FUNDAMENTALS OF SPACE BIOLOGY

Research on Cells, Animals, and Plants in Space

Gilles Clément
Klaus Slenzka
Editors

Space Technology Library

Fundamentals of Space Biology

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Cover photo of Earth from Space: View of Africa and the Indian Ocean taken in December 1972 by Apollo 17, the last of the Apollo missions to explore the Moon. Photo courtesy of NASA.

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The authors dedicate this book to the memory of the crewmembers of the Space Shuttle Columbia STS-107 mission: Rick D. Husband, William C. McCool, David M. Brown, Kalpana Chawla, Michael P. Anderson, Laurel Clark, and Ilan Ramon who were tragically lost on February 2, 2003. The STS-107 mission was a multi-disciplinary microgravity and Earth science research mission with several space biology investigations. A number of these investigations are described in this book, and photographs of some of the STS-107 astronauts conducting these experiments in orbit are also included.

Contents

FOREWORD

Fundamentals of Space Biology is the third textbook addressing Space Life Sciences in this *Space Technology Library* series. The first of these books focused on the psychological and psychiatric issues that affect people who live and work in space (Volume 16, *Space Psychology and Psychiatry*). The second book described the physiological and medical issues of living in a space environment (Volume 17, *Fundamentals of Space Medicine*). The objective of this third book was to review the effects of spaceflight on less complex biological systems, from single cells to animals and plants.

Indeed, to better understand the changes at the function level, it is necessary to comprehend the changes at cellular and tissue levels. Studies of cell cultures, for example, allow the investigation of the *indirect* effects of gravity; i.e., those which occur not because of changes in the stimulation of dedicated gravity-sensing organs, but because of the new physical properties resulting from the reduction in gravitational force within the cell.

Furthermore, studies of animals and plants in space allow investigations of the effects of gravity on development, a research field that is not open to human subjects. The International Space Station era promises opportunities to observe and test various features of animal and plant development during long-term exposure to microgravity, with access to centrifuges capable of exposing the specimens to fractional g-loads. Recent space missions, such as Neurolab, have provided evidence for the existence of critical periods when gravity is necessary for the normal development of frogs and rats. The ability to raise animals and plants on the International Space Station over several life cycles should allow scientists to establish precisely the how, when, where, and why of this gravity dependence.

There is no doubt that the Earth's gravitational field influences the morphology, physiology, and behavior of life in virtually all its manifestations. The common motivating force for the space biologists is a genuine desire to understand the role that gravity has played in the evolution of life on our planet, whether a single-celled microorganism, or a complex multi-cellular organism, such as a plant or an animal. Space biology research encompasses a broad range of biological sub disciplines, including gravitational, developmental, and radiation biology. It also focuses on advanced technologies that include research in genomics, molecular and nano-technologies, DNA arrays, gene-array technologies, and cell culture and related habitat systems; thus, reflecting the evolving nature of biological research as well as the ever-increasing linkage between science and technology.

A fundamental understanding of how living organisms perceive and respond to gravity and adapt to the space environment can make a significant contribution to our understanding of life. For example, contributors to this book have tried to answer the following questions: How does acute or chronic exposure to altered gravity and other space-related factors affect normal physiology, metabolism, and function of mature organisms? How do responses differ among a wide diversity of organisms? What do we explicitly know about organism development in microgravity? Or about the development of gravity receptor systems at the cellular level and above, and their adaptation to microgravity? Can Earth-based species adapt progressively to a gravitational environment of less than 1 g?

There is a need for a comprehensive textbook in the area of space biology. It is evident that more and more people are interested in space life sciences, given the growing number of students enrolled in our department year after year. Yet most of them do not have a solid background in life sciences, nor do they have access to specialized journals, conference proceedings, or agency brochures where most of the relevant space research is documented. The challenge for this book was to make space biology easily accessible and comprehendable for students or teachers whose background may not necessarily be in the field of life sciences. Therefore, a scholarly approach has been taken. The first chapter defines the various disciplines of space biology and the rationale for conducting this research. The second and third chapters provide a list of animals and plants flown in space, and the biological facilities used on board the various space laboratories, including those being developed for the International Space Station.

In the subsequent chapters, space biologists have broadly summarized the status of their own research in sub disciplines such as cell biology, development biology in animals and plants, and radiation biology. Each chapter begins with a section on basic physiology to bring the reader up to speed. This is followed by an overview of experiments performed in space or during relevant ground-based studies, summarizing what is known. The authors then conclude their chapters by providing some speculation about what is still to be learned.

The last chapter lists the research efforts made in the field of biotechnology, which exploits microgravity as a tool for separation and processes and techniques, and for the production of cells for medically valuable proteins, hormones, enzymes, and vaccines.

Each chapter includes references to relevant published works in that topic area. However, due to page limit constraints, we acknowledge that our reference list is not comprehensive. We made every attempt to include the most up-to-date and solid peer-reviewed publications when compiling this work. We apologize to our colleagues whose work may have been omitted.

The other challenge for this book was to stimulate ideas for future space research and capture the imagination of the general public who will then be more likely to support this type of research. We hope that *Fundamentals of Space Biology* will achieve these goals.

Gilles Clément,
Athens, 25 November 2005

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We are grateful to Mr. Philippe Tauzin, from the Service Commun Multimédia of the Université Paul Sabatier in Toulouse, for his help with some of the artwork. We also appreciate the great work done at NASA to document, catalogue, and give access to its archives, as well as video and pictures gallery. Many photographs in this book come from this gallery, and the astronauts themselves during their space missions took most of them.

Finally, thanks to Dr. Harry (J.J.) Blom and to Dr. James R. Wertz who offered us the opportunity to disseminate the legacy of space biology research in the *Space Technology Library* series.

Chapter 1

INTRODUCTION TO SPACE BIOLOGY

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Research in Space Biology is aimed at addressing the basic questions regarding the extent to which gravity plays a role in growth, morphology, and function of cells in the space environment (Cell Biology), and from the early development of animals and plants to several life cycles (Developmental Biology). More applied aspects of Space Biology research also include the biological effects of space radiation and radiation standards (Radiation Biology) and the production of cells for medically valuable proteins (Biotechnology).

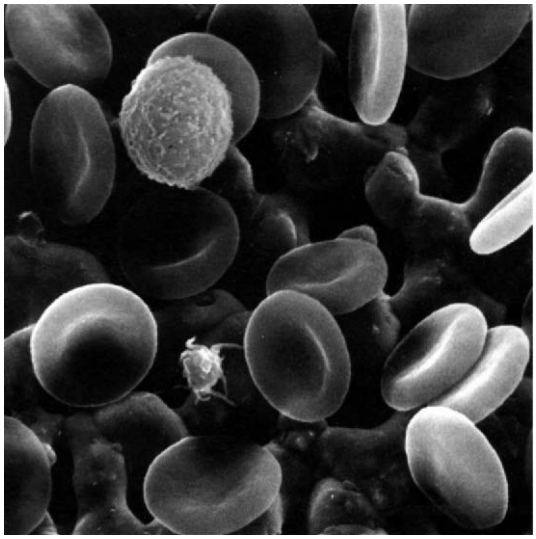

Figure 1-01. Blood cells are mammal cells the most studied in the space environment.

1 SPACE BIOLOGY: WHAT IS IT?

The space environment inside a pressurized module in orbit is characterized by the absence of effective gravity with the associated condition of microgravity[1], the part of the cosmic radiation spectrum that penetrates the

[1] The term *microgravity, or 0 g,* is used to describe the environment inside a space vehicle in orbit around the Earth. The experiments inside this vehicle do not

walls of the modules and interacts with its materials and inhabitants, and the absence of circadian (24-hour) rhythms. These unique conditions constitute hazards to the safety and proficiency of astronauts and cosmonauts during spaceflight, as reviewed in an earlier book in this Space Technology Series, entitled *Fundamentals of Space Medicine* (Clément 2005). At the same time, they create unique conditions for the study of vital functions in organisms of varying complexity, from single cells to the most organized species (Figures 1-01 and 1-02).

Transitioning from a terrestrial environment to the space environment has long been known to cause adaptive or maladaptive (i.e., pathological) changes in the human body. Astronauts endure nausea, disorientation a shift in body fluids, disruption of their sleep pattern, depression of their immune reaction, and other conditions as they adjust to life in space. When they return to Earth, most of these conditions disappear, some immediately, some gradually. Some condition, the loss of bone mass for example, can take several years to repair itself, and for some astronauts, the damage may be permanent. The same changes, e.g., modifications in the calcium balance, seem to occur at cellular level.

Neither the 0-g environment nor the complex natural spectrum of space radiation can be produced or effectively simulated in ground-based laboratories. Consequently, studies of the influences of these factors on living organisms can only be studied in space. Both factors have at least two characteristics of exceptional biological interest. Firstly, they have not been encountered by living organisms throughout the entire history of their terrestrial existence and evolution. Secondly, various living organisms display varying degrees of tolerance to each factor, permitting varied and systematic quantitative experiments to determine the nature and extent of their actions. The space environment therefore represents a new and powerful research tool in biology: it makes possible experimental investigations into problem areas in which theory is in no position to make trustworthy predictions (Bjurstedt 1979).

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experience a perfect free-fall state. As the vehicle orbits the Earth, it is subjected to small decelerations from atmospheric drag. The location of experiments inside the vehicle is another important factor. Since they are not usually located at the spacecraft center of gravity, a slight mismatch is created between the path of the vehicle orbit and the orbit of an experiment inside. This combination of off-alignment and atmospheric drag alters the free-fall of the experiment. Therefore, near-weightlessness on the order of 10^{-4} to 10^{-6} g is more typically experienced in today's spacecraft and is usually referred to as microgravity (with "micro" defined either literally as 10^{-6}, or figuratively as "very small"). For a detailed description of the physics of microgravity and Space Shuttle flight trajectory, see Clément (2005).

Figure 1-02. Belgian Astronaut Frank DeWinne is pictured near a plant growth experiment on board the International Space Station. Photo courtesy of NASA.

1.1 Definitions

Space Biology is a fundamental component of *Space Life Sciences*. Space life sciences include the sciences of physiology, medicine, and biology, and are linked with the sciences of physics, chemistry, geology, engineering, and astronomy. Space life sciences research not only helps to increase new knowledge of our own human function and our capacity to live and work in space, but also explores fundamental questions about the role of gravity in the formation, evolution, maintenance, and aging processes of life on Earth.

As with *Space Physiology* and *Space Medicine*, Space Biology experiments have a goal of using the space environment as a tool to help in the understanding of the influence of gravity on fundamentals biological processes. Space Biology focuses on smaller organisms, such as cells, animals and plants, whereas Space Physiology looks at systems level in humans. In addition, Space Medicine must assess the problems and dangers with which humans will be required to cope during prolonged spaceflights, and suggest solutions, or countermeasures, to those problems (Clément 2005).

Space Biology reflects the evolving nature of biological research as well as the ever-increasing linkage between science and technology. As such, scientific research in space biology encompasses a broad range of biological sub disciplines. *Gravitational Biology* examines the role of gravity in the

evolution and development of terrestrial organisms and ecological systems as well as how plants and animals react and adjust to the effects of different gravity levels. Research spans multiple levels of biological processes, from molecular and cellular through tissue and organism to ecosystem and evolutionary.

More specifically, *Cell Biology* investigates the physical effects of spaceflight at the cellular level, i.e., exploring whether gravity has a direct effect on cells, or if their aspect and function are modified due to changes in gas exchange mechanisms, heat transfer, or fluid physics in the absence of gravity. At the molecular level, scientists study the expression of genetic information in response to exposure to the space environment.

At the organism level, researchers compare responses of a wide variety of organisms to the spaceflight environment. *Developmental Biology* evaluates how spaceflight affects the development of multicellular organisms. Investigators study what happens during critical stages of development to ascertain whether altered gravity levels or related spaceflight factors induce profound effects that change, limit, or block normal development, and whether or not these effects are reversible. At the molecular level, researchers work to identify what genes, gene products, and metabolic changes serve as markers for such developmental polymorphism or plasticity.

Research in *Radiation Biology* looks for reliable ways to predict and measure the effects of ionizing radiation on living tissues. Radiation biologists work in active cooperation with clinicians to refine our knowledge of the varying effects of radiation on the living body. Both *in vitro* and *in vivo* observations from biological tissues exposed to space radiation have enriched our understanding of these processes, and point to a future, which includes cellular and genetic promise in radiation response modifiers and increasing precision in applying radiation to disease.

As in any research discipline, a distinction needs to be made between the factors that are of *applied* nature, and those that concern basic aspects of biology as studied in the space environment. The basic research objectives of space biology are to make use of the unique properties of the space environment to study the fundamental nature and properties of living organisms (Table 1-01).

• *Understand the effects of gravity on cells, animals and plants*
• *Determine the combined effects of microgravity and other environmental stresses (e.g., radiation, absence of day/night cycles) on biological systems*
• *Improve the quality of life on Earth through the use of the space environment to advance knowledge in the biological sciences*

Table 1-01. Goals of Space Biology.

The applied aspects, however, include the better characterization, purification and possible culture of medically valuable substances here on Earth, by taking advantage of the effects of microgravity on the growth of these substances (Figure 1-03). These applied aspects are also referred to as the area of *Biotechnology*. Biotechnology includes those techniques, equipment, and procedures that are developed and used in support of near-term and long-term science goals. Space biotechnological research programs use "genomic technologies", molecular and nano-technologies, cDNA[2] arrays, gene array technologies, and cell culture and related habitat systems. It is also necessary to develop sensors, signal processors, biotelemetry systems, sample management and handling systems, and other instruments and platforms for real-time monitoring and characterization of biological and physiological phenomena while the research is being conducted on board the space laboratory. In particular, there is a need for automated acquisition, processing, analysis, communication, archiving, and retrieval of biological data with interfaces to advanced bioinformatics and biocomputation systems. Finally, scientists require advanced bioimaging systems, with real-time capabilities for visualization, imaging, and optical characterization of biological systems. These technologies include multidimensional fluorescent microscopy, spectroscopy systems, and multi- and hyperspectral imaging.

Figure 1-03. Close-up view of sodium chloride crystals in a water bubble within a 50-mm metal loop photographed by a crewmember in the Destiny laboratory on the International Space Station. Photo courtesy of NASA.

[2] *cDNA* (for copy DNA) is a DNA strand synthesized (copied) from mRNA (messenger RNA) rather than from a DNA template. This type of DNA is used for cloning or as a DNA probe for locating specific genes in DNA hybridization studies.

There is also a practical need to study plant and microbial interactions in varying gravitational environments. This is essential to our ultimate ability to sustain humans for a year or more on the surface of extraterrestrial bodies or in spaceflight missions of long duration where re-supply is not possible and food must be produced in situ. Experiments during long-term space missions will determine which plants are most efficient and best suited for our needs. For instance, can soybeans germinate, grow normally, and produce an optimum crop of new soybeans for food and new seed for ensuring future crops? All of this biological cycling along with the development of equipment for water and atmosphere recycling and waste management will also yield important benefits for terrestrial applications.

1.2 Objectives of Space Biology

Space life sciences research has now been conducted for more than four decades. The continuing interest in studying the way living systems function in space derives from two main benefits of that research. First, in order for humans to engage in long-term space travel, we must understand and develop measures to counteract the most detrimental effects of spaceflight on biological systems (Table 1-02). Problems in returning to normal gravity environment on Earth must also be kept to a manageable level.

- *Vestibular changes leading to space motion sickness*
- *Redistribution of body fluids*
- *Loss of blood cells and blood volume*
- *Changes in blood levels of several hormones*
- *Loss of muscle mass*
- *Loss of bone mass and increase in calcium excretion*
- *Decrease in immune system reaction*
- *Sleep disorders*
- *Radiation exposure*
- *Psychological and social issues*

Table 1-02. Physiological changes which could endanger the crewmembers during long-duration space missions (see Clément 2005 for review).

Ensuring the health and safety of astronauts is a continuous priority to which the study of different organisms makes valuable contributions. Evaluating the risks posed by conditions in space and developing effective countermeasures requires an understanding of the short and long-term effects of exposure to such unique conditions. For example, how much shielding is required to reduce the risk of genetic mutations as a result of ionizing radiation? What causes the process of bone demineralization observed in humans and animals during spaceflight and is it possible to control its gene expression? Controlled experiments using different organisms complement

human studies to address these questions and reduce risk. In addition to the chronic effects of the space environment, acute medical care of astronauts is also important, particularly to support missions to the Moon and Mars. An understanding of how healing processes such as bone and muscle repair and immune responses to infection are affected by the spaceflight is critical, as is pharmacological knowledge of how space travel influences responses to drugs such as anesthetics and antibiotics (Figure 1-04).

Figure 1-04. On the Space Shuttle Discovery's flight deck, astronaut Stephen K. Robinson uses a cotton swab to collect a saliva sample for microbial analysis. Photo courtesy of NASA.

Second, increasing our understanding of how organisms function in reduced gravity gives us new understanding of fundamental biological processes. Throughout its evolution, life on Earth has experienced only a 1-g environment. The influence of this omnipresent force is not well understood, except that there is clearly a biological response to gravity in the structure and functioning of living organisms. To better understand a system, the scientific method set by the physiologists in the 19th century consists of studying the consequences of its exclusion. So, the removal of gravity is a desirable, even necessary, step toward understanding its role in living organisms. Although techniques exist on Earth to increase the gravitational force by using centrifuges, or to reduce its effects by immobilization, slow rotation, or unloading, the effects of prolonged microgravity and cosmic radiation cannot be studied on Earth. While the effects of gravity are fairly obvious at the total organism or system levels, as observed in astronauts, they are not immediately

apparent at the cellular level. In addition to this fundamental question of the role of gravity at cellular level, it is also interesting to know if the responses observed in organs or individuals can result from changes produced at the cellular level. The information gained from this research can be used to improve human health and the quality of life on Earth (Planel 2004).

Finally, studying the way in which various plants, animals, and microorganisms interact in closed ecosystems will be essential for developing the advanced life support systems needed for long duration missions. In Earth's orbit, spacecraft can be continuously replenished. Exploring other planets and celestial bodies, however, will require the development self-sustaining ecosystems. The bio-regenerative properties of plants and microbes will be essential factors in such systems, performing critical functions such as producing food, absorbing CO_2, and recycling waste. In addition to supporting space exploration, research into these properties should produce many benefits here on Earth.

1.3 Why Send Cells, Animals, and Plants in Space?

The question could also be formulated this way: Why not just study humans in space?

Studies using humans as subjects produce the most direct assessment of how spaceflight affects a particular aspect of human physiology or of the efficacy of a particular countermeasure. But there are a number of reasons why humans are often not viable or appropriate research subjects. First, from the ethical point of view, as in research on Earth, many experiments in space cannot be ethically performed on humans, such as those investigating embryonic development or genetic mutation.

Second, there is a practical advantage in using smaller organisms. Compared with humans, organisms selected for study in space are small and easy to handle and maintain. They also grow quickly and have short life cycles, allowing rapid assessment of the effects of the space environment at different development stages and across multiple generations, often within single missions. Small size also means statistically significant numbers can be studied in a single experiment.

Finally, biology research is reinforced by a substantial amount of ground-based data. Many established laboratory organisms have a high degree of genetic homogeneity, allowing accurate comparison with replicate experiments and ground-based control populations. Studying diverse organisms also contributes to comparative and evolutionary biology, increasing our knowledge of how different organisms are related.

The benefits of studying different organisms in space—plants as well as animals—are therefore comparable with their use in Earth-based research, with additional advantages related to risk assessment and the logistical and operational constraints of spaceflight.

1.4 Justification for Animals Models

Scientists want to know how the body reacts to microgravity. Many experiments can be conducted on humans while they work on board the Space Shuttle[3] or the *International Space Station* (ISS), but many others interfere with daily activities. That's where the use of animals comes into play. And while scientists may not really care how a rat reacts to space conditions, animal data can transfer to human models and help prevent or solve physical issues people face today.

Due to the housing needs and the practicalities of space travel, the lowest form of life is most suitable for space travel. Often, experiment results using snails and fish can be applied to human conditions: inner ear exams can be done in snails rather than highly evolved mammals, and genetic studies can be conducted in fish. While there is not a one-to-one transfer, the similarities are enough to gain necessary knowledge (Souza et al. 2000).

Animals go into space to help conduct scientific research only when absolutely necessary. Researchers prefer to execute research with computer models, or by involving the astronauts directly. For some experiments, however, only animals will work. Sometimes the situations need to be closely controlled, such as a monitored diet. Human astronauts generally aren't willing to agree to eat the same amount and type of food every day, so this experiment would be a burden to them. Animals, however, are, and their feeding can be automatically monitored.

With the use of animals in space biology research comes the responsibility to conduct that research in an ethical and humane fashion. Regulations for animal research are stricter than those for using people in research because people can give consent. Animals can't object, so people need to work on their behalf. Animals that travel in space are cared for ethically and humanely. All research organizations are required to submit all animal protocols for approval by an *Institutional Animal Care and Use Committee* (IACUC). The IACUC verifies that studies using animals are both necessary and that all experiment protocols and animal care procedures meet federal animal welfare guidelines (Souza et al. 2000).

The IACUC and the funding space agencies also make sure that the number of specimens used is the minimum required to obtain valid scientific results, and that the experimental protocols are designed to minimize distress, pain, and suffering. Animal housing rules are more extensive than the requirements for human children day care centers. NASA facilities that house

[3] The Space Shuttle is also referred to as the *Space Transportation System* (STS). Shuttle missions are numbered sequentially, e.g., STS-78, STS-79, STS-80, at the time they are planned. However, because of launch delays due to weather, technical glitches, and a host of other issues, missions are often postponed, schedules are shuffled, and the flights are sometimes "out of order".

animals for research are accredited by an organization that requires proof that animals are cared for in a facility that meets those standards (Figure 1-05).

The professional standards of good science also guarantee animal health and welfare. Not only are researchers responsible for the health and welfare of the animals they use, but also it is in their professional interest to make sure the animals are well cared for. Sick or ill-treated animals are not good research subjects. They can compromise the quality of resulting data or contribute to the failure of an experiment. This hinders the scientists' ability to publish their research. Spaceflight opportunities are hard to come by and competition for slots is extremely intense. A failed flight experiment may never have the opportunity to be reflown.

Finally, the magnifying glass of the public eye is strongly focused on space research. The public, the media, and politicians watch with interest. They want to be sure that things are done properly and that their tax dollars are not wasted (Souza et al. 2000).

Figure 1-05. Ham, the first chimpanzee ever to ride into space is shown off by his animal trainer at Cape Canaveral, Florida. Photo Courtesy of NASA.

1.5 Frequently Asked Questions

1.5.1 How Many Animals and Plants Have Flown in Space?

Throughout the history of space life sciences, the combination of research priorities and practical constraints has led to a veritable menagerie of organisms orbiting the Earth. Some of the more exotic include African clawed frogs, Japanese quails, tobacco hornworm pupae, flour beetles, sea urchin eggs, parasitic wasps, and pepper plants (see Ballard and Mains 1990). A more complete list appears in Chapter 2, Section 2.

1.5.2 How Do Animals React to Microgravity?

Through millions of years of evolution, most terrestrial organisms have adapted to function optimally in the presence of a constant, unidirectional 1-g gravitational field. It is therefore not surprising that considerable changes occur at the organism level when the gravitational force is virtually removed.

Do the animals like living in microgravity? Does floating instead of walking confuse them? Amazingly, they adapt very quickly. Within five minutes, mice are floating in their living spaces, grooming themselves, and eating, just as they would on Earth.

Can fish swim in microgravity? Do bees make honey in space? Can ant farms exist on the Space Station? Fish and tadpoles swim in loops, rather than straight lines, because there are no up or down to orient them. If a light shines, the fish use that as their guide source and swim towards the light. Baby mammals have a hard time in space because they normally huddle for warmth. In space, it's hard to huddle when bodies drift and float. It is also difficult for babies to nurse when they can't locate their mother's nipple.

1.5.3 Why Study Microbes in Space?

Microbes are thought to make up more than 60% of the Earth's biomass. They have survived and evolved for over 3.7 billion years and have been found in almost every environment (for a review of microorganisms with particular physiological and nutritional characteristics, see Clément 2005, Chapter 2, Table 2-01). The diversity and range of environmental adaptations exhibited by microbes makes them a natural choice for studying how terrestrial life adapts to unique environmental pressures, such as those found in space. In addition, they are easy to grow and handle and most microbes are not responsible for diseases in humans, animals, or plants, making them relatively safe to study in a closed environment. Though microbes such as the bacteria *Escherichia coli* (*E. coli*) (see Chapter 2, Section 3.1) are used extensively to study basic biological processes in space, including cell growth and metabolism, they are also of considerable interest to bioengineers keen to

exploit their waste recycling potential in bio-regenerative life support systems.

Microbes are also making a contribution to nanotechnology advances being developed to support space exploration. For example, NASA scientists are using a genetically modified strain of *E. coli* to produce large quantities of proteins that sticks to gold or semi-conductors. The protein can be crystallized to form nano-templates about 5,000 times smaller than the width of a human hair (Figure 1-06).

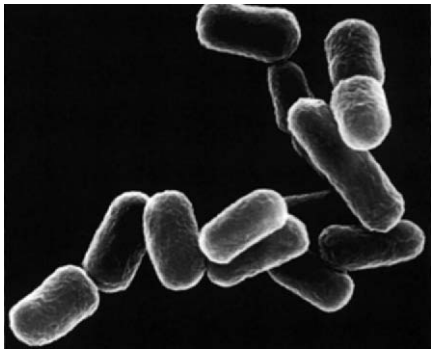

Figure 1-06. Certain organisms, such as E. coli shown here, have become "model organisms" in research laboratories because of advantages in studying them. E. coli reproduces rapidly (under optimal situation 0.5 h/generation) such that results for a number of experiments can be quickly obtained. Certain mutants of E. coli have been defined that cannot express certain proteins at saturation growth, and, therefore, die. Also, it is easy to manipulate both genetically and biochemically. E. coli's ability to take up exogenous genetic material under the procedure known as DNA-mediated cell transformation has also made it a popular model for studies using recombinant DNA. Most importantly, it shares fundamental characteristics, such as DNA and messenger RNA, with all other organisms. Photo courtesy of NASA.

1.5.4 Why Grow Plants in Space?

Plants respond to gravity and to other environmental factors in fundamental ways. By studying plants in the microgravity environment of space, we can begin to understand basic concepts in plant biology, such as perception, signal transduction, and response to stimuli. The spaceflight environment can also be used to specifically elucidate gravity-mediated events, such as gravitropism and circumnutation.

The same qualities that make plants essential to life on Earth—food production, absorption of CO_2, and release of O_2 and water vapor—make them highly desirable on long-term human space missions. Investigations with a variety of organisms also have practical implications to developing life support systems. A *Controlled* (or *Closed*) *Environmental* (or *Ecological*) *Life Support System* (CELSS) may be required for space missions of increasing durations and numbers of crewmembers. Such missions may require life support systems relatively independent of re-supplying consumable items, such as food, water, and oxygen. These substances may need to be recycled or regenerated. On Earth, life support systems including higher plants have been studied for at least 20 years and tests with some components have been

conducted on the Russian *Mir* station and the ISS (for a review, see Eckard 1996).

On an aesthetic level, plants have been shown to have a positive psychological effect on astronauts, providing some relief from the prolonged confinement of spaceflight and the sterility of the spacecraft environment.

In the sections that follow, the fundamental questions being asked in the various areas of space biology, and the approach to answering them are described.

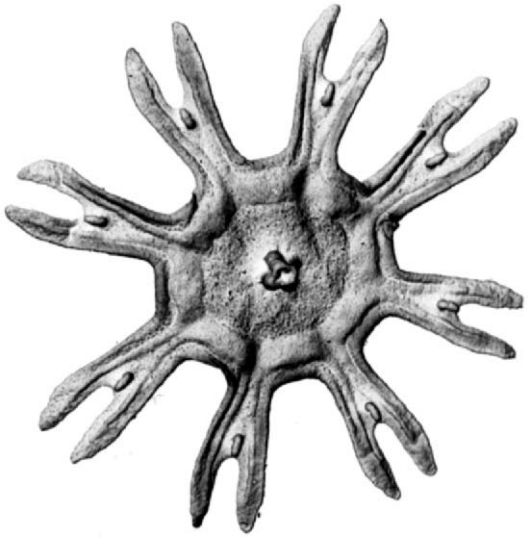

Figure 1-07. Jellyfish are aquatic organism with one primary radial axis of symmetry. This photograph of a jellyfish Ephyra shows the organism's gravity-sensing rhopalia. Another common species of jellyfish is Physalia. By reference to the simplistic orientation behavior of this animal, our first series of experiments studying human spatial orientation on board the Russian Mir space station was named "Physalie". Photo courtesy of NASA.

2 GRAVITATIONAL BIOLOGY

In "The Symposium", Plato has suggested that there was a time when the human body was completely spherical, with the features more or less evenly distributed around its circumference. Although humans were never spherical, our primitive ancestors were indeed arranged on a radially symmetrical plane. When an animal is circular, restrictions are imposed on both its movement and its perceptions. For example, the bell-shaped jellyfish can only pulse and drift, and the sea anemone can only retract and expand (Figure 1-07). When one's repertoire of movement is as limited as this, there is no particular advantage in having an extended field of perception. Consequently, the gravity-sensing organs are very simple. At a relatively early stage in the evolution of life, there appeared elongated forms which were clearly oriented in all three dimensions: gravity gave them a top and a bottom, bilateral symmetry gave them a right and left; motivating the same direction on every occasion gave them a head an a tail. *They had not only an attitude but an approach to the world*, and their instruments of action and perception modified themselves accordingly. The rear end became an organ of

locomotion, whilst the front became the organ of feeding and finding, with the development of more and more sophisticated sensory organs that became elaborate instruments of exploration, orientation, and anticipation (Miller 1978).

2.1 Principles of Gravitational Biology

Gravity influences the design of terrestrial organisms, an observation that was originally made by Galileo in 1638. He recognized that large animals have relatively thicker weight-supporting bones than smaller animals. As animals increase in body size there is an increase in relative skeletal size, a property known as the *scale effect* (Table 1-03). These differences are due to gravitational influence, since marine mammals have lesser skeletal size than terrestrial mammals with a comparable body size (Smith 1975). Consequently, large animals are more dramatically affected by gravity than small animals. Smaller organisms, such as insects, are more affected by such forces as surface tension, whereas viscosity, Brownian movement, and other intermolecular phenomena principally affect microorganisms like bacteria.

Animal	***Body***	***Skeleton***
Mouse	*0.02*	*8*
Dog	*5*	*13-14*
Man	*75*	*17-18*

Table 1-03. Increase in body mass (in kg) and relative skeletal mass (in % of body mass).

However, it is controversial as to whether living systems perceive gravity at cellular levels, hence a *direct* effect, or if the effect of gravity translates to physiologically important phenomena at multicellular or tissue levels, i.e., an *indirect* effect. In fact, at the cellular level, structures remain generally insensitive to forces on the order of Earth's gravity. For example, to selectively remove the organelles of animal cells, a centrifuge must rotate at speeds generating centrifugal forces in the order of 1,000 g. To separate large molecules, such as proteins, forces in the order or 100,000 g are required. By the same principle, the tolerance to high acceleration is inversely correlated to body size. Small animals are intrinsically more tolerant to high g than large animals (Figure 1-08). The tolerance limits are also affected by posture, which results in great part from the structure of the vascular apparatus, especially a column of blood. A significant fraction of the total blood flow (defined as the cardiac output) is directed to the brain and is necessary for the brain to function. The pressure drop in a blood column is proportional to the height of the column, the fluid density, and the acceleration. For prolonged acceleration exposures, body fluid shifts become relatively important and tend to dominate the deleterious effects of acceleration.

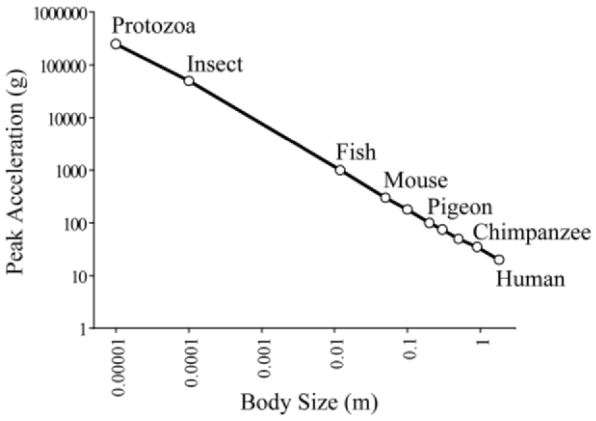

Figure 1-08. Body size is inversely related to the peak acceleration that the animals can sustained before serious injury occur, Other studies have found that body weight is also inversely related to the threshold g-value at which animals are resistant to a prolonged acceleration. By comparison, small, young plants can easily withstand 10 minutes at 30-40 g without noticeable structural change and can even endure several hundred g without evident structural breakage (Smith 1975).

The development of terrestrial organisms resulted in an increased susceptibility to gravity. Life originated in unicellular organisms in an aquatic environment wherein gravity had little direct effects. Certain animals became terrestrial at a relatively small size, after an intermediate step of developing increased body mass and a skeleton. In becoming terrestrial and increasing their size, such animals had to adapt and conform to the more stressful requirements imposed by gravity-induced loads. It is perhaps significant in this regard that the largest terrestrial animals became extinct. Or that the largest animals that ever lived, the blue whales, returned to an aquatic existence, where gravitational influence is greatly reduced (Smith 1975).

We know that organic form and metabolism are adapted to body size, but the relative importance of gravity and genetic factors in such adaptations is not fully understood. By the use of sensitive methods it should now be feasible to study the role of gravity in the manifestation of scale effects in animals, and also in plants, removed from the gravitational stimulus.

A significant challenge is that Space Biology grew so fast and in such a sporadic fashion that basic whole-organism biology has barely been studied in microgravity. Richard Wassersug (2001) advocated the fundamental importance of studying "Integrative Biology" in space, i.e., not just fly in-vitro experiments, but whole organism. *The whole is more than the sum of the parts.* Knowing how cells perform in a culture in space may reveal little about how whole organisms will respond in microgravity. Evidence today suggests that cells and tissues studied individually in space may not reflect how whole organisms respond in the same environment. Ultimately, in the crucial area of astronaut health, it is the whole organism that most assuredly counts most. Also, the public likes whole organisms and can identify with and understand biology at that level more so than most any experiment in cell biology. In fact,

the more carefully scientists observe the behavior of the developing system, the more they see evidence of altered behavior. These alterations are organized, adaptive responses to microgravity. Research on board the International Space Station must incorporate the expertise of behavioral scientists to attain accurate, meaningful results.

Another issue is that animals were often flown in confined systems to prevent them from endangering themselves during launch and reentry or damaging sensors or instrumentation during the flight. If the animals cannot float freely within their habitats while in the microgravity environment, then their responses, particularly those related to muscle atrophy, bone loss, and spatial orientation, cannot be directly compared to those in astronauts. Before the ISS, no spacecraft, Mir included, has had the space or equipment onboard for anything but simple studies of highly confined vertebrates. It is now time to move from studies using *passive* (restrained) exposure to studies allowing *active* (free-floating) exposure to microgravity.

A comprehensive understanding of microgravity effects will require analyses of both the whole organism as well as some of its component cells, tissues or organs. In fact, the first microgravity effects to be recognized in whole organisms may have been preceded by prior effects, more subtle, which were not detected. Most organisms contain effective homeostatic mechanisms for masking environmental challenges such as changes in the amplitude of the gravitational vector.

One thing is sure: a variety of organisms have flown during spaceflight and most of them have survived in the space environment. After extended stays in space, can those organisms grow, develop, and reproduce normally? Such questions are much more difficult than questions of survival because they require sophisticated scientific experimentation in order to understand just how the living organisms are affected by the conditions of spaceflight, including microgravity.

2.2 Cell Physics

Hardware to support living organisms is designed to accommodate the conditions of spaceflight, but microgravity poses special engineering challenges. Plants are usually flown attached to a substrate so that nutrients and water can be provided through the root system. Cultured cells are flown in suspensions of renewable media contained within specialized hardware units. However, fluids behave differently in microgravity. On Earth, fluids are dominated by *pressure* (i.e., the weight of fluid above) and *buoyancy*, whereas in microgravity physical properties such as *surface tension* (Figure 1-09), *Marangoni effects* (Figure 1-10), and *diffusion* are dominant. Furthermore, in normal gravity, objects in a fluid sink or float depending on whether they are more or less *dense* than the fluid. The sinking or rising speed is dependent on

the fluid viscosity. In microgravity, density differences do not cause objects to move in a fluid because buoyancy and sedimentation effects do not exist.

Within a single cell on Earth, density-dependent streaming and separation, or at least orientation, occur along the gravitational vector. The cell interior behaves in part like a fluid having only modest viscosity. Therefore active and passive cell deformations can be easily modified or governed by gravity. For cells in suspension, such as in blood where no interstitial meshwork prevents movement, the more dense elements will be pulled "downwards", or sedimented, in the direction of gravity. In fluid-filled compartments, changes in the gravitational force will lead to fluid shift redistribution, varied hydrostatic load across the walls, and passive deformation of the tissues.

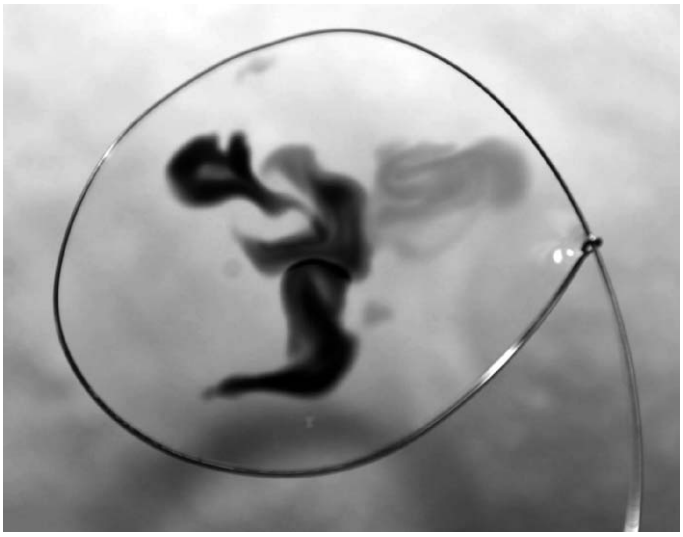

Figure 1-09. Astronaut Donald R. Pettit, during his stay on board the ISS, photographed this surface tension demonstration in microgravity using food coloring added to the water that is being held in place by a metal loop. Photo courtesy of NASA.

It is important to distinguish between the *indirec*t and the *direct* effect of microgravity. Among the indirect effects are the absence of *sedimentation* and the loss of gravity-driven *convection*. The latter include density-driven phase separation (vinegar goes to bottom and oil goes to top), and thermal convection (heated liquids rise), which are caused by buoyancy. In normal gravity, thermal convection establishes a current that rapidly dissipates heat, renews nutrient supplies, and removes waste materials. This factor is most important when no fluid flow (like blood flow) exists to dissipate metabolic products and exchange nutrients around the cell. Without convection, slow diffusion processes are the only means for heat and nutrient exchange. This factor is likely to be most relevant in plants and single celled microorganisms such as bacteria that have no motile structures like cilia or flagella. Although

blood flow in animal tissues mostly overcomes this effect, it could still be a factor in cells localized where blood flow is minimal.

Many cell-based systems rely on being anchorage-dependent, or at rest against a surface. By means of a solid-liquid interface they have access to nutrients, metabolize, lay down matrix material, and reject wastes. When the cell's ability to interface with a surface is taken away, such as in microgravity where sedimentation is absent, the role of that particular interaction can be investigated. Interestingly, when cells are prevented from sedimenting against a surface, they make their own surface with which to work.

Cells are also exposed to *hydrodynamic shear*. Hydrodynamic shear is a force created by fluid moving past a fixed object, objects moving at a faster or slower relative rate, or an object moving in a direction opposing the flow. The red and white blood cells, as well as the endothelial cells that line the blood vessels, are exposed to a fairly violent environment of hydrodynamic shear. In fact, endothelial cells need these hydrodynamic forces to express certain sets of genes that allow them to mature. Hydrodynamic shear facilitates renewal, by removing old cells. We do this every morning when we take a shower. We are shearing cells off of our bodies and sending them back to the recycle system. However, too much shear results in death, or in substantial changes in membrane composition, because cells then produce large amounts of extra-cellular material. The ability of a cell to respond to a specific ligand and transduce a signal to the nucleus may then be substantially affected (Pellis 2005).

On Earth, when maintaining an upright posture, there is a considerable *hydrostatic pressure* gradient along the body axis. Various cells within the body respond to and rely upon hydrostatic pressure gradients for normal function. For example, bone growth, maintenance, and renewal depends on physical force profiles that include hydrostatic pressure along the body axis. In microgravity, the pressure gradient is redistributed in such a way that it is essentially homogeneous throughout the long body axis. One hypothesis for the bone loss that occurs in microgravity conditions is the absence of stimulation by hydrostatic pressure of the cells responsible for bone formation. By the same principle, it is necessary to put shear back into the process to grow pieces of biological tissues, for example, that have endothelium in them (Pellis 2005).

