its inorganic ionic constituents, and that membranes of the endolymphatic compartment are freely permeable to water. The endolymph must consequently gain or lose water to maintain osmotic equilibrium with surrounding extracellular fluids. Alterations of endolymphatic volume are accompanied by sensations of vertigo and dizziness.

Endolymphatic ion transport mechanisms may play an important part in the etiology of "space sickness" if the redistribution of extracellular fluid and electrolytes known to occur upon first exposure to weightlessness should disturb the osmotic equilibrium of the endolymph. Vestibular dysfunction might then result from fluctuations in endolymphatic volume, when the rate of potassium transport is maintained at a level incompatible with a new osmotic equilibrium. Alternatively, the sensory dysfunction may reflect compensatory alterations in the rate of potassium transport, subserving endolymphatic volume maintenance at the expense of endolymphatic potassium concentration.

The only putative mechanism for transepithelial potassium transport yet identified in vestibular tissues is the enzyme  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase. Appreciable enzymatic activity has been detected in microdissected samples of vestibular tissues by Thalmann and his colleagues, using sensitive biochemical assay techniques. Such methods, however, do not conclusively resolve the cellular origin of the enzymatic activity in tissues like these, which display considerable cellular heterogeneity.

We have employed two different, but complementary, procedures for cellular localization of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in the guinea pig vestibular system. One of these techniques, devised by Stirling, depends upon the well-documented ability of the specific inhibitor ouabain to bind selectively to  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, blocking catalytic activity. Microdissected vestibular tissues are incubated with tritium-labelled ( $^3\text{H}$ -) ouabain, and regions with a high concentration of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase are subsequently identified by light microscope autoradiography. A second method, originated by Ernst, detects inorganic phosphate released from an artificial substrate (nitrophenyl phosphate) by catalytic activity of the

enzyme. In the presence of strontium ion, phosphate is precipitated near regions of high activity, then converted to a product which may finally be visualized in the electron microscope. This cytochemical enzymatic reaction is inhibited by ouabain.

A light-microscopic survey of vestibular tissues incubated with  $^3\text{H}$ -ouabain shows that the highest densities of ouabain binding sites are associated with the dark-cell epithelium of the ampullae (Figure 2), and to a lesser extent, the dark-cell epithelium in the wall of the utricle. Substantial numbers of binding sites are also found in nerve fibers penetrating the connective tissue beneath the sensory epithelium of the ampullae and otolithic organs. Moreover, autoradiographic label is distributed in the deep portion of the sensory epithelium, both in the ampullae and in the otolithic organs. These latter binding sites may be associated with nerve terminals on the receptor cells.

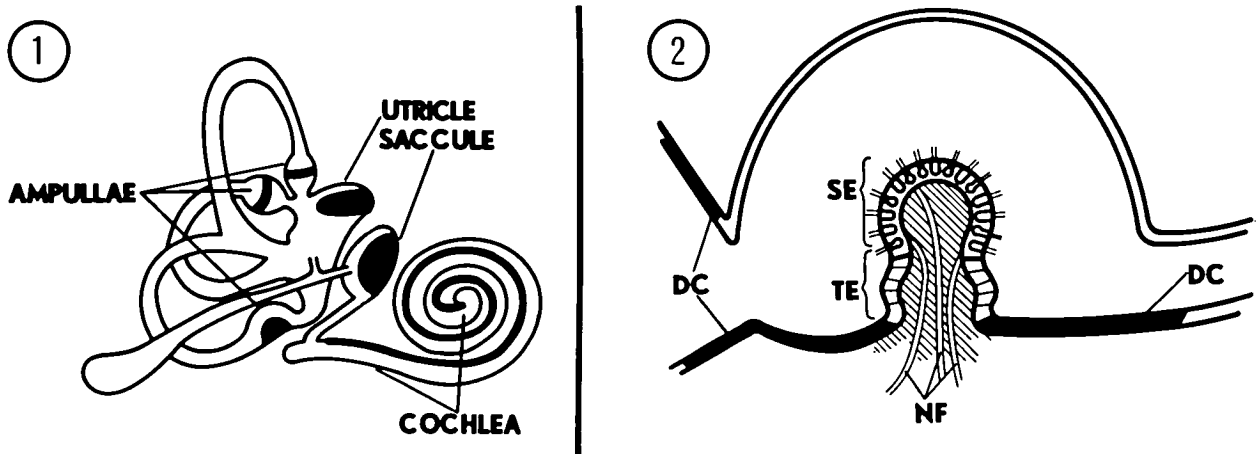
At the ultrastructural level, the dark cells exhibit extensive basolateral membrane infolding, a morphological hallmark of cells engaged in transepithelial ion transport. Cytochemical reaction product is restricted to the basolateral membrane extensions, with little or no product on the luminal membrane. The extent of membrane infolding in dark cells of the utricle was often less pronounced than that of the ampullar dark cells, and the intensity of the cytochemical reaction appeared to correlate with the extent of membrane infolding.