Cells are also submitted to *mechanical forces*. Chronic abrasion induces cells to proliferate in those abraded areas. This occurs artificially in cell culture just by the stirring mechanism. A spinner flask used in a lab has a stirring bar suspended in it, which stimulates cell growth. However, the selective role of vibration on cells is largely unknown. Some injuries result from repetitive use, such as tennis elbow. On the other hand, there are also reparative mechanisms that seem to be invoked by certain frequencies of vibration and change in activities within the endothelial cells.

The direct effects of microgravity include no surface attachment, the tendency toward a change to a spherical shape unless previously attached to a surface, and the disorganization of microtubules organizing the skeletal array within the cells. Cells in microgravity have also less of a tendency to apoptose (die), which could potentially lead to them become cancerous and increase the risk for an autoimmune event. Finally, the increase in *surface tension* forces and *Marangoni convection*, i.e., the surface tension driven convection induced by a temperature or concentration gradient, presumably tends to favor cell-cell interaction. Indeed, the Marangoni convection is masked by gravity-driven convection on Earth, but becomes the dominant form of convection in microgravity, where it facilitates mixing. *Diffusion*, i.e., the mixing of liquids by random molecular movement, can also be a dominant force in mixing of microgravity bulk liquids. The pure effects of diffusion are masked by buoyancy convection and sedimentation in Earth's gravity (Pellis 2005).

In summary, microgravity affords a unique environment for cells. It is unlike anything that cells have experienced before. The response of cells to the space environment must undergo a careful analysis to understand its direct and indirect contribution. What sensory device within the cell is triggered for gravity, if there is one? The direct effects of microgravity on cell shape probably reset many of its functions. Inversely, the restoration of mass transfer, shear, and/or vibration could favor cell culture in microgravity, thus offering promising possibilities for space bioprocessing.

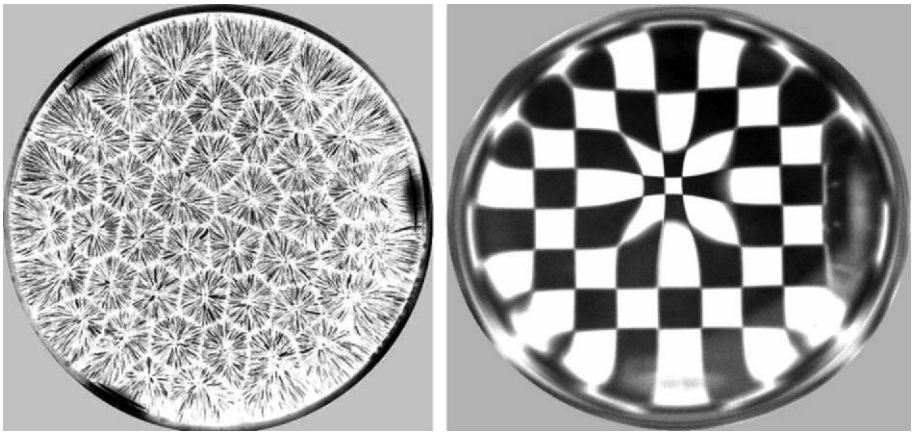

Figure 1-10. Marangoni convection can be observed with a bowl shallow filled with silicon oil and graphite and heated from below (left). The reflection of a chessboard, in which the surface of the oil is used as mirror, shows the deformation on the surface. Adapted from Jäger (1996).

2.3 Research Questions

The only way in which cells can make themselves independent of the conditions of the surrounding environment is by forming communities, which are large enough to include a self-made climate, and then developing mechanisms for stabilizing that climate to the advantage of all the constituent members. The mechanism defending and regulating the constancy of this climate is called *homeostasis* (Figure 1-11). The efficiency of living cells depends on the composition of the fluids by which they are surrounded. Single-celled animals can counteract small fluctuations in these fluids, but multi-cellular animals are much more resilient: they can enclose and defend an inland sea whose chemical composition is supervised and regulated by the kidneys and other organs. The survival of cells also depends on the stability of the materials from which they are made, and the energetic material surrounding them. The mechanisms responsible for maintaining life are virtually indistinguishable from the structures they support (Miller 1978). Fluid dynamics change in microgravity, it is therefore expected that microgravity exposure will affect the mechanical, biochemical, and physiological processes at cellular level. The flight environment presents a unique opportunity to study cell morphology and growth, as well as cell metabolism and interactions, in response to a change in the physics of the environment (Table 1-04).

Figure 1-11. The homeostatic mechanism preserves a constant internal environment in the face of wide variations in the outside world. But there is a limit to the physiological powers of compensation. If the external environment varies beyond what is physiologically expected, it is necessary to provide the subject with a portable version of his normal surroundings. This photograph shows an astronaut in a space suit and wearing a portable life support system when outside the space vehicle. Photo courtesy of NASA.

2.3.1 Effects of Gravity on Cell Shape, Function, and Growth

The modern view of adaptation is that the environmental forces set certain "problems" that organisms have to "solve", and that evolution, by means of natural selection and mutations, is the result of these solutions. By which mechanisms do cells sense gravitational forces and what is the role of gravity on growth and development? The use of the weightless state for unmasking factors operating at normal gravity is of decisive importance in the study of these problems. As of today, knowledge is still sparse in this research area, largely due to the constant gravity on Earth and the longstanding inability to reduce or null it.

A central feature of gravitational biology is the search for the presence of gravity sensors at *cellular level*, and the study of whether gravity affects molecular forces in living systems. For example, in the cytoplasm of cells, a continuous movement, referred to as *protoplasmic streaming*, and a binding of particles to cellular skeleton elements normally tend in some degree to oppose gravity-dependent stratification of particles according to their densities. The force of gravity undoubtedly affects the spatial relations among cellular organelles and structures. However, it is still unclear if prolonged weightlessness has an effect or not on the function of simple cells grown in culture.

Also, many cells are organized in a *polarized* manner, so that their contents are distributed non-uniformly along an axis, with different cytoplasmic components oriented towards either pole. Examples of highly polarized mammalian cells are the white blood cells and especially the leukocytes in which centrioles tend to occupy the geometric center of the cell, and pancreatic secretory cells in which the nucleus is found in a basal locality. The pathways of biosynthesis with the cell are orderly and related to the spatial arrangement of the various cell organelles. Complex processes such as DNA replication, RNA transcription (see this Chapter, Section 3.2) and the migration of proteins between organelles are strongly related to cell polarity.

Modification of molecular transport within cells by the force of gravity can be expected since transport affects morphology and vice-versa. Changes in pressure gradient and transport processes involving convection or diffusion might therefore result from an alteration in either magnitude or direction of the gravitational vector. Basic life processes in the cell, such as the regulation and distribution of water and ions, the turnover of cell membranes, secretion, absorption, division, and hormone and molecular interactions can also be assumed to be potential targets for such influence (Figure 1-12).

2.3.2 Specialized Cells *in Vitro*

In the recent years there has been rapid progress in the methods for maintaining the viability of specialized *in vitro* cell cultures. In many cases, the behavior of cells cultures in vitro may provide useful models for the study of cellular events as they occur *in vivo* in tissues and organs.

Loss of bone calcium and muscle mass and a decrease in red blood cell count are examples of disturbances occurring in humans during prolonged exposure to the microgravity environment. The availability of *in vitro* cultures of isolated bone and skeletal muscle cells provides a unique possibility for analyzing how bone and muscle formation, destruction, and regeneration are affected by microgravity.

Lymphocytes are the cells responsible for the immune response (see Figure 1-17). Previous studies on lymphocytes of crewmembers have shown delayed reactivity to agents, or *mitogens*, that normally stimulate cell proliferation. Experiments performed on lymphocyte cells from human blood allow evaluating the effect of microgravity on this mitogen-induced proliferation (see Chapter 4, Section 3.2).

Figure 1-12. Model of cell division. The cell cycle is an ordered set of events, which results in cell growth and division into two identical daughter cells (somatic cell division). This sequence of specific events includes cell growth, protein, DNA, and organelle replication, and nuclear (chromosomes separate during mitosis) and cellular (the cytoplasm divides during cytokinesis) division. The phases of the cell cycle are: the G_0 phase where cells exist in a quiescent state; the G_1 phase, which is the first growth phase; the S phase, during which the DNA is replicated; the G_2 phase, which is the second growth phase, also the preparation phase for the cell; and the M phase or mitosis, i.e., the actual division of the cell into two daughter cells (as shown here). A surveillance system monitors the cell for DNA damage and failure to perform critical processes. If this system senses a problem, a network of signaling molecules instructs the cell to stop dividing. They can let the cell know whether to repair the damage or initiate programmed cell death, a form of which is called "apoptosis".

2.3.3 Gravity-Sensing Mechanism

Complex organisms have special *gravity-sensing organs*, by which the direction of the gravitational vector is used for orientation. In mammals, these are the *otolith* organs of the vestibular apparatus in the inner ear. In vertebrates, the gravity-sensing system has the same basic structure from fish to humans. It consists of tiny calcium carbonate crystals, the *otoliths*, resting on a layer of specialized nerve cells, the *macula*. The relative weight and movement of the otoliths resting on them stimulate these hair cells. They respond to linear acceleration in three planes and signal information to the brain concerning the position of the head relative to gravity. In the adult, there is a precise interplay between these signals and those arising from many other receptors involved in the reflex muscular control of balance, posture and locomotion.

During the Neurolab mission it was found that, upon arrival in microgravity, the macular receptors compensate for the weightless calcium crystals by increasing the number of connections among hair cells. In other words, the nerve cells in the macula make new connections in space. After return to Earth, the additional connections generated in space were eventually deleted, as they were no longer needed in normal gravity (Ross et al. 2003). This result shows evidence for neuronal plasticity, or learning, at cellular level in a gravity-sensing area of the nervous system.

Gravity-sensing organs are also present in lower vertebrates and insects. These are even simpler systems, which are easier to analyze and develops faster. In snails, for example, the gravity-sensing component is lined with hair cells that send signals to the brain when they are triggered. The "triggers" are small particles of calcium carbonate, referred to as *otoconia* or *statoliths* (Figure 1-13). With gravity, these triggers weigh down upon and bed different groups of hair cells, which then send orientation signals to the brain. Ground-based experiments in which snails were developed in a centrifuge have shown that the size of the statoliths is determined by their weight. Somehow the pull of gravity signals the developing statolith when an appropriate level of growth is reached. In space, however, without this signal, these grain-like particles should develop to a larger size than they do on Earth. A related question is the following: If indeed statolith and otolith size increases in microgravity, will the behaviors of the snails and fish change also?

Crickets have even simpler gravity sensors, which are connected to a simple and well-studied nervous system. They develop rapidly, making them ideal for studies during spaceflight. Crickets roll their head when tilted, and this reflex is activated by the gravity-sensing system. By measuring this head movement, scientist can determine the efficiency and accuracy of the synaptic connections that have developed in the cricket's gravity sensors in microgravity (see Chapter 5, Section 4.1). Also, investigators can determine if

the microgravity-exposed animals can regenerate the gravity sensor, as they accomplish it on Earth.

Similarly, jellyfish would serve as excellent subjects for research on gravity-sensing mechanisms, because their specialized gravity-sensing organs have been well characterized by biologists. Jellyfish and other invertebrates use structures called *rhophalia* or *statoliths* to maintain their correct orientation in water (see Figure 1-07). To determine the function of the statoliths and their adaptation to microgravity, investigations could compare, for example, their morphology and the swimming behavior of tiny jellyfish metamorphosed in space with those metamorphosed on Earth.

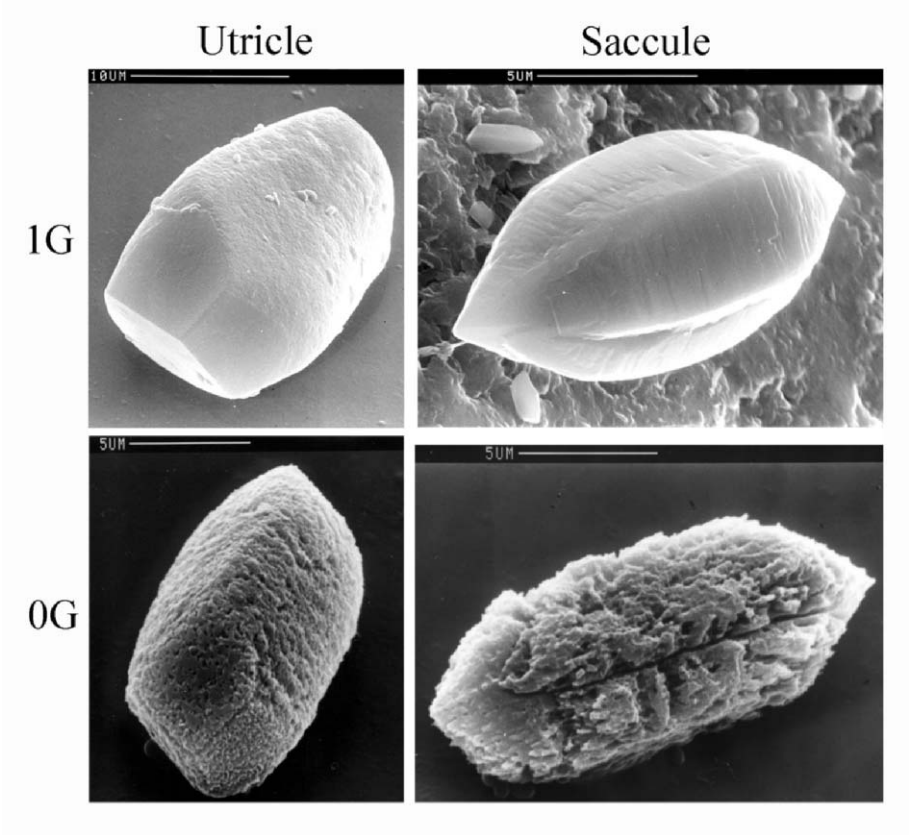

Figure 1-13. Adult amphibians have otoconia located in their vestibular labyrinth. These otoconia are made of calcium carbonate in two crystal forms, with calcite in the utricle (left) and aragonite in the saccule (right). Compared to ground (top), there are changes in the appearance, morphology, and crystallographic structure of the otoconia in Pleurodeles walt developed in microgravity (bottom). Adapted from Oukda et al. (2001).

Modern genetics research has made available a variety of engineered mutations that affect the gravity-sensing systems of the brain and body. Space research provides the only way to gain a full picture of how these systems develop in microgravity, and will be key to unraveling some of these basic mysteries of gravity-sensing.

Some questions of fundamental interest are the following: Will the morphology of the vestibular apparatus or the sensorial functions of orientation, equilibrium, and locomotion be permanently affected by the loss of the gravitational stimulus? If the gravity-sensing otolith organs serve as a keystone in the development of posture and locomotion, what will be the consequences of a complete deprivation of the gravitational stimulus at the time of development of these functions? Will other functions related to the action of gravity, such as blood pressure control and bone formation at the cellular level, develop normally? Since critical stages in the functional development may also occur after birth, will only the animals that have been exposed to the spaceflight environment during certain phases of the postnatal period exhibit those changes? These questions are also addressed by research in Development Biology, as described in the section below.

Current
• *Cell structure/morphology*
• *Growth and differentiation of vertebrates' cell cultures*
• *Understand the effects of gravity on cells, animals and plants*
Future
• *Cell reproduction, development, genetics*
• *Cell characteristics/integrity*
• *Cell metabolism and products*
• *Cell-cell and cell-body interactions*
• *Response to foreign agents*
• *Other environmental factors than microgravity*

Table 1-04. Current and future space research in Cell Biology.

3 DEVELOPMENTAL BIOLOGY

Common to all living systems, from the primitive single cell to human, life is a matter of a continuing regeneration of kind, dynamically influenced through time by mutation and natural selection. Studies of the different parts of a life cycle involve virtually all of the major biological disciplines: molecular biology, genetics, and cell physiology. These disciplines all deal with information transfer in fertilization and reproduction through physical and chemical separations and bondings.

Embryology begins with development from the fertilized egg through growth and morphogenesis to differentiation and specialization of tissues and

organs. As living organisms mature, reproduce, age, and die, their physiological and behavioral response and adaptation to the environment change. Success or failure through natural selection of the organisms' adaptation influences the genetic and social inheritance of each succeeding generation. Consequently, the organisms' ability to deal with the environment is also affected. Through the life cycle, an organism is variously vulnerable to its ambient conditions, being perhaps most sensitive in early development. The spaceflight environment includes several potential hazards such as radiation, alterations in atmospheric pressure, prolonged toxic exposure and weightlessness, which may affect developmental processes.

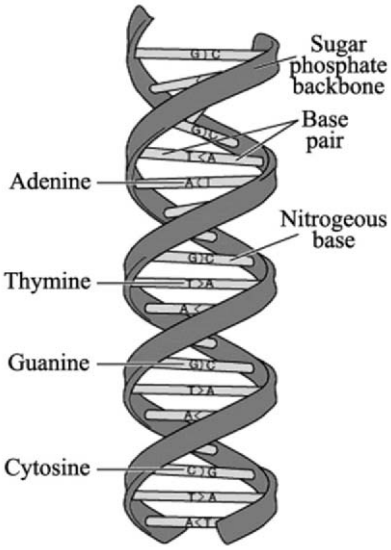

Figure 1-14. Deoxyribonucleic acid (DNA) is a nucleic acid that contains the genetic instructions specifying the biological development of all cellular forms of life. Pieces of DNA are pairs of molecules, which entwine like vines to form a double helix. Each vine-like molecule is a strand of DNA, i.e., a chemically linked chain of nucleotides (adenine, thymine, guanine, cytosine). The sequence of nucleotides along a DNA strand defines a messenger RNA sequence, which then defines a protein that an organism is liable to manufacture or "express" at one or several points in its life using the information of the sequence.

3.1 Principles of Development Biology

3.1.1 The Making of a Living Organism

The structure and composition of living organisms varies greatly, from single-celled bacteria to complex multi-cellular organisms with differentiated cell types and interconnected organ systems. Regardless of the complexity, every living entity contains a blueprint for its construction in the form of a double-helical chain of molecules called *deoxyribonucleic acid* or DNA (Figure 1-14). DNA is an amazingly simple chemical structure, yet it contains an entire library of information on how to make, maintain, and reproduce an organism, and also keeps a record of clues to the organism's evolutionary history. The entire sequence of DNA in an organism is called its genome. A genome can be as small as the 9,750 bases of the *human*

immunodeficiency virus (HIV; the cause of AIDS) or, as large as the +3 billion bases in mouse, human, and frogs.

DNA encodes the unique nature of different organisms by specifying the precise structure of each protein in a cell. In analogy to DNA, proteins are made from a linear sequence of amino acids, and the exact sequence of amino acids is what determines the function of the protein. A DNA sequence is translated into the protein sequence by a code, where a triplet of bases (a codon) specifies a single amino acid; some codons specify the end of the protein. Humans are built from an estimated 20,000-35,000 proteins, yet, only a small percentage of the 3 billion bases in the human genome codes for these proteins. The discrete sections of DNA that encode proteins are referred to as *genes*.

Protein production is a highly regulated process. For example, a cell does not want to waste energy making the proteins needed for cell division if it is busy with other functions, such as secreting a hormone. This process of turning a gene on and off depending on the cell's need for a particular set of proteins is referred to as regulation of *gene expression*. Certain proteins, and even other regions of DNA, physically bind to the DNA sequence surrounding a gene to affect its expression. This interaction occurs at regions of the DNA with apt names, such as promoters or enhancers of gene expression.

Gene regulation is an essential part of life. Since every cell in an organism contains the same genetic blueprint, turning on different genes at different times during development creates different cell types. In fact, it is differential gene expression that allows stem cells to become unique cell types. Gene regulation is also critical for cellular response to metabolic needs.

The entire genome is not read all in one piece. Instead, cells make copies of selected genes at selected times via the process of transcription. These copies are transported out of the nucleus to a cellular factory, or organelle, called the *ribosome*, where translating the original DNA code makes proteins.

3.1.2 Reproduction

Fertilization activates the egg and brings together the nuclei of the sperm and egg. Fertilization forms the diploid zygote and triggers the onset of embryonic development. The sperm nucleus swells and merges with the egg nucleus to form the zygote and DNA replication begins with the first division occurring in about 90 minutes.

Duplication of a cell's DNA is required for both cellular replication to replenish dying cells, and for sexual reproduction. In unicellular organisms, these two processes are the same. DNA is duplicated before the cell divides to produce two separate organisms, each with the original amount of DNA. This asexual method of reproduction is known as binary fission. In multi-cellular

organisms, a similar process called *mitosis* is used to replenish lost cells (Figure 1-15). However, reproduction is more complex and begins with specialized cells called gametes (eggs and sperm). Through the process of meiosis, these cells have only half of the DNA of other cells; that is, only one copy of each of the 23 chromosomes.

Maintaining genetic variability in successive generations can be achieved through recombination of genetic material. The order of the genes on the chromosome remains the same, but the specific versions of the genes become shuffled. Recombination is one of the primary reasons that offspring from the same parents do not look alike, as new combinations of alleles are formed in every gamete.

Viruses are simply a strand of genetic material, either DNA or RNA, encapsulated in an outer protein shell, and sometimes a membrane. They cannot reproduce on their own or perform basic cellular tasks like protein synthesis, so most people do not consider them to be living organisms. Viruses reproduce by hijacking the *replication* machinery of a host cell. They transfer their genome into the cell of another organism, integrate their DNA sequence into the host DNA, and let the host cell replicate, transcribe and translate their genes. Viral genomes contain genes for directing the replication and packaging of complete copies of the virus, so that eventually, the host cell bursts open and releases new viruses to infect other cells.

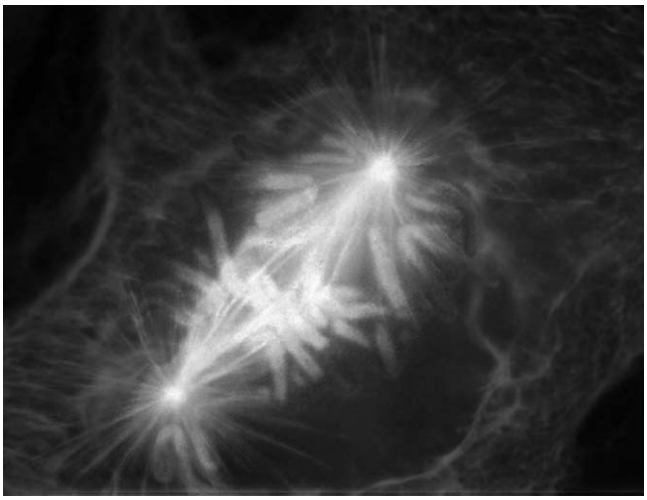

Figure 1-15. This image depicts a cell in mid-prometaphase. In this cell, the spindle is forming between the well-separated centrosomes. Some of the chromosomes have established connections to both poles and are aligned at the spindle equator, while others are still connected only to one pole. Photo courtesy of NASA.

Cell division, the process by which our cells grow and multiply, is normally tightly controlled. In embryos and young children, cell division is required primarily for growth. However, its main role in adults is to repair and replace old cells. Cell division is a very complex process, and it involves a very ordered sequence of events. For example, cancer occurs when a cell

breaks free from normal constraints and starts multiplying uncontrollably. Tens, if not hundreds, of molecules are involved in cell division, and many of these have been implicated in cancer.

Cleavage is a succession of rapid mitotic cell divisions following fertilization and produces a multi-cellular embryo, the *blastula*. A definite polarity results is shown in the egg caused by the concentration of cellular components as mRNA[4], proteins, and yolk. The yolk is a key factor in determining polarity and influencing cleavage in frogs and other animals. The vegetal pole of the egg has the highest concentration of yolk. The animal pole has the lowest concentration and is the area where polar bodies bud off of the cell. The animal pole marks where the most anterior part of the animal will form. The animal hemisphere is gray due to the presence of the pigment melanin. The vegetal hemisphere is slightly yellow due to the yellow yolk. Cleavage in the animal hemisphere is more rapid than in the vegetal hemisphere. If there is little yolk in the vegetal hemisphere cleavage will proceed equally. The first two cleavage divisions are vertical and divide the embryo into four cells. The third cleavage plane is horizontal and produces an eight-cell embryo with two levels. Continual cleavage produces a solid ball of cells called the *morula*. A fluid-filled cavity, called the *blastocoel*, develops within the morula forming a hollow ball of cells called the *blastula* (Figure 1-16).

Gastrulation then rearranges the blastula to form a three-layered embryo with a primitive gut. The three layers produced by gastrulation are embryonic tissues called embryonic germ layers. These three germ layers will eventually develop into all parts of the adult animal.

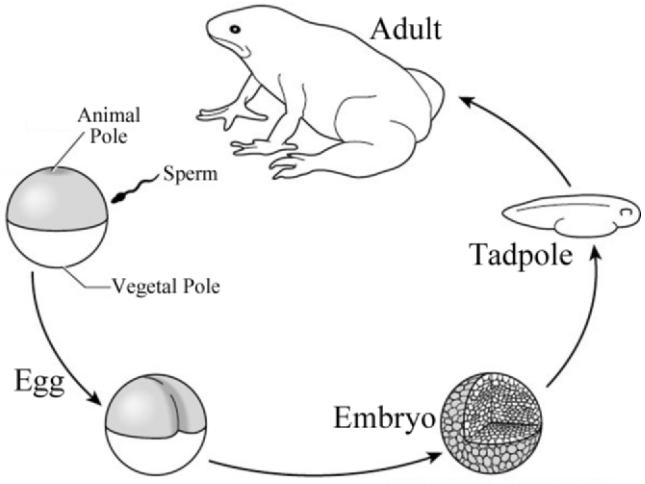

Figure 1-16. Fertilization of a Xenopus egg is followed by cleavage (a succession of cell divisions that partition the large fertilized egg cell in smaller cells), differentiation, and organogenesis. After hatching from the egg, the tadpole will exist in an aquatic stage with gills and a tail, until complex hormonal changes transform it into an adult frog. Source NASA.

[4] *mRNA* stands for *messenger RNA*. The DNA of a gene is transcribed into mRNA molecules, which then serve as a template for the synthesis of proteins.

3.1.3 Differentiation and Embryogenesis

Multi-cellular organisms develop from a single cell into a complex entity replete with a variety of cell types, such as skin, muscle, nerve, and bone. Different cell types require different sets of enzymes, structural proteins, and regulatory proteins to drive their specific chemical processes and support their unique needs. Embryonic cells differentiate into new cell types by regulating gene expression turning on and off the transcription and translation of individual genes.

Biologists have found that the organism's development is mostly determined by the genome and the organization of the egg's cytoplasm. As the zygote undergoes cleavage, the cytoplasm is compartmentalized causing the nuclei of the different cells to be exposed to different cytoplasmic environments. These different cytoplasmic environments result in the expression of different genes in different cells. Inherited traits then emerge, in an orderly fashion, in space and time by mechanisms controlling gene expression.

Cell differentiation occurs very early in embryogenesis. In mammals, after just a few divisions of the original fertilized egg, cells begin to migrate and form defined ends of the organism and a narrow grove that will become the spinal cord. Cells quickly begin to differentiate, expressing genes that are specific to their future cell type. Gene regulation not only guides differentiation, but also allows an organism to respond to a changing environment as well as to the needs of a developing fetus versus those of an adult.

Complex organisms have three general types of cells: somatic, stem, and germ cells. *Somatic cells*, from the Greek "soma" or body, make up most of the organism. When fully differentiated, somatic cells become damaged or worn out, many are replaced by simple mitosis. However, some are replenished from a pool of stem cells. *Stem cells* exist in an earlier, less differentiated state and have the ability to mature into a variety of cell types depending on the extracellular signals they receive. Stem cells identified in humans include brain, bone marrow (Figure 1-17), blood, blood vessel, skeletal muscle, skin, and liver. The term *germ cell* refers to the reproductive cells or gametes, sperm, and eggs.

Organogenesis forms the organs of the animal body from the three embryonic layers. The first evidence of organogenesis is morphogenetic changes (folds, splits, condensation of cells) that occur in the layered embryonic tissues. The neural tube and notochord are the first organs to develop in frogs and other chordates. The notochord stretches the embryo lengthwise and forms the core around witch the mesoderm cells will develop the muscles of the axial skeleton. As organogenesis continues, other organs and tissues develop from the embryonic germ layers. *Morphogenesis* includes the final physical processes that give shape to the animal's body and organs.

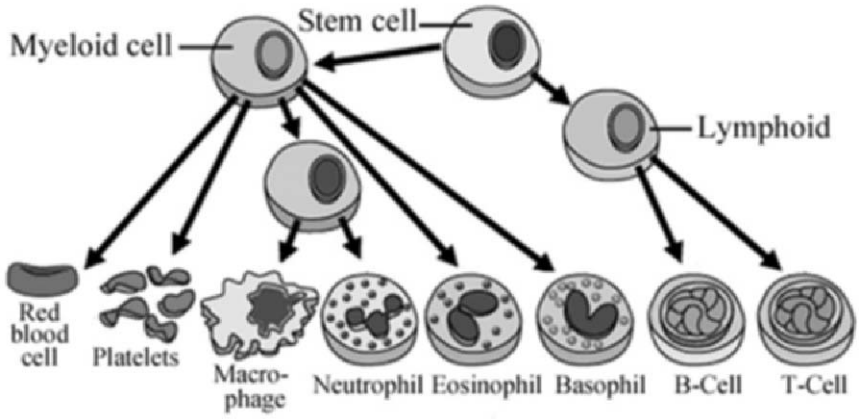

Figure 1-17. This graphic illustrates the variety of blood cells that are derived from a single stem cell in bone marrow of mammals. The red blood cells contain hemoglobin for the transportation of oxygen. Platelets are not cells, but fragments of megakarocytes that form clumps to assist in clotting. Macrophages are white blood cell that ingests foreign substances in the body. The other white blood cells contain granules that are tiny sacs of enzymes for digesting microorganisms that invade the body. B-cells produce antibodies (proteins) directed against specific antigens (foreign bodies). Some mature into "memory cells" that recognize re-infection by the same foreign body and stimulates further production of antibodies. T-cells are responsible for the destruction of foreign bodies.

3.2 Genetic Analyses

Since the early 1950's, laboratory methods to perform genetic analyses have rapidly advanced. Significant additions include the discovery of the three dimensional structure of DNA, technologies for sequencing and synthesizing DNA, and the development of laboratory reagents and procedures to identify expressed genes.

DNA can be extracted from almost any tissue type. A small number of live cells can also be used as seed stock to grow an immense number of cells in tissue culture. Such cells are grown in dishes or flasks at controlled temperatures and fed specific nutrients, eventually producing an identical population of cells. Thus, tissue cultures provide an unlimited and renewable source of DNA.

To preserve cells grown in culture, they are frozen in liquid nitrogen at temperatures below -196 C°, where they can remain indefinitely. The freezing solution contains an inert cryopreservative that prevents the formation of crystals during freezing. Frozen cell cultures provide a long-term source of DNA, which is invaluable in cases where the availability of tissue or blood samples is limited, such as for endangered species.

Scientists often need to obtain large amounts of DNA containing specific genes, or unique regions of genes. Two procedures are critical to rapidly producing large quantities of a target DNA sequence: cloning and polymerase chain reaction.

Cloning is the process of duplicating genetic information. Cloning occurs in nature and is a common form of reproduction in single-celled organisms and even some plants. In the laboratory, cloning can refer to the replication of any specific DNA sequence, or to the duplication of a whole organism for the purpose of producing a genetically identical copy. Most laboratory cloning benefits from the assistance of bacteria, an easily maintained, rapidly reproducing organism.

Genetically Modified Organisms (GMOs) are a derivative of single-sequence cloning as described above. In the case of GMOs, the target gene is transferred to a plant or animal so that they can produce chemicals or proteins that are useful for agricultural production or human medicine. For example, goats and sheep are being engineered to produce bioactive molecules in their milk for medical treatments such as human serum albumen; tomatoes have been developed containing a gene insert to delay ripening and extend shelf life, and researchers are exploring the possibility of producing cancer drugs from modified tobacco plants.

Polymerase Chain Reaction (PCR) was developed in the mid 1980's and revolutionized the study of genetics because it allows accurate production of millions of copies of a target sequence in a matter of hours. In addition, a single target sequence within a mixture of millions of other sequences can be faithfully amplified (replicated). The method mimics the same process that cells use to copy DNA.

Another technique to separate DNA is *electrophoresis*. Electrophoresis is used to separate pieces of DNA that vary in length. DNA is essentially sifted through a thin slab of "gel", similar in consistency to over-hardened Jell-O. The gel can be made of different materials, agarose or acrylamide, depending on the size of the DNA fragments to be separated. Liquid gel is poured into a mold leaving small slits or wells in one end where DNA samples (suspended in a buffer and dye) are placed once the gel solidifies.

DNA separated by electrophoresis is used for a variety of applications. For cloning or DNA sequencing, DNA bands are cut out of the gel and extracted into the appropriate solution. Other analyses rely on DNA *hybridization*, a process whereby complementary strands of DNA bind to each other, to determine if the separated DNA contains a sequence of interest.

3.3 Research Questions

What can we learn about evolutionary adaptation to gravity by exploiting the weightlessness of spaceflight and the relative plasticity of early developing organisms?

The lower life forms, e.g., bacteria, protozoa, and invertebrates, are relatively independent of the force of gravity in their development, at least as determined by hypergravity studies using centrifuges. They are also relatively insensitive to radiation. The vertebrate embryo, on the other hand, is more sensitive to disturbing environmental parameters. Even very slight increases in gravity are known to affect vertebrate development. Satisfactorily experimental designs can now be implemented on board the Space Shuttle or the ISS to answer questions concerning the effect of microgravity on very early development of the more primitive vertebrates, the cold blood animals. In fact, the results of some of these experiments are reported in Chapter 5.

The warm blood animals, i.e., the birds and mammals, demand more time for developmental studies than the present Space Shuttle mission durations ranging from 11-14 days. The chicken egg takes 21 days to hatch, with a total of about 23 days after fertilization. Many rodents have about a 22-day gestation period while the more advanced mammals have month-long gestation periods. The minimum requirements for a rat development experiment are not even met by the 21-day flight of a Russian biosatellite. Some answers for vertebrate development questions can only be obtained by experiments on board the ISS.

Life cycle studies of lower forms, e.g., insects, can benefit from the shorter missions of the Space Shuttle. However, longer duration flights can afford exposure of many more generations to the space environment. Thus the prospects of adaptation and expression of mutations increase. The longer the mission, the more advanced the life forms that can experience a complete life cycle.

Even more interesting from the standpoint of gravity effects would be the use of the more gravity-sensitive, larger, and more advanced animals. A one-year long mission, for instance, would permit the successive development of as many as four generations of a rodent. The importance of the mechanics of post-gestation development, e.g., nursing, feeding, drinking, and maternal behavior, can be effectively studied under low-g conditions. If mating behavior can take place in a microgravity environment becomes also an important question in the frame of very long duration mission or space colonization.

The ISS provides ideal conditions with a long-term exposure to microgravity, associated with closely controlled systems of isolation and sensory deprivation with tight environmental tolerances (light, temperature, noise). Therefore, of primary interest are the studies on circadian rhythms,

which are cycles of about a day, as for example the sleep and rest cycle. Most animals have some kind of biorhythms, and studies describing and attempting to explain them with diverse animal and plant species are relevant to the biomedical implications of human circadian rhythmicity, such as blood pressure cycles, drug susceptibility, sleep/wakefulness, and jet lag. Of more basic interest are those studies concerned with effectors, cues, and inherent oscillators operant in circadian control of physiological and behavioral function. Logical candidates for flight biorhythm studies are *Drosophila*, mice, and rats.

The effects of spaceflight on the neurons of the brain that constitute the internal clock can be studied by measuring *Immediate Early Gene* (IEG) activation in response to light/dark stimuli. IEGs, a newly discovered class of intracellular messengers, contain instructions for the production of proteins. These genes are unique in that they respond very early to stimuli. While there are many families of IEGs, the action of each family is evoked by different stimulation. Two families, known as c-fos and jun-B, are of interest because they are activated by neuronal activity, and, as such, serve as early markers of neuronal plasticity. By identifying the presence and production of IEGs in microgravity, scientists can determine where in the brain plasticity is occurring. In biorhythm studies, scientists can measure the levels of these early genes products, as well as the expression of IEGs in the brain structures involved in the regulation of the sleep-wake cycle (Fuller et al. 2003).

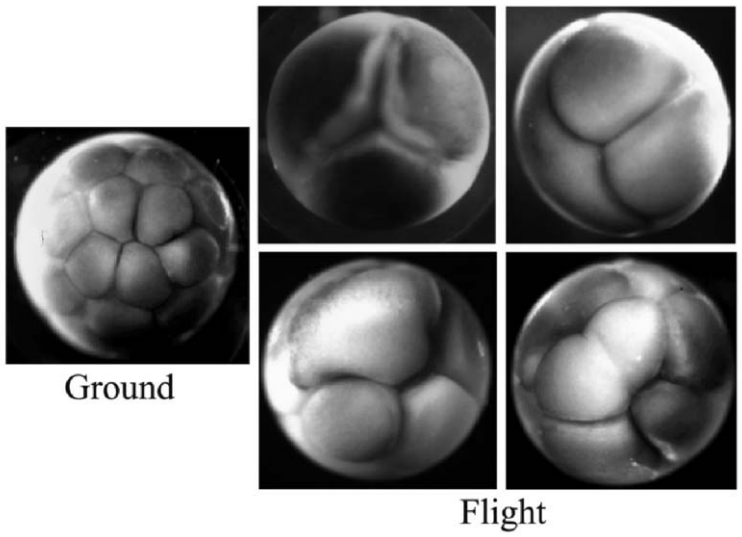

Figure 1-18. Division in amphibian (pleurodele newt) eggs, showing some abnormalities (larger sillons, odd number of cells) in the flight specimens by comparison to ground controls. Adapted from Gualandris-Parisot et al. (2002).

Consequently, developmental biology research in space is one of the most promising areas of investigation for both the ISS program and for biomedical concerns on Earth. In fact, there are presently a variety of exciting and innovative studies in the area of development. For example, space research has proven that rapidly developing and growing bodies are even more sensitive to the effects of weightlessness than are mature adults, thus providing more dramatic and rapidly established models of how gravity affects the formation and maintenance of bone, muscle, and cardiovascular function. We know that calcium crystals in the otoliths of the inner ear form differently in the weightless environment of space. New questions about the developmental programs that form this sensory system and others, and their connections to the brain can be investigated. So, it is of great importance to make use of the microgravity environment for studying the role of gravity-sensing mechanisms in the normal anatomical, physiological, and behavioral development.

Also of great interest is the influence of gravity during and after egg fertilization, and over the early course of embryonic development. Many of the mechanisms underlying early developmental processes are still unknown. The same holds true for influences exerted by environmental factors in critical stages of embryogenesis. For example, radical changes in the structure and connections of neurons occur during the development of the nervous system. From the tissue layers found in embryonic animals, cells increase in number and eventually differentiate and migrate to their appropriate function and position in the developing nervous system. In all, up to 75% of neurons are lost by the process of apoptosis, or programmed cell death, during development. Those that remain must form synapses with communicating neurons to serve their function in the adult nervous system. Because these processes are regulated by both chemical and mechanical factors, gravity may play a crucial role as a stimulus for proper development of the nervous system.

The notion that environmental input is essential during critical periods of development is not new. Young animals deprived of opportunities to see or walk during the first one to two weeks of life never see or learn to walk correctly, respectively. Thus experience can dictate development. By studying the development of mammals, it is possible to learn how genetic determination and experience interact to define the capabilities of the adult nervous system (Walton et al. 2003).

Certain well-known effects on amphibian eggs induced by changes in the gravitational vector make it important to also investigate whether early mammalian development processes are gravity-sensitive and thus may be disturbed in the 0-g environment. Of special interest is the possibility that gravitational forces might modify the morphogenetic pattern in its earliest and most fundamental manifestations, such as polarity and bilateral symmetry.

Manipulation of fertilized frog eggs, by which the heavy yolk is forced to maintain an upward instead of downward position, has been shown to initiate abnormal development (Figure 1-18). Also, exaggeration of the force of gravity by centrifugation has been shown to interfere with morphogenesis in amphibians when applied at a sensitive stage of the developmental process.

Thus, the space environment, and particularly microgravity, is a unique tool in the study of early developmental mechanisms (Table 1-05). Indeed, we do not know if normal embryonic development is possible in this condition. Is the spatial orientation of the plane of cleavage division affected by the line of reference provided by the gravitational vector? If the predominance of a given initial plane of cleavage is weakened by the absence of effective gravity, could this lead to an abnormal embryonic development?

Current
• *Amphibian early embryogenesis*
• *Mammalian fertilization*
• *Gravitosensing organs morphology and development*
Future
• *Cell differentiation, cell/tissue competence*
• *Gravitosensing organs/tissues:*
- *Threshold of sensitivity*
- *Developmental period of sensitivity*
- *Information processing*
- *Chemistry and physical properties*
- *Evolution*
• *Developmental timing*
• *Organ development*
• *Gametogenesis*
• *Birth and mating in microgravity*
• *Effect of gravity on life cycle*
• *Maturation*

Table 1-05. Current and future space research in Developmental Biology.

4 PLANT BIOLOGY

The Earth is host to more than 400,000 documented species of plant life. In turn, our planet depends upon these plants to nurture and sustain all living things. Plants play a critical role in the complex food web. Powered by light from the sun, CO_2 from the air, and nutrients from the soil, plants pass on this energy to the life forms that consume them. And for the human species, plants bring aesthetic pleasure, delighting the senses with their beauty and variety.

On Earth, plant roots as a rule grow downward toward gravity, while stems grow up and away from gravity, a phenomenon known as *gravitropism*.

By studying plants in microgravity on board spacecraft, biologists seek to understand how plants respond to gravity. Also, plants respond to environmental stimuli such as light, temperature, water, wind, and magnetic or electric fields. These responses are masked on Earth by the overriding response of plants to gravity. In addition, any strategy that visualizes a long-term sustained human presence in space absolutely requires the ability to continuously grow and reproduce various plant species over multiple generations, for food and controlled environmental life support systems.

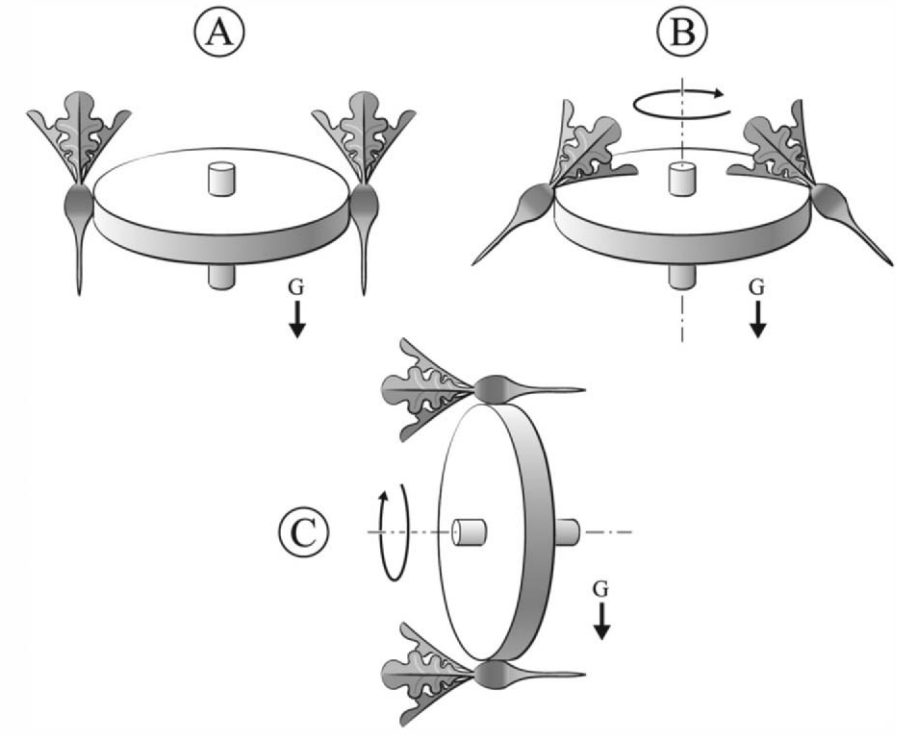

Figure 1-19. Knight's experiment showing the angle of orientation with respect to gravity assumed by roots and shoots of seedlings when they are rotated in a horizontal plane. Seedlings, originally pinned vertically (in A), grow in the direction of the resultant of the gravitational and centrifugal force vectors (in B). When the clinostat in oriented vertically and slowly rotates (in C), during a complete turn of the disc, the gravitational force acts laterally from all directions. As the plant is kept rotating, it orients itself in the horizontal direction, with no gravity-dependent curvature in roots and shoots. Adapted from Bjurstedt (1979).

4.1 Gravitropism

As a seedling germinates, the shoot grows upward toward the light of day, and the root grows downward toward water and nutrients. Clearly, gravity is the stimulus that directs that growth, but the mechanism is less clear. In 1806, Knight conducted his now classic but simple experiment with seedlings on rotating wheels and showed that roots grow at different angles with respect to the imposed gravitoinertial force. The roots grow away from, and the shoots towards, the center of the wheel (Figure 1-19).

The mechanism by which the different plant parts perceive a change in their position with respect to the gravitational vector is still a matter of uncertainty. Several possibilities seem likely. All appear to be associated with a gravity-induced sedimentation within the cytoplasm of *statoliths*, i.e., particles or organelles that are present in limited numbers of special sensor cells. In certain algae, these particles are crystals of barium sulphate, but in higher plants the sedimenting organelles are membrane-bound starch-containing *amyloplasts*. It is unclear whether it is the movement of these particles with the cytoplasm, or the pressure that they exert upon membrane complexes in particular areas of the cell, that is the fundamental basis of the gravity-sensing system.

4.2 Development of Plants

A large variety of plants with short life spans have flown in space: algae, carrots, anise, pepper, wheat, pine, oat, mung beans, cress, lentils, corn, soybeans, lettuce, cucumbers, maize, sunflowers, peas, cotton, onion, nutmeg, barley, spindle trees, flax, orchids, gladiolas, daylilies, and tobacco. Due to this variety, for the most part, observations on plants exposed to microgravity have been anecdotal. It has been demonstrated repeatedly that plants do grow in microgravity. However, whether plants can grow and develop normally over several generations remains to be determined.

Few higher plants are able to complete their life cycle within 30 days. Some plants, e.g., *Arabidopsis thaliana* are reported to do so (see Figure 6-22), and for this reason these plants have been used in many studies during relatively short-duration spaceflights, such as Spacelab missions (see Chapter 3, Section 1.1.8). However, the reproducibility of these very short life cycles appears to be questionable. Furthermore, the exclusive use of these species would probably limit any interpretations to these rather distinctive plants.

Past space studies suggest that the reproductive phase is complete in microgravity when the culture conditions (gas and liquid exchanges) are adequate. However, until recently, whether or not a seedling growing from the beginning in microgravity could flower and produce normal seeds, which could eventually lead to normal plants, remained a matter of debate. Long-term flight experiments are required to determine if a variety of plant species

can grow normally over a long period in microgravity and, in particular, if they can produce viable seeds (Oser and Battrick 1989). Some preliminary data obtained during experiments on board the ISS are encouraging (Figure 1-20) (see Chapter 6, Section 5).

4.3 Research Questions

Can plants survive and thrive in space? Can they grow, develop, reproduce, and orient themselves normally in the virtual absence of gravity. Will the space environment affect their metabolism and photosynthesis, which are so dependent on the 12-hour cycling of day and night? How will space affect seed viability and seed germination? A number of factors such as pollen viability, aspects of fertilization, and floral development influence reproduction. These are important aspects for space horticulture (Table 1-06).

Knowledge of physiology, cell biology, biochemistry and molecular biology of plants coupled with biotechnology advances contributes to our fundamental knowledge of plants and provides impetus for a new era of plant investigations. The opportunity to experiment in microgravity provides a new dimension that enables interdisciplinary plant research to answer important questions about the plant's reception of the gravity signal, the plant's biochemical interpretation of that signal, and how that interpretation causes a developmental reaction. It appears that this reaction system, in general, interacts with receptor systems that detect both internal and external signals. It is for this reason that understanding the role of mechanical signals, such as gravity, assumes such significance for plant science: these investigations could begin to reveal the precise control mechanisms involved in dictating plant form, structure, and function.

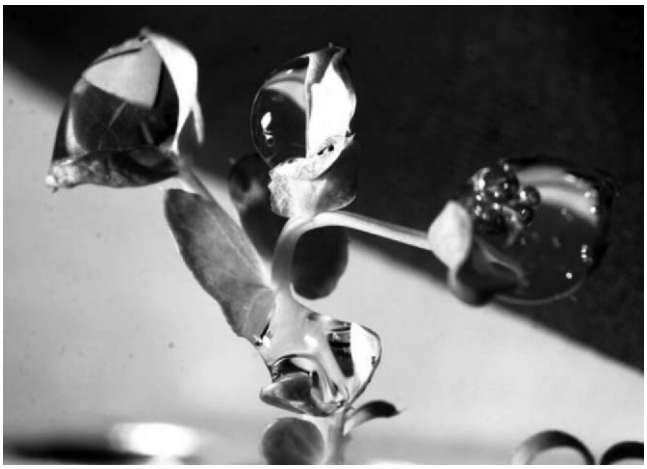

Figure 1-20. A close-up view of water droplets on leaves on a Russian plant growth experiment on board the International Space Station. Photo courtesy of NASA.

Understanding how basic processes can be manipulated and put into use in new ways that develop new products and increase productivity is the basis for biotechnological applications in agriculture, horticulture, and forestry. For example, understanding the interaction between gravity and light could be the basis for genetic engineering of plants resulting in increased crop productivity while minimizing the required growing space. Application to horticulture could include the ability to control plant form, and forestry could benefit from faster methods of regeneration of lost forest areas (McClain and Scott 1997).

Years of research in space have demonstrated that plants, as well as humans and animals, are affected by spaceflight. Researchers have found that changes detected by plant gravity sensors result in alterations of growth patterns, biomass production, and development in plants during spaceflight. Cell division is decreased in space-grown plants and chromosomal abnormalities such as breakage and fusion are reported to occur more frequently in plants grown in space than in those grown on Earth.

Understanding these changes is critical because the *Closed Ecological Life Support Systems* (CELSS) needed to support humans during future long-term space travel depend on the ability to grow plants reliably and efficiently in space. Regular resupply of air and food constitutes a major cost of operating a space station. So, in plant research, applied questions result from the need to maximize food production while minimizing the required onboard spatial volume or from the need to raise plants in an entirely closed environment.

Obviously, the use of plants as food in space requires that the effects of gravity on the morphology of the organism to be known. This requirement must be placed upon all plants considered as food sources for CELSS. It

Current
• *Gravity perception*
• *Morphology/structure*
• *Development/genetics*
• *Growth*
Future
• *Cell/tissue competence, differentiation*
• *Developmental timing*
• *Organ development*
• *Cellular function*
• *Metabolism/metabolites*
• *Photosynthesis, respiration*
• *Fluid dynamics, transport*
• *Interaction of gravity with light, radiation, other forces*

Table 1-06. Current and future space research Plant Biology.

involves investigation of gravity effects during all of the developmental phases of the plants, from germination to maturation and fruiting. Also, studies are required to assure that culture and harvesting techniques appropriate on Earth will be applicable in space. Finally, experiments should assure that the nutritional composition (and the taste) of the organisms does not differ appreciably from that found on Earth.

5 RADIATION BIOLOGY

The Earth is continuously bathed in high-energy ionizing radiation known as *Galactic Cosmic Radiation* (GCR), emanating from outside the solar system, and sporadically exposed to bursts of energetic particles from the Sun referred to as *Solar Particle Events* (SPEs). The main source of GCR is believed to be supernovae (exploding stars), while occasionally a disturbance in the Sun's atmosphere (solar flare or coronal mass ejection) leads to a surge of radiation particles with sufficient energy to penetrate the Earth's magnetic field and enter the atmosphere (Figure 1-21).

Outside the Earth's atmosphere, GCR consists mostly of fast-moving protons (hydrogen nuclei) and alpha particles (helium particles). GCR is 98% atomic nuclei and 2% electrons. Of the energetic nuclei, 87% are protons, 12% are helium ions and 1% is heavier ions. So, GCR, along with other forms of radiation presents a problem for space biology. In terms of biological development, space radiation is a major factor that must be understood in order for humanity to move deeper into space.

Ionizing radiation refers to subatomic particles that, on interacting with an atom, can directly or indirectly cause the atom to lose an electron or

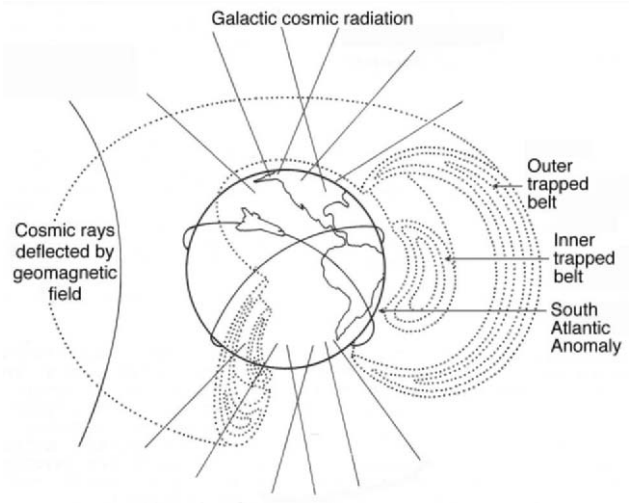

Figure 1-21. Schematic representation of the sources of ionizing radiation in low Earth orbit.

even break apart its nucleus. It is when these events occur in body tissue that health effects may result if the cell's self-repair mechanism fails. The ionization process in living tissues consists of ejecting bound electrons from the cellular molecules, leaving behind chemically active radicals that are the source of adverse changes. Many of the radicals resulting from radiation injury are similar to those produced in normal metabolic processes, for which the cell has developed recovery mechanisms needed for long term survival. The substantive target of radiation injury is considered to be the DNA structure, which may be changed or injured directly by a passing ionized particle. The ability of the cell to repair the effects of ionization depends in part on the number of such events occurring within the cell from the passage of a single particle, and the rate at which such passages occur. During the repair process, gene translocation and other chromosome aberrations may occur.

A number of studies have identified an increased rate of unstable chromosomal aberrations in flying crewmembers and related these to cosmic radiation exposure. However, there is no epidemiological evidence to link these aberrations with the development of cancers. Nevertheless, a cell may become cancerous as a result of being irradiated, the likelihood being dependent upon the energy and the dose received. Also, a child conceived after exposure of a parent to ionizing radiation is at risk of inheriting radiation-induced genetic defects. These may take the form of anatomical or functional abnormalities apparent at birth or later in life.

Studies involving various animal models have been conducted. It has been determined that exposure to radiation during spaceflight induced mutations in *Drosophila* (Ikenaga et. al. 1997). Furthermore, the mutation rate in space for *C. elegans* was twice or three times as great as what would have been anticipated on the ground (Hartman et al. 2001).

Studies have also been conducted on primates. In one large experiment, 2-year old primates, *Macaca mulatta*, were subjected to varying levels of proton irradiation. Seventeen years later, mortality studies were conducted. The investigators determined that one of the leading causes of increased mortality was a significant increase in the incidence of endometriosis[5] (48% in irradiated females compared to 0% in controls).

In a similar experiment done by Wood (1991), female rhesus monkeys were given total body exposures of protons of varying energies. The doses and energies of the radiation received were within the range that could

[5] *Endometriosis* is a common medical condition where the tissue lining the uterus (the endometrium, from *endo*, "inside", and *metrium*, "mother") is found outside of the uterus, typically affecting other organs in the pelvis. The condition can lead to serious health problems, primarily pain and infertility. Endometriosis primarily develops in women of the reproductive age.

be received by an astronaut traveling in low Earth orbit (LEO) during a random solar flare event. The frequency of developing endometriosis was highly significant in the irradiated group versus the non-irradiated controls. The minimum latency period was found to be 7 years. The scientists concluded that the risk of endometriosis could not be ignored when weighing the importance of a mission versus the risk of delayed radiation effects in female astronauts. Other scientists too have the same opinion after having obtained similar results in their experiments.

Plant cells are affected by radiation, just like any other cells. Chromosome damage and abnormalities are seen in a variety of plants in space. In general, seeds are less sensitive than developing embryos or growing plants. This may be because their cells are not actively dividing.

Several studies have been done to try and determine which radiations are most damaging, or even whether the chromosome damage was solely due to radiation at all. Some studies showed that standard radioprotectant chemicals like cytosine, aminoethyliothiourea, and 5-methoxytryptamine did not stop the damage. Some of the flights on which damage was found were short enough that GCR dosages were low. Some of the chromosomal damage and abnormalities could be due to higher energy particles. Some other environmental factors, like microgravity, also remain a possibility (Thora and Scott 1990).

6 BIOTECHNOLOGY

6.1 Definition

Biotechnology is the application of engineering and technology to life sciences research. It is a set of techniques and equipment for rearranging and manufacturing biological molecules, tissues, and living organisms. Biotechnology uses living systems or derivatives of living systems to make a product or to perform a specific service. For example, the beer industry, which uses yeast to get a fermented brew, is, in a sense, a biotech industry that produces a product. The sewage disposal plant is also a service that is biotechnologically provided because microorganisms are used to remove a number of organics from the water to make the water drinkable once again, or at least amenable to reprocessing.

In the space environment, biotechnology uses microgravity or the technologies developed for the space environment to make new products or perform a specific service. We have seen that gas exchange and fluid flow in biological systems are greatly affected by fluid dynamics in a gravitational environment (see this Chapter, Section 2.2). Consequently, products that are extremely pure, flawless and/or contain smooth mixes of materials with different density are difficult or impossible to produce on Earth. But this is possible in microgravity. As described above, there are many different forces

that act on liquids. In normal gravity, buoyancy-driven convection is the major mixer. In microgravity, however, surface tension, Marangoni convection, and diffusion can be the major forces of mixing. Also, containers are often not needed for microgravity processing. Instead, surface tension is used as the container. Consequently, there is no container contamination, and the heat or solution can be applied anywhere into the liquid being processed; hence, a better, purer product.

The major space applications in biotechnology are in the areas of protein crystals studies, tissue culture, and the improvement of technology such as electrophoresis.

6.2 Protein Crystal Analysis

The human body contains thousands of different proteins, which play essential roles in maintaining life. The structure of a protein determines the specific role that it plays in the human body. To learn how various proteins function, scientists construct computer models that reveal the complex three-dimensional structures of these large biological molecules. To solve a protein's structure, scientists must first crystallize the protein and analyze the resulting crystals by a process called X-ray diffraction. Precise measurements of thousands of diffracted intensities from each crystal help scientists map the probable positions of the atoms within each protein molecule.

A major bottleneck for the drug industry and research groups continues to be the inability to obtain high-quality, diffractable crystals of proteins for structure determination. Analysis of these crystals helps us better understand the nature of proteins, enzymes, and viruses, which could lead to the development of new drugs and a better understanding of the fundamental building blocks of life. With an improved understanding of the molecular structures and interactions of proteins, drug designers may be able to develop new drug treatments that target specific human, animal, and plant diseases. The microgravity environment of space currently produces most of the best quality protein crystals. The ideal crystal should be able to grow slowly in all directions without interference. Crystals grown on Earth are subject to sedimentation affect, uneven protein solution concentration, and a faster diffusion rate, which cause imperfections in the structure of the growing crystal (Figure 1-22). In microgravity, crystals are straighter and larger, without defects due to gravity-induced deformation.

Protein crystals are an important application area for drug design. The atomic structure of a crystallized protein can be determined from X-ray diffraction. This information allows a better understanding of its chemical interactivity. The higher the resolution, the better the analysis. Compared to a ground-based environment, the resolution of crystals grown in space usually increases from about a half to a full angstrom. This does not sound like much, but at molecular level, that is a huge increase in resolution.

Figure 1-22. Natural quartz crystals, here shown in cluster, are the most common forms of mineral crystals.

Some experiments have been conducted on the Space Shuttle, although most are limited by the short duration of these flights. The ISS experiments will lead to studies influencing possible treatments for cancer, diabetes, emphysema, and immune system disorders.

6.3 Tissue and Cell Culture

More than 200 types of cells make up the human body. They are assembled into a variety of tissues, such as skin, bone, and muscle. Most tissues contain a mixture of cell types. Cells are small and complex, which makes it difficult for scientists to see their structures, to discover their molecular composition, and especially to find out how their various components function.

What can be learned about cells depends on the available tools. Culturing (or growing) cells is one of the most basic techniques used by medical researchers. The growth of human cells outside the body enables the investigation of the basic biological and physiological phenomena that govern the normal life cycle and many of the mechanisms of disease. In traditional research methods, mammalian cells are cultured using vessels in which cells settle to the bottom surface of the vessel under the influence of gravity. This gravitational influence results in a thin sheet of cells, with the depth of a single cell, called a monolayer. Cells in human tissues, however, are arranged in complex, three-dimensional structures. When cells are grown in a monolayer, they do not perform all the functions that the original tissue does. Although much valuable information can be gained from monolayer cell cultures, further understanding of the processes that govern gene expression and cellular differentiation is limited because the cells are not arranged as they are in the human body.

When the influence of gravity is decreased, the cells are able to grow in more tissue-like, three-dimensional aggregates, or clusters. On the ground,

cells sediment to a surface and interact with it. In space, they do not. That is an advantage because there is a minimization of the cell interaction with inert surfaces. Another advantage is there is no surface to confine the direction in which the cells will grow. This allows for three-dimensional growth, more like actual tissues in the body.

A *bioreactor* is a device that is used to grow tissues in three-dimensions. The bioreactor allows cells to be cultured in a continuous free-fall state, simulating microgravity and providing a unique cell culture environment on the ground. This allows for cell aggregation, differentiation, and growth. The bioreactor affords researchers exciting opportunities to create three-dimensional cell cultures that are similar to the tissues found in the human body. Using both space- and ground-based bioreactors, scientists are investigating the prospect of developing tissues that can be used in medical transplantation to replace failed organs and tissues (Figure 1-23). Bioreactors in space are not only interesting for the production of cells or tissue. They will also be the key element for the treatment of waste products (such as water and CO_2), the production of food, or the decontamination of the life support system from unwanted bacteria and other microorganisms (Walther 2001).

In addition, investigators are striving to produce models of human disease to be used in the development of novel drugs and vaccines for the treatment and prevention of diseases, to devise strategies to reengineer defective tissues, and to develop new hypotheses for the progression of diseases such as cancer. Growing cultures for long time periods on board the ISS will further advance this research. Finally, cells exposed to simulated and true microgravity respond by making adaptations that give new insights into

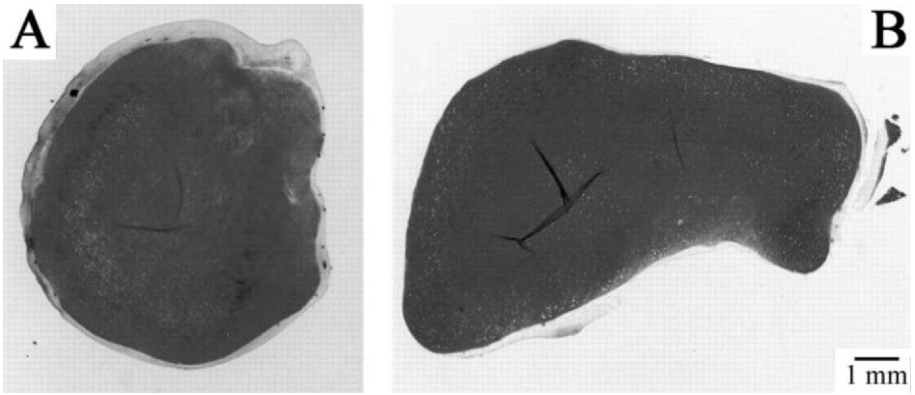

Figure 1-23. Specimens of tissue-engineered cartilage grown in space tend to become spherical in space, demonstrating that tissues can grow and differentiate into distinct structures in microgravity. The flight samples (A) were smaller, more spherical, and mechanically weaker than Earth-grown control samples (B). Adapted from Freed et al. (1997).

cellular processes, establish a cellular basis for the human response to microgravity and the space environment, and pave the way for cell biology research in space regarding the transition of terrestrial life to low-gravity environments (Figure 1-24).

Figure 1-24. Space biology research benefits humans back on Earth, as crewmembers on board the ISS perform long-duration research that could lead to medical advancements, new materials, and breakthroughs in technology, including the development of countermeasures to the symptoms of the aging process. Photo courtesy of NASA.

6.4 New Technology

Revolutionary technology enhancements have radically changed the world of biological research. As a result, technology now plays a central role in accomplishing scientific goals. *Electrophoresis* can be used for the separation of materials using a weak electrical field. There are many different kinds of electrophoresis. All of these carry with them a potential corrupting risk that involves convective forces, i.e. the convection created by the accumulation of heat in certain areas. In gravity, ions will be moved by gravity as well and may not evenly distribute over the plate. In microgravity, however, the ions are drawn directly to the plate and give a more even distribution. This is useful for getting a faster separation of materials and

products of higher purity. However, this process is still somewhat in its infancy as far as space projects are concerned.

Further research into the physiology of plants could lead to new categories of plant-based technologies. Work in gravitational biology and ecology could help us design hardier crops for space farms and onboard food production, as well as more efficient biologically based waste management systems. Crystals grown in space can help improve catalysts used to extract oil, enhancing the yield of petroleum products. The use of microgravity and vacuum production techniques in space might trigger the next generation of highly pure and accurate semiconductors for use in electronics. Demands for resources such as power and data communications may spur private space development investments to provide commercial services to space experimenters. For all these reasons and more, the ISS will serve as a laboratory and testbed for the development of new processes, products, and services to benefit life on Earth and in space (Figure 1-25).

Figure 1-25. Astronaut Peggy A. Whitson holds a soybean plant growth experiment in the Destiny laboratory on the International Space Station. Photo courtesy of NASA.

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Chapter 2

ANIMALS AND PLANTS IN SPACE

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Animals and plants have been invaluable in space biology and have contributed greatly to the current database of knowledge in this field. This chapter presents an overview of the historical involvement of animals and plants in space, describes the hardware and logistics of flying live specimens on board spacecrafts, and discusses future plans for animal and plant experiments in space.

Figure 2-01. Chimpanzee Ham is greeted by recovery ship Commander after his successful flight on the Mercury-Redstone rocket in 1961. Photo courtesy of NASA.

1 SPACEFLIGHT HISTORY

Since the beginning of aeronautics and space exploration, animals have accompanied and sometimes preceded humans as space travelers. Historically, extensive animal experimentation was used in both the United States and Soviet/Russian space programs to collect the medical knowledge and test the engineering design concepts that would be required to support human spaceflight. A variety of animal models were used as substitutes to test

the suitability of the space environment for human presence, including launch systems, radiation and microgravity exposure, life support systems, and recovery procedures (Borkowski et al. 1996). At first, small organisms, such as insects and plant seeds, were sent in space, primarily to evaluate the effects of cosmic radiation. Later, test flights involved mammals and primates for investigating the physiological effects of acceleration and microgravity. These early animal flights evaluated the basic medical risks of short-duration spaceflights, paving the way for human missions. Once it was determined that complex biological organisms could survive in orbit, humans ventured into space, and took animals along as experimental subjects. With humans spending longer and longer periods in space, biological specimens continue to be flown during manned or unmanned missions to better understand the long-term physiological effects of the space environment, including microgravity and cosmic radiation (Balard and Souza 1991).

Figure 2-02. Photograph of Laika in her space capsule. Image courtesy of Alexander Chernov and the Virtual Space Museum in Russia. Source: http://www.nasa.gov

1.1 Preparation for Human Spaceflights

1.1.1 Suborbital Flights

Non-human organisms have played a leading role throughout the history of technological flight. In 1783, a duck, a rooster, and a sheep became the first passengers in a hot air balloon, since no one knew whether a human could survive the flight. All three animals survived the flight, although the duck was found with a broken leg, presumably due to a kick from the sheep after landing. Humans followed soon after and experienced for the first time the symptoms of hypoxia.

In the 1950s, when the decision was made to explore space, some people were skeptical. Could human beings live in a world virtually without

gravity? Did the space environment harbor dangerous organisms? Just like for balloon flights, animals were sent up in rockets before humans to ascertain whether a living being could withstand and survive a journey into space. Instruments monitored various physiological responses as the animals experienced the stresses of launch, reentry, and the weightless environment.

The first animals intentionally sent into space were fruit flies that were sent with corn seeds inside the nose cone of a captured German V-2 rocket in July 1946. These were the same models as those used during World War II. The purpose of the experiment was to explore the effects of radiation exposure at high altitudes. Some further V-2 missions carried biological samples including moss. During subsequent tests, vertebrate animals flew (Table 2-01). The first was a 4-kg anaesthetized rhesus monkey (*Macaca mulatta*) named Albert. Unfortunately, Albert died as a result of breathing difficulties in the cramped capsule before his rocket left the ground. In the second flight, in 1949, the capsule was redesigned to allow the subject (Albert II) assume a less cramped position. Albert II died at impact, however, but respiratory and cardiological data were successfully recorded up to that moment. Thus, it was established that a monkey had lived during an entire flight which reached an altitude approximately 133 km above the surface of the Earth, following accelerations of 5.5 g at lift off and 13 g at the opening shock of the parachute recovery system. The evolution of engineering techniques was making possible greater success in the scientific exploration of physiological factors related to spaceflight.

One of the following V-2 experiments used a mouse as the subject and no attempt was made to record heart action or breathing. Unlike the monkeys, the mouse was not even anesthetized because the purpose of the experiment was to record the conscious reactions of an animal to changing gravity conditions. For this purpose, the mouse capsule was equipped with a camera system to photograph the mouse at fixed intervals. Again, the recovery system failed and the mouse did not survive the impact. But the photographs came through successfully and showed that the mouse retained "normal muscular coordination" throughout the very brief period of microgravity, even though "he no longer had a preference for any particular direction, and was as much at ease when inverted as when upright relative to the control starting position" (Dempsey 1985).

Flights of more advanced Aerobee rockets in late 1951 and 1952 carried an ark full of animals to space and brought them all back alive. Included in the menagerie were a monkey instrumented to record heart beat, respiration and blood pressure; nine mice who went along simply to be exposed to cosmic radiation; and two other mice in a rotating drum for the photographic observation of their reactions to short-term microgravity. One of the two mice had undergone a prior operation removing the vestibular apparatus, and was already accustomed to orient itself by vision and touch

exclusively. Interestingly enough, she did not seem affected by loss of gravity during the flight. The other mouse, which was normal, clawed at the air and appeared definitely disturbed during the microgravity phase of the trajectory (Dempsey 1985).

On December 13, 1958, a Jupiter ballistic missile was launched from Cape Canaveral, Florida with a U.S. Navy trained South American squirrel monkey named Gordo onboard. The nose cone recovery parachute failed to operate and Gordo did not survive the flight. Telemetry data sent back during the flight showed that the monkey survived the 10 g of launch, 8 minutes of weightlessness and 40 g of reentry at 16,000 km/h. The nosecone sank and was not recovered. A rhesus and a squirrel (*Saimiri sciureus*) monkey, named Able and Baker, respectively, became the first monkeys to survive spaceflight after their 1959 flight on board a Jupiter missile. They withstood forces 38 times the normal pull of gravity and were weightless for about 9 minutes. A top speed of 14,000 km/h was reached during their 16-min flight. The monkeys survived the flight in good condition.

The Soviet Union, which began the space race with Sputnik in 1957, also launched dogs during suborbital flights. In other countries, France flew rats into space in 1961 and 1962, and two cats in 1963. The cats had electrodes implanted into the head to measure neural impulses. The first cat was recovered alive but the next cat in space was not. The final French animal launches were of two monkeys (Martine and Pierrette) in March of 1967. China launched mice and rats in 1964 and 1965 and two dogs in 1966 (Table 2-01).

Spacecraft	***Date***	***Animals (number)***	***Apogee (km)***
V-2 No 37	*1948 Jun 11*	*Rhesus monkey*	*63*
V-2 No 47	*1949 Jun 14*	*Rhesus monkey*	*133*
V-2 No 32	*1949 Sep 16*	*Rhesus monkey*	*1?*
V-2 No 31	*1949 Dec 08*	*Rhesus monkey*	*127*
V-2 No 51	*1950 Aug 31*	*Mouse*	*?*
Aerobee -12	*1951 Apr 18*	*Rhesus monkey*	*61*
R-1V	*1951 Jul 22*	*Dog (2)*	*100*
R-1B	*1951 Jul 29*	*Dog (2)*	*100*
R-1B	*1951 Aug 15*	*Dog (2)*	*100*
R-1V	*1951 Aug 19*	*Dog (2)*	*100*
R-1B	*1951 Aug 28*	*Dog (2)*	*100*
R-1B	*1951 Sep 2*	*Dog (2)*	*100*
Aerobee-19	*1951 Sep 20*	*Rhesus monkey; Mice (11)*	*71*
Aerobee-26	*1952 May 22*	*Phillipine monkeys (2)*	
		Mice (11)	*26*
R-1D	*1954 Jul 2*	*Dog (2)*	*100*
R-1D	*1954 Jul 7*	*Dog*	*100*
R-1D	*1954 Jul 26*	*Dog*	*100*
R-1E	*1955 Jan 25*	*Dog*	*100*
R-1E	*1955 Feb 5*	*Dog*	*100*
R-1E	*1955 Nov 4*	*Dog*	*100*

R-1E	*1956 May 14*	*Dog*	*100*
R-1E	*1956 May 31*	*Dog*	*100*
R-1E	*1956 Jun 7*	*Dog*	*100*
V-2A (R-2)	*1957 May16*	*Dog (2)*	*220*
V-2A	*1957?*	*Dog*	*220*
V-2A	*1957?*	*Dog*	*220*
V-2A	*1957?*	*Dog*	*220*
V-2A	*1957 Aug 31*	*Dog*	*220*
Thor Able	*1958 Apr 23*	*Mouse*	*0?*
Thor Able	*1958 Jul 10*	*Mouse*	*(45 min)*
Thor Able	*1958 Jul 24*	*Mouse*	*?*
V-5A (R-5)	*1958 Aug 27*	*Dog (2)*	*450*
V-5A (R-5)	*1958 Sep 17*	*Dog (2)*	*450*
V-5A (R-5)	*1958 Oct 31*	*Dog (2)*	*450*
AM-13 Bio-1	*1958 Dec 13*	*Squirrel monkey*	*?*
AM-18 Bio-2	*1959 May 28*	*Rhesus & Squirrel monkeys*	*600*
V-2A	*1959 Jul 2*	*Dog (2),*	*220*
V-2A	*1959 Jul 10*	*Dog*	*220*
AM-23 Bio-3	*1959 Sep 15*	*Mice (12)*	*0?*
LJ-2	*1959 Dec 4*	*Rhesus monkey*	*85*
LJ-1B	*1960 Jan 21*	*Rhesus monkey*	*15*
V-2A	*1960 Jun 15*	*Dog (2)*	*220*
V-2A	*1960 Jun 24*	*Dog (2)*	*220*
V-2A	*1960 Sep 16*	*Dog (2)*	*220*
V-2A	*1960 Sep 22*	*Dog (2),*	*220*
Atlas 71D	*1960 Oct 13*	*Mice (3)*	*(20 min)*
MR-2	*1961 Jan 31*	*Rhesus monkey*	*253*
Veronique-24	*1961 Feb 22*	*Rat*	*110*
SP Pod 13	*1961 Nov 10*	*Squirrel monkey*	*1*
SP Pod 6	*1961 Dec 10*	*Rhesus monkey*	*500?*
Veronique-37	*1962 Oct 15*	*Rat*	*?*
Veronique-36	*1962 Oct 18*	*Rat*	*?*
Veronique-47	*1963 Oct 18*	*Cat*	*?*
Veronique-50	*1963 Oct 24*	*Cat*	*?*
T-7A-S	*1964 Jul 19*	*Rats and mice*	*?*
T-7A-S	*1965 Jun 1*	*Rats and mice*	*?*
T-7A-S	*1965 Jun 5*	*Rats and mice*	*?*
T-7A-S2	*1966 Jul 14*	*Dog (m.)*	*100*
T-7A-S2	*1966 Jul 28*	*Dog (f.)*	*?*
Vesta 4	*1967 Mar 7*	*Pigtailed macaque*	*233*
Vesta 5	*1967 Mar 13*	*Pigtailed macaque*	*240?*

Table 2-01. List of vertebrate animals flown during suborbital flights between 1948 and 1967. Source: http://planet4589.org/space/book/astronauts/astronaut/bio.html

1.1.2 Orbital Flights

In 1957, a dog named Laika the first organism to orbit the Earth (Figure 2-02). Dogs were used because scientists believed they could endure long periods of inactivity better than other animals and because the Soviets

had a long history of research with canines. The dogs were trained using centrifuges to simulate the extreme g forces of take off. Laika had been equipped with a comprehensive array of telemetry sensors, which gave continuous physiological information to tracking stations. The cabin conditioning system maintained sea-level atmospheric pressure within the cabin, and Laika survived six days before depletion of the oxygen stores caused asphyxiation. Laika's flight demonstrated that spaceflight was tolerable to animals. The Soviet Union launched mice and for the first time, guinea pigs and frogs in the Vostok-3A flights of March 1961, just preceding the historical flight of Yuri Gagarin in April 1961.

Twelve other dogs, as well as mice, rats and a variety of plants were then sent into space for longer and longer duration between 1960 and 1966 (Table 2-02). In 1966, a Soviet biosatellite Cosmos mission carried two dogs in orbit for 23 days. The dogs were observed via video transmission and biomedical telemetry. Their spacecraft landed safely. The first tortoise in space and the first animal of any kind in deep space was launched in 1968 by the Soviet Union. The Horsfield's tortoise was sent on a circumlunar voyage along with flies, worms, and other biological specimens, and the capsule was recovered at sea.

Figure 2-03. This photograph shows the biosensors used for monitoring the physiological parameters of monkeys during spaceflight. Photo courtesy of NASA.

During this period, in the U.S., in preparation for the human flights, Sam, a rhesus monkey, was launched in a Mercury capsule in the late 1959. He returned safely to Earth after a suborbital flight (Table 2-01). He flew again later, becoming the first animal to fly in space twice. Ham preceded Alan Shepard into space, in January of 1961 (Figure 2-01). He enjoyed a

ballistic flight of about 16 minutes and landed in the Atlantic Ocean, again with no untoward effects. He was trained to pull levers to receive rewards. His flight demonstrated the ability to perform tasks during spaceflight. The chimpanzee Enos flew in Mercury Atlas-5 immediately preceding John Glenn's orbital flight. He was the first primate to orbit the Earth, during which he was carefully monitored (Figure 2.03), with no change in his physiology. He performed tasks as he would have on Earth, which indicated that humans would not be incapacitated by the environment of space.

1.2 Beginning of Systematic Biological Investigations

All the precursor flights showed that humans could survive weightlessness and the effects of high gravitational forces. After several more flights, the number of animals sent into space decreased. Indeed, most experiments could be conducted in space on humans directly, without involving animals. The longer missions of Gemini, Apollo, and Skylab were operations oriented, often confounded by higher g loads than minimum, and, in any case, carried only a very few, relatively unattended, uncontrolled "piggyback" biology experiments.

Although other animals, such as mice, had been included on early research flights, it wasn't until 1966 and the U.S. Biosatellite programs that the first effort to systematically study basic biological processes in space was made. The objectives of the Biosatelitte-I and -II were to study the influence of microgravity on the growth, form, development, morphology, and biochemistry of selected organisms, and to determine their sensitivity to ionizing radiation in microgravity. The flown organisms included plants, bacteria, fruit flies, and frog eggs. Although the Biosatellite-I mission was unsuccessful, Biosatelitte-II carried replica experiments on a successful three-day mission, establishing the feasibility of operating an unmanned biology laboratory in space (Sanders 1971).

However, from the standpoint of space biologists, the unmanned Biosatellites missions were unsatisfactorily short, and limited by automatic operations. The unmanned experiment designs were constrained by the inflexibility of automatic operation. In addition, the inflight 1-g control centrifuge, when functional, was too small to permit satisfactory interpretation of 0-g effects (Ballard and Connolly 1990).

During the Apollo era (1960-1972), most of the missions did not include animal payloads, as it had already been shown that animals could survive in space. However, two bullfrogs were launched on a one-way mission on the Orbiting Frog Otolith satellite on November 9, 1970 to better understand space motion sickness (Figure 2-03). Apollo-16 carried nematodes and Apollo-17, the last mission to the Moon in 1972, carried five pocket mice housed in self-sustaining, hermetically sealed, cylindrical aluminum canister to study exposure to cosmic particle radiation hazards (Table 2-02).