Cytochemical reaction was also observed in a non-epithelial cell type, characterized by prominent membrane infoldings, and situated in the connective tissue beneath the sensory epithelium. These specialized cells seemed particularly numerous in the connective tissue beneath the transitional epithelium of the ampulla. Similar cells occur in the lateral wall of the cochlea; while their function is not known, they are probably not directly concerned with endolymphatic ion transport.

These studies represent the first cellular localization of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in the mammalian vestibular system, and the first cytochemical demonstration of vestibular  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase at the ultrastructural level. The observation that enzymatic activity in the dark cells is restricted to basolateral membrane extensions is of particular importance. The same pattern of activity has been found in several other tissues (e.g., kidney) where the enzyme is known to mediate transepithelial transport of cations and salt-coupled transport of water. The present results may therefore be taken as evidence for participation of the vestibular enzyme in endolymphatic potassium transport.

Research during the remainder of this year will quantitate  $^3\text{H}$ -ouabain binding sites in various vestibular tissues by the use of liquid scintillation spectrometry. Further ultrastructural studies will explore the distribution of enzymatic activity in vestibular sensory epithelia and nerve fibers.

FIGURE 1: The membranous labyrinth. Sensory epithelia indicated by black shading.  
FIGURE 2: The ampulla. SE, sensory epithelium; TE, transitional epithelium; NF, nerve fibers; DC, dark cell epithelium; cross-hatching, connective tissue.



Bioassay, Isolation and Studies on the Mechanism of Action of  
Neurite Extension Factor

Douglas Kligman  
Laboratory of Clinical Science, NIMH  
Sponsor: David Jacobowitz

The identification and purification of molecules active in promoting neurite outgrowth requires a sensitive reproducible bioassay. A quantitative bioassay was utilized to purify a neurite extension factor (NEF) based on counting the number of phase bright neurons with processes at least equal to one cell body diameter after 20 hrs. in culture in defined, serum-free medium. Using a combination of heat treatment DEAE cellulose chromatography and gel filtration, an acidic protein of  $M_r = 75,000$  has been highly purified. Upon reduction, it yields subunits of  $M_r = 37,000$ . Purified fractions are active half-maximally at 100 ng/ml in inducing neurite outgrowth in this bioassay. Additionally, HPLC is being utilized to achieve a more rapid purification protocol for NEF from brain.

Currently, monoclonal antibodies to NEF are being produced. Female Balb C mice have been immunized with the antigen and fusions with mouse myeloma cells will be performed to yield hybridoma cells. Hybridomas will be screened for binding to NEF. These hybridomas will be extremely useful in the characterization of the biological and biochemical actions of NEF.

The mechanism of action of NEF is currently under investigation. Phosphorylation of membrane proteins in target tissues has been demonstrated to be an important regulatory step in the mechanism of action of several other growth factors, and thus the effect of NEF on phosphorylation of proteins from rat brain (a tissue rich in NEF) was tested. Using ATP as donor, it has been discovered that in rat brain membranes NEF greatly stimulates phosphorylation of an  $M_r = 90,000$  protein in a dose-dependent manner. This reaction is cyclic nucleotide-independent, appears to have both a calcium-dependent and calcium-independent component, and is not stimulated by calmodulin. Subcellular fractionation studies indicate that the 90K substrate is greatly enriched in plasma membrane fractions, and tissue distribution studies indicate that nerve tissue is a rich source of this substrate. These findings may represent some of the earliest effects of NEF on cellular metabolism and may also provide a sensitive biochemical assay for NEF. The 90 K substrate will be hydrolyzed and the phosphorylated amino acids separated by high voltage paper electrophoresis to determine which amino acid is being phosphorylated. This information will be useful in the characterization of the NEF-stimulated kinase in rat brain membranes. Additionally, membrane receptors for NEF may interact with this kinase, and thus the study of the NEF receptor may be aided.

Isolation and characterization of  $\beta$ -glucan synthase:  
A potential biochemical regulator of gravistimulated  
differential cell wall loosening.

Name: Dr. Konrad M. Kuzmanoff

Affiliation: Stanford University, Dept. of Biological Sciences

Sponsor: Dr. Peter M. Ray

In plants, gravity stimulates differential growth in the upper and lower halves of horizontally oriented organs. Auxin regulation of cell wall loosening and elongation is the basis for most models of this phenomenon. Auxin treatment of pea stem tissue rapidly increases the activity of Golgi-localized  $\beta$ -1,4-glucan synthase, an enzyme involved in biosynthesis of wall xyloglucan which apparently constitutes the substrate for the wall loosening process.