Spacecraft	***Date***	***Species (number)***	***Duration***
Sputnik-2	*1957 Nov 3*	*Canis familiaris*	*1 d?*
Corona 3	*1959 Jun 3*	*Mus (4)*	*0 d*
Corona 4	*1959 Jun 24*	*Mus (4)*	*0 d*
Vostok-1 No. 1	*1960 Jul 28*	*Canis familiaris (2)*	*0 d*
Vostok-1 No. 2	*1960 Aug 19*	*Canis familiaris (2)*	*1 h*
Vostok-1 No. 3	*1960 Dec 1*	*Canis familiaris (2)*	*1 h*
Vostok-1 No. 4	*1960 Dec 22*	*Canis familiaris (2)*	*0 d*
Vostok-3A No. 1	*1961 Mar 9*	*Canis familiaris (2),*	
		Mus (?),	
		Cavia porcellus (?)	*0 d*
Vostok-3A No. 2	*1961 Mar 25*	*Canis familiaris (2),*	
		Mus (?),	
		Cavia porcellus,	
		Rana pipiens	*0 d*
Mercury SC9	*1961 Nov 29*	*Pan troglodytes*	*0 d*
Voskhod-3KV5	*1966 Feb 22*	*Canis familiaris (2)*	*23 d*
Biosatellite-I	*1966 Dec 14*	*Bacteria, Drosophila*	*0 d*
Biosatellite-II	*1967 Sep 7*	*Bacteria, Drosophila*	*3 d*
L-1 No. 7	*1968 Apr 22*	*Testudo horsfieldi (2*	*0 d*
L-1 No. 9	*1968 Sep 14*	*Testudo horsfieldi (2)*	*7 d*
L-1 No. 12	*1968 Nov 10*	*Testudo horsfieldi (2)*	*7 d*
L-1 No. 13	*1969 Jan 20*	*Testudo horsfieldi (2)*	*0 d*
Biosatellite-III	*1969 Jun 29*	*Macaca nemestrina*	*9 d*
L-1 No. 11	*1969 Aug 7*	*Testudo horsfieldi (4)*	*7 d*
*OFO**	*1970 Nov 9*	*Rana calestiana (2)*	*8 d*
Apollo-17	*1972 Dec 7*	*Perognathus longim. (5)*	*12 d*
Skylab-3	*1973 Jul 28*	*Perognathus longimembris (6),*	
		Fundulus heteroclitus (2),	
		Araneus diadematus (2)	*59 d*
Bion-1	*1973 Oct 21*	*Testudo horsfieldi (?),*	
		Rattus norvegius (?)	*21 d*
Bion-2	*1974 Oct 22*	*Testudo horsfieldi (?),*	
		Rattus norvegicus (40)	*20 d*
Apollo-Soyuz	*1975 Jul 15*	*Fundulus heteroclitus (2)*	*9 d*
Soyuz- 41	*1975 Nov 17*	*Testudo horsfieldi (?)*	*90 d*
Bion-3	*1975 Nov 25*	*Rattus norvegicus (?),*	
		Testudo horsfieldi (?),	
		Fundulus heteroclitus (?)	*19 d*
Almaz OPS-3	*1976 Jun 22*	*Testudo horsfieldi (?),*	
		Danio rerio (1)	*?*
Bion-4	*1977 Aug 3*	*Rattus norvegicus (?)*	*18 d*
Bion-5	*1979 Sep 25*	*Rattus norvegicus (37)*	*18 d*
Columbia (STS-8)	*1983 Aug 30*	*Rattus norvegicus (?)*	*7 d (AEM)*
Bion-6	*1983 Dec 15*	*Macaca mulatta (2)*	
		Rattus (?), Fish (?)	*5 d*
Spacelab-3	*1985 Apr 29*	*Saimiri sciureus (2),*	
		Rattus norvegii (24)	*7 d (RAHF)*
Bion-7	*1985 Jul 10*	*Macaca mulatta (2),*	
		Rattus (10, plus litter),	
		Triturus (10),	
		Danio rerio (1500)	*7 d*

Bion-8	*1987 Sep 29*	*Macaca mulatta (2)*	*13 d*
Discovery (STS-29)	*1989 Mar 13*	*Rattus norvegius (4)*	*5 d (AEM)*
Bion- 9	*1989 Sep 15*	*Macaca mulatta (2), Rattus, Stick insect, Drosophila Triturus (?), Fish (?)*	*14 d*
*FSW-1 No. 3***	*1990 Oct 5*	*Cavia porcellus (?)*	*8 d*
Soyuz 7K No. 61	*1990 Dec 2*	*Japanese tree frogs (6)*	*8 d*
Columbia (SLS-1)	*1991 Jun 5*	*Rattus norvegicus (30)*	*9 d (RAHF)*
Discovery (STS-48)	*1991 Sep 12*	*Rattus norvegicus (8)*	*6 d (PARE-01)*
Spacelab J	*1992 Sep 12*	*Cyprinus carpio (2), Rana pipiens*	*8 d (E01-L2)*
Bion- 10	*1992 Dec 29*	*Macaca mulatta (2), Rana pipiens (?) Drosophila*	*12 d*
Endeavor (STS-54)	*1993 Jan 13*	*Rattus norvegicus (?)*	*6 d (PARE-02)*
Discovery (STS-56)	*1993 Apr 8*	*Rattus norvegicus (?)*	*9 d (PARE-03)*
Columbia (SLS-2)	*1993 Oct 18*	*Rattus norvegicus (24)*	*14 d (RAHF)*
Columbia (IML-2)	*1994 Jul 8*	*Triturus (?), Oryzias latipes (4)*	*15 d (AAEU)*
Atlantis (STS-66)	*1994 Nov 3*	*Rattus norvegicus (10)*	*10 d (PARE)*
Foton-9	*1994*	*Shrimp Artemia*	*?*
Foton-10	*1995*	*Drosophila, Beetle*	*?*
SFU	*1995 Mar 18*	*Triturus (?)*	*?*
Discovery (STS-70)	*1995 Jul 13*	*Rattus norvegicus (?)*	*9 d (NIH-R-2)*
Columbia (STS-78)	*1996 Jun 20*	*Rattus norvegicus (12)*	*16 d (AEM)*
Bion-11	*1996 Dec 24*	*Macaca mulatta (2), Triturus (?)*	*12 d*
Foton-11	*1997*	*Drosophila, Artemia, Beetle*	*?*
Columbia (STS-90)	*1998 Apr 17*	*Rattus (152), Mus (18), Snails (135), Oyster toadfish (4), Swordtail fish (229), Crickets (1500)*	*14 d*
Foton-12	*1999 Sep 9*	*Bacteria, Yeast*	*12 d*
Soyuz-Taxi-Flight	*2001 Nov 12*	*Clawed toad tadpoles (50), Xenopus laevis*	*10 d (Aquaris)*
Columbia (STS-107)	*2003 Jan 16*	*Swordtail fish (16), Cichlid fish (50) Snails (18) C. elegans (>100), Rattus (>100), Mice (?)*	*16 d*
Soyuz-Taxi-Flight	*2005 Apr 15*	*Acheta domesticus (200)*	*10 d (CRISP)*
Foton-M2	*2005 May 31*	*Scorpion (2)*	*16 d (Biokon)*

Table 2-02. List of animals flown during orbital flights between 1957 and 2005. The following table excludes invertebrates, plants, and unicellular organisms.

*Note: *OFO = Orbiting Frog Otolith Satellite; ** Chinese biosatellite. For the common names of the listed animal species, please refer to Table 2-03.*

Source: http://planet4589.org/space/book/astronauts/astronaut/bio.html, updated.

In 1973, two major research spacecraft were launched into Earth orbit, the U.S. Skylab space station and the first Russian Bion mission. Although Skylab's primary life sciences objectives involved studying extended-duration effects of the space environment on humans, mice and insects, it also included the first student experiment in space. A garden spider named Arabella was flown to see if spiders could spin webs in microgravity. Arabella spun a perfect web, although postflight examination of web samples indicated that the thread was significantly finer than that spun preflight, suggesting that spiders use a weight-sensing mechanism to size the thread. The mice were used to study circadian rhythm.

The Soviets flew a number of Bion program missions, which consisted of satellites with biological payloads. The Bion satellites were a series of unmanned research missions spanning more than 20 years. Bion-1, launched in 1973, carried tortoises, rats, insects, and fungi. Other missions have carried monkeys, plants, quail eggs, fish, frogs, and seeds. Over time, the Bion missions became increasingly international cooperations, with American and European countries contributing experiments. The last mission, Bion-11, which carried monkeys as well as frogs and fruit flies, was launched in 1996 (Marbarger 1998).

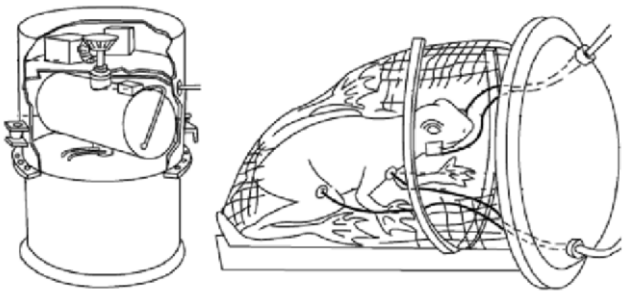

Figure 2-04. The Orbiter Frog Otolith experiment hardware contained all apparatus necessary to assure survival of two frogs. Specimens were housed in a water-filled, self-contained centrifuge, which supplied linear acceleration to stimulate the otolith organs in orbit (left). Electrodes were implanted in the vestibular nerve to measure its firing rate, while other sensors measured the animal body temperature and heart rate (right). Adapted from Souza et al. (2000).

1.3 Orbital Space Biology Laboratories

With the end of the space race, the space program evolved. The Russians opted for longer and longer human missions on board their space stations *Salyut* and *Mir*. The Americans developed the Space Shuttle program, which included a more suitable environment for space biology research. Animals and plants were flown in the middeck and in the pressurized Spacelab and SpaceHab modules. These modules included animal holding facilities, refrigerator/freezers, small and large mass measurements devices, and special workstations for manipulating the specimens.

On Soyuz-20, launched November 17, 1975, tortoises set the duration record for an animal in space when they spent 90.5 days in space. Salyut-5 in 1976 carried tortoises and a *Zebra danio* fish.

The Spacelab missions were conducted in a reusable space laboratory developed by the European Space Agency, which flew 19 times in the cargo bay of Space Shuttle Columbia between 1983 and 1998. Spacelab-3, in 1985, carried 24 rats and two squirrel monkeys, orbiting for seven days. In addition to pioneering a new generation of hardware for studying animals in space, one of the crewmembers (veteran U.S. astronaut Norm Thagard) had the distinction of becoming the first person ever to clean out animal cages in orbit.

The last Spacelab flight, the Neurolab mission, was a dedicated neurology research payload. Research subjects included baby rats, pregnant mice, snails, crickets and prehistoric looking oyster toadfish (Figure 2-05). Neurolab reinforced the value of using diverse organisms to address different biological questions. For example, crickets were included by virtue of their external gravity-sensing apparatus, the development of which can be easily studied (see Figure 5-01).

Figure 2-05. An oyster toadfish flown on the Neurolab STS-90 Mission. Each fish is between 20-30 cm long. This fish is an excellent model for looking at vesti-bular function because the archi-tecture of its vestibular apparatus in the inner and middle ear is similar to those of mammals. Photo courtesy of NASA.

The last flight of Space Shuttle Columbia in 2003 carried a SpaceHab module including silkworms, golden orb spiders, carpenter bees, harvester ants, and Japanese killifish. Nematodes (*C. elegans)* from one experiment were found still alive in the debris after the Space Shuttle Columbia disaster.

From 1995 through 1998, NASA collaborated with the Russian Space Agency to use the Mir space station, conducting numerous animal and plant life science experiments. These ranged from studying the effects of the space environment on developing quail embryos to its impact on basic plant processes such as photosynthesis and water use. A notable milestone came in 1997 when seeds harvested from plants grown in microgravity were successfully germinated in orbit. This first "seed-to-seed" growth of plants in space proved that gravity is not required for plant reproduction

As the International Space Station (ISS) assembly proceeds, on-orbit space research capability will grow as additional experimental facilities are deployed. The first ISS laboratory was placed on orbit in 2000. Other laboratories, including the European *Columbus* and the Japanese *Kibo* modules will presumably be launched in the near future. Experimental facilities that fit inside these laboratories and on attachment sites will follow in accordance with the rhythm of Space Shuttle delivery flights.

The ISS laboratory areas will have equipment such as general-purpose workstations, surgical areas, facilities for breeding and maintenance of animals, aquatic facilities for marine experiments, and multigenerational plant facilities (see Chapter 3, Section 3). Research could also be conducted on biologically regenerative life support systems. The laboratory procedures conducted on board the ISS will allow that data be available in real time on the ground, a considerable improvement compared to the earlier space stations.

Many milestones have yet to be achieved. One of the most significant will be the first birth of a mammal in space, leading to multi-generational mammalian studies. The ability of humans to reproduce and develop normally over multiple generations beyond Earth is an essential part of plans to explore and inhabit other worlds. Mammalian studies will be a critical milestone step in assessing this ability.

2 ORGANISMS STUDIED IN SPACE

Since the first orbital flight of Laika, spaceflight has become more sophisticated and more frequent. We have studied a diverse and perhaps surprising array of organisms in space. These investigations include studies on the development of various functions from the fertilized egg to the adult individual, alterations in metabolism of various organs, and modification in the genetic materials. Table 2-03 provides the list of organisms studied in space. It is by no means exhaustive.

Bacteria	***Invertebrates***
Aeromonas proteolytica	*Acheta domesticus (Cricket)*
Bacillus mycoides	*Araneus diadematus (Spider)*
Bacillus subtilis	*Biomphalaria glabrata (Snail)*
Bacillus thuringiensis	*Caenorhabditis elegans (Nematode)*
Burkholderia cepacia	*Cynops pyrrhogaster (Newt)*
Chaetomium globosum	*Drosophila melanogaster (Fruit fly)*
Deinococcus radiodurans	*Habrobracon juglandis (Wasp)*
Escherichia coli	*Manduca sexta (Tobacco hornworm)*
Nematospiroides dubius	*Pelomyxa carolinensis (Amoeba)*
Rhodotorula rubra	*Pothetria dispar (Gypsy moth)*
Salmonella typhimurium	*Scorpio maurus (Scorpion)*
Trichophyton terrestre	*Tribolium confusum (Beetle)*
	Trigonoscelis gigas (Beetle)
Vertebrates–Aquatic species	***Vertebrates–Birds***
Arbacia punctulata (Sea urchin)	*Coturnix coturnix (Quail)*
Aurelia aurita (Jellyfish)	*Gallus gallus (Chicken)*
Fundulus heteroclitus (Killifish)	
Lytechinus pictus (Sea urchin)	***Vertebrates–Mammals***
Opsanus tau (Toadfish)	*Canis familiaris (Dog)*
Oreochromis mossambicus (Cichlid fish)	*Felix maniculata (Cat)*
Oryzias latipes (Medaka fish)	*Macaca mulatta (Rhesus monkey)*
Rana catesbeiana (Bullfrog)	*Macaca nemestrina (Macaque monkey)*
Rana pipiens (Frog)	*Mus musculus (Mouse)*
Strongelocentrotus pupuratus (Sea urchin)	*Oryctolagus cuniculus (Rabbit)*
Xenopus laevis (Frog)	*Pan troglodytes (Chimpanzee)*
Xenopus laevis Daudin (South African toad)	*Perognathus longimembris (Pocket mouse)*
Xiphophorus helleri (Swordtail fish)	*Rattus norvegicus (Rat)*
	Saimiri sciureus (Squirrel monkey)
	Testudo horsfieldi (Tortoise)
Plants	
Aesculus hippocastanum L. (Horse chestnut)	*Helianthus annuus L. (Sunflower)*
Arabidopsis thaliana (Thale cress)	*Hemerocallis (Daylily)*
Avena sativa (Oat)	*Lepidium sativum (Garden cress)*
Brassica rapa (Field mustard)	*Linum usitatissimum (Flax)*
Capsicum annuum (Ornamental pepper)	*Lycoperscion esculentum (Tomato)*
Ceratodon (Moss)	*Neurospora crassa (Fungus)*
Ceratopteris (Fern)	*Nicotiana tabacum (Tobacco)*
Ceratophyllum demersum (Hornweed)	*Oryza sativa (Rice)*
Cucumis sativus (Cucumber)	*Physarum polycephalum (Slime mold)*
Dactylis glomerata L. (Orchard grass)	*Pseudotsuga menziesii (Douglas fir)*
Daucus carota (Carrot)	*Pseudotsuga taeda (Loblolly pine)*
Digitalis lanata (Foxglove)	*Saccharomyces cerevisiae (Yeast)*
Digitalis purpurea L. (Foxglove)	*Tradescantia (Spiderwort)*
Elodea (Waterweed)	*Triticum aestivum (Wheat)*
Flammulina velutipes, Agaricales (Fungus)	*Triticum vulgare (Wheat)*
Glycine max (Soybean)	*Vigna radiata (Mung bean)*
Haplopappus gracilis (Haplopappus)	*Zea mays (Corn)*

Table 2-03. Some organisms studied in space. This list was generated in part with the help of the following referenced documents: Leonard and Hughes (2004), Souza et al. (2000), and Asashima and Malacinski (1990).

3 MODEL ORGANISMS

Over four decades of space biology research has seen an impressive diversity of organisms studied in space, generating a wealth of valuable data. But, why so many?

Determining the most appropriate subject for studying a particular biological question in space is not always straightforward. Scientific objectives must be reconciled with the operational and logistical constraints such as size, mission duration, and maintenance requirements. Different classes of organism have been used to study different biological areas. Bacteria and cell cultures are useful for studying genetic changes in response to microgravity radiation, as they are relatively easy to maintain and have short life cycles. Aquatic species such as sea urchins serve as models of fertilization and embryonic development. Insects have been used to study circadian rhythms while mammals such as rats are used frequently to address human adaptations to spaceflight and its health implications. Many species of plant have also been studied to investigate altered growth patterns of roots and shoots in response to gravity. Other, more exotic species were chosen because they have physiological systems already well studied by biologists. For example, jellyfish are excellent subjects for research on gravity-sensing mechanisms because their specialized gravity-sensing organs, or statoliths, are already well characterized.

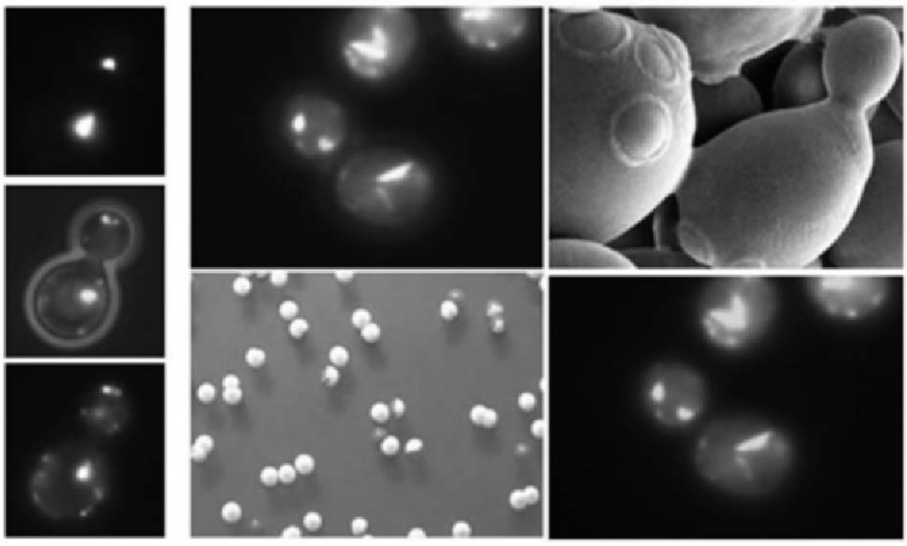

Figure 2-06. Various images of the yeast Saccharomyces cerevisiae. It is the common yeast used in baking and brewing. Yeasts are used as model systems for the understanding of both applied and fundamental aspects of cellular biology.

However, current trends are towards consolidation, and recent advances in cell and molecular biology have led to a more focused research strategy. This has involved the use of a smaller selection of highly characterized organisms, known as *model organisms*, to study the underlying mechanisms of adaptations to the space environment. There is also a desire to conduct detailed "reference studies" using different multiple model organisms and conditions that can be used to more accurately assess the biological risks of long-term spaceflight, in preparation for human missions beyond Earth orbit (Souza et al. 2000).

Researchers selected a small assortment from tens of millions of possibilities because they have common attributes as well as unique characteristics. They are practical: A model must be cheap and plentiful; be inexpensive to house; be straightforward to propagate; have short gestation periods that produce large numbers of offspring; be easy to manipulate in the lab; and boast a fairly small and (relatively) uncomplicated genome.

Model organisms have emerged out of the genome-sequencing projects of the late 1990s. These relatively simple organisms historically used for research are now understood at the genetic level and are providing new insights into the essential biological properties (genes, proteins, metabolic pathways) that they share with each other and, more importantly, with humans (Bahls et al. 2003). For example, because its genetic blueprint is known and gene changes can be detected, the nematode roundworm *Caenorhabditis elegans* is a good model for studies of the effects of radiation and weightlessness on genetic material. Complete genetic sequences, or genomes, are also known for the fruit fly *Drosophila melanogaster*, the yeast cell *Saccharomyces cerevisiae*, and the bacterium *Escherichia coli* (see Figure 1-06).

The genome sequence for the mouse, a valuable mammalian model, also was recently completed and extends the list of model organisms accessible for modern biological research. There are also lab rats of a new field, nicknamed "space genomics", in which scientists study the way weightlessness and space radiation affect an organism's genes. Since diverse organisms share many of the same genes, such studies may give scientists a better understanding of how space travel may affect human genes.

Selection of living organisms as models to work with depends largely upon the experimental purpose. For space biology, the constraints of spaceflight must also be considered. Model organisms believed to have the necessary characteristics are briefly described in the following sections.

3.1 Bacteria

Escherichia coli (*E. coli*) is a common bacterium that has been studied intensively because of its small genome size, normal lack of pathogenicity, and rapid growth in the laboratory. It is considered the

archetypal model organism to study the fundamental principles of cell metabolism, macromolecular synthesis, and gene regulation. This organism is considered to be a good candidate for studying the effects of spaceflight on metabolic pathways and "normal" growth.

3.2 Yeast

Yeast (*Saccharomyces cerevisiae*) is the first eukaryote (organism with a nucleus) to have its genome completely sequenced, in 1996 (Figure 2-06). The cellular activities of yeast are much more like ours than a prokaryote like *E. coli*. But like *E. coli*, it can be cultured easily, it grows rapidly, and, since its entire genome is known, it can be easily transformed with genes from other sources. In fact, many core cellular processes (e.g., cell cycle and control, DNA repair, telomere maintenance) that are important in cancer are conserved between yeast and mammals. These similarities make yeast a particularly good model organism for radiation and aging studies in space.

3.3 Nematodes

The tiny (roughly one millimeter) soil nematode (worm), *Caenorhabditis elegans*, was chosen as a model in the early 1960's. It is genetically amenable and transparent (Figure 2-07), so that every cell division and differentiation event can be followed directly under the microscope. In addition, these tiny worms are hardy: they can survive storage by freezing, and the ones taken on the doomed Space Shuttle Columbia were found alive nearly four months after the crash.

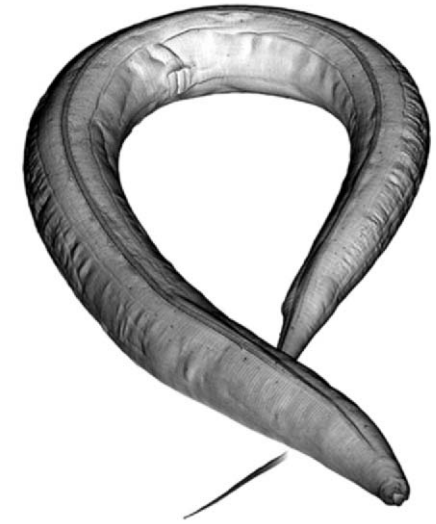

Figure 2-07. The worms C. elegans are mostly transparent at all stages. So, investigators using a simple microscope can view their internal organs/cells without any sort of invasive techniques. Experiments are in planning to examine worms on the Space Station with a video camera so that investigators on Earth can view their development and examine them for differences between Earth-grown worms. Photo courtesy of NASA.

When the genome *C. elegans* was deciphered in 1998, it was found that approximately one third of the worm's proteins—more than 6,000—are similar to those of mammals. Since then, more than 1,400 gene functions have been identified. More than 65% of human disease genes have homologues in the *C. elegans* genome, and essential aspects of mammalian cell biology, neurobiology and development are faithfully recapitulated in this organism. Specifically, a gene that governs the rate of aging in worms has been found to be active in both yeast and mice, and may have a counterpart in humans. This finding is relevant to space biology since during spaceflight astronauts rapidly experience some of the physiological changes associated with the aging process on Earth.

Since the life span of a worm is about three weeks, catapulting worms into low-Earth orbit will allow multigenerational studies. Nematodes have a generation time, a period from birth to reproduction, of about three days. Each individual worm produces 280 offspring through self-fertilization. They are ideal for genetic studies because the ancestry of every cell in their bodies is known from the time of fertilization. The majority of the nematodes flown on the Space Shuttle were maintained at a dormant larval stage known as *dauer larva*. Dauer larvae do not feed and require minimal levels of oxygen and care. Other nematodes were launched as young larvae and were allowed to develop for up to two generations during the flight.

C. elegans development has been extensively studied in terrestrial environments and this species offers the advantage of having a genome that has been completely sequenced. A complete map of its development is available with tracking of each cell division from egg to adult. Like *Drosophila*, it shares extensive homology with vertebrates at the molecular level, has a short life cycle, is small, develops externally, can be placed into cold storage and a large database of mutants is available. Also, the developmental patterns of several genes are known and GFP-marker lines[1] are available.

3.4 Drosophila

Drosophila melanogaster is a species of the fruit fly and, from the standpoint of genetics and cytology, is one of the most studied organisms. Many of the genes known to be associated with disease in humans have equivalents in flies, in particular p53 (a tumor-suppressor gene). Similarities such as this make *Drosophila* of interest to space biology to study, for

[1] *GFP* stands for *Green Fluorescent Protein*. It allows to look directly into the inner workings of cells. In genetic engineering, the cell is engineered with the genetic instructions for building the GFP protein, and GFP folds up by itself and starts to glow.

example, the molecular events that underlie the radiation damage in space. It is also a good model for developmental and neurobiology research.

Drosophila development has been extensively studied in terrestrial environments and this species offers the advantage of having a genome that has been completely sequenced. Therefore, a great deal of information about its genetics, including developmental mutations is available. Additionally, its genetic homology to vertebrates, short life cycle, external development, small size, large number of offspring, large number of indicator lines and mutants, and ability to be transported in cold storage to the desired environment (in this case an altered gravity environment) offer important advantages to space developmental biology. Drosophila can also be used for research on circadian (day/night sleep) rhythms.

The results obtained during the last successful flight of the Space Shuttle Challenger, in early November 1985, indicate that oogenesis and embryonic development of *Drosophila melanogaster* are altered in the absence of gravity. Two hundred forty females and ninety males, wild type Oregon R *Drosophila melanogaster* flies were flown in the Space Shuttle. The results showed an increase in oocyte production and size, a significant decrease in the number of larvae hatched from the embryonic cuticles in weightlessness and alterations in the deposition of yolk (Vernos et al. 1989).

In connection with these results, at least 25% of the living embryos recovered from space failed to develop into adults. Studies of the larval cuticles and those of the late embryos indicate the existence of alterations in the anterior, head and thoracic regions of the animals. There was a delay in the development into adults of the embryos and larvae that had been subjected to weightlessness and recovered from the Space Shuttle at the end of the flight.

No significant accumulation of lethal mutations in any of the experimental conditions was detected as measured through the male to female ratio in the descendant generation. It seems that even *Drosophila melanogaster* flies are able to sense and respond to the absence of gravity, changing several developmental processes even during very short spaceflights. The results suggest that weightlessness interferes with the distribution and/or deposition of the maternal components involved in the specification of the anterio-posterior axis of the embryo.

There is little data behavior of Drosophila in space, but movement seems to increase in microgravity. There is also some indication that spaceflight affects aging in fruit flies. Video images, large sample sizes, and studies over multiple generations should help confirm these results.

3.5 Mammals

Although mammalian cell cultures are more difficult to maintain, they are, nevertheless, essential to space biology. Human cell lines from muscle,

bone, lymphoid, kidney, liver, and other tissues can help us understand, at the molecular and cellular levels, the tissue degradation observed in previous spaceflights.

Laboratory rats and mice have long been used for studying normal and disease processes in the human, primarily because of an extensive body of knowledge of rodent physiological mechanisms, a significant number of rat models that mimic human diseases, the ease of breeding, and the ability to generate inbred congenic rat strains. Rodents are very close to humans in terms of their genome and more than 90% of proteins identified so far show similarities to known human proteins. The mouse genome has been sequenced which brings research one step further toward elucidating mechanisms underlying physiological changes experienced by astronauts during spaceflight (Figure 2-08).

When investigations address human adaptation to spaceflight and its health implications, the use of mammalian species often becomes necessary when humans are not appropriate subjects. The rat is the mammal employed most frequently for space research. Its well-demonstrated biochemical and structural similarity with humans makes the rat an appropriate subject with which to test new drugs and investigate many disorders experienced by astronauts during and after spaceflight. Because of their phylogenetic proximity to humans, nonhuman primates, such as Rhesus monkeys, have occasionally served as research subjects in space biology, but only when the need has been clearly demonstrated (Souza et al. 2000).

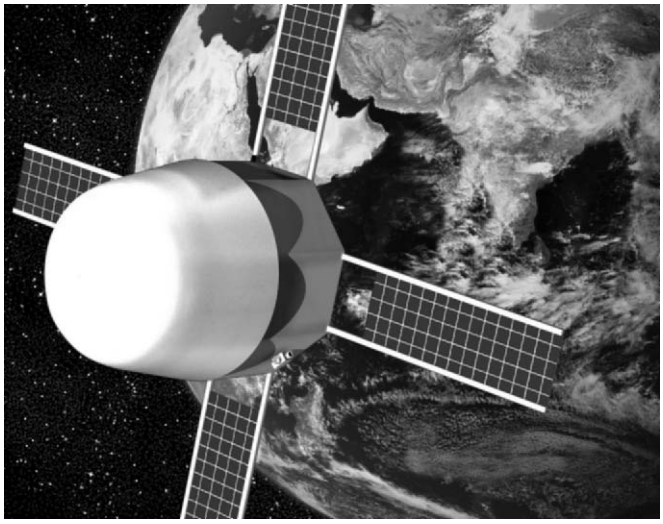

Figure 2-08. The Mars Gravity Biosatellite is a project from MIT to study the effects of Mars gravity on mammals. A 400-kg biosatellite carrying will rotate about its central axis, providing 0.38 g outwards against a curved floor. After 5 weeks in low Earth orbit, the re-entry capsule will separate from the primary spacecraft to return the mice safely to a landing zone in the Australian desert. The biosatellite provides autonomous life support capabilities and data telemetry or storage from onboard experiments. Credit: MarsGravity.org

When working with higher organisms, such as mammals, stress caused by unfamiliar conditions can impact science results. To prevent this, the animals must be habituated to their flight habitat, life support hardware, and biosensors. Some animals, such as rats and rhesus monkeys, must be trained to use inflight feeding and watering devices. When performance and behavior is studied, as is sometimes the case with rhesus monkeys, the animals must be trained to perform particular tasks in response to automated stimuli.

Also, when mammals are used as research subjects, microbiological testing of the animals is mandatory to ensure that they are free of pathogens that could be transmitted to crewmembers. Organisms that are part of the science payloads must be isolated from the humans onboard so that possible contaminants and odors do not affect crew health, comfort, or performance. Hardware for housing the experiment subjects is typically custom-built for this purpose and kept sealed or filtered for the duration of the mission.

As in the case of amphibians and zebrafish, a genome project is proceeding for mice, genetic information related to developmental mutations is available, there is a great deal of homology developmental patterns. Mice also have a short developmental cycle (21-day gestation) and short life cycle (4 months). Additionally, since adults are small, habitats take less space than those of other mammals. Mice are typically used in experiments requiring large numbers of individuals. On the other hand, rats are used in experiments requiring a minimum of six individuals per treatment. However, this number of 6 can increase to 12 or 24 with additional requirements, such as the need for an onboard 1-g centrifuge control group, or the need for sacrificing the animals inflight.

Rats have a developmental cycle similar to mice and, as in the case of mice, some flight data are available and a genome project has begun. Additionally, some well-developed rat models for human disease and pathophysiology are available as is a significant rat database of maternal fetal behavior.

The results of reproductive studies done on mammals in the space environment are probably the best ones from which to extrapolate in order to estimate human limitations in this area. In one of the early experiments conducted on board the Soviet Cosmos biosatellites, flown male rats were allowed to mate with non-flight females 5, 75, and 90 days postflight. Litters of the 5 days postflight rats had a significant increase in the number of abnormalities as compared to the controls. These abnormalities were mainly in the development of the various organs. Some of the offspring also showed growth retardation though the overall infant mortality was same as the controls. Later postflight mating showed no differences in both samples (5 versus 75 and 90 days postflight samples), thereby suggesting that only the mature spermatozoa were affected during the flight (Serova 1989).

In addition to the above, male and female rats were allowed to mate in space. No pregnancies resulted, but postflight laparotomy showed that the ovulation and fertilization did occur in the rats, though for some reason embryogenesis did not proceed in the normal way. Later, the same rats were mated with nonflight rats and all the litters were found to be normal (Denisova et al. 1989).

Ground-based experiments have shown decreased number of embryos and increased embryo mortality in immobilized female rats. In the clinostat experiment, very few oocytes reached the second meiotic division. Similarly, oocytes were not found in the oviduct of female rats that had been subjected to centrifugal forces up to 3 g for 3 hours a day during the first and second days of pregnancy. However, samples that were subjected to the centrifugal force during the fifth and sixth day of pregnancy showed developed embryos but most of these were found to be morphologically abnormal.

Video footage of adult animals during centrifugation indicates that behavioral activity within the environment is reduced and limited in range. However, the behavior of animal subjects in microgravity has not been investigated, and the influence of activity-dependent stimulation on development is presumably important (Alberts and Ronca 1999). For example, neonate rats flew on board several Space Shuttle flights. The results involved losses of animals or compromised weight gain, presumably related to inadequate maternal care and limited nursing interactions with the dams. Results from these studies also suggest altered motor behavior and neuromuscular development during spaceflight.

Figure 2-09. Haplopappus gracilis has been utilized in space to test whether the normal cell division in the root tip can be sustained in microgravity, and to determine whether the fidelity of chromosome partitioning is maintained during and after spaceflight.

3.6 Plants

Like bacteria, plants were exposed to spaceflight very early in the space program. Seeds of five species were first sent up on Sputnik-4 in 1960. Since then there has been a bias to send a variety of plants into space rather than picking one or two species and studying them in detail over the decades. In part this is because different scientists have “specialty” systems that they

work on, or they pick certain plants as best for particular tests. In part, it is because of practical concerns (e.g., a need for plants with short life spans to match short spaceflights) or a desire to see whether a variety of possible foodstuffs would do well. A few of the plants types sent so far have included algae, carrots, anise, pepper, wheat, pine, oat, mung beans, cress, lentils, corn, soybeans, lettuce, cucumbers, maize, sunflowers, peas, cotton, onion, nutmeg, barley, spindle trees, flax, orchids, gladiolas, daylilies, and tobacco.

Shoots of cell culture-derived daylily (*Hemerocallis* cv. *Autumn Blaze*) and haplopappus (*Haplopappus gracilis*) have been selected because they represent both major groups of the plant kingdom. The daylily represented herbaceous monocotyledonous plants and the haplopappus represented annual dicotyledonous plants. *Haplopappus* is valuable for studies of chromosome behavior because it has only four chromosomes in its diploid state (Figure 2-09). Daylily was chosen for the study because it has special karyotypic features (features related to the number, size, and configuration of chromosomes seen in the metaphase portion of mitosis) and it is a species for which a great deal of culture technology has been developed.

Arabidopsis plant has been chosen because it has many genes in common with humans. This flowering herb is a member of the mustard (*Brassicaceae*) family that is widely used as a model organism in plant biology. This species is a flowering herb that is widely used for research in plant genetics. With a small, completely sequenced genome, rapid life cycle (about 6 weeks from germination to mature seed), prolific seed production and easy cultivation in restricted space, it offers important advantages for basic research in genetics, development, and molecular biology in the space environment. In addition, this plant uses a chemical, glutamate, much as it is used in the human brain, that is, as a chemical messenger. However, as mentioned earlier, the reproducibility of these very short life cycle plant appears to be questionable. *Mouse-ear cress* was also chosen because its small size allowed it to fit easily into the experiment hardware (Musgrave et al. 1995).

Woody plants, such as conifer, can also be used to evaluate the effect of microgravity on the ability of plants to form a reinforcement tissue known as *reaction wood*. On Earth, woody plants produce this distinctive reinforcement tissue when their stems are bent contrary to their normal orientation. The reaction wood formation restores the stems to its upright position, which contributes to the plant's survival, but it has an adverse effect on wood quality and texture. Conifer seedlings placed in a plant growth facility (see Chapter 3, Section 3.3) can be used for this research.

Plants can be harvested and preserved chemically to stop their growth and development at predetermined intervals (Figure 2-10), and then frozen for postflight analysis. Electron and light microscopic study of the samples can define the time and place or reaction wood formation, while biochemical

analysis enables the scientists to study the regulatory enzymes and genes involved.

Figure 2-10. On the Space Shuttle Columbia's middeck, an astronaut works with the Brassica Rapa plants being grown for comparing changes in ultrastructure, biochemical composition and function induced by the spaceflight environment on the photosynthetic apparatus of its seedlings at different stages of vegetative development. Photo courtesy of NASA.

4 THE CLASSICS

Amphibians, fish, and birds have long been used during spaceflight for studies on developmental biology. In addition, some features of their gravity-sensing mechanisms (otoliths) are similar to those of mammals. Also, they share the characteristics of using both horizontal and vertical space in their habitat.

4.1 Amphibians

Amphibians were the first vertebrates to come on land and to resist gravity by supporting their weight. Frogs and toads typically start life in water, and become terrestrial, arboreal, or even remain aquatic after metamorphosis. They have various morphological, physiological, and behavioral adaptations to the diverse environments in which they are found.

Like *Drosophila* and *C. elegans*, amphibians have short developmental periods, using only a few days to proceed from fertilization to larva and this is an advantage when studying this period in altered gravitational environments. The life cycle through adulthood, however, is relatively long in *Xenopus laevis*, an aquatic frog, but comparable to mouse in *Xenopus tropicalis* (Figure 2-11).

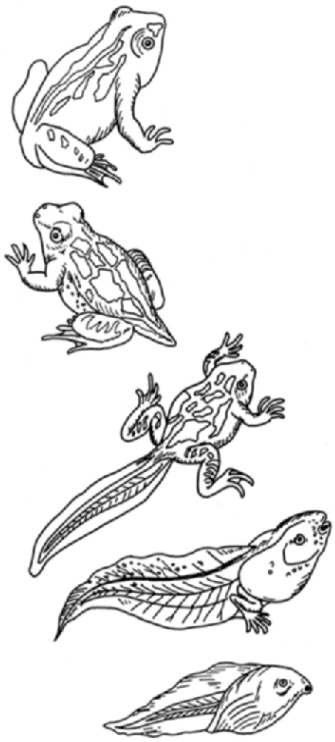

Figure 2-11. Eleven weeks after the egg was laid, a fully developed frog with lungs, legs, and no tail emerges from the water. From bottom to top: After its 21-day development period, the embryo leaves its jelly shell and becomes a tadpole. After about five weeks, the tadpole begins to change. It starts to grow hind legs. Behind their heads bulges appear their front. Their tails become smaller. Lungs begin to develop, preparing the frog for its life on land.

Amphibians also share homology to mammals at the molecular level and for the mechanisms of tissue induction, developmental patterns of several genes are known and some GFP-marker lines are available. Amphibians produce durable eggs and embryos in large numbers, fertilization and development are external (so as in the case of *C. elegans* and *Drosophila*, development can be videotaped), a genome-mapping project has begun and some mutations are available. While embryos are opaque, tadpoles of some species are semi-transparent.

Preliminary flight data have been collected from several amphibian species showing that eggs are stratified based on the gravity vector and that cytoplasmic localization of maternal factors (necessary for formation of the germ line and initial axes) are potentially affected by gravity (see Chapter 5). The unfertilized frog egg appears to be radially symmetric about its animal

vegetal axis. Establishment of bilateral symmetry, dorsal-ventral axis specification, requires a 30-deg rotation of the vegetal yolk mass relative to the egg surface during the first cell cycle. One well-known external influence on frog eggs is gravity. There are observations that eggs of the frog *Xenopus laevis* tilted 90 deg off-axis during in vitro maturation do not have true radial symmetry (Smith and Neff 1986).

To test whether gravity is required for normal amphibian development, *Xenopus laevis* females were induced to ovulate on board the orbiting Space Shuttle. Eggs were fertilized *in vitro*, and although early embryonic stages showed some abnormalities, the embryos were able to regulate and produce nearly normal larvae. These results demonstrate that a vertebrate can ovulate in the virtual absence of gravity and that the eggs can develop to a free-living stage (Souza et al. 1995, Danilchik and Savage 1994). However, recent intriguing work suggests that there are subtle developmental changes in the *Xenopus laevis* embryos subjected to novel gravitational fields. These changes include the position of the third cleavage plane, the dorsal lip of the blastopore and the size of the head and eyes. The eggs fail to undergo the cortical/cytoplasmic rotation that specifies dorsoventral polarity, and they lack an array of parallel microtubules associated with the rotation (Elinson and Pasceri 1989).

Additionally, it has been shown that *Xenopus laevis* larvae fail to inflate their lungs in a weightless environment suggesting that a complete life cycle in weightlessness would not be possible for such an airbreathing amphibian (Wassersug 2001).

4.2 Fish

Most space data regarding fish come from zebrafish, but some are from studies involving other species. As in the case of amphibians, a genome project is proceeding for zebrafish, genetic information related to developmental mutations is available, there is a great deal of homology to mammals at the molecular level, developmental patterns of several genes are known, indicator lines are being developed, they have a short life cycle, fertilization and development are external and eggs and embryos are hardy and produced in large numbers. As opposed to amphibians however, zebrafish embryos are transparent. Additionally, significant flight data are available for vestibular system development.

The fish *Medaka* is particularly suited for systematic evaluation of vertebrate development and growth since it is a hardy fish, whose embryos tolerate reduced temperatures well, allowing researchers to subject the embryos to low temperatures and slow embryonic development (Figure 2-12). This provides more time to study each stage of vertebrate development and maximizes the effects of weightlessness on each stage. The *Medaka* fish are one of the shortest life-cycle vertebrate animals: eggs become sexually mature

fish to lay eggs (egg-to-egg) within 3 months. Also, the embryos are optically clear, which allows investigators to visually examine molecular markers and the development of the internal organ systems Fixations of embryos can occur at different stages so that all phases of growth and development can be compared and studied. Rates of development for some key organs, such as the eyes and the heart, can be established before the mission, and molecular probes can be used to establish relationships between specific pattern-regulating genes and the development of specific organs (Crotty et al. 1995). One of the direct molecular genetic studies consists in cloning of the *Medaka* homeobox-containing gene Hoxa-4. The Hoxa-4 gene is a marker of embryonic development for analyzing the effect of weightlessness stress on embryonic segmentation.

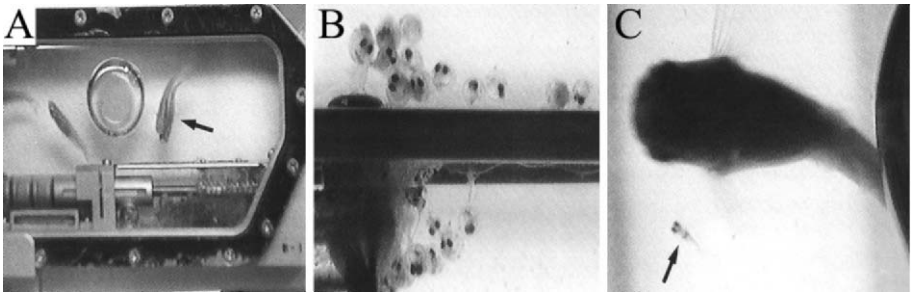

Figure 2-12. In the Summer of 1994, four Japanese killifish (Medaka) flew for 15 days on board the Space Shuttle Columbia, IML-2 STS-65). These fish mated in space for the first time among vertebrate animals (A) and laid eggs (B), which developed normally and hatched as fry (C). Adapted from Ijiri (1995).

4.3 Avian

Birds share extensive homology with mammals at the cell, tissue and molecular level. Developmental patterns of many genes are known, a genome project is proceeding, and some mutants are available. They have short developmental cycles (21 days for chick, 16 days for quail), though with relatively long life cycles. Avian species can be studied in large numbers and early embryos can be stored at cool temperatures and subsequently re-warmed to in order to restart development at desired times.

Gravity also affects the early stages of development in birds. In the oviduct, the fertilized egg is subjected to repeated rotations. Numerous experiments have shown that the direction of this rotation and the position of the egg relative to the vertical will determine the orientation of the embryo's bilateral plane of symmetry (Planel and Oser 1984).

Preliminary results indicate no adverse effects of vibration and g force (at least those experienced during a Space Shuttle launch) on avian development. Flight data on early embryogenesis exist as well, indicating that there are some sensitive periods during which these embryos do not do well in the flight environment. To date, only few quail embryos have survived in weightlessness and only two of these embryos survived to the latter stages of development, i.e., to the 16th days of incubation. These flight experiments could indicate that gravity may be needed during the earliest stages of avian embryogenesis, but is not important for the latter stages of development (Bellairs 1993).

However, interpretations of the results were made more difficult by the fact the synchronous control showed a similar lack of viability. Retrospective analysis of onboard flight recording data suggests that the incubator temperature control malfunctioned and the eggs were being incubated at 42ºC instead of the programmed 37.5ºC. Also, an egg incubator within a centrifuge can allow determining if lack of gravity is the reason for the death of young avian embryos in space.

On future spaceflights experiments will attempt to determine the effect of weightlessness on embryonic development initiated after the launch, the fecundity of adult quail during orbit, and the assessment of their hormones and reproductive tissues after orbit. Other objectives include the regeneration potential of quail in weightlessness based on primordial germ cell migration and differentiation, gametogenesis, ovulation, fertilization, embryonic development, and hatching.

Figure 2-13. Japanese quail chick on board the Russian space station Mir. Quail eggs that underwent two thirds of embryonic development on Earth were incubated. They hatched during the spaceflight and were returned back to Earth for postflight analysis.

These experiments will provide substantial basic information about the effects of weightlessness on embryonic differentiation and development, as well as important information about adult avian endocrinology and physiology. Other experiments will investigate the acute response of birds to

the absence of microgravity. One predictable and commonly observed response of animals that find themselves in microgravity is to react as if they were upside-down, and they begins to roll over and over to "right" them up. It is still unknown if birds will be disoriented or will quickly lean to fly in microgravity (Figure 2-13).

Figure 2-14. Astronaut Shannon Lucid checks on wheat plants on board the Russian Mir space station. Photo courtesy of NASA.

5 CONCLUSION

Model organisms are being used to investigate some of the most up-to-date areas in biological research. Each model organism is distinctively suited as a simplified model to the study of complex aspects of biology. Researchers are repeatedly surprised that discoveries in simple organisms are relevant to human biology, which encourages transposition of results from one model system to another, and highlighting the extent of conservation and commonality of life forms. The differences hold value as well, as they provide important insights to understanding cell physiology and pathology (Blair Hedges 2002).

Animal and plant model organisms have proven particularly useful for space biology, because of their advantages for experimental research, such as rapid development with short life cycles, small adult size, ready availability, and because of the large number of ground-based studies carried out on them.

A large amount of genetic information can then be derived from these organisms, providing valuable data for the analysis of normal human development and gene regulation, genetic diseases, and evolutionary processes. It is now known that microgravity induces certain physiological changes that may produce useful experimental models for studies of Earth-based diseases such as osteoporosis, immune dysfunction, vestibular disorders, wound healing impairment, anemia, and aging (see Clément 2005 for review). The judicious use and application of experimental animal models to the study of complex biomedical and pathophysiological problems will continue to provide new insights into biological mechanisms that influence our lives on Earth and in space (Borkowski et al. 1996).

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FURTHER INFORMATION:

International Flight Experiments Database: http://www.mainsgate.com/IFE/index.html

NASA Education Resource on Space Biology: http://www.spacebio.net/index.html

NASA Life Sciences Data Archive: http://www.lsda.jsc.nasa.gov

Chapter 3

FACILITIES FOR GRAVITATIONAL BIOLOGY

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This chapter describes the laboratory facilities used to conduct experiments in the domains of cell and developmental biology on board various space transportation systems, such as the International Space Station, Space Shuttle, Space-Hab, Soyuz, as well as the unmanned Bio-satellites and sounding rockets. Equipment used in laboratories on Earth for performing ground-based control experiments, like clinostats and centrifuges, and the rationale for conducting inflight and ground control experiments, is also developed.

Figure 3-01. Astronauts Carl E. Walz, (left) and Jerome Apt (right) work on a biotechnology experiment on board the SpaceHab module in the Space Shuttle Atlantis. The cartilage experiment investigated tissue formation and basic cell-to-cell interactions in a low-gravity, stationary-cell-culture environment. Photo courtesy of NASA.

1 TOOLS TO STUDY THE EFFECTS OF GRAVITY

Some biology experiments require periods of days, weeks or months of microgravity, and therefore need to be carried out on orbiting spacecraft. However, there are situations in which much shorter microgravity periods can be useful to the biological investigator. Under such conditions, the critical

operation of hardware devices, a fixation system for example, can be verified; the feasibility of operations such as fluid transfer can be checked; and short-term biological studies on processes such as membrane function and cytoskeleton activity can be performed.

Also, the costs of research on manned missions can be attributed largely to the extensive testing of the experiment hardware and the need to meet crew safety requirements. Unmanned missions are generally much less expensive, with most of the cost going for hardware automation. Experiments performed on such missions must accommodate for the lack of crew to conduct support procedures or intervene if equipment malfunctions.

Periods of microgravity can be provided in a number of ways. Table 3-01 shows a summary of the gravity levels attainable and the duration for which they can be achieved by these different facilities, as well as the size and mass of the embarked payload.

Facility	***Gravity***	***Duration***	***Payload Size***	***Payload Mass***
Drop Tower	*10^{-6} g*	*2-5 s*	*up to 1 m^3*	*+100 kg*
Parabolic Flight	*10^{-2} g*	*20-25 s*	*several m^3*	*+1000 kg*
Sounding Rocket	*10^{-6} g*	*5-15 min*	*up to 1 m^3*	*up to 500 kg*
Biosatellites	*10^{-6} g*	*10-15 days*	*up to 4.3 m^3*	*up to 700 kg*
Soyuz	*10^{-6} g*	*12 days*	*0.5 m^3*	*4-12 kg*
Space Shuttle	*10^{-6} g*	*12-16 days*	*1 m^3*	*+100 kg*
SpaceHab	*10^{-6} g*	*12-16 days*	*1 m^3 per rack*	*700 kg per rack*
Space Station	*10^{-6} g*	*45 days or +*	*1 m^3 per rack*	*700 kg per rack*

Table 3-01. Quality and duration of microgravity.

1.1 Microgravity Facilities

1.1.1 Balloon Flights

While balloon flights cannot provide a microgravity environment directly, they can be used to expose samples to radiation similar to those encountered in orbit. They can therefore be used to provide information to complement results from orbiting experiments. While it is highly desirable to have a control 1-g centrifuge on every microgravity mission in order to separate the effects of the space environment from those of microgravity (see this Chapter, Section 2.3), this is not always possible. The more easily available, and considerably cheaper, stratospheric balloon can then be used as a means of providing a 1 g, cosmic-ray-irradiated control. It has the additional advantage that larger and more numerous samples can be carried than on a space mission. Flights lasting up to 24 h, carrying payloads of 2,000 kg up to altitudes of 40 Km, have been used to expose biological samples to cosmic ray levels close to those experienced in low Earth orbit (LEO) (Planel 2004).

1.1.2 Drop Towers/Shafts

The most economical and easily available way to provide microgravity are drop towers or shafts up to 100 m in height wherein the air can be evacuated so that an experiment capsule can fall freely for a short time before being decelerated. Drops can typically provide 2-5 sec of microgravity, but this period can be doubled by using a catapult system at the base of the shaft, such as the one at the ZARM scientific institute in Bremen, Germany.

However, not all types of scientific inquiry are appropriate for the drop facilities. Meaningful microgravity research in biology and biotechnology can seldom be conducted in drop experiments, because the duration is very short and the deceleration too violent. Living organisms are not used. Crystals grow too slowly for such short-term microgravity exposure. On the other hand, these conditions are sufficient for many physics and material sciences experiments.

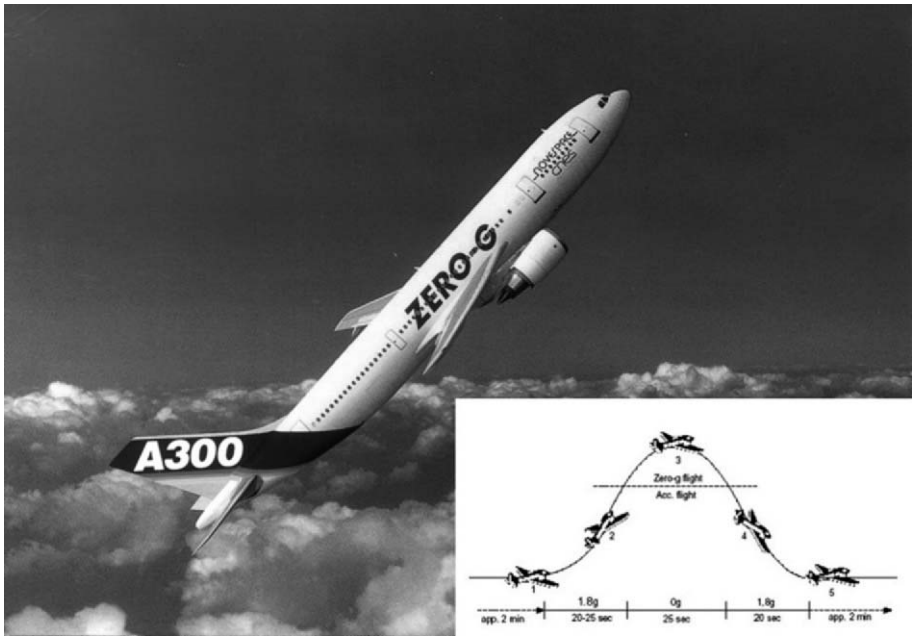

Figure 3-02. The ESA-CNES Airbus A300 Zero-G initiating a parabola. The insert shows the complete trajectory. Photo courtesy of Novespace.

1.1.3 Parabolic Flight

To achieve a parabolic trajectory from a steady horizontal flight, an aircraft gradually pulls up its nose and starts climbing at an angle of approximately 45 deg (Figure 3-02). This “injection” phase lasts for about 20

seconds, during which the aircraft experiences an acceleration of about 1.8 g. The engine thrust is then reduced to the minimum required to compensate for air-drag, and the aircraft then follows a free-fall ballistic trajectory, i.e., a parabola, lasting approximately 20 seconds, during which weightlessness is achieved. At the end of this period, the aircraft must pull out of the parabolic arc, a maneuver which gives rise to another 20-s period of 1.8 g on the aircraft, after which it returns to normal level flight attitude.

Sequences between 30 and 40 parabolas are normally flown on each mission, so allowing repetition of experiments. Relatively large pieces of apparatus can be carried and operated by the experimenter on these flights. Parabolic flights have been used extensively to investigate human and animal physiology, and gravitational biology under low gravity.

1.1.4 Sounding Rockets

Sounding rockets are sub-orbital rockets that carry a payload above the Earth's atmosphere for period of up to 15 minutes, but which do not place the payload into orbit around the Earth. Typically, such rockets reach an altitude of 250-350 km at which point the payload is separated and undergoes stabilized free-fall, finally landing by parachute. Payloads can be quite large, thus containing a number of individual experiment modules.

One of the benefits of this type of carrier is that late access to the payload is available until about 2 hours before launch, thus allowing studies on time-critical biological processes, or samples requiring complex preparation. Such facilities have been used to study gravity-sensing mechanisms in a number of plants and animals.

Not only are sounding rocket missions carried out at very low cost, but the payload can also be developed in a very short time frame, sometimes as quickly as three months. This rapid response enables scientists to react quickly to new observed phenomena and to incorporate the latest, most up-to-date technology in their experiments.

1.1.5 Biosatellites

Some space biology studies require the presence of human to carry out the experiments in orbit. Others simply require that animals be kept in space for some period of time. The latter "passive" type of study is a very efficient utilization of animals providing many investigators with specific tissues from the same animals. The Russian Biosatellite flight series, which began in 1966 with Voskhod (see Chapter 2, Table 2-02), is currently the only facility dedicated to biological experimentation using unmanned, Earth-orbiting satellites for missions lasting for up to 15 days.

The earlier Biosatellites used by the former Soviet Union were the so-called *Cosmos* or *Bion* biosatellites. Their design was based on the famous Vostok spacecraft, which carried Yuri Gagarin as the first man into space in

1961. Unmanned recoverable capsules of the *Foton* type were introduced in 1985 (Figure 3-03). Foton was envisaged as a microgravity platform for physics and materials science to complement the very similar Bion capsules that were aimed at life science studies. However, in later years an increasing number of biology and non-microgravity experiments were transferred to Foton, while the Bion program was discontinued.

Foton capsules are pressurized and temperature-controlled, can host a payload of 700 kg in a volume of 4.3 m^3 with 800 W of electrical power provided for the entire duration of the mission. The capsule is composed of three compartments: the landing module, the instrument assembly compartment, and a hermetically sealed unit that contains additional chemical sources of energy. The landing module is a complex, autonomous spherical compartment that can house plants, animals, and cell cultures. The samples can be loaded in the capsule only 14 hours before the launch. After the flight, the biological specimens are immediately removed from the capsule by a ground team and placed in refrigerated containers. The Foton capsule is then transported, first by helicopter, then by aircraft. The samples are then dispatched to the participating science teams via Moscow. The scientific instruments are removed from the capsules a few days later and transported back in the investigators laboratories.

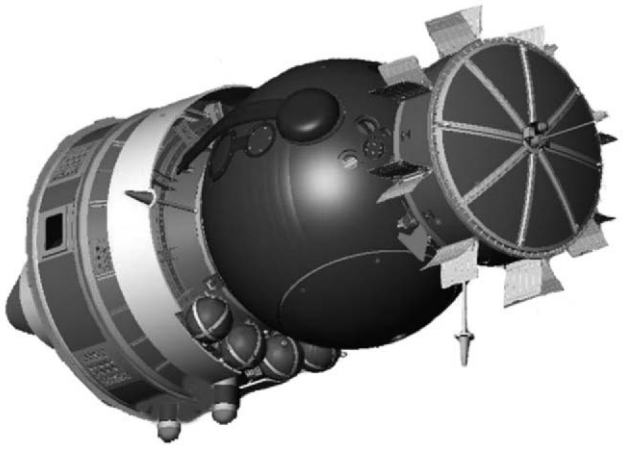

Figure 3-03. Artist drawing of a Foton capsule. The spherical compartment in the middle is the one that is housing the biological specimens. Source ESA.

The Foton capsules provide unique opportunity for flying biological specimen (animals, cells, and plants) when no crew activity is needed. Telemetry can be used to activate some procedures during the flight, such as fixation of cells, or turn on or off the light. Small onboard centrifuge generating centripetal accelerations of up to 1 g can also be utilized to provide comparison with ground controls and make sure that the observed effects of the flight on the specimen are not due to the stress of launch and landing or to

atmosphere changes. The samples are loaded in the capsule up to a few hours prior to launch.

Because the spacecraft is an unmanned biosatellite, all experiment operations, spacecraft subsystems, and life support systems for experiment subjects must be automated. Experiment materials and subjects cannot be directly manipulated during the flight, and viewing is possible only by means of video. Malfunctioning hardware cannot be repaired during a mission, and life support equipment cannot be manually regulated. These limitations place special demands for quality and reliability of flight hardware and allow the experiments somewhat less flexibility than those flown on manned vehicles.

There are, however, significant advantages to conducting life sciences experiments on unmanned spacecraft. The cost of flying an unmanned mission is markedly less than that of a manned mission. Hardware can be built relatively inexpensively, using a wider range of materials, without jeopardizing crew safety. Similarly, missions can often be extended or shortened to maximize science return and animal welfare, since crew requirements do not have to be considered. Also, they allow mission management to control the launch date and thereby allow payload readiness to be a significant factor.

1.1.6 Soyuz

The *Soyuz* "Taxi" flights are dedicated to exchange the Soyuz emergency return vehicle on ISS and are therefore planned exactly every six months. Two to three cosmonauts (with one seat being commercially available for about 20 million dollars!) participate in such flights, offering approximately five effective experiment days on board the ISS.

The total length of flight is around 10 days with 2 days in orbit before docking with ISS. The time between de-orbit and recovery is in the order of a few hours. The Soyuz capsule has a pressurized volume of about 4 m^3. The mass of payload that can be carried to the ISS is about 250 kg, and the mass that can be brought back to Earth is about 150 kg. However, most of this mass is used to carry supplies to the ISS cosmonauts (water, food, and personal items). Therefore, only a limited number and simple experiments can be accommodated. Based on the European experience, a passenger of a Soyuz "Taxi" mission is allowed to carry 12 kg of equipment or samples up (volume 0.4 x 0.4 x 0.4 m), and to return 4 kg of equipment (e.g., tapes, films) or samples down to Earth. However, scientific equipment can be sent in advance to the ISS using a *Progress* unpressurized vehicle.

The experiments are usually performed in the Russian section of the ISS. Crew time can also be used to perform experiments using hardware already on board the ISS, in the Russian module, or brought up by the *Progress* cargo re-supply ship. Sample return immediately after termination of the mission is extremely limited and without temperature control capability

(room temperature only). These flights are best suited for the activation of automatic experiments.

1.1.7 Space Shuttle

For the period until complete construction of the ISS, all Space Shuttle flights are dedicated to assembly and operation of the ISS. Therefore, opportunities for Shuttle-based experiments are limited. Nevertheless, the Space Shuttle can accommodate flight experiments with typical flight duration of 8 to 12 days. Equipment can be stored in the storage lockers (up to 27 kg and 0.36 m^3) on the forward bulkhead of the middeck (Figure 3-04). Each drawer has foam-rubber spacers to hold the contents in place. The experiments themselves must require only limited crew training and involvement to execute. Experiment hardware occupying or requiring a large volume to operate will not likely be accommodated. Experiments that do not require Shuttle power (i.e., battery-operated) are more easily accommodated, since in general there is no power available in the middeck lockers during ascent and reentry.

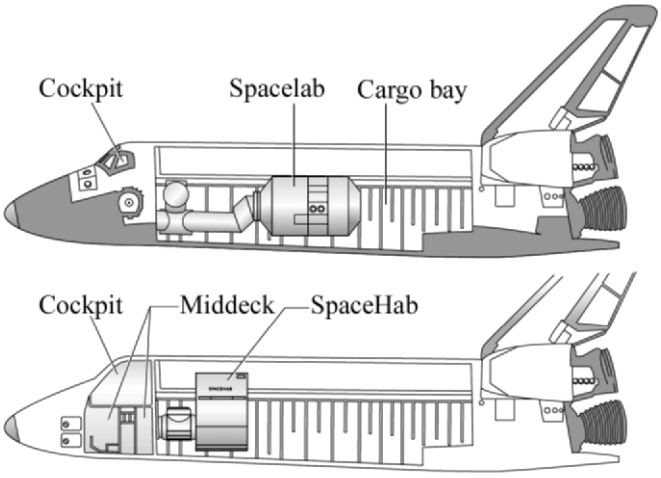

Figure 3-04. The Spacelab and SpaceHab modules are pressurized laboratory facilities that can be placed in the Space Shuttle cargo bay. The middeck contains pressurized living quarters for the crew as well as locker space for holding small payloads. Source NASA.

The *Small Self-Containers* (SSC), or "Getaway Special" payloads, can also be used to conduct space life science experiments. The SSC are small (90 kg, 1.4 m^3) cylindrical containers attached to the inside wall of the Space Shuttle cargo bay. There are placed on board when allowed by space and weight restrictions. These containers must contain their own systems for power, handling data, and environmental control. Some of the systems may be turned on or off from the flight deck, but otherwise they are completely automatic. They must, however, adhere to flight safety guidelines. Many biological experiments proposed by students have flown in these containers.

Time-critical supplies or specimens can be loaded in the Space Shuttle between 40 and 20 hours before launch. It is possible to retrieve equipment, supplies, and data that have time- or temperature-critical sensitivities after landing plus 3 hours. Note that there are periods of time before the flight and after landing when no access to the experiment is possible and maintenance of the equipment integrity must be assured. The availability of Shuttle resources for experiments that require animals as subjects is also extremely limited for short-duration experiments.

1.1.8 Spacelab & SpaceHab

Spacelab was built by the European Space Agency for use in the Space Shuttle cargo bay. Spacelab was a pressurized module, 4 m in diameter and 7 m in length equipped with standard experimental racks (0.48 m) that held up to 290 kg of equipment and instruments. Spacelab mainly flew during dedicated life and material science missions of the Space Shuttle *Columbia* (Figure 3-04). A Spacelab module was even flown as a cargo carrier, during the first docking of the Space Shuttle with the Russian space station Mir. The first Spacelab flew on STS-9 in 1983, and the last on STS-90 in 1998. Over its 15-year flight history, the Spacelab program hosted payloads for practically every space research discipline. In all, 19 Space Shuttle missions carried life and microgravity sciences research into orbit and resulted in more than 750 experiments and more than 1,000 peer-reviewed articles, as well as numerous talks, abstracts, and Master's and Doctoral theses. The International Microgravity Laboratory missions (IML-1 and -2) carried not only international research but also international crews. One mission was dedicated solely to Japanese research (Spacelab-J) and two missions dedicated to German research (Spacelab D-1 and -2).

SpaceHab is designed for housing a four-person crew in a pressurized laboratory within the Space Shuttle cargo bay. This laboratory includes temperature and moisture control, and power supply with AC and DC current supplied to all experiment locations, and high-data rate communications. The Research Double Module (RDM) is proposed on a commercial basis to microgravity experiments: it includes six standard double-rack locations, and storage lockers (up to 27 kg and 0.36 m^3), for a total payload capacity of approximately 4,000 kg. The crew has access to the RDM through a pressurized tunnel connected to the Shuttle middeck airlock.

Like its older brother Spacelab, SpaceHab relies on the high bandwidth Ku-band signal processing of the Space Shuttle. However, during periods of communication blackout (*Loss of Signal*, or LOS) data can also be stored onboard and downlinked later.

Up to the STS-107 mission, SpaceHab was particularly useful to conduct life and material sciences experiments during dedicated missions while waiting for the completion of the ISS (Figure 3-05). None of these

missions are currently manifested. Today, SpaceHab modules are added to Space Shuttle missions visiting the ISS to carry supplies requiring a pressurized environment.

Figure 3-05. Photograph showing an astronaut inside the SpaceHab module on board the Space Shuttle Columbia STS-107 mission. Photo courtesy of NASA.

1.1.9 International Space Station (ISS)

While we are writing these lines, the entire Space Shuttle fleet is grounded following the foam problems that occurred again during the launch of the STS-114 Return-to-Flight mission. So, it is difficult to predict the final state of the ISS. The following are the supposed capabilities of the ISS after completion, as of September of 2005.

More than four times as large as the Russian Mir space station, the completed International Space Station will have a mass of about 450 tons and more than 1200 m^3 of pressurized space in six laboratories. The United States will provide two laboratories (the *United States Laboratory* and the *Centrifuge Accommodation Module*). There will be two Russian research modules, one Japanese laboratory referred to as the *Japanese Experiment*

Module (JEM) named *Kibo* (for "Hope"), and one European Space Agency (ESA) laboratory called the *Columbus Orbital Facility* (COF).

All six laboratories together will provide 37 *International Standard Payload Racks* (ISPR). An ISPR, about the size of a home refrigerator, holds research equipment and experiments. Additional research space will be available in connecting nodes and the Russian modules. The JEM also has an exterior "back porch" with 10 spaces for mounting experiments that need to be exposed to space. The experiments will be set outside using a small robotic arm on the JEM. There are also four attached payload sites on the truss and two spaces on the COF for mounting external experiments.

ISS Flight Equipment	***Research Area***
Advanced Animal Habitat (AAH)	*Development*
Aquatic Animal Experiment Facility (AAEF)	*Gravitational; Development*
Aquatic Habitat (AQH)	*Gravitational; Development*
Biolab	*Cell; Radiation*
Biopack	*Cell; Biotechnology*
Biotechnology Mammalian Tissue Culture Facility (BMTC)	*Cell; Gravitational; Biotechnology*
Biotechnology Research Facility (BRF)	*Biotechnology*
Cell Biology Experiment Facility (CBEF)	*Cell; Gravitational; Radiation*
Cell Culture Unit (CCU)	*Cell; Gravitational; Radiation*
Centrifuge Accomodation Module (CAM)	*Gravitational; Development*
Compound Microscope	*All*
Crew Health Care System (CHeCS)	*Biomedical research*
Dissecting Microscope	*All*
Egg Incubator (EI)	*Development*
European Physiology Modules (EPM)	*Human physiology; Biomedical*
Gravitational Biology Facility (GBF)	*Gravitational; CELSS*
Habitat Holding Racks	*Gravitational; Development*
Human Research Facility (HRF)	*Biomedical*
Insect Habitat (IH)	*Gravitational; Development*
Life Sciences Glovebox	*All*
Matroshka	*Radiation*
Microgravity Sciences Glovebox	*Biotechnology*
Modular Cultivation System (EMCS)	*Cell; Gravitational; Development*
Plant Research Unit (PRU)	*Gravitational; Development*
Small Centrifuge	*Gravitational; Development; CELSS*
Space Station Incubator	*Cell; Biotechnology*
2.5-m Centrifuge	*Gravitational, Development; CELSS*
X-Ray Crystallography Facility	*Biotechnology; Gravitational*

Table 3-02. Equipment dedicated for space life sciences research on board the ISS after assembly phase is complete, and the corresponding research areas.

Table 3-02 lists the experimental facilities that will fit inside the science laboratories of the ISS, and the research area concerned by this equipment. Some of these facilities, along with their pictures, are detailed in the Section 3 of this Chapter. A list of Internet websites describing these

facilities in more details is also provided at the end of the References list (this Chapter, Section 5).

There are severe limitations for operating science facilites in space. For example, there is a minimum storage period of 5-6 days before starting an ISS experiment, since the Shuttle has to travel to (2 days) and dock with the ISS after which the experiment must be transferred to the ISS facility. The experiment can then stay on board the ISS for the duration of one or several increments. After the last increment, the samples will be transferred back into the Space Shuttle for a minimum of 5-6 days, and then returned to Earth, where they will be made available to the scientists approximately 3 to 5 hours after landing. Samples and specimens can also be transferred to and from the ISS using the Soyuz "Taxi" flights.

Today, the two-person crew allowed on board the ISS is so small that the astronauts spend the vast majority of their time on maintenance, leaving little room in their schedule for actual experiments. Once completed, the ISS will house an international crew of up to seven for stays of approximately three months. Emergency crew-return vehicles will always be docked with the ISS while it is inhabited, to assure the return of all crewmembers.

1.2 Ground-Based Simulations

All organisms evolved on Earth under a constant 1-g gravitational force. To determine the gravity sensitivity of multiple physiological systems, the scientific method dictates exposing species to gravity levels both above and below 1 g, and to study the resultant effects. Centrifuges are used to increase gravitational forces on Earth. A number of models *simulating* various aspects of decreased gravity have been designed and developed.

For example, the effects of microgravity may be simulated by removing the gravitational load on a particular portion of the body. *Bed rest* is the most commonly used method for simulating microgravity when the research subjects are humans or non-human primates. Studies of muscle and bone atrophy are sometimes conducted using this method. *Tail suspension* is also used to simulate microgravity in rats: the gravitational load to the hind limbs is eliminated by suspending rats by their tails, leaving them free to move about on their forelimbs. Horizontal, rotating *clinostats* that apply a constantly changing vector acceleration force canceling out the vector force of gravity are often used to simulate microgravity in plants (Figure 3-06).

However, these systems minimize the effects of gravity, but gravity cannot be reduced on Earth. Although the models are not perfect, they allow faster accumulation of data under more ideal conditions than is currently possible during spaceflight. Some studies are also carried out on Earth in order to obtain pilot data for flight experiments or to verify the results of flight experiments. These models are useful not only for predicting adaptation to spaceflight and readaptation upon reentry to Earth but also for studying

countermeasures and for increasing our understanding of the role of fluid distribution, mechanical loading, and posture (horizontal vs vertical) in normal physiology (Morey-Holton 2004).

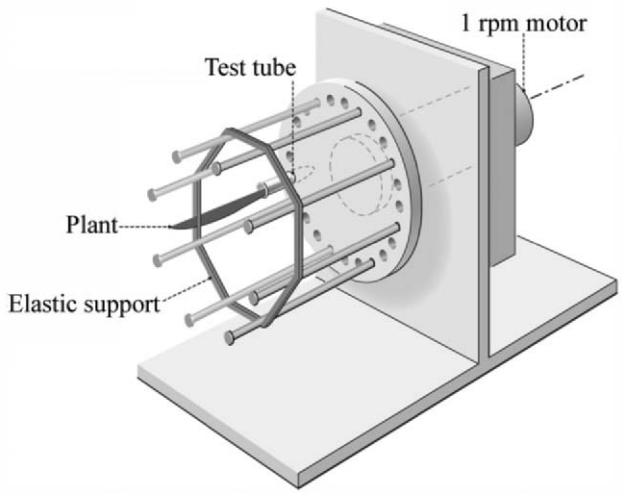

Figure 3-06. A do-it-yourself clinostat. Single-axis clinostats are used for cells, small specimens, and plants. The radius from the center of rotation to the specimen and the rotation rate must be adequate so that the forces generated are not be perceived by the specimen as a gravity stimulus. The single-axis clinostat continuously changes the direction of the gravity vector so that orientation to a specific direction becomes impossible.

1.2.1 Clinostat and Bioreactor

On Earth, plant scientists have conducted many experiments to try to unravel the apparently simple responses of plants to the Earth's gravity. The basic problem is how to modify the amplitude of gravity so that developmental responses under different gravitational force levels can be studied. On of the earliest experimental devices for approaching this problem was to rotate material as Knight (1806) has done, but oriented horizontally on the periphery of a disc rotating in a vertical plane (see Figure 1-19C). The apparatus is called a *clinostat*, and the objective is to compensate the effect of the directional component of the gravitational force vector by having each part of the plant experience a multidirectional gravitational pull.

Plant organs can perceive small changes in orientation of the gravity vector. The length of time required to detect a response to a change in orientation is called the presentation time and ranges from 0.3 to 10+ minutes. The clinostat continuously changes the direction of the gravity field and the plant does not have sufficient time to respond to a change in direction. It is important that the number of revolutions per minute is great enough to assure that the gravitational force exerted upon the tissue from any direction is not sustained for long enough to permit an asymmetric growth response. As the disk in Figure 1-19C revolves, the plant will also rotate, causing the gravitational force vector to move relative to the plant with its direction always perpendicular to the long axis of the plant, but continually progressing

around it. In this way, a small seedling originally placed horizontally will continue to grow in a horizontal direction despite that its long axis is oriented at right angles to the gravitational force vector (Figure 3-06). If the rotational rate is too slow, however, the seedling will again show gravity-evoked bending of root and shoot towards its preferred positions.

It is clear that once a seedling has developed beyond the state of a single root and shoot and produces lateral roots, leaves or branches, a simple clinostat can no longer provide directional compensation of the force field to all parts of the plant. It is for this reason that some very sophisticated tumbling clinostats have been developed in attempts to maintain the fully compensated condition in growing plants over prolonged periods of time. A *three-dimensional clinostat*, for example, is a combination of two or three clinostats that keeps the specimen tumbling in the middle (Figure 3-07). Also, the fast clinostat, which rotates anywhere from 55-120 rpm, can be very useful for very small systems, such as a few cells, because it prevents the internal components of the cell from settling out or continually tumbling about. However, the organism must be kept in the very center of the clinostat stage, or there will be centrifugal forces. Some scientists have also used a tilted clinostat. If the clinostat is at a slight angle off horizontal, say at 9°, that will create roughly 1/6 g or 0.18 g, which is the level of gravity on the Moon. If it's tilted at about 17.5 deg, that will give 1/3 g, the level of gravity on Mars (Morey-Holton 2004).

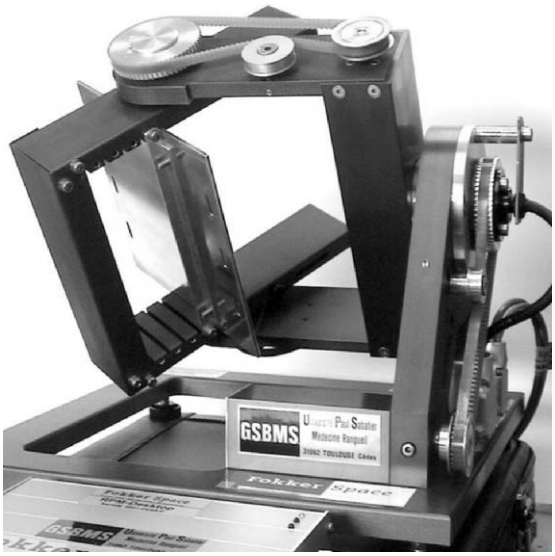

Figure 3-07. The three-dimensional clinostat, also called Random Positioning Machine (RPM), creates a condition in which the weight vector is continually reoriented in all directions. Photo courtesy of Gilbert Gasset, GSBMS, Toulouse.

As seen above, botanists have used the slow clinostat for many years to study mechanisms of gravity perception. Recently animal investigators have begun to use the clinostat to study possible gravity responsiveness in embryonic structures. Clinostats have also been used for cultured cells: these

are called *rotating wall vessels* or *bioreactors* (see Figure 4-12). These devices are also based on the concept that the g vector must act for at least a few seconds in a constant direction in order to generate an effect in cells. The bioreactor allows cells or bacteria to be cultured in a continuous free-fall state, simulating microgravity and providing a unique cell culture environment on the ground. Shaped like a cylinder, the growth medium–filled cylindrical vessel rotates about a horizontal axis, suspending the cells in a low-shear[1] culturing environment. Specimens in the bioreactor never hit bottom, they hang suspended in their liquid growth medium, much as they would in Earth's orbit. This allows for cell aggregation, differentiation, and growth.

The bioreactor provides a low-turbulence culture environment that promotes the formation of large, three-dimensional cell clusters. Cell constructs grown in the bioreactor more closely resemble tumors or tissues found in the body. Cell constructs grown in a rotating bioreactor on Earth eventually become too large to stay suspended in the nutrient medium. In the microgravity of orbit, however, the cells can stay suspended. But because there is a liquid medium, streaming during slow rotation generates Coriolis forces that may confound the experiment results. Also, coexisting objects of varying density will not be equally balanced within a rotating system as they are in a state of true freefall. The parameters affecting sedimentation and buoyancy differ for a bioreactor and actual free fall (Klaus 2001). These differences may contribute or be responsible for the altered behavior of biological systems observed to occur in space or under clinorotation.

Clinostats therefore provide "gravity compensation", not "zero gravity". Even the best clinostat technology on Earth cannot provide a facility to either investigate if there is an absolute dependence of plants or cells upon a gravitational force, or determine with certainty the mechanisms by which this force is perceived and responded to. In fact, experiments of 20-day clinostat rotation have identified problems with this method, indicating that the clinostat is a questionable simulator of weightlessness for long-duration studies. Some experiments on plant development showed that inflight plants did not appear significantly different from clinostat controls but the flight specimens took several hours to revert to normal gravitropism, whereas the Earth-clinostat controls rebounded immediately when removed from the rotating clinostat. Furthermore, clinostat rotation increased nuclear volume in some wheat seedling roots, but did not duplicate flight results at the cellular level. These results suggest that some spaceflight effects can be predicted with clinorotation, while others cannot.

For these reasons, many critical experiments have remained undone, and the ISS now offers the opportunity for tests in the 0-g environment with the proper inflight controls.

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[1] *Shear* is the force caused by the cells sliding against one another.

Figure 3-08. The ground-based gondola centrifuge used in the University Paul Sabatier in Toulouse to assess the long-term effects of microgravity on mice development. Four groups of animals, born and raised under conditions of normal gravity, are placed inside the gondolas to live for 1-2 months under 2 g. The light/dark cycle is provided inside the box. Food and water is available at will. However, the centrifuge rotation is stopped briefly every other day for housekeeping and maintenance. Photo courtesy of Gilbert Gasset, Groupement Scientifique en Biologie et Médecine Spatiales, Toulouse.

1.2.2 Centrifuge

On Earth, centrifuges have been used to obtain effective g values greater than unity for relatively short periods. Radial acceleration produced by a centrifuge induces a force that is superimposed upon Earth's gravitational force. The two forces interact to give a net resultant vector, whose amplitude is larger than 1 g, and whose direction is tilted relative to gravity. When centrifuges are used in space, the Earth's gravitational force is counterbalanced by free fall, allowing centrifugation to produce a radial acceleration unencumbered with another directional force.

All centrifuges have a drive system connected to a center spindle with a rigid rotating arm. The length of the centrifuge arm varies from a few cm to several meters. The longer the centrifuge arms, the slower the rotation rate required to achieve a given g level. Gravity gradients are a significant design driver in determining arm length for centrifuges. Some centrifuges have the capability of placing cages at various positions along the arm to achieve multiple gravity levels in a single experiment. Other centrifuges have the cage or gondola located at the end of the arm. The position of the cage or gondola along the arm determines the rate of rotation required to achieve a specific gravity level. The gondola or cages on centrifuges usually rotate freely in the roll axis to maintain the resulting gravity vector perpendicular to the cage's floor, which is the normal gravity direction (Figure 3-08).