The primary objective of this project is to determine if auxin induces de novo formation of Golgi glucan synthase and increases the level of this glucan synthase mRNA. This shall be accomplished by (a) preparation of a monoclonal antibody to the synthase, (b) isolation, and characterization of the glucan synthase, and (c) examination for cross reactivity between the antibody and translation products of auxin-induced mRNAs in pea tissue. The antibody will also be used to localize the glucan synthase in upper and lower halves of pea stem tissue before, during and after the response to gravity.

RHEOCEPTIVE MEDIATORS OF GRAVIPERCEPTION IN A WATER FLEA:  
MORPHOLOGICAL IMPLICATIONS OF ANTENNAL-SOCKET SETAE IN DAPHNIA MAGNA.

DEWEY G. MEYERS, Biology Department, Millsaps College, Jackson, MS 39210

Aquatic microcrustaceans of the genus Daphnia are known to orient to light during the day. At night, in the absence of visual cues, daphnids were suspected of maintaining equilibrium by monitoring the direction of gravity through their swimming antennae. Recent investigations in our laboratory using simulated, weightlessness conditions coupled with absence of illumination have revealed hair-like structures or setae on the basal, articulating socket of the antennae that, when surgically removed, resulted in disorientation. Given the simulated-weightlessness or neutrally buoyant condition that eliminated sinking of the normally negatively buoyant Daphnia, it was proposed that the antennal-socket setae function as rheoreceptors stimulated by the upward rush of water currents during gravity-induced, sinking phase of daphnid swimming movements.

This rheoreceptively mediated, gravity perception hypothesis is further supported by morphological investigations. Scanning electron micrographs indicate that antennal-socket setae are anatomically similar to proprioceptors used by higher crustaceans to monitor gravitational direction. Similarities noted were: i) size; 40-70  $\mu\text{m}$  long, 5-10  $\mu\text{m}$  in diameter, ii) location; associated with antennae, iii) structure; paired slender shafts, iv) surface area; increased by feathering or a row of setules on either side of the setal shaft, v) position; elevated on a prominent base, vi) axial orientation; lateral, perpendicular to the line of sink and vii) comparative length vs position; the anterior seta of each pair is longer and more prominent. Initial transverse sections, preliminary to transmission electron microscopy studies, have also revealed the structural differences of individual seta of each pair that may indicate selective sensitivities. On-going studies are aimed at understanding setal innervation and sensitivity.

EFFECTS OF SUSPENSION ON TISSUE LEVELS OF GLUCOCORTICOID RECEPTORS

Name: J.M. Steffen  
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Differential atrophy of rat hindlimb skeletal muscles has been documented as a result of exposure to conditions of weightlessness. These atrophic alterations have been ascribed to a combination of both hypokinesia (decreased muscle activity) and hypodynamia (reduced mechanical loading). These differential muscle responses can be simulated by hypokinetic/hypodynamic (H/H) suspension of rats with complete unloading of the hindlimb muscles. Since mechanism(s) underlying these atrophic effects have not been clearly elucidated, experiments were initiated to investigate a possible role for glucocorticoids in the physiological and biochemical responses to H/H. The principal objective of these experiments was to assess the potential for alterations in peripheral responsiveness to glucocorticoids in response to H/H. Studies have initially focused on the determination of tissue levels of glucocorticoid receptors as one index of hormonal sensitivity at the cellular level. Four hindlimb muscles (soleus, gastrocnemius, plantaris and EDL), previously demonstrated to exhibit differential responses to H/H, were investigated. Receptor levels in other glucocorticoid sensitive tissues (heart, liver, and kidney) have also been determined. Male rats (180-200g) were suspended for 7 or 14 days, sacrificed by cervical dislocation, and the tissues excised. Cytosolic tissue fractions were obtained by homogenization and centrifugation. Glucocorticoid receptors were assayed by incubating cytosol with increasing concentrations of <sup>3</sup>H-dexamethasone at 2°C for 20 hours. After correction for nonspecific binding, maximum ligand binding and the apparent dissociation constant were determined from Scatchard plots. These investigations indicate that H/H suspension results in elevated glucocorticoid receptor levels in muscles exhibiting atrophic responses. The increase in receptor levels parallels the differential nature of the muscle atrophy. These effects appear to be specific, as there were only minor alterations in receptor levels in the heart, liver and kidney. There were no significant changes in the apparent dissociation constant of the receptor in any tissue in response to H/H suspension. While these studies document tissue specific effects of H/H on glucocorticoid receptor levels, they do not clearly demonstrate a cause and effect relationship between receptor levels and muscle atrophy. Further studies will be required to elucidate this relationship.



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