A confounding factor in using centrifuge is the presence of Coriolis forces when the animals or the samples move out of the plane of rotation. In

addition, centrifugation introduces unwanted inertial shear forces[2] to the sample. Depending on the centrifuge and the geometry of the experiment hardware used, these shear forces contribute significantly to the total force acting on the cells or tissues. For example, in a typical ISS plant research facility, like the *European Modular Cultivation System* (EMCS), the radius of centrifugation in the center of the experiment container is 200 mm. When we consider *Arabidopsis thaliana* grown in this facility, the gravity variation over an adult plant is 0.6-1.43 g while the lateral inertial shear force ranges from 0 to 0.153 g. When such a structurally unbalanced plant is transferred from a microgravity environment into a centrifuge to study subsequent gravitropic responses the plant will, besides gravity, experience a lateral shear force within its structure. A small deviation of the stem or leaves from an exact alignment along the line of radial acceleration will result in forces generated within the plant that are different from that on Earth. It might be expected that this will be partially, or fully, compensated by the plant's active internal gravitropic response, but this is a completely different and more complex field of forces and responses compared to the on-ground situation. This makes the interpretation of the effect of "gravity" on a plant in such a system very difficult (van Loon et al. 2003).

The relative influence of inertial shear force may be limited by using large radii centrifuges such as the Centrifuge Accommodation Module, as is currently foreseen for the ISS.

1.2.3 Muscle Unloading

The rodent model of hind limb unloading has been successfully used to simulate some of the effects of spaceflight conditions, especially on muscle, bone, and renal responses (see Figure 5-22, left). The hind limb unloading system seems adequate for developmental studies since rats gain weight similar to controls (as long as controls are fed daily with the same mean food consumption of the experimental rats) and adult rats maintain their body mass.

Comparison of hind limb unloading data with flight suggests that responses during the first week of unloading, whether on Earth or in space, are very similar, but continued unloading on Earth appears to return to normal levels within two weeks while spaceflight may require longer for adaptation (the same observation hold true for experiments with plants on clinostats). Total musculoskeletal unloading occurs during flight, but only partial

[2] Shear forces can be brought about by inertia (inertial shear) and/or fluid flow (fluid shear). In cells both fluid shear stress and inertial shear stress will generate cell deformation, i.e., strain. In centrifuges an essential difference between inertial shear force and the force of gravity is that inertial shear acts perpendicular to the gravity acceleration vector.

unloading is possible in the ground model as the humerus, cervical vertebra, and skull continued to bear some weight. In fact, the load-bearing front limbs serve as an internal control for the ground experiments, but these limbs are unloaded during spaceflight. Also, the stress of reentry following spaceflight and the time delay between reentry (with reloading) and sampling of flight specimens can create difficulty in comparing data with model experiments; tissues can be taken from animals on the model without a time delay at the end of an experiment (Morey-Holton and Globus 2002).

2 ISSUES IN CONDUCTING SPACE BIOLOGY EXPERIMENTS

Space life sciences research is critical to preparing for the eventuality of long-term space exploration. Along the way, this same research increases our knowledge of basic biological processes and provides insight into the mechanisms and treatment of various medical conditions on Earth. However, these scientific results are not achieved easily. The study of living organisms in space poses many challenges that may be negligible or nonexistent in ground-based research. The following considerations were extracted from a review by Souza et al. (2000).

2.1 Choice of Species

Spaceflight imposes several unique operational constraints that must be addressed in addition to scientific selection criteria. The size, weight, and ease of maintenance of an organism, and the availability of flight-qualified support hardware are issues that become more central when conducting life sciences research in space rather than on the ground.

Species are often selected on the basis of their capacity to undergo some physiological adaptation process or life cycle stage within a short period of time. For example, Japanese red-bellied newts were selected for experiments on the Second International Microgravity Laboratory (IML-2) payload because their vestibular systems would undergo most of their development within the planned duration of the Shuttle flight. Other organisms are chosen because they are resilient and can be easily cared for in an automated setting where food, water, and appropriate environmental conditions can be provided but where human caretakers may not be available. Some, such as rats of the Wistar strain, are valuable research subjects not only because of their genetic homogeneity, but also because of their extensive use in research makes them a known quantity. Furthermore, their small size and ease of maintenance allows them to be flown in relatively large numbers in the limited space available in a spacecraft. Finally, their genetic similarity throughout the strain allows for statistical significance with a small sample (Borkowski et al. 1996).

2.2 Loading and Retrieval

Space life sciences experiments often require that research subjects be installed in the spacecraft in a precisely timed manner. For instance, if germination of plant seeds is to occur in space, or embryos are to undergo a particular stage of cell division, they must be in a specific stage of development at the time of launch. If the launch is delayed because of inclement weather or a system malfunction, research subjects frequently must be unloaded from the spacecraft and a fresh group of subjects installed once a new launch time is set. To accommodate such an eventuality, researchers must have several backup subject groups, in varying stages of development, prepared for flight.

In order to prepare the spacecraft itself for launch, all payloads, including those accommodating live research subjects, must be integrated into the spacecraft as early as several months before launch. Only critical items, such as the subjects themselves, can be loaded up to several hours prior to launch. Installation of habitats with living organisms may require special handling, depending on the structure and orientation of the spacecraft. Installation of research subjects into the Space Shuttle, which is oriented vertically during the prelaunch period, can involve lowering the organisms in their hardware units through a tunnel into the holding racks in the Spacelab or SpaceHab (Figure 3-09).

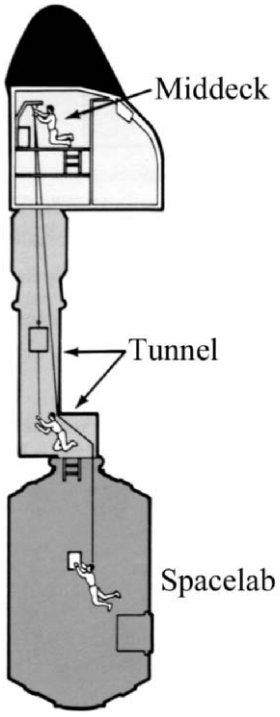

Figure 3-09. Biological specimens and perishable items may be loaded in the Space Shuttle middeck or pressurized module only a few hours before launch. When it is necessary to install scientific samples and specimens in the module, it can be entered through a vertical access kit. Source ESA.

Because organisms begin to readapt to Earth's gravity immediately upon landing, dissection and tissue preservation in orbit or quick access postflight is critical to the value of the science. Organisms can be removed from manned spacecraft such as the Space Shuttle within a few hours after touching down. Removal from the unmanned Foton Biosatellite occurs several hours postflight because mission personnel must first locate, and then travel to, the landing site. Transport from the spacecraft to ground laboratories may be time-consuming when the biosatellite lands some distance away from Moscow. In such instances, a temporary field laboratory is set up at the landing site to allow scientists to examine the subjects before readaptation occurs. The issue of postflight readaptation highlights the value of inflight data and tissue collection.

2.3 Control Groups

Space biology experiments make extensive use of control groups in part because limited flight opportunities may not allow for replication of a given experiment. Employing control groups is essential to increase the statistical validity of the results of an experiment with a relatively small number of subjects in the experiment group. Control groups help researchers isolate the effects of microgravity and the vibration, acceleration, and noise of spacecraft launch and landing from the effects of other conditions that research subjects may encounter inflight, such as altered environmental conditions, and the stress that can be associated with confinement, isolation, implantation of sensors, and biosampling procedures.

Several types of control groups are often employed in space biology experiments. The *synchronous* control consists of organisms that are identical in type and number to those flown on board the spacecraft. They are housed in identical habitats and kept within a simulated spacecraft environment in a ground laboratory. Conditions within the simulated spacecraft environment, such as humidity and temperature, are set to levels expected to occur within the actual spacecraft during flight. The synchronous control procedures last for a period identical to that of the flight. This control is used to determine whether the effects that may be seen in the flight organisms are the result of anomalous environmental conditions, such as increased temperature, that may have occurred during the flight. Due to time or resource constraints, the synchronous control may be delayed in time compared to the actual spaceflight. This *asynchronous* control is then similar to the synchronous control except that procedures begin several hours or days after the flight. For the asynchronous and delayed synchronous controls, conditions within the simulated spacecraft environment are identical to those that prevailed within the actual spacecraft throughout the flight.

A *vivarium* control is usually conducted to determine whether effects that may be seen in the flight organisms could be due to the stress of being

confined or isolated or of being housed in flight hardware units. In this control, a group of organisms similar to the flight group is housed in standard laboratory conditions for duration identical to the length of the flight.

Important inflight controls include the use of *onboard 1-g centrifuges*. Some experimental set-ups also include an on-ground control centrifuge. Scientists have indeed observed some differences between samples on an inflight centrifuge and non-rotated specimens (Schmitt et al. 1996). Although these differences might be resulting from launch effects, cosmic radiation or a pre-exposure of inflight centrifuge samples to microgravity, it is also possible that centrifuge inertial shear artifacts might have caused these differences (see this Chapter, Section 1.2.2). The relative role of the inertial shear forces can be evaluated by comparing the results between the inflight 1-g and the on-ground (1.41-g) centrifuges since, due to Earth's gravity, the on-ground centrifuge generated higher shear accelerations compared to the inflight centrifuge.

Additional controls may be conducted as indicated by specific research concerns. For instance, when the flight research subjects are mammals implanted with biosensors, a control group of similar animals without implanted sensors may be studied to determine whether any effects observed could be the result of the implants.

Figure 3-10. Cosmonaut Salizhan S. Sharipov, Expedition-10 flight, prepares to set up the European-built "Kubik" biological incubator in the Zvezda Service Module of the International Space Station. Photo courtesy of NASA.

3 SPACE BIOLOGY FACILITIES

The ISS will include multiple habitats to support a variety of organisms, a centrifuge with a selectable rotation rate to house specimen habitats at a variety of gravity levels, and a fully equipped workstation/glovebox. The laboratory will also provide microscopes, freezers, and other research equipment to conduct experimental procedures.

3.1 Cell Biology Facilities

The ESA *Biopack* facility, which flew during NASA-Mir and Spacelab missions, was primarily designed to carry out automated biology and biotechnology experiments. *Biopack* included an incubator with three centrifuges (300 mm diameter; 0.001 g to 2 g), a cooler, and a freezer. Occupying about the volume of two Space Shuttle middeck lockers, the *Biopack* was designed to accommodate small biological samples, e.g., mammalian cell and tissue cultures, small plants or insects. This device was later reconfigured as a portable incubator, named *Kubik*, for flying on the Soyuz "Taxi" missions to the ISS (Figure 3-10).

Biolab is a facility that will be on board the European *Columbus Orbital Facility* (see Figure 4-02). This double rack is designed for continuation of space research on cell cultures, unicellular organisms, plants and small animals, as earlier flown on ESA's *Biorack* on board Spacelab, now with larger specimens and longer duration. In contrast to earlier incubators like *Biorack* or *Biobox,* which were only temperature-controlled, *Biolab* provides a fully controlled life support system for biological specimens. *Biolab*'s major capability includes cell culture, stowage, automated sample processing, and imaging. It also hosts two 1-g control centrifuges (600 mm diameter; 0.001 g to 2 g), six experiment containers, with several container designs for accommodating a large variety of specific experiments. Research objectives include studies of regulatory mechanisms of proliferation and differentiation, the role of the cytoskeleton, mechanical loading, graviperception and thresholds, mechanisms underlying radiation damage, and repair mechanisms in cells and tissues.

The *Biotechnology Mammalian Tissue Culture Facility* (BMTC) is a new facility for tissue engineering being considered by ESA for research on cell and tissue culture on board the ISS. The core of the system will provide robust control of concentration gradients and of mechanical forces thanks to the integration of fluid distribution tools, microsensors, and microactuators.

The *Cell Biology Experiment Facility* (CBEF) will provide a controlled environment for fundamental life science research in space using cells, tissues, small animals, plants, or microorganisms. The CBEF will be equipped with a centrifuge providing variable gravity for reference

experiments at g levels from 0.1 to 2 g. This is a JAXA Space Station Facility that will be located in the Japanese Experiment Module *Kibo*.

The NASA *Cell Culture Unit* (CCU) will be used to support basic research in cell and tissue biology on board the ISS. The CCU will have the capability to grow and maintain animal, microbial, and plant suspension cultures, attachment cultures, tissues less than 4 mm in length, and non-feeding aquatic specimens. The CCU allows automated sampling and video microscopy. It can also be mounted on the 2.5-m diameter centrifuge in the Centrifuge Accommodation Module (see Section 3.4.1 of this Chapter).

The *Space Station Incubator* can house specimens for up to 135 days on orbit or can be used for short-term events such as heat shock. The temperature can be adjusted at any time during the 135-day increment.

The NASA *Biotechnology Research Facility* (BRF) is the primary scientific facility for conducting mammalian cell culture, tissue engineering, biochemical separations and protein crystal growth on ISS (Figure 3-11). The BRF consists of one rack that provides support services for a variety of sub-rack payload experiments developed by investigators. Facility services include power, thermal management, video signal switching and processing, distribution of research quality gases and bulk 37° C incubation. The BRF will provide a centralized command and data-handling interface to the Space Station, as well as some data and video storage.

Figure 3-11. Astronauts participating in a biotechnology experiment on water treatment system during the STS-111 Shuttle mission. Photo courtesy of NASA.

Future projects for cell biology research in space seek beyond the basic culture capabilities. Scientists and engineers are searching for ways to mix solutions and add these solutions to the cell cultures, image cells with microscope, and measure cell responses to microgravity *as they grow*. Of great interest would be to count cells (with cytometer and fluorescently activated cell sorter) or measure gene expression.

Pertinent to the field of Radiation Biology, the *Matroshka* human phantom body is composed of various tissue substitutes simulating the human body in terms of size, shape, orientation, mass density, and nuclear interactions. At the sites of the body organs of interest, spaces are provided at the surface and at different depths within the phantom to accommodate dosimeter packages to measure any ionization levels. Matroshka is affixed to the exterior of the ISS (see Figure 7-01). Dosimeters are either active (e.g., solid-state nuclear track detectors, radiation-sensitive crystals, polymers) and are returned periodically to the ground, or active (e.g., silicon detectors) and connected to a computer for online sensor data processing.

3.2 Animal Research Facilities

Taking animals into space requires special considerations. For mice, the traditional aquarium-style cages don't provide enough traction for the animals to walk around. Instead, space mice have wire mesh cages so their toes can grip a rougher surface. Wood chips couldn't be used for bedding; they wouldn't stay in place. Gravity-feed water bottles wouldn't work; pressurized water containers are needed instead. Bowls of dry food aren't practical, so compressed food bars are provided instead. As for how to clean the cages, a special waste containment system has been created to keep everything in its place.

Suitable habitats and adequate life support systems for each research subject are essential for experiment success. Hardware to support living organisms is designed to accommodate the conditions of spaceflight, but microgravity poses special engineering challenges. Fluids behave differently in microgravity. The relative importance of physical properties such as surface tension increases, and convective air currents are absent or reduced (see Chapter 1, Section 2.2). Plants are usually flown attached to a substrate so that nutrients and water can be provided through the root system. Cultured cells are flown in suspensions of renewable media contained within specialized hardware units. Nonhuman primates are often flown in comfortable confinement systems to prevent them from endangering themselves during launch and reentry or damaging sensors or instrumentation during the flight. Other organisms such as rodents are typically flown without confinement so they can float freely within their habitats while in the microgravity environment. With the use of implanted biotelemetry hardware, however, small primates can be flown unconfined.

The comfort and safety of research subjects is a high priority. Because trauma or stress can compromise experiment results, humane care and good science go hand in hand. Animals may be singly or group-housed, but group-housed animals tend to remain healthier and exhibit fewer signs of stress. For nonhuman primates, environmental enrichment is provided in the form of behavioral tasks or "computer games", which can double as measures of behavior and performance. Such enrichment helps to prevent stress and boredom, a possible result of confinement and isolation.

Light within habitats is usually regulated so as to provide a day/night cycle similar to that on Earth. Air circulation and heating or cooling ensures that temperature and humidity are maintained at comfortable levels. Food is provided according to the needs of the species in question and the requirements of the experiments. Generally, a continuous water supply is available. Waste material, which includes not only excreta, but also particulate matter shed from the skin and debris generated during feeding activities, is eliminated using airflow systems engineered for the purpose (Souza et al. 2000).

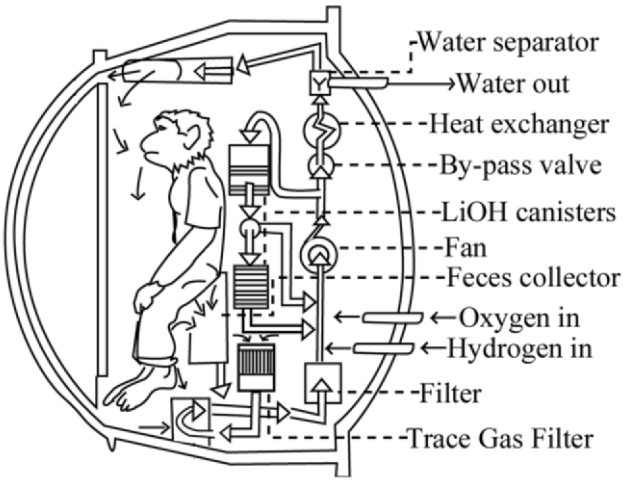

Figure 3-12. Drawing showing the life support system for a rhesus monkey during a flight on board the Russian Cosmos-Bion unmanned biosatellite. Adapted from Souza et al. (2000).

3.2.1 Primate Habitats

Between 1975 and 1990, NASA participated in seven missions flown on board the Russian Cosmos-Bion spacecraft. Five of these biosatellite missions have been dedicated to research with rhesus monkeys lasting up to 14 days. All ten monkeys from these Bion missions were recovered from orbit. The animals were housed in two capsules within the spacecraft's landing module (see Figure 3-03). The capsules, each containing life support and experiment equipment, were oriented within the spacecraft so that the

monkeys could view each other. Couches inside the capsules supported and confined the monkeys and provided adequate cushioning when the capsule impacted the ground at landing. A lightweight bib prevented the monkeys from disengaging leads emerging from the implanted sensors. Unidirectional airflow moved excreta toward a centrifugal collector beneath each couch. Monkeys could obtain juice and food, in paste form, from dispensers located in each capsule by biting on switches in the delivery tubes. Primate access to the dispensers could be controlled remotely from the ground. A video camera in each capsule monitored animal behavior during flight (Figure 3-12). The last flights with rhesus monkeys, Bion-11, took place in 1996. One monkey died the day after the capsule recovery during his post-landing medical operation and checkup. This death raised new questions regarding the ethics of using primate animals for research, and NASA dropped out of participation in a planned Bion-12 mission. Also, the cost of purchasing a monkey, its housing and care, and the training and associated hardware made monkey flights almost as complex and costly as human experimentation.

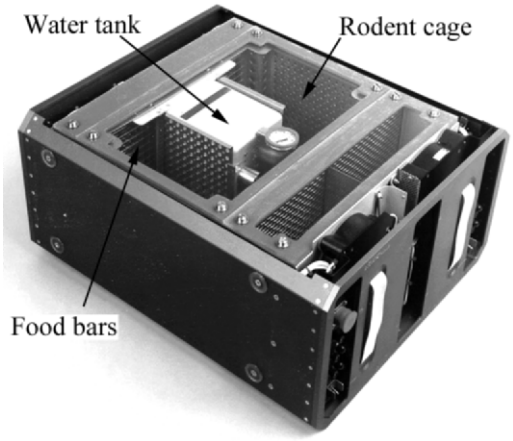

Figure 3-13. The Animal Enclosure Module (AEM) is a rodent housing facility that supports up to six 250-g rats. The unit fits inside a standard Shuttle middeck locker with a modified locker door. A removable divider plate can provide two separate animal holding areas. The AEM remains in the stowage locker during launch and landing. On orbit, the AEM may be removed partway from the locker and the interior viewed or photographed through a Lexan cover on the top of the unit. Photo courtesy of NASA.

3.2.2 Mice and Rat Habitats

In Shuttle and SpaceHab, rats can be housed in two habitat types: the *Animal Enclosure Module* (AEM) and the *Research Animal Holding Facility* (RAHF).

The AEM is a self-contained animal habitat, storable in a Shuttle middeck locker, which provides ventilation, lighting, food, and water for a maximum of six adult rats (Figure 3-13). Fans inside the AEM circulate air through the cage, passively controlling the temperature. A filtering system controls waste products and odors. Although the AEM does not allow handling of contained animals, a clear plastic window on the top of the unit permits viewing or video recording.

The RAHF was a general use animal habitat designed for the Spacelab or SpaceHab modules. Animal-specific cage modules were inserted, as needed, to provide appropriate life support for rodents (Figure 3-14). Cages could be removed from the RAHF to accommodate inflight experiment procedures. Each cage assemblies carried two rats separated by a divider. Life support systems ensured environmental control, delivery of food bars and water, and waste management. Activity monitors in each cage recorded general movement using an infrared light source and sensor. However, a basic life support system such as RAHF appeared insufficient for maintaining rodents on long-duration flights. The need for a control 1-g centrifuge and a programmed waste collection system (collection and preservation at discrete time intervals) becomes particularly significant in long-duration flights.

On board the ISS, the *Advanced Animal Habitats* (AAH) will provide a research environment for laboratory rats and mice in orbit for up to 90 days. The AAH is internally modularized so that it can be reconfigured for a wide range of rodent experiments to accommodate mice in all stages of their life cycle (pregnancy, birth, nursing, post-weaning, and adult), and rats from weanlings (neonates once separated from their mothers) to adults.

ESA is also developing a design concept for a facility able to support experimentation with mice on board the ISS. The so-called *MISS* facility is composed of two main parts: a rack dedicated to the habitat and the scientific equipment, and a container to transport the animals from ground to the MISS rack in orbit and vice-versa. A total of 30 mice with a mean weight of 30 grams/animal could be housed inside the MISS rack for a period of up to 3 months, while 10 mice could be transported within each container.

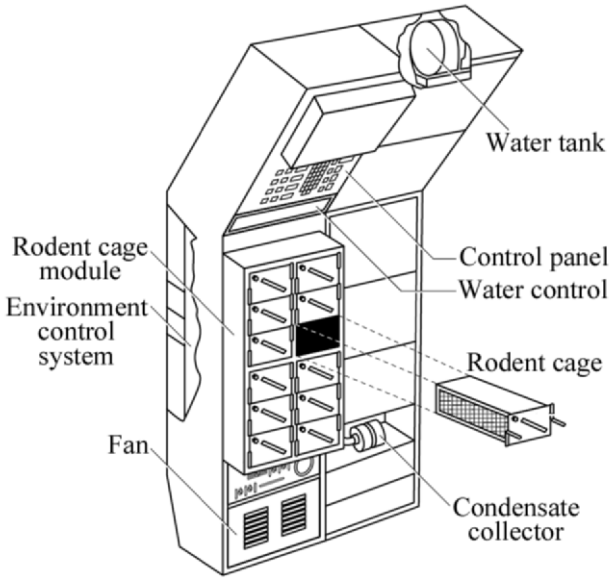

Figure 3-14. The Research Animal Holding Facility (RAHF) was an animal habitat for general use within the Spacelab module. Animal-specific cages were inserted, as needed, to provide appropriate life support for rodents. Cages could be removed from the RAHF to allow inflight experiment procedures to be conducted. Adapted from Souza et al. (2000).

3.2.3 Aquatic Habitats

It has been realized since the early days of space biology that aquatic organisms were prime candidates for research in gravitational biology. During the third Skylab mission and then the Apollo-Soyuz Test Project, hundreds of killifish were sent into space in plastic bags filled with water and oxygen. High survival rates were obtained using that simple method (Baumgarten et al. 1975, Scheld et al. 1976, Hoffman et al. 1977). Killifish and guppy fish flew on board the Russian biosatellites Cosmos-782 and -1514 using the same technique, with the amazing result of the fish swimming with their back always turned towards the oxygen bubble (Krasnov 1977, Gazenko and Illyin 1984).

With the onset of the Space Shuttle era, new technologies could be implemented and thus new hardware development occurred based on the science requirements. The first pressurized container, called STATEX (from STATolith EXperiment) flew on the Spacelab D1 German mission. It allowed the investigation *Xenopus* tadpoles (Neubert et al. 1983). Inside the pressurized container was a control centrifuge and additional room for experiment-specific hardware. Both the centrifuge and the experiments could be equipped with small water tanks, like Petri dishes, with a bottom biofoil providing gas/oxygen transfer to the water. A modified STATEX container next flew on board the Space Shuttle Columbia in 1993, where both tadpoles and cichlid fish larvae were investigated (Neubert et al. 1991) (Figure 3-15).

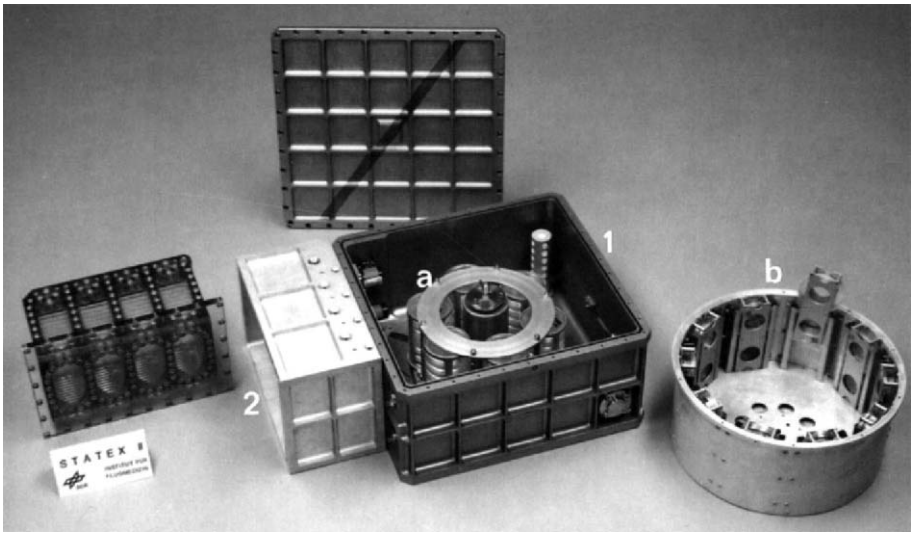

Figure 3-15. Photograph of the STATEX-II hardware. 1: Main container with reference centrifuge (b) and the microgravity stacks (a). 2: Observation and fixation unit. Photo courtesy of Institute of Aerospace Medicine, DLR, Germany.

A more sophisticated hardware for aquatic specimens, the *Aquatic Research Facility* (ARF), was then developed by the Canadian Space Agency (Figure 3-16). In contrast to STATEX, which was bound to fly within Spacelab, the ARF fits in a standard Space Shuttle middeck locker and can therefore be used during more flight opportunities. Embryonic starfish and other aquatic animals have flown in this facility, and many more species should fly in the future, including a series of experiments on tadpole development (Snetkova et al. 1995). The life support system is also based on biofoil.

Another aquatic research, known as the *Aquatic Animal Experiment Unit* (AAEU), was developed by Japan for the Spacelab-J and IML-2 missions. The AAEU provided larger volumes for animal habitats, as well as an artificial lung and an automatic feeding system. A major step in studies on developmental biology in space was achieved when the first mating of Medaka fish in space occurred in this facility on board IML-2 (see Figures 2-12 and 5-02) (Ijiri 1995). The AAEU also hosted investigations on Japanese koyfish during both orbital and parabolic flights (Mori et al. 1994).

The second generation of the AAEU is the *Vestibular Function Experiment Unit* (VFEU), developed by NASDA for the Neurolab mission in April 1998. It also flew on a subsequent Shuttle mission, carrying two marine Oyster toadfish as experiment subjects (see Figure 2-05). Housed in the VFEU, the fish were electronically monitored to determine the effect of gravitational changes on their balance system. The electrophysiological activity of the otolith nerves of freely moving fish was recorded through a specially designed array of implanted electrodes. Measurements of afferent and efferent activity were made before, during, and after the flight.

Figure 3-16. Canadian astronaut Marc Garneau performs a status check on the Canadian Aquatic Research Facility (ARF) during the STS-77 Space Shuttle mission in May 1996. Photo courtesy of the Canadian Space Agency (CSA).

The systems described above supported animal life support by either passive oxygen transfer via biofoil or, in case of the AAEU and VFEU, by active transfer using a water purification system and an artificial lung. Another approach is to create a life support system which resembles the biosphere used naturally on Earth. Basic research into bio-regenerative life support systems in aquatic habitat was executed at the University of Bochum, Germany. A miniaturized version of their full-scale *Closed Equilibrated Biological Aquatic System* (CEBAS) was then developed for space conditions under contract of the Germany Aerospace Center DLR (Blüm et al. 1994).

The CEBAS minimodule is able to house aquatic species in a large volume of water (8.6 liter), either in different compartments or as a community, in a closed ecological system. Hornweed plants are used for oxygen production, a biofilter allows for water recycling, and fish and snails evolving freely in this environment can be used as experimental research subjects (Figure 3-17).

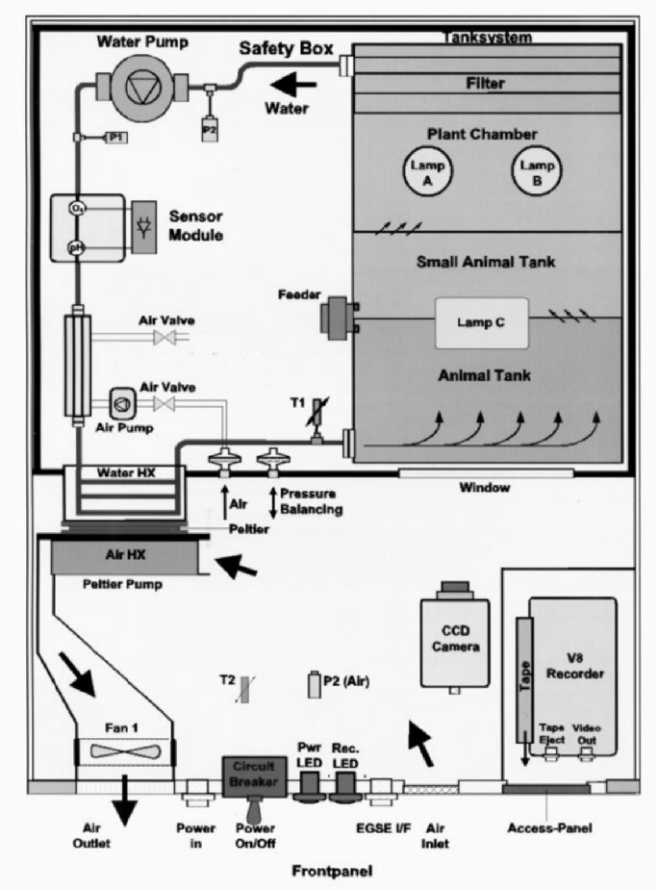

Figure 3-17. Diagram showing the architecture of the CEBAS (Closed Equilibrated Biological Aquatic System) Mini-module.

The CEBAS minimodule flew twice in 1998 on board the STS-89 and STS-90 missions, with adult swordtail fish, newborn swordtail fish, and several pond snails. A CEBAS with cichlid fish flew also on board the last flight of the Space Shuttle Columbia (STS-107) in January 2003, for an investigation on the development of the fish otoliths in microgravity.

A video subsystem enabled the scientists from various disciplines, including neurobiology, develop-mental biology, and bone physiology, to view the animals and analyze their behavior. The CEBAS Minimodule allowed the first investigations of eco-physiological research under space conditions in an almost complete self-sustaining mode. Indeed, only light and food (although in small amounts) were provided to the plants and animals.

New, state-of-the-art hardware is being developed for the ISS, enabling much longer exposure to microgravity. The *Aquatic Animal Experiment Facility* (AAEF) will accommodate freshwater and saltwater organisms in microgravity on board the ISS. The facility will be designed to accommodate experiments for up to 90 days, making it possible to conduct research ranging from early development and differentiation to individual responses in the microgravity environment. Access to the Centrifuge Accommodation Facility will provide the onboard 1-g controls, as well as acceleration forces from 0 to 2 g to identify the gravity response threshold for particular cellular and physiological processes. This JAXA Space Station facility will be located in the Japanese Experiment Module.

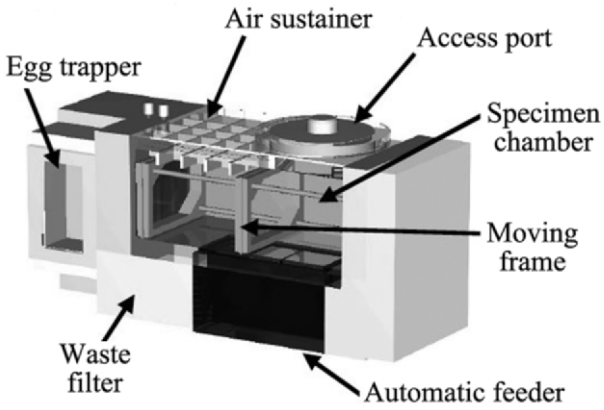

Figure 3-18. Schematic drawing of the Aquatic Habitat (AQH). The access port is for specimen sampling and the moving frame is for inner wall cleaning. The egg trapper is for egg collection by water flow. Photo courtesy of JAXA.

The *Aquatic Habitat* (AQH) will accommodate both freshwater and marine organisms Three-generations of small freshwater fish (killifish *Medaka* and zebrafish *Dario*), and egg through metamorphosis of amphibians (*Xenopus*) could be experimented by AQH. Invertebrate organisms, such as sea urchins and snails, and aquatic plants species will eventually be supported by this habitat. Various experimental functions such as automatic feeding, air-water interface, day/night cycle, video observation, and specimen sampling

mechanism will be also available (Figure 3-18). The water circulation system was improved from the past aquatic facilities for Space Shuttle experiments under the consideration of the long life-time, and a brand-new specimen chamber was developed to equip the above various experimental functions.

ESA's *Biolab* (see Figure 4-02) will also allow research with small aquatic animals. However, the volume of the experiment containers is limited to about hundred milliliters.

A new generation of CEBAS minimodule, housing aquatic species in a completely closed environment, is also being planned for ISS. Ground-based research and development have already started on a commercial basis (Slenzka et al. 2001, 2003, 2006). The development of complete bioregenerative systems helps our understanding of ecophysiology and ecology in general. So the results of this research and development will ultimately benefit our understanding of Earth's ecology.

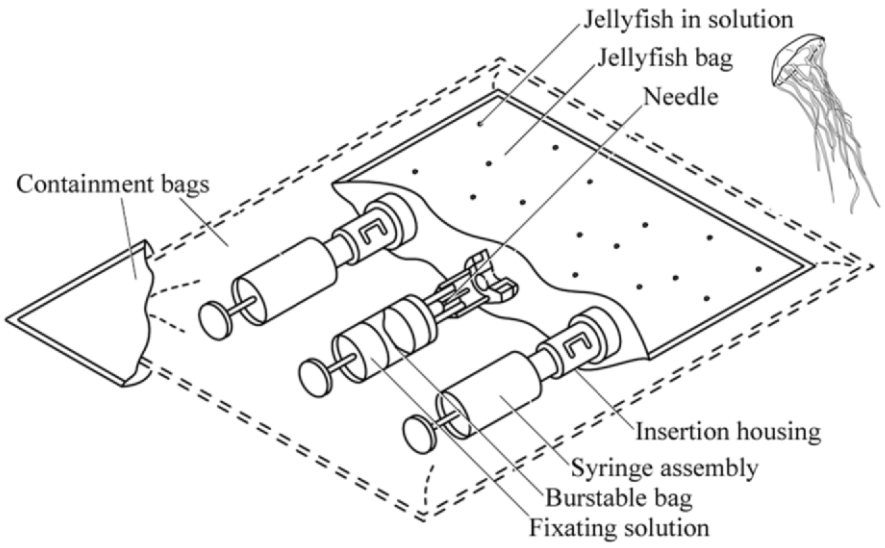

Figure 3-19. The four Jellyfish Kits flown on Spacelab missions contained the necessary materials to maintain jellyfish during flight, measure the radiation dose, and apply fixative to the specimens. Adapted from Souza et al. (2000).

3.2.4 Other Habitats

The *Egg Incubator* (EI) for ISS is designed to support experiments utilizing non-mammalian amniotic eggs such as chicken and Japanese quail eggs. Anticipated experiments include studies in embryo orientation and mortality, embryogenesis, and development of bone and muscular tissue. The

EI fits into one Space Shuttle Orbiter middeck locker, which allows for late access prior to launch and early access upon return.

The *Insect Habitat* (IH) System developed by CSA for ISS consists of a Transport Element, a Science Element and an Insect Container Element. The IH is designed to support a variety of insect species. However, during the initial increments the IH will be devoted to experiments using *Drosophila melanogaster*.

Other animal habitats can be much simpler. For example, jellyfish polyps are usually contained in bags and flasks of artificial seawater (Figure 3-19). At the beginning of the flight, crewmembers can inject controlled amounts of thyroxine or iodine into the bags, inducing the polyps to metamorphose into free-swimming ephyra, a tiny form of jellyfish. After several days in space, crewmembers cam inject again some fixatives and stow the bags in the onboard refrigerator. Some of the other bags and flasks are filmed to observe the animal's swimming behavior for example. After the mission, investigators can then examine both sets of live and fixed jellyfish and compare them for changes in morphology, calcium, and statolith size, shape, and number.

Figure 3-20. Photograph of plant shoots inside the Plant Growth Unit. Photo courtesy of NASA.

3.3 Plant Research Facilities

Plant development studies require a minimum of 36 plants per experiment. However, plant physiology studies vary considerably in their requirements and also may require experiment-unique hardware.

Plants such as culture-derived daylily (*Hemerocallis* cv. *Autumn Blaze*) and haplopappus (*Haplopappus gracilis*) were flown in the *Plant Growth Unit* (PGU) located in the middeck of the Space Shuttle. The PGU occupied a single middeck locker and had a timer, lamps, heaters, and fans to provide temperature regulation and lighting. The unit also has a data

acquisition system and displays, which allow the crew to monitor equipment status and environmental parameters (Figure 3-20).

For long-duration flights, a plant growth chamber with a minimum total growing area of 0.5 to 1 m^2 is required. This chamber requires its own environmental control system with subsystems for the control of light, temperature, gas composition, and water/nutrient delivery. The *Commercial Plant Biotechnology Facility* (CPBF) in the US Lab Module provides a large enclosed, environmentally controlled plant growth chamber designed to support commercial and fundamental plant research on board the ISS for continuous operation of at least one year without maintenance.

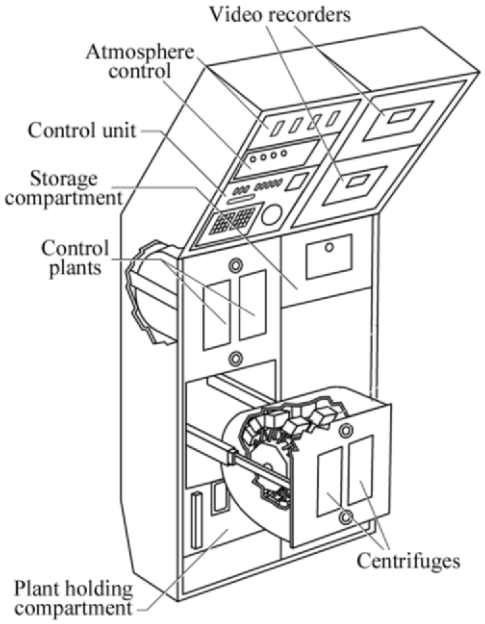

Figure 3-21. The Gravitational Plant Physiology Facility (GPPF) was a double-rack supporting plant studies within the Spacelab. Capabilities include two 1-g centrifuges to simulate Earth's gravity, various lighting conditions, and visible and IR video monitoring. Adapted from Souza et al. (2000).

Three other plant facilities are dedicated to fundamental research on plants on the ISS. The NASA *Plant Research Unit* (PRU) and two ESA facilities: the *European Modular Cultivation System* (EMCS) and *Biolab* (see above). All facilities use experiment containers that can be mounted on the 2.5-m diameter centrifuge, thus allowing to expose the specimens to centrifugal accelerations between 0 and 2 g. Transparent covers allow illumination and observation (also near-infrared) of the internal experiment hardware containing the plant specimen. Standard interface plates provide each container with power and data lines, gas supply (controlled concentrations of CO_2, O_2, and water vapor; ethylene removal), and connectors to water reservoirs. There is a difference in container size (Table 3-03) and in the degree of automation. All these facilities are designed to support plant growth in space for up to 90 days, for studies on protoplasts,

callus cultures, algae, fungi and seedlings, as earlier flown on Spacelab using *Biorack* and the *Gravitational Plant Physiology Facility* (GPPF) (Figure 3-21), and new experiments with larger specimens of fungi, mosses and vascular plants.

Facility	*Volume*	*Height*
Biolab	*0.36 l*	*60 mm*
EMCS	*0.58 l*	*160 mm*
PRU	*20 l*	*380 mm*

Table 3-03. Container sizes for the plant growth facilities on board the ISS.

3.4 Multipurpose Facilities

3.4.1 Animal and Plant Centrifuge

According to the current plan, a 2.5-m centrifuge will be housed in the *Centrifugation Accommodation Module* (CAM) developed jointly by JAXA and NASA for the ISS (Figure 3-22). This centrifuge will produce artificial gravitational forces upon attached habitats that house various biological specimens, from cells to rodents to large plants. It will be capable of generating controlled, artificial gravity levels ranging from 0.01 g to 2.0 g. The centrifuge will also provide life support resources and electrical power to the habitats as well as data transfer links to computers on the ISS. The habitats that are available to researchers for use on the CAM include the *Cell Culture Unit* for cell and tissue cultures, the *Plant Research Unit* for small plants, as well as the *Egg Incubator*, the *Insect Habitat*, the *Aquatic Habitat*, and the *Advanced Animal Habitat* for rats and mice described above.

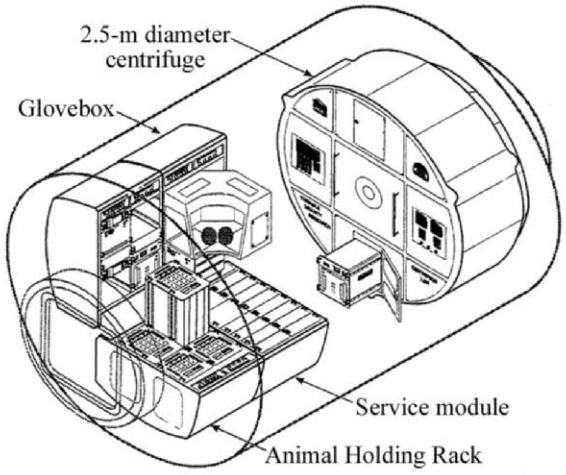

Figure 3-22. The Centrifuge Accommodation Module built by JAXA for the ISS is 8.9 m long, 4.4 m in diameter. The CAM has 14 rack locations, 4 of which house experimental hardware (including the Life Sciences Glovebox, a Habitat Holding Rack, and a Cryo-Freezer) with the remaining 10 dedicated to stowage. The 2.5-m diameter Centrifuge Facility is located in the module's endcone. Source JAXA.

3.4.2 Workstation and Glovebox

Experiments not requiring manipulations during flight consist of radiation biology experiments and some plant biology experiments. Other types of both plant and animal experiments require manipulations at such short-time intervals that an unmanned, long-duration flight is unsuitable. Crewmembers may support experiments by monitoring research animals or plants visually on a periodic basis or performing contingency procedures made necessary by hardware malfunction or unexpected experiment performance. The crew may also replenish water and food supplies, substantially reducing the need for automation, and conduct inflight experiment procedures directly on research specimens. These manipulations may include operations such as taking blood samples, perform dissections, fixating preparations, and harvesting plants.

Figure 3-23. The glovebox provides a sealed work area where crewmembers can perform experimental procedures such as subsampling cultures, fixating preparations, fluid chemical handling, and harvesting plants. Photo courtesy of NASA.

The difficulty of carrying out these apparently simple procedures in microgravity should not be underestimated. For example, spores or seeds released from a plant must be collected (they will not fall to the ground) and then actively distributed onto or into the surface of a new growth medium. The action of fixation by pouring a large volume of fixative solution onto dissected structures is impossible, all the components of such a system must

be contained and controlled. Direct access to the biological organisms is accomplished using a workstation that maintains biological isolation of the organisms. The so-called *glovebox* is a closed, retractable cabinet for laboratory activities that require the crew to handle chemicals and manipulate samples (Figure 3-23). Crewmembers can introduce samples through a side access door and handle the specimen through gauntlets in the front of the enclosure. A mesh grill and forced airflow keep solid particles, liquid spills, and gaseous contaminants within the cabinet. The spacecraft environment is also monitored for escaping contaminants by an air sampler, photography, and crew observations and comments.

3.4.3 Microscopes

A Zeiss *Compound Microscope* with system magnification up to 1000X will allow performing cellular and subcellular observations on board the ISS. It is designed to operate inside the work volume of the Life Sciences Glovebox and provides differential interference contrast, phase contrast, fluorescence, brightfield and darkfield microscopy for fresh, live, fixed, and stained sample observation.

A Leica stereo *Dissecting Microscope* with 4X to 120X zoom magnification will be used for microscope-aided inspections and manipulations. It is also designed to operate inside the work volume of the *Life Sciences Gloveboxes* and provides large depth-of-field with long working distance optics to facilitate specimen dissections and similar operations.

3.4.4 Life Sciences Laboratory Equipment

Life Sciences Laboratory Equipment (LSLE) is an inventory of equipment available for utilization in space biology or human physiology. This equipment is currently available and most has been utilized on flights integrated into the middeck, Spacelab, and SpaceHab facilities on board the Space Shuttle, or the Bion biosatellite. An online catalog of the LSLE equipment can be found at the following URL:

http://fundamentalbiology.arc.nasa.gov/PI/PI_flthdw.html

4 BASELINE DATA COLLECTION FACILITIES

In order to understand the changes induced by spaceflight, it is essential that testing and sample collection be done on a well-considered and rigorous schedule, with a sufficient number of points preflight and postflight. A series of ground-based, preflight measurements is generally made for each flight experiment to establish a set of normal or “baseline” values for the biological system studied. The variability in these measurements is necessary to determine the significance of the changes observed inflight. Early postflight

measurements are also necessary to define adequately the time course of recovery for inflight changes.

The investigator's team and the support personnel participate in the *Baseline Data Collection* (BDC) in facilities located at the launch and the landing sites. These facilities are also designed for preparing biological experiments for flight, for doing ground control experiments simultaneously with flight experiments, and for analyzing data. Data are transmitted from the space laboratory to these work areas, and are used to adjust the timeline and environment conditions for the ground-based controls.

Because the number of flight samples will necessary continue to be small, and individual variations in response are often large, a large number of testing needs to be done on the ground before the flight, in order to select the most representative specimens. In addition, some studies require animals or plants to be at a well-defined period of their development at the time of launch. Given the possibilities for multiple launch delays, it is necessary for these studies to anticipate those delays and always carry the right number of specimens at the right time. This can lead to very large numbers of biological specimens for a given mission (Figure 3-24).

Postflight collections of biosamples are also carried out for many life sciences experiments. Because readaptation to Earth's gravity reverses many of the changes that occur in tissues in space, it is imperative that biosamples be obtained as soon as possible postflight. To facilitate this, ground laboratories are usually prepared to implement such experiment procedures at the time of landing.

Figure 3-24. Swordtail fish (Xiphophorus helleri) in their holding tanks in the Operations and Checkout Building at the NASA Kennedy Space Center, before being selected for flying as part of the Neurolab payload on Space Shuttle Mission STS-90. Photo courtesy of NASA.

In the nominal sequence of post-landing operations of the Space Shuttle, living specimens can be handed over to investigators within 1-2 hours after wheel-stop for postflight analysis. For specimens located in the SpaceHab, the earlier access can be 4-5 hours. Stored data (e.g., plates, films, tapes) and samples brought back by the Space Shuttle can be in hands of the users within a few hours after the landing. The science racks within the SpaceHab are disassembled and the equipment is shipped to the postflight science facility or to the investigators' laboratory within a few days of landing.

At the NASA Kennedy Space Center, a Space Life Sciences Laboratory has recently been built, featuring a variety of biological specimen holding areas and laboratories, including controlled environment chambers for plants, habitats for rodents, aquatic species, avian species and insects. It is also equipped with biological imaging techniques and analytical chemistry, and can support biomolecular and microbial ecology research, as well as developmental, physiological, and molecular experiments.

In the case of the biosatellites or sounding rockets, biosample collections are carried out in mobile field laboratories set up at the landing site. Indeed, unlike the Space Shuttle, the biosatellites and sounding rockets do not land at a specific site. As the module descends under a parachute, a radio direction finding equipment is used to locate the biosatellite. Once the ground personnel recover the biological subjects, immediate postflight operations are conducted in a temperature-controlled field laboratory erected at the landing site. Animals flown on Russian biosatellites are examined upon recovery and then shipped to Moscow for testing. Processing of other biospecimens begins three or four hours after landing. Tissue samples requested by investigators are preserved or frozen according to instructions, and later shipped to the investigators' laboratories. If required, postflight testing is performed after the subjects have been transported to Moscow.

Unused tissues from the organisms flown in space may be fixed or frozen and stored in archives for later use by scientists. Access to these sample databases can be made through study proposals in response to solicitations for research experiment from the space agencies. So, analysis of the data may continue for several years. As results are analyzed, investigators prepare for publication, sharing the information with other investigators of the space mission or the science community at large. After publication of scientific peer-reviewed articles, the results of the space experiments are stored in life sciences databases, such as the *International Flight Experiments Database* (IFED). This database can be accessed through the following URL:

http://www.mainsgate.com/IFE/index.html

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FURTHER INFORMATION:

NASA Space Station Biological Research Project:
http://brp.arc.nasa.gov/Home/home_index.html

Life Science Hardware on board the International Space Station:
http://www.spaceref.com/directory/exploration_and_missions/human_missions/international_space_station/life_science_hardware/

JAXA Life Science Hardware for the International Space Station:
http://idb.exst.jaxa.jp/edata/02310/199908E02310000/199908E02310000.html

ESA Life Science Hardware for the International Space Station:
http://www.spaceflight.esa.int/users/file.cfm?filename=iss01-esaf

ISS Payload Information Resource:
http://stationpayloads.jsc.nasa.gov/F-facilities/index.html

International Space Station Reference:
http://spaceflight.nasa.gov/station/reference/index.html

Chapter 4

CELL BIOLOGY

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The discipline of Cell Biology examines biological processes at the levl of the basic unit of biology, the cell. Cell Biology focus principally on events intrinsic to individual cells and on cellular responses to environmental factors. Cell Biology therefore provides the underpinning for other disciplines relevant to Space Biology, including Development Biology, Radiation Biology (see individual chapters of this report), as well as Space Physiology and Medicine. Each of these areas of research at the tissue and organism levels ultimately depends on the normal function of individual cells and their integration into physiological networks. This section reviews the effects of spaceflight on bacteria, unicellular organisms, and human cells. Although the majority of these experiments are primarily of fundamental interest, the effects of gravity and microgravity on various processes at a systemic and cellular level reveal basic phenomena also relevant in non-spaceflight related sciences, such as in the area of biotechnology.

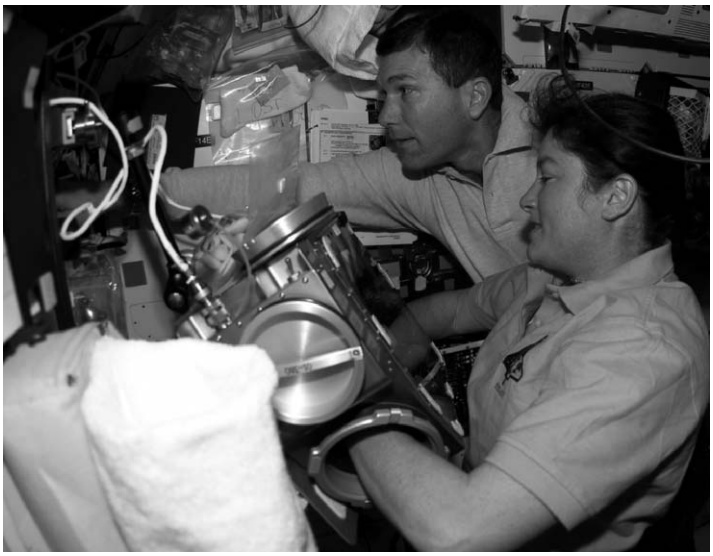

Figure 4-01. STS-107 Astronauts Laurel B. Clark and Rick D. Husband conducting an experiment in the Biopack incubator on the middeck of the Space Shuttle Columbia. Photo courtesy of NASA.

1 INTRODUCTION

1.1 Prologue

Nearly 30 years ago, shortly after humans made their last excursions to the surface of the Moon, I discovered in the coffee room an envelope addressed to the Institute of Biochemistry of the ETH Zurich where I was working at that time. After five or six days in which the envelope lay neglected on a table, I decided to open it. It was an invitation form ESA to submit experiment proposals to be flown in the first Spacelab mission. The announcement contained a brief summary of biological and medical investigations hitherto conducted in space mainly by U.S. and Soviet scientists.

A systematic bibliographic research was difficult because the few data available were published in U.S. and Soviet agency reports or in obscure journals difficult to find in a library. However, three books turned out to be very helpful: *Biomedical Results of Apollo* (Johnston et al. 1975), *Gravity and the Organism* (Gordon and Cohen 1971), and *The Experiments of Biosatellite-II* (Sanders 1971).

What triggered my interest were findings like changes in cell differentiation and the depressed reaction of human lymphocytes from space crewmembers when activated *in vitro* by mitogens, thus mimicking the reaction of the immune system *in vivo* against an infection. At that time I had just terminated a postdoctoral year at the Weizmann Institute of Science in Rehovot, Israel, where I learned to work with lymphocytes and I became interested in the differentiation mechanisms leading to T-cell activation.

I thought that it might have a good chance to be selected by answering the call of ESA proposing a simple experiment consisting of activating human peripheral blood lymphocytes with a mitogen under weightless conditions. My proposal was accepted in December 1976 and that was the beginning of my "space biologist" career. In 1986 my wife, also a chemist and biologist, joined my team. Since then we are working together in this exciting new field of research.

1.2 History of Research on Cell Biology in Space

The purpose of this chapter is to present the results obtained in nearly thirty years of research with single cells in space laboratories and platforms. I have tried to select and report only those findings sustained by solid facts, based on proper controls and experimental conditions, and published in international peer-reviewed journals. In fact, still nowadays, a great many of biological experiments that are conducted in space lack of inflight 1-g controls, or of sufficient number of samples to provide statistical significance, or of reliable environmental control. Most of such investigations are reported

in agency brochures or presented at meetings as short abstracts. I firmly believe that it is deleterious to science to conduct such crippled investigations: the data are not reliable and support the skepticism and the hostility of part of the scientific community towards space biology.

The focus of this chapter is on gravitational effects, including those observed with Earth-bound devices providing vectorless gravity conditions, like clinostats and rotating wall vessels. The effect of cosmic radiation on living systems is discussed in another chapter of this book.

The development of space biology can be subdivided in four phases. In the first phase, from the early seventies to the mid-eighties, living systems were studied at random to look for detectable effects of the space environment. During the second phase, which lasted until the mid-nineties, several important effects on cellular mechanisms were discovered and characterized. We are presently in the third phase, which consists of the use of microgravity as a tool for basic research and medical diagnosis. Major topics addressed are genetic expression, cell-cell interactions, membrane properties (lipid rafts in particular), cytoskeleton changes and signal transduction. The fourth phase is at its beginning and is characterized by attempts to develop processes of biotechnological and medical importance. Space cell biology obviously develops in parallel with the progress of scientific achievements and of analytical techniques like the microarray, cyto-fluorimetic and specific markers technologies.

Figure 4-02. Within the Biolab facility, experiments on board the International Space Station can be incubated, stored at cool or cold temperatures, microscopically or photometrically analysed and freshly prepared, if required, allo-wing most of these features without crew interaction, but with telescience capability. Source ESA.

By now, about three hundred experiments in space have shown that single cells from all steps of the evolutionary ladder can live and proliferate in space, but that at the same time dramatic changes can occur. Unfortunately the catastrophe of Columbia's flight STS-107 has put on hold dozens of investigations that were selected for flights from 2003 onwards. Moreover, due to the hold of the Space Shuttle program, the beginning of the operations of the major biological facilities on the International Space Station (ISS) like *Biolab* (Figure 4-02) and the *European Modular Cultivation System* have been delayed by at least two to three years. Therefore, the use of ground-based facilities, like the fast rotating clinostat, the random positioning machine and the rotating wall vessel, which provide conditions simulating microgravity (see Chapter 3, Section 1.2), has gained great importance. As a consequence, many interesting data in gravitational cell biology of the last three years have been obtained on the ground.

1.3 Phase One

The first phase of space biology was characterized by very simple experiments, naïve hypotheses, and rather primitive instrumentation. The most common of such simple but not trivial hypotheses said that "the behavior of a biological system is altered in space and in microgravity in particular". No better arguments were available at that time as there was no history of biological experiments in space. There were, however, old data from experiments performed almost 200 years ago in centrifuges showing that the development of plant seeds was altered at centrifugal forces higher than 1 g. A common speculation was that monocellular organisms like algae, protozoa, and single cells from multicellular organisms, humans included, would change their behavior when exposed to the weightless environment. The rationale for such speculation was that all living beings developed on Earth throughout millions of years under steady gravitational conditions. The lack of proper instrumentation in space and the limited knowledge of cellular signal transduction mechanisms[1] allowed only simple experimental approaches at a time when the technologies and the products of genetic engineering were not yet developed. The consequence was that the focus of the experiments was on the determination of so-called *end points*[2] of cellular

[1] The most important cell functions like mitosis, expression and secretion of specific products are controlled by "biochemical signals", mainly hormones, growth factors, cytokines, that are taken up by receptors located on the cell surface. Once a signal is recognized, its message is transmitted, i.e., transduced, into the cell to organelles. A major effect is a change of genetic expression which is triggered in the cell nucleus and that leads to cell differentiation.

[2] The endpoint of signal transduction in a cell is preceded by several intermediate steps that are being identified with the modern techniques of molecular biology, such

processes that followed an initial activation step. Examples are cell proliferation assays, light microscopy and electron microscopy observations of morphological changes, biochemical analysis of the secretion, and consumption of metabolites in the culture medium. A major problem was the lack of proper controls like onboard 1-g centrifuges[3]. A variety of organisms were flown in rather simple devices in batch containers. For example, Yuri Gagarin, the first human in space, carried containers with bacteria on board his space vessel Vostok in 1961. I remember what one of the U.S. investigators of those days once told me. Containers with *E. coli* cells were installed inside the upper tip of a rocket to be launched on a cold winter day without any thermal control. To prevent freezing of the cells the wife of the investigator knitted a "pullover" that enveloped the rocket tip to protect the cells from freezing. One exception was the experiment called *Woodlawn Wanderer*, described in more detail below. This experiment flown on board Skylab in 1973 used a sophisticated fully automated instrument with culture medium exchange, microscope, time-lapse cinematic camera, and sampling device.

In spite of their simplicity, such experiments delivered important information indicating that indeed gravity interacts with critical cellular functions. This was the basis of the research conducted in the following years. Highlights of this period were the flights of two U.S. biosatellites in 1966 and 1967, of several Russian Cosmos-Bion biosatellites between 1974 and 1989, three missions on board Skylab in 1973-1974, and the first flight of Spacelab on board the Space Shuttle Columbia in 1983.

1.4 Phase Two

The second phase started in 1985 with the flight of *Biorack* on board the Spacelab D-1, a mission organized by the German space agency DFLVR[4]. *Biorack* was a multi-user facility developed in Europe by ESA (Figure 4-03).

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as the *Reverse Transcriptase-Polymerase Chain Reaction* (RT-PCR) and the microarray technologies. The RT-PCR permits to identify qualitatively and quantitatively the genes expressed following a specific signal perception and transduction.

[3] A crucial part of every experiment is its *control*. For instance, it can be argued that effects detected in a space laboratory may be due not only to the exposure to 0 g and/or cosmic radiation but also to other factors of spaceflight like the launch acceleration (3-4 g on the Space Shuttle, 16 g on a sounding rocket), difference of the composition or the incubation temperatures between ground and space laboratory. Therefore, it is important to have controls under artificial gravity conditions at 1 g. This is achieved by means of centrifuges installed in the same incubator in which the *static*, i.e., the 0-g samples are kept.

[4] DFVLR stands for *Deutsche Forschungs- und Versuchsanstalt für Luft und Raumfahrt*. Today, the name of the Agency is DLR.

This facility was exclusively dedicated to the study of small organisms like bacteria, slime moulds, fungi, small plants and animals, as well as single plant and animal cells. The main feature of *Biorack* was that nearly all experiments had inflight 1-g controls in a centrifuge installed within the same incubator as the 0-g samples. Basic biology became a priority and it was the privilege of European scientists to be the first users of Biorack. After D-1, Biorack flew on board the Spacelab International Microgravity Laboratory missions (IML-1 in 1992, IML-2 in 1994) and three times on board SpaceHab in 1996-1997. Biorack was followed by other biological multi-user facilities like *Biolabor* flown in Spacelab D-2 in 1993, *NIZEMI*[5], a slow rotating microscope flown in Spacelab IML-2, and *Biobox*, an automated incubation facility installed on Russian biosatellites.

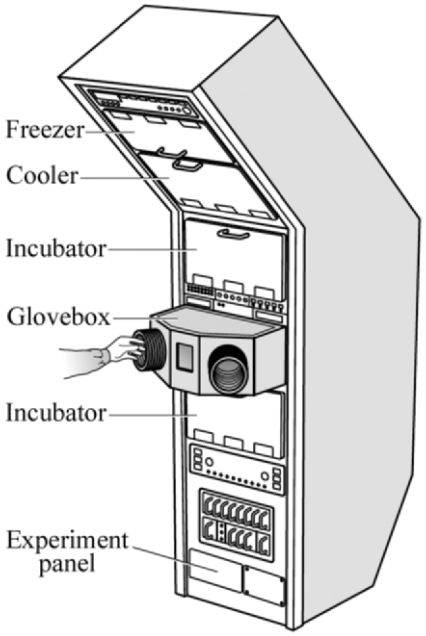

Figure 4-03. Biorack as installed in the Spacelab: two incubators (22°C and 37°C) above and below the glovebox provided the thermal environment and 1-g reference centrifuges for the experiments. A freezer accommodated frozen samples. A laptop computer was used as an interface for facility and experiment data. A camcorder allowed on-line video observation or recordings of the glovebox activities. Source ESA.

This second phase began with more systematic investigations and is characterized by the transition from tests of naive hypotheses to investigations of molecular mechanisms at the cellular level. It is accompanied by studies on signal transduction on board sounding rockets delivering few minutes of microgravity. Large amounts of data were collected showing that even single cells undergo dramatic changes in microgravity. The discovery of dramatic

[5] NIZEMI, for *Niedergeschwindigkeit Zentrifuge Mikroskop,* consists of a rotating microscope on which samples could be observed at 20-400x magnifications at accelerations from 0 g (static) to 200 g.

gravitational effects in mammalian cells, the progress of techniques like the reverse transcriptase polymerase chain reaction and of fluorocytometry with fluorescent monoclonal antibodies as markers, gradually directed the focus of the research towards the intermediate steps of intracellular signal transduction. Examples are the studies of the genetic expression of early oncogenes and the activation of the G-proteins-inositol-triphosphate or the protein kinase C pathways. A prominent task is the discrimination between direct and indirect effects of gravity, as we will review it below.

1.5 Phase Three

The third phase consists of experiments selected by the major national and international space agencies according the criteria established by international peers of scientists and based on the most actual trends and findings of basic research and applied technology. It started in 1996-1997 with three flights of *Biorack* during three Shuttle missions to the Russian space station Mir, followed by the Spacelab mission Neurolab in 1998.

Hopefully, this phase will continue in the next decade with investigations on sounding rockets and with the use of *Biolab* on board the ISS. There, the potential benefits of bioprocessing in space will be investigated in addition to basic research. The gap between the last flight of *Biorack* and the first flight of *Biolab* should have been bridged by *Biopack* (see Figure 3-10), a small and flexible instrument that was installed on board the Columbia STS-107 mission, which ended catastrophically on February 1st, 2003 (Figure 4-01).

A comprehensive review of the most important biological experiments of the first three phases has been presented in Moore and Cogoli (1996).

1.6 Phase Four

The fourth phase is beginning now and it is driven by two main factors. One is the technological and scientific knowledge achieved in thirty years of gravitational cell biology research in space and in machines providing vectorless gravity. The other is given by the costs of the space activities. In fact, the average price of an experiment on board a manned space laboratory is of the order of magnitude of ten million dollars. In order to justify such sums in front of the taxpayer, the space agencies are supporting the development of commercial applications on board the ISS. Such programs shall involve scientists from universities and non-aerospace industries interested in medical, biotechnological, and material applications.

One example is the *Microgravity Application Program* (MAP) of ESA. Several projects have been approved that confirm the industrial and

scientific interest to make use microgravity as a new tool for research and development.

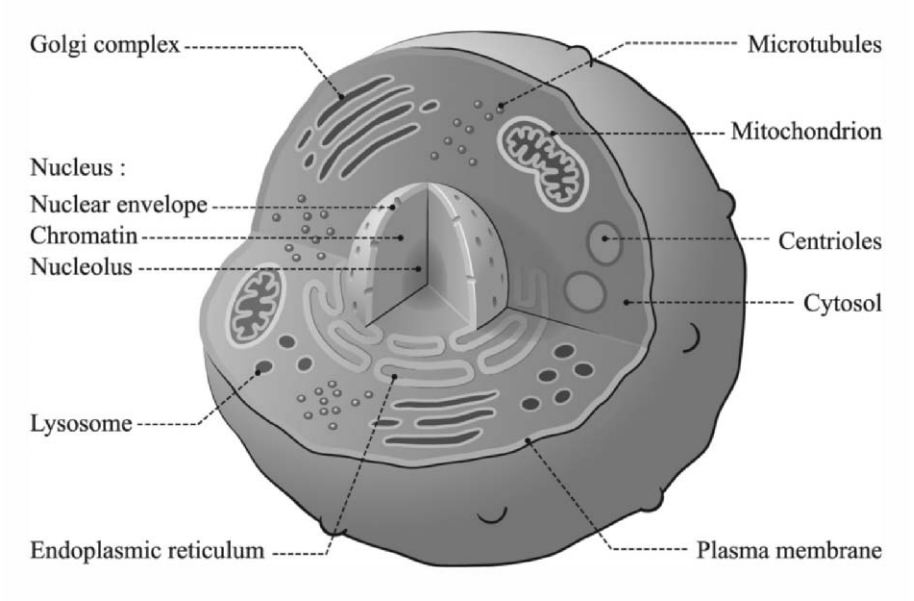

Figure 4-04. A cell is a complex living system in which organelles of different densities perform specific vital functions.

2 CRITICAL QUESTIONS IN CELL BIOLOGY

The following and other intriguing questions were asked when automated and crew-tended space laboratories became available tree decades ago:

a. Are unicellular organisms, from bacteria to mammalian blood cells, sensitive to gravitational forces? If yes, how and why?
b. Are the effects direct or indirect?
c. Which are the structures that perceive gravity?
d. What are the medical and physiological implications for humans in space?
e. Can microgravity become a useful tool for biotechnological processes of commercial importance?

We shall keep in mind that gravitational forces are ubiquitous in our Universe with the exception of very few special points, for example the Lagrange point where Earth's and Moon's gravity vectors have opposite

directions and compensate each other. What we call *microgravity*[6], or weightlessness, or 0 g, are in fact free-fall conditions analogous to those we would experience in an elevator that precipitates to the ground when the cable breaks. A satellite circling the Earth is permanently falling back to it. However, the satellite's speed is such that the fall is endless and just nearby the Earth.

Another aspect to consider is that evolution of life on Earth and probably in other regions of the Universe has always been conditioned by exposure to gravitational accelerations. On Earth, the gravitational force acting on a body depends on its mass and corresponds to the product of such mass by Earth's gravitational acceleration (1 g = 9.81 m/s^2). Consequently, all living beings have developed their physiological and biological functions to work optimally at 1 g. Therefore, we have a skeleton to sustain our body against gravity, a heart to pump the blood from the lower to the upper extremities, muscles to move the mass of our body up and down, forward and backward, and a vestibular system to control our posture.

But what happens to a single cell? Did Mother Nature decide that a cell should also adapt to gravity and therefore develop its own gravity-perception mechanism? How can such questions be addressed? Which aspects shall be investigated first? A few theoretical aspects outlined in the next Section should help to choose the adequate experimental approaches.

2.1 Theoretical Considerations

First we have to consider whether single cells possess structures that can sense gravity as a signal to be transduced into a biological response. Let's call these structures *gravity receptors*. The best example is the plant gravitropism transmitted by the pressure of dense organelles, the statoliths on the cell membrane, of the statocyte in the root (see Chapter 6, Section 2.1). Statocytes are "professional" gravisensing cells designed by evolution to drive the growth of the plant perpendicular to the Earth's surface. There are also unicellular organisms, like the protozoan *Loxodes*, having specific gravity receptors, the Müller's bodies (see below). Therefore, it is reasonable to distinguish between "professional" and "amateur" gravisensitive cells. While in the former a gravity-dependence is clearly identified (e.g., the plant statocytes with their statoliths), in the latter it is difficult to find cellular structures that may interact with gravity (e.g., in lymphocytes or fibroblasts). In addition, we must distinguish between single cells in culture (*in vitro*) and

[6] The word *microgravity* was invented in 1977 by the "Founding Fathers" of ELGRA, the *European Low Gravity Association*. Microgravity does not mean 10^{-6} g as the term *micro* may suggest but rather the fact that real 0-g conditions are not existing. In fact, the mass of the satellite itself generates a gravitational force on the objects on board.

cells as constituents of a multicellular organism (*in vivo*). In the last case, cells can be either part of an organ or a tissue, or may circulate in a fluid as blood cells do.

Organelles	***Diameter (µm)***	***Density (g/ml)***
Nuclei	*5-10*	*1.4*
Mitochondria	*1-2*	*1.1*
Ribosomes	*0.02*	*1.6*
Lysosomes	*1-2*	*1.1*
Peroxisomes		*1.06-1.23*

Table 4-01. Density of various cell organelles

In principle, any mass is subject to the force of gravity and consequently can be regarded as a gravity receptor (as indeed the statolith is). Table 4-01 indicates that the density of certain organelles can be significantly higher than one, which is the approximate density of cytoplasm. Consequently, at 1 g the organelles will apply a certain pressure on the filaments of the cytoskeleton. Such pressure disappears at 0 g with possible effects on the interactions between the players of the signal transduction chains that are embedded in the cytoskeleton (Figures 4-04, 4-05, and 4-06).

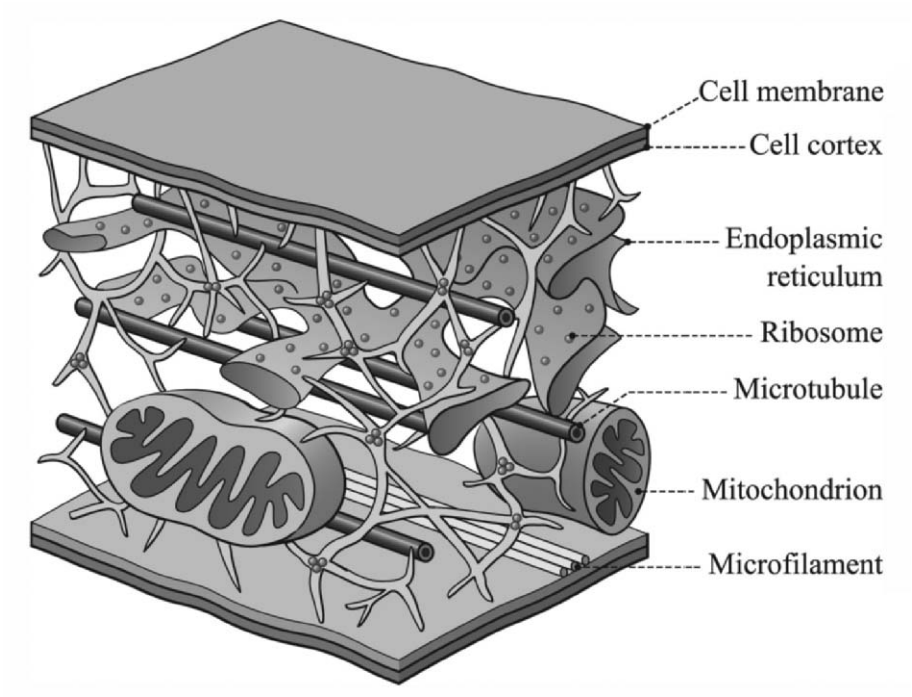

Figure 4-05. The organelles within a cell are embedded in and interact with the cytoskeleton.

Crucial is the identification of *direct* gravitational effects at the cellular level. Direct effects are those caused by the interaction of the force of gravity with cellular structures and organelles or by its absence, respectively. *Indirect* effects are those caused by changes in the cell microenvironment under altered gravitational conditions. Indirect effects may be due to the absence of convection and sedimentation at 0 g that causes a change of the distribution of nutrients and of waste products around the cells.

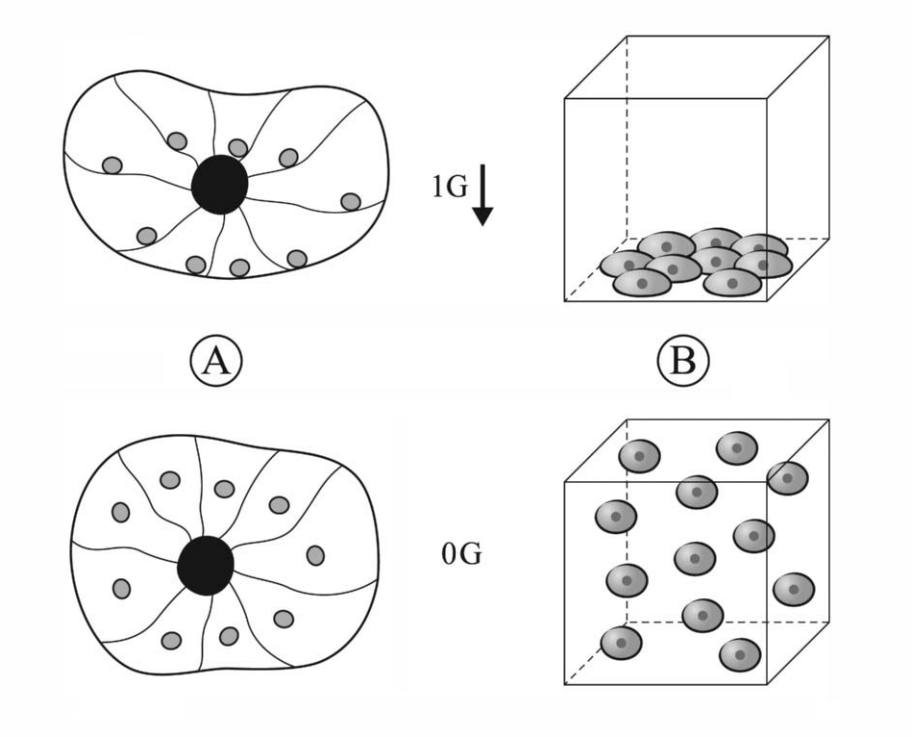

Figure 4-06. How gravity affects cells. A. Organelles having densities higher than the surrounding cytoplasm exert pressure on the cytoskeleton filaments when in normal gravity (1 G). Such pressure disappears in microgravity (0 G). B. In normal gravity, cells sediment to the bottom of the culture flask within minutes, thus living and interacting in a two-dimensional environment. In microgravity, cells remain suspended in the medium in a three-dimensional environment.

In a world of molecules embedded in fluids and loaded with electrical charges dominated by viscosity and electrostatic forces, gravity is an extremely week force. If one calculates the impact of such forces considering the cell as a static system one comes to the conclusion that the effect of gravity is negligible compared to that of the other forces. However, most, if not all, biological systems are not static, but in a non-equilibrium status. The

principle of "small cause/large effect" applies. In fact, in a biological process consisting of many subsequent steps, a small perturbation of one of the steps is sufficient to provoke dramatic changes downstream till the *endpoint*. Such effects are predicted by the *bifurcation theory*, as described theoretically by Prigogine and Stenger (1984) and experimentally by Tabony et al. (2002). This theory states that at determined bifurcation points a biological system may choose between two pathways leading to completely different endpoints (Figure 4-07). In fact, Stenger and Prigogine argue that "non-equilibrium amplifies the effects of gravity". In other words, it is conceivable that at 1 g the evolutionary pressure drove the system towards one of the two paths. Conversely, 0 g conditions may favor the second path, thus upsetting all the predictions based on a static model at 1 g. In fact, as described in the following sections, several surprising findings resulted from the "fishing experiments" conducted at the early stages of space biology.

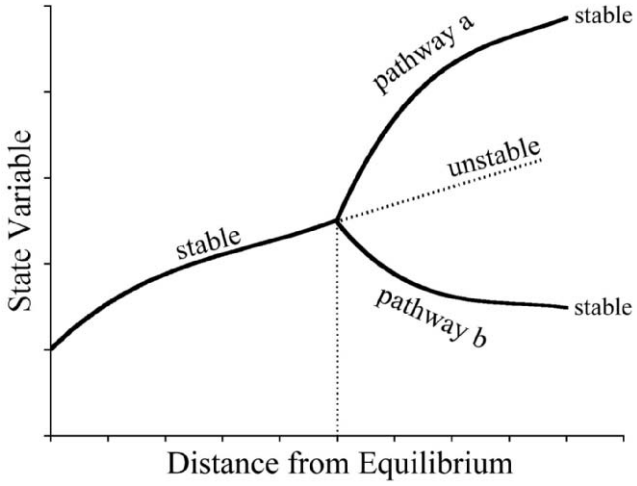

Figure 4-07. During a biological process, for example during T-cell activation, a system moves from an equilibrium state (e.g., a T-cell in the G_0 phase) towards an unstable, non-equilibrium state (e.g., when a T-cell is activated). The vertical axis represents a state variable (e.g., the gravitational acceleration). At a certain point of the biological process, called bifurcation point, the system becomes unstable and can follow multiple pathways. Two pathways are shown here. Adapted from Tabony et al. (2002).

Important changes like the loss of sedimentation, density-driven convection and hydrostatic pressure are occurring in a weightless cell culture. For a cell immersed in a fluid, as it is the case in a culture, this is a completely new situation. First, in 1 g, mammalian cells sediment within a few minutes to the bottom of the flask, where many of them may spread and adhere. In 0 g, instead, cells remain in suspension. Going from 1 g to 0 g is a change from a two- to a three-dimensional environment and has a remarkable impact on cell interactions, cell movements and, due to the lack of a substratum on which to spread and adhere, on cell shape (see Figure 4-06B).

Second, density-driven convection (due to changes in the concentration of nutrients and waste products in the medium) does not occur in microgravity, thus preventing mechanical diffusion. Thermodynamic diffusion is not affected, however.

Third, a new convection, predicted at the beginning of the last century by Marangoni, and not detectable at 1 g, becomes relevant in microgravity. The lack of buoyancy prevents gas bubbles (e.g., CO_2 developed by the metabolism of living cells) to rise to the surface of a culture, thus favoring the formation of larger bubbles in the middle of the liquid phase rather than a separation of the liquid and gas phases. For more details on the physics of fluids in microgravity, see Chapter 1, Section 2.2.

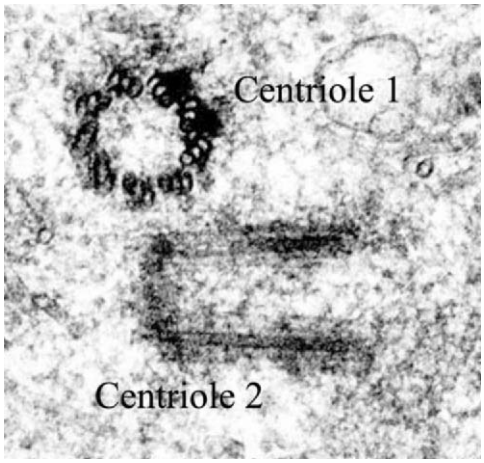

Figure 4-08. Centrioles are cylindrical structures that are composed of groupings of microtubules arranged in a 9 + 3 pattern. The pattern is so named because of a ring of nine microtubule "triplets" arranged at right angles to one another. They are present in animal cells and play a role in cell division. Courtesy of Albrecht-Bühler, Northwestern University Medical School, Chicago.

2.2 Further Considerations

2.2.1 Cell Shape and Structure

Gravity may induce polarization of the cell. The *centriole*, the structure giving origin to the spindle at the beginning of mitosis, consists of two *centrosomes* formed by tightly assembled microtubule. The centrosomes are not only dense structures, but they are perpendicularly oriented to each other, thus defining a plane (Figure 4-08). One could speculate that they may function as a kind of compass of the cells. Consequently, in microgravity the cell might lose its orientation.

The density of the nucleus and other organelles is higher than that of the cytosol, i.e., the fluid portion of the cytoplasm. Thus, organelles are expected to sediment inside the cell. For instance, it has been calculated that at 1 g the nucleus might sediment at a rate of 10 μm/hour. Conversely, at 0 g such sedimentation would not occur. In addition, factors other than microgravity may be responsible for the lack of sedimentation and three-

dimensional structure: the viscosity of the cytosol or by Brownian motion and cytoplasmic streaming might prevent sedimentation.

We can assume that the network formed by the cytoskeleton is responsible of maintaining the structure and shape in eucaryotic cells by linking together the membrane, the nucleus, and the organelles. The most important components of the cytoskeleton (actin filaments and microtubuli) are made of globular proteins subunits that can rapidly assemble and disassemble in the cell. As elegantly demonstrated by Tabony et al. (2002), assembly and disassembly of such labile structures is governed by gravity.

2.2.2 Biochemistry

The physiology of the cell may also be influenced by gravity. While passive transport of small molecules through the lipid bilayer is governed by diffusion (a gravity-independent process), active transport of ions and charged molecules, in which protein channels and transient membrane invaginations are involved, may be influenced by gravity.

Gravity may also play a role in intercellular transport processes. In fact exothermic metabolic processes generate continuously warmer micro-regions that are less dense than the neighborhood. Thus, thermal convections are produced by gravity with consequent ultra-structural rearrangements. Such convections are obviously absent in microgravity.

Also the energy turnover in the cells can be influenced by gravity. According to calculations made by Nace in 1983, gravity causes an uneven distribution of the organelles that gives rise to a torque capable to modify the shape and the structure of the cell. Energy is required to maintain its shape against gravity. In microgravity, such energy may be saved for other processes, such as proliferation or biosynthesis.

Finally, free-swimming cells consume energy to swim against gravity to avoid sedimentation. Such energy is not required at 0 g.

I conclude this section with a fundamental consideration: All living systems react in one way or another to changes of the environmental parameters like temperature, illumination, pressure, concentrations of nutrients, or activators/inhibitors. Gravity is a mechanical force. Change of the gravitational environment, i.e., changes of the forces acting on the cell, is a significant environmental change. It should therefore be no surprise that single cells also react and adapt to changes from 1 g to 0 g conditions.

3 RESULTS OF SPACE EXPERIMENTS

The long-term effects of microgravity can be investigated on board orbital spacecraft and sounding rockets, whereas the short-term effects are usually studied on board aircraft during parabolic flight. For some biological responses, ground-based methods or theoretical models can simulate the

conditions of weightlessness. However, we should keep in mind that these are just simulations of some of the effects of spaceflight. Nevertheless, in many cases, the results of experiments using clinostats were found to be in good agreement with those of space experiments and vice versa. In the last ten years a new device developed by Hoson et al. (1997) at the Osaka University, called *random positioning machine*, or *three-dimensional clinostat*, has been introduced in cell biology to provide a better conditions simulating the effects of microgravity (see Figure 3-07).

Ground-based investigations can be also carried out in centrifuges. However, in some cases, responses are not only related to the hypergravity level, but also to the experimental conditions, such as the size of the flasks, the angular velocity of the centrifuge, or the Coriolis forces resulting from motion of the samples while being centrifuged.

3.1 Results by Kinds of Cells

About 300 experiments have been carried out in space using various kinds of cells like bacteria, algae, fungi, protozoa and mammalian cells. Main changes were observed on cell proliferation, cell morphology, cell shape, cell membrane, cell metabolism, signal transduction, and gene expression. Particular attention is dedicated to the effect of microgravity on T-cells (see Figure 1-17). Indeed, the activation of T-lymphocytes during spaceflight turned out to be one of the most intriguing stories of space cell biology.

Unfortunately, the catastrophe of Columbia has caused a long delay in the space biology program. Therefore, important investigations in space could not be carried out in the last three years. Nevertheless, ground-based investigations led to interesting results.

3.1.1 Enzymes and Microtubuli

It is difficult to figure out how gravity may interact with subcellular structures or even at the level of macromolecules. Two fundamental studies have shown that this is indeed possible. In a series of brilliant experiments conducted on sounding rockets, Tabony et al. (2002) have shown that the self-assembly of microtubules is gravity dependent. Microtubules are formed *in vitro* when solutions of monomeric tubulin and GTP[7] are warmed at 35°C. GTP delivers the energy required for this process: when one molecule of tubulin is added to the microtubule, one molecule of GTP is hydrolyzed to GDP. Once formed microtubules undergo a complex dynamic process, called *treadmilling* by Tabony, in which tubulin is added at one end of the microtubule and lost from the other end. If such process is conducted at high

[7] *GTP* (Guanosine Triphosphate) is a chemical compound (nucleotide) that is incorporated into the growing RNA chain during synthesis of RNA and used as a source of energy during synthesis of proteins.

concentrations of tubulin (10 mg/ml), the microtubules tend to assemble in a stable structure that can be easily detected in polarized light. In their sounding rocket experiments, Tabony and collaborators discovered that such self-assembly of microtubuli does not take place in microgravity, whereas identical samples kept in a onboard 1-g centrifuge do (Figures 4-09 and 4-10).

Such gravity-dependence is convincingly attributed to density fluctuation occurring during the self-assembly process. This process being a

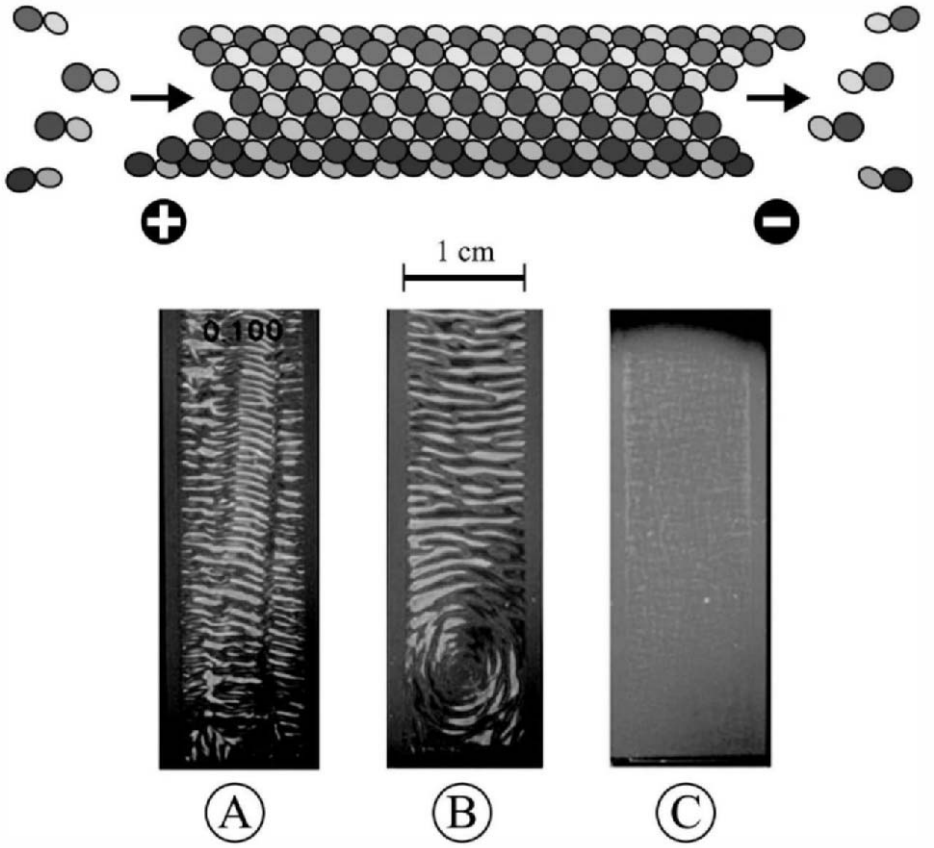

Figure 4-09. Top. A microtubule in a solution of tubulin and GTP is undergoing a dynamic process called "treadmilling". Tubulin is added to one end of the microtubule (+) and is removed from the other (-), at the expenses of GTP that is hydrolyzed to GDP. In such a way, the microtubule grows on one side and shrinks on the other. Bottom. Results of an experiment conducted on a sounding rocket in microgravity. The samples with tubulin and GTP contained in spectrophotometer cells were photographed in polarized light. The samples were kept inside an onboard 1-g centrifuge with the centrifugal force directed along (in A) or perpendicular to (in B) the long axis of the cell. The patterns show the self-organization of the microtubules. In the samples kept in 0 g (in C) almost no self-organization is occurring. Adapted from Tabony et al. (2002) (see color insert).

highly dynamic non-equilibrium process, even small gravitational effects on density changes are magnified according to the bifurcation theory. If we keep in mind that microtubules are the constituents of the centrioles, we may speculate that the formation of the spindle at the beginning of mitosis is also gravity-dependent.

Density fluctuations are also responsible for changes of the enzymatic activity of lipoxygenase in microgravity conditions according to Maccarrone et al. (2003). Lipoxygenases are a family of enzymes playing a role in important cellular functions like signal transduction, apoptosis, and metabolism. Experiments performed during parabolic flights showed that the affinity for the substrate (given as k_m value) was increased four times in 0 g, whereas the maximum velocity (V_m) of the enzyme remained unchanged.

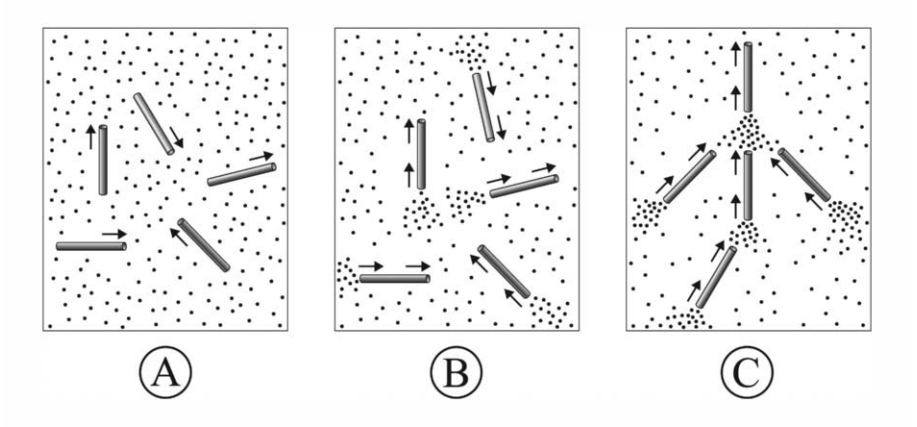

Figure 4-10. Mechanism proposed by Tabony to explain that the self-assembly of microtubules is gravity-dependent. The growing and shrinking of microtubule generates regions of low and high concentrations of tubulin at their ends, respectively (A). When more microtubules are coming closer to each other they will preferentially grow where the tubulin concentration is higher (B). This leads to the formation of tubulin trails and consequently to macroscopic density gradients in the system (C). Gravity interacts with such density gradients and leads to microtubules self-assembly. Adapted from Tabony et al. (2002).

3.1.2 Viruses

Viruses can be regarded as an assembly of macromolecules that may reproduce themselves under favorable conditions. Therefore they are at the boarder between chemical compounds and living beings. Viruses and bacteria have been used in space experiments to study the potential risks of infectious diseases affecting crewmembers. Other studies were dedicated to the crystallization of tobacco mosaic virus in 0 g in order to determine the three-dimensional structure by X-ray crystallography. The working hypothesis was

that the crystal structure developed in microgravity is of higher quality than that obtained in normal gravity (see Chapter 8).

Monomeric virus proteins may self-assemble to form pentameres, the *capsomeres*. In the presence of calcium ions the capsomeres self assemble to form larger symmetrical structures called *capsids*. Consigli and collaborators (Chang et al. 1993) conducted an interesting study in 1991 on board the Space Shuttle with polyomavirus protein VP1. In microgravity, VP1 formed capsomeres of homogeneous size, which did not assembly to form capsids, while the ground-based controls formed capsomeres of heterogeneous size that assembled to capsids. The failure to form capsids in 0 g may have similar causes as the failure of self-assembly of the microtubules reported by Tabony and thus further supports the bifurcation theory.

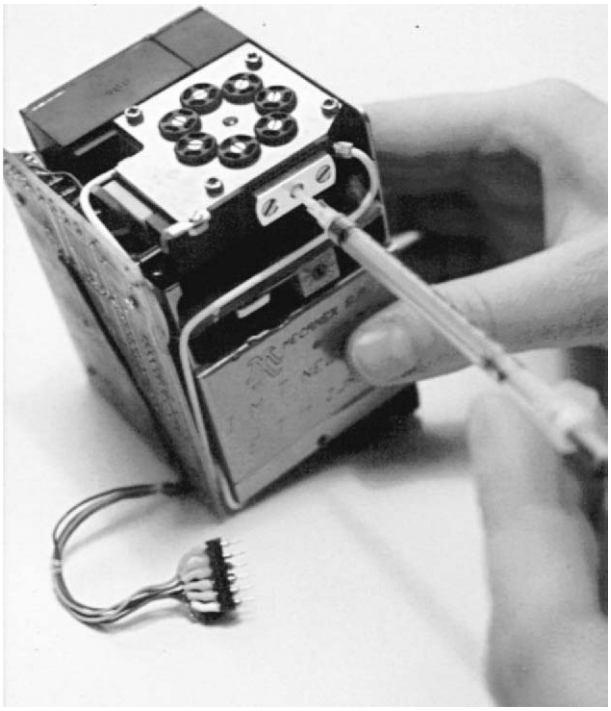

Figure 4-11. Space bioreactor developed in collaboration by the ETH Zurich, Mecanex, Nyon and the University of Neuchâtel. Photo courtesy of Isabelle Walther.

3.1.3 Bacteria

I remember well that when I was preparing my proposal for my first experiment in space I found in the library a book edited by Gordon and Cohen in 1971 entitled *Gravity and the Organism*. One chapter by Pollard was dedicated to a study conducted on *Escherichia coli* in an ultra rapid centrifuge at 50,000 g. I was disappointed to learn that neither DNA, RNA, nor protein synthesis was altered. But in a later work published by Montgomery et al. (1978), centrifugation at 110,000 g increased lag phase and prolonged

generation time in *E. coli*. I thought that if some changes occur at high-g there is a good chance that qualitatively opposite changes could happen at 0 g. Indeed a few years later, in 1967, cultures of *E. coli* flew on board the U.S. Biosatellite-2. Mattoni et al. (1971) reported that after a 45-hour orbital flight, the flight populations grew significantly faster than the Earth controls. This effect is probably due to the fact that, in absence of gravity and sedimentation, cells are homogeneously distributed in the culture medium and thus not subject to the waste and nutrient gradients existing in populations of cells laying on the bottom of the culture flasks. A stimulating effect on cell growth rate was also noted in experiments performed in the rotating clinostat. Such changes appear to be typical indirect effects of gravity caused by changes of the microenvironment of the cells.

Planel and collaborators (2002) discovered an increased resistance of *E. coli* and *Staphylococcus aureus* to antibiotics when cultured in several experiments in space. The effect was attributed to an increase of the thickness of the cell membrane, observed in electron micrographs, with consequent decrease in the membrane permeability.

More recently Klaus et al. (1997) conducted experiments with *E. coli* during seven Space Shuttle flights and reported a decrease in lag phase, an increased duration of exponential phase of cell growth, and an approximately twofold increase in final cell population density compared to ground controls. It is a pity that such experiments did not have an inflight 1-g control. Nevertheless, Kacena et al. (1997) reported similar results.

However, other experiments with *E. coli* gave contradictory results. Bouloc and d'Ari (1991) reported that cell growth did not increase during an investigation flown on a Russian Cosmos biosatellite. Gasset et al. (1994) came to the same conclusion with an experiment conducted on board the Space Shuttle.

Quite new and original was the experimental approach adopted by Ciferri et al. (1986) for an experiments on *E. coli* conducted in the *Biorack* facility on board the D-1 Spacelab mission. There are three types of interactions between bacteria: the exchange of chromosomal DNA via sex pili (conjugation), the transfer of short stretches of DNA by bacteriophage (transduction), and the uptake of extracellular DNA fragments (transformation). No gravity effect was found with respect to transduction and transformation. Conversely, conjugation was enhanced three- to four-fold in microgravity compared to the inflight 1 g control.

Two experiments were performed during the IML-2 Spacelab mission to examine the effects of microgravity on *E. coli* cell microenvironment and signal transduction through the cell membrane (Thevenet et al. 1996). Although the investigators are very cautious in the interpretation of their data and honestly describe the difficulties commonly encountered in space experimentation, their results are interesting. They used two *E. coli* strains,

namely the K12 prototrophic strain, i.e., a wild strain capable of synthesizing by themselves all required biochemicals like the amino acids, and the non-motile mutant *motB::*Tn10. While cell growth of the wild type did not change at 0 g, the lag phase appeared considerably shorter in the non-motile mutant compared to the inflight 1-g control. In the second experiment, signal transduction was studied by subjecting the cells to osmotic shock with 0.1 and 0.2 M sodium chloride. Cells respond to osmotic shock by turning on a specific set of genes, among them is the *ompC* gene. The induction depends on a two-components regulatory system, EnvZ/OmpR. The data show that this signal transduction system worked even better under microgravity conditions than in the 1-g control.

Also, *Bacillus subtilis* showed a higher growth rate in 0 g in an experiment performed using *Biorack* during the Spacelab D-1 mission with an onboard 1-g centrifuge (Mennigmann and Lange 1986).

What can be concluded from all these, in part disagreeing, data? As said above and due to their simple structure, small size, and lack of organelles it is difficult to believe in a direct effect of gravity on bacteria. Nevertheless, it is clear that microgravity is a favorable condition for cell growth and signal transduction. In addition to basic research, the high interest in such studies is driven by the importance to assess the risk of bacterial infections on board space vehicles. In fact, gravity seems to be an environmental signal affecting bacterial virulence. Another aspect, discussed in Chapter 7, is the use of *E. coli* to study of the effect of cosmic radiation on living systems.

I would like to end this section by mentioning the theory of panspermia proposed in the seventies by the British astronomers Hoyle and Wickramasinghe, according to which life expanded in the Universe by means of bacteria as constituents of the cosmic dust.

3.1.4 Yeast

Saccharomyces cerevisiae, the yeast used to bake bread and cakes, is a highly appreciated organism to study several aspects of eukaryotic cell, like signal transduction, genetic expression, and adaptation to environmental stress. It has the great advantage of being resistant to rough environmental conditions like freezing or lack of nutrients. It also has biological properties and behavior analogous to those of mammalian cells that are, by contrast, much more sensitive to the environment and therefore much more difficult to keep alive in space experiments. The analogy with mammalian cells permits to investigate crucial biological processes and even to carry out cancer research with yeast cells. In addition, it is widely used in biotechnological processes, in particular in genetic engineering. Therefore, it is not surprising that yeast cells have been extensively chosen for experiments in space. As in the case of *E. coli*, several studies were dedicated to the effects of cosmic radiation.

With the increasing interest in bioprocessing in space (see Chapter 8) the need for sophisticated cell culture and tissue engineering facilities, also known as *bioreactors*, to be installed in space laboratories, in particular on ISS, became evident. It was clear that the technological challenge due to the constraints imposed to space instrumentations suggested to start the development of space bioreactors using yeast cells that are easy to cultivate and to preserve instead of delicate and sensitive mammalian cells. Only once the instrumentation has proven adequate can the experimentation with mammalian cells and tissue begin.

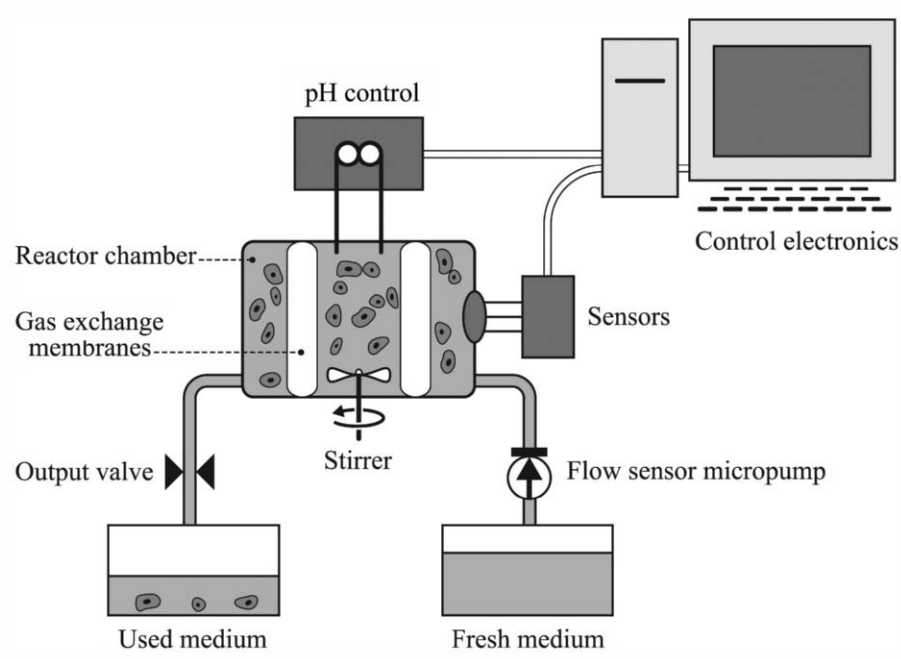

Figure 4-12. Elements and interconnections of the space bioreactor of the ETH Zurich. Courtesy of Isabelle Walther.

A first step in this direction was the development of a bioreactor for the culture of yeast cells that flew during three Space Shuttle missions (Figures 4-11 and 4-12). The experiments were conducted by Isabelle Walther from our laboratory (Walther et al. 1994, 1996, and 2003). When a daughter cell is generated, a typical scar, called *bud scar*, is left on the envelope of the mother cell. Normally, the scars left by several daughters are symmetrically distributed at two poles of the mother. A significant difference in the distribution of the bud scars was observed between cells cultured in 1 g and in 0 g. In fact the percentage of randomly distributed bud scars was higher in the

0-g (17%) than in the 1-g (5%) cells (Figure 4-13). However, no significant differences were noted in the cell cycle, ultrastructure, cell proliferation, cell volume, ethanol production, and glucose consumption.

NASA is also strongly supporting a project of a large bioreactor facility for ISS, called the *Cell Culture Unit* (CCU). The preliminary tests will be conducted with yeast cells. Further information is available on the Internet at the following URL sites: http://brp.arc.nasa.gov/GBL/Habitats/ccu.html and www.payload.com

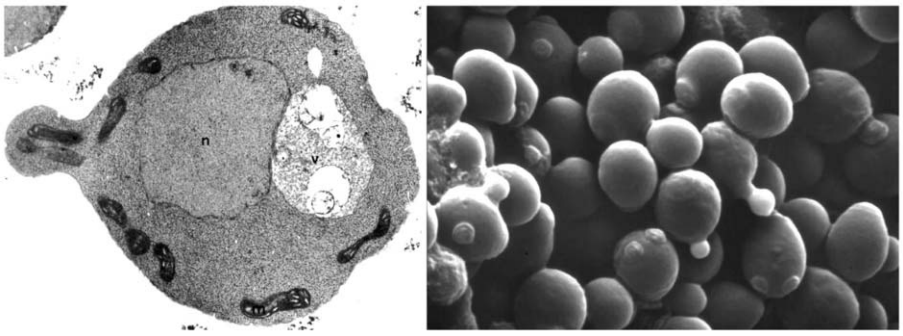

Figure 4-13. Saccaharomyces cerevisiae cells cultivated in a space bioreactor. Left: Transmission electron micrograph of one cell, 27.000 x. Right: Scanning electron micrograph of cells showing budding scars and buds, 7.000 x.

3.1.5 Ciliates and Flagellates

There are unicellular organisms that are particularly interesting for studies in gravitational physiology and space biology due to the display of swimming properties like negative and positive *gravitaxis* and *gravikinesis*. Pioneering studies were conducted by Planel et al. (1982), Häder et al. (1996), and Hemmersbach et al. (Hemmersbach and Häder 1999, Hemmersbach and Bräucker 2002). All have published several review articles on the subject.

The swimming behavior of ciliates and flagellates may be driven by gravity, light irradiation, oxygen, and nutrient concentration. This implies that they have structures and organelles sensing gravity. *Positive gravitaxis* is swimming in the same direction as the gravity vector, whereas negative gravitaxis is swimming in the opposite direction.

Gravikinesis describes an active regulation of the swimming velocities in order to compensate at least part of the cell's sedimentation: acceleration during upward swimming and deceleration during downward swimming. Such postulated changes in swimming velocities can be measured, and the values can be used for calculation of gravikinesis (Machemer et al. 1991).

Gravitokinesis can be calculated using the following formula:

$$\text{Gravikinesis} = \frac{\textit{Downward}\ \text{Swimming Velocity} - \textit{Upward}\ \text{Swimming Velocity}}{2} - \text{Sedimentation Velocity}$$

The study of the regulation of motion of ciliates and flagellates and the question of their gravisensing combined with the easiness of experimental observation has attracted scientists as early as in the late nineteenth century. This is also the reason why most of what we know today has been achieved during ground-based experiments (see Häder et al. 2005 for review).

I will describe here three unicellular systems in which gravisensitivity has been deeply investigated, as reviewed by Hemmersbach and Bräuker (2002) and Häder et al. (2005): the ciliates *Paramecium* and *Loxodes*, and the flagellate algae *Euglena*. In all these organisms, gravisensitivity has been attributed to mechanosensitive ion channels. In case of the ciliates *Paramecium* and *Stylonychia*, electrophysiological studies revealed the existence of such kind of channels and their bipolar distribution in the cell membrane. It has been postulated that the mechanical load activates these "gravisensitive" channels, i.e., weight, of the cytoplasm, which exceeds the density of the medium by about 4%.

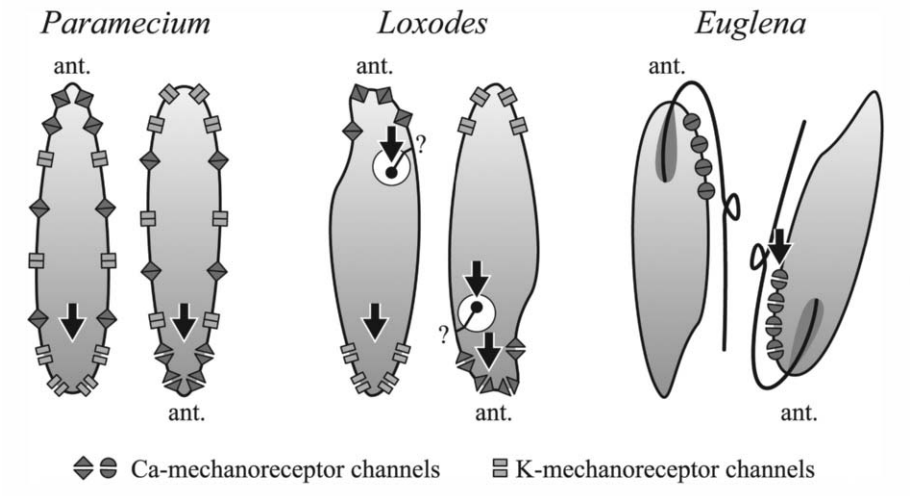

Figure 4-14. Models of graviperception in three protist species (ant. = anterior cell pole). Ca- and K-mechanoreceptor channels are incorporated in the cell membrane. These channels are activated by the mechanical load of the cytoplasm (forces symbolized by arrows; see text for details). Additionally, Loxodes bear specialized gravireceptors, the Müller vesicles (not to scale). Adapted from Hemmersbach and Bräucker (2002).

In *Paramecium*, potassium (K) channels are located mainly at the posterior site of the organism, whereas calcium (Ca) channels are located at the anterior site (Figure 4-14). Stimulation by the weight of the cytoplasm leads, according to Machemer et al. (1991) either to hyperpolarization (K-channels) or depolarization (Ca-channels) of the membrane potential, which in turn increases or decreases the swimming rates, respectively.

In fact, if a *Paramecium* or *Stylonychia* cell is tuned upside down, a distinct gravireceptor potential can be measured: hyperpolarization (stimulation of the posterior mechanosensitive K-channels) and depolarization (stimulation of the anterior mechanosensitive Ca-channels), depending on the orientation of the cell (Gebauer et al. 1999) (Figures 4-14 and 4-15).

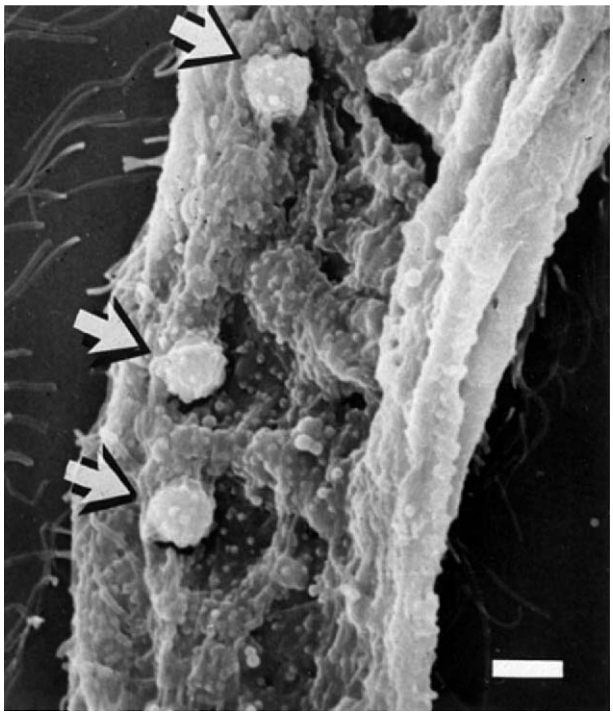

Figure 4-15. Scanning electron micrograph of a replica of a ruptured Loxodes striatus. Arrows indicate the barium sulfate granula of three Müller vesicles. Scale bar: 1 µm. Courtesy of R. Hemmersbach, Institute of Aerospace Medicine, DLR, Cologne.

In *Euglena* gravisensing is also based on mechanoreceptors in the membrane. According to Häder et al. (2005) mechanosensitive Ca-channels are located at the anterior part of the cell, and are activated by the load of the cytoplasm. Their stimulation induces a signal transduction cascade where cAMP[8], calmodulin[9], and possibly phosphorylation[10] processes are key players (Streb et al. 2002).

[8] *cAMP* is a small, ring shaped molecule that acts as a chemical signal in signal transduction.

In contrast to the cell membrane-located gravisensing mechanism described above, an intracellular gravity receptor has been identified in case of *Loxodes*. This ciliate bears statocyst-like organelles, the *Müller vesicles*. This is a vacuole of 7-µm diameter containing a dense granulum of barium sulfate fixed to a microtubular stick. The stimulus for graviperception, provided by the movement of the cell, causes mechanical shear to the stick. This stimulus triggers changes in membrane potential and in ciliary activity, which induces cell movement. Destruction of the Müller vesicles by means of laser beams leads to the loss of orientation capacity in *Loxodes*. However, 12-day cultivation in space did not affect their morphology. Although there were indications of less mineralization of the Müller vesicles in 0 g, this protist showed normal gravitaxis after flight. Such organelles appear to be an exclusivity of the family *Loxodidae* among protozoa, and show some analogy to the statoliths in plants and the otoliths in humans and other vertebrates.

Several pioneering experiments were carried out with *Paramecium* in space by the team of Hubert Planel at the University of Toulouse (Planel et al. 1981, Planel et al. 1982, Planel 2004). The main results were: higher cell growth rate, increase in cell volume, decrease in total cell protein content, and lower cell calcium content. It was postulated that the higher cell proliferation is related to changes in the energetic metabolism. Indeed, in microgravity it seems likely that the ciliary movement and the swimming of paramecia should require less energy expenses than on Earth. A fraction of the ATP, the component used for the ciliary movement, could be saved and used for cell metabolism and cell division. In hypergravity, the swimming, which is reduced, should require more energy. Therefore, less ATP is available, which could explain the lower cell growth rate. The changes in ATP content in *Paramecium* exposed to hypergravity or simulated microgravity are in good agreement with this mechanism.

The data on the increasing proliferation rate of *Paramecium* in 0 g are intriguing in view of the discovery of clock genes. One can speculate, as we will discuss it later in this chapter, that gravity may interact with certain cellular functions regulated by such genes.

The question of sensitivity threshold has been addressed by using a sophisticated slow rotating centrifuge microscope, called NIZEMI (for *Niedergeschwindigkeit Zentrifuge Mikroskop*). NIZEMI has been developed by the German Space Agency based on ideas and initial concepts by

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[9] *Calmodulin* is a small calcium-binding protein that is the most important transducer of intracellular calcium signals.

[10] *Phosphorylation* is the process of adding a phosphate group to a protein or another compound (e.g., the formation of ATP from ADP). This process modifies the properties of neurons by acting on an ion channel, neurotransmitter receptor, or other regulatory molecule.

Wolfgang Briegleb, and flew on board the IML-2 mission in 1994. The acceleration threshold inducing graviresponse has been determined by increasing the acceleration profile from 0.0001 to 1.5 g. The following values were obtained: *Paramecium*, 0.35 g; *Euglena*, 0.16 and 0.12 g; and *Loxodes*, less than 0.15 g. Interestingly, the results were similar when the cells were subjected either to increasing or decreasing accelerations, and the effect was independent of the previous exposure to microgravity up to 12 days, although the cells underwent several division cycles.

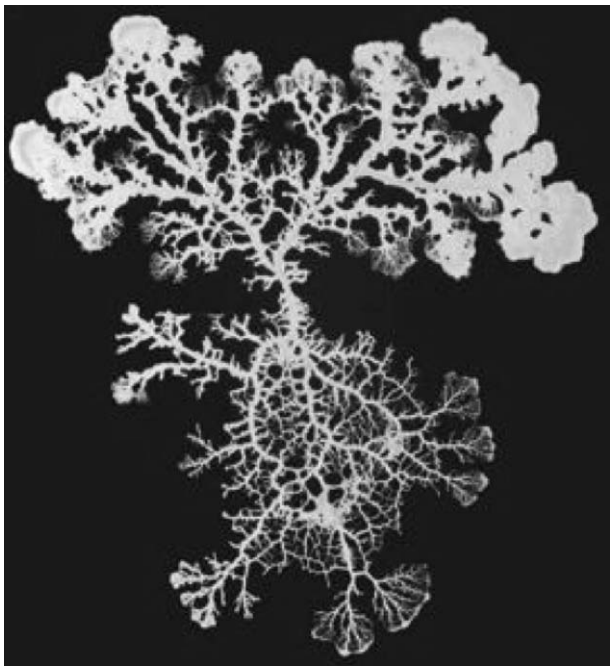

Figure 4-16. Physarum polycephalum. Courtesy of I. Block, Institute of Aerospace Medicine, DLR, Köln, Germany (see color insert).

3.1.6 Slime Mold

Physarum is a unicellular organism that lives in forests on rotting wood and can grow to cover areas up to one square meter (Figure 4-16). It is characterized by a system of communicating cytoplasmic veins, in which a rhythmic cytoplasm streaming distributes nutrients and disposes of waste metabolites. Also, the streaming is involved in cellular signaling. Wolfgang Briegleb and collaborators studied the cytoplasmic streaming and the underlying contraction rhythm of the veins by means of cine- and video cameras under actual microgravity and in a fast-rotating clinostat (Block et al. 1986, 1994a). Significant increases in the frequency of the contraction rhythm and the streaming velocity were observed.

Follow-up studies (Block et al. 1994b, 1996, 1999) allowed to:

a. Demonstrate a simultaneous processing of different stimuli (acceleration, light) in the same signal-transduction pathway;
b. Determine the acceleration-sensitivity threshold to be 0.1 g in *Physarum*;
c. Show the all-or-none-law to be valid in the acceleration-stimulus response;
d. Imply the existence of internal gravireceptors (dense cell organelles, nuclei or mitochondria, both numbering to the million in one Physarum cell);
e. Detect the involvement of second messengers (cAMP) in the first steps of the acceleration-signal transduction chain.

3.1.7 Mammalian Cells

Three pioneering experiments conducted in the early days of space biology have inspired my own research. One was a Soviet-Hungarian study on human lymphocytes that were activated with polynucleotides on board Salyut-7 (Talas et al. 1984). Although the conditions of this space experiment were not ideal, the results showed that lymphocyte function changed in 0 g. A five-fold increase of the interferon-α production was observed. The second investigation was performed on WI38 human embryonic lung cells by a U.S. team on board Skylab (Montgomery et al. 1978). In what is probably the most sophisticated instrument for cell biology ever used in a space laboratory, the cells were cultivated over weeks under controlled conditions. A microscope and a camera permitted cinematographic recording. However, cinematographic recording, phase, electron and scanning microscopy indicated no observable differences in ultrastructure and in cell migration between flight and ground controls. The third study was conducted independently by both U.S. and Soviet scientists, and was dedicated to the study of the immune system of humans in space. Lymphocytes taken from crewmembers of Skylab and Salyut prior to and after flight were activated with mitogens. Kimzey (1977) reported that the rate of RNA synthesis was significantly decreased after flight. Konstantinova and collaborators (1973) obtained similar results. Although these last investigations were not true cell biology experiments, they showed that it was possible to simulate an immune reaction *in vitro* and thus to study a very intriguing differentiation process.

In the following years, more experiments were carried out on animal and human cells in space as well as on Earth in devices simulating conditions of microgravity. It became clear that microgravity affects the morphology and important cellular functions. As described in the following sections, changes were noted in cell proliferation, in the cytoskeleton, in signal transduction, and in genetic expression.

The most extensively studied cell systems were lymphocytes and bone cells. The lymphocyte studies were conducted mainly by our team in Zurich, Didier Schmitt and collaborators (Hatton et al. 1999) in Strasbourg, Neal Pellis and Ben Hashemi at the NASA Johnson Space Center (Sundaresan et al. 2002, Hashemi et al. 1999), Steve Chapes at Kansas State University (Chapes et al. 1992), and by Marian Lewis at the University of Alabama in Huntsville (Lewis 2002).

The bone cells work was performed by Jackie Duke at the Texas University in Houston, Yasuhiro Kumei now at the Tokyo Medical Dental University (Kumei et al. 1996, 2004), and by Millie Hughes-Fulford at the University of California in San Francisco (Hughes-Fulford 2002).

A pioneering space study on genetic expression was performed on human renal cortical cells by Tim Hammond of the Tulane University (Hammon et al. 1999).

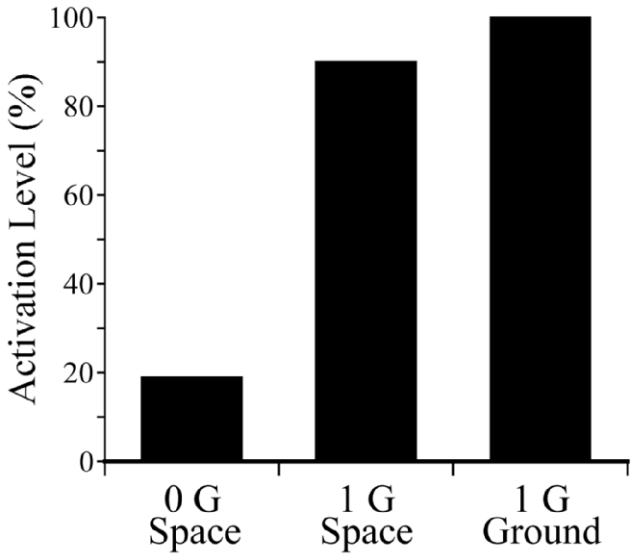

Figure 4-17. Mitotic index, determined as amount of tritiated thymidine (2 h pulse) incorporated into DNA, of T-lymphocytes activated for 72 h with concanavalin A. The data are expressed as percent of the control processed on the ground. There is a 80% decrease in activation in 0 g (0 G Space) compared to Earth (1 G Ground). The slightly reduced activation of samples kept in an onboard 1-g centrifuge (1 G Space) compared to Earth is most probably due to the stops of the centrifuge to operate other experiments.

3.2 Results by Cell Functions

In the following section, I am reviewing the relevant findings subdivided in the major cell functions affected.

3.2.1 Cell Proliferation

One of the most dramatic effects discovered so far is the nearly total loss of response to mitogenic activation by human T-lymphocytes *in vitro* (Figure 4-17). This was the result of an experiment conducted during

Spacelab-1 in 1983 by a team of my laboratory (Cogoli et al. 1984). Another unexpected result was the 100% increase in the mitotic index when the same cells were attached to microcarrier beads. These results were later confirmed in a series of experiments performed using *Biorack* during the Spacelab D-1, IML-2, and SLS-1 missions. Part of the work was conducted in collaboration with the team of Proto Pippia of the University of Sassari in Italy. These surprising data triggered similar investigations with lymphoid cell lines and other mammalian cells in several other laboratories.

Due to their role in cellular immunity and to the complexity of their activation mechanism, T-cells are objects of extensive investigations since decades. As said before, in the early seventies Russian scientists were the first to report that the activation of lymphocytes from astronauts by mitogens was depressed after flight. A little later, U.S. investigators reported similar results. This may point to a higher risk of infection during and after spaceflight. To study the problem in more detail it was suggested to test lymphocyte activation in cell cultures in space. Three lines of experiments were conducted: *in vitro*, *ex vivo*, and *in vivo* studies.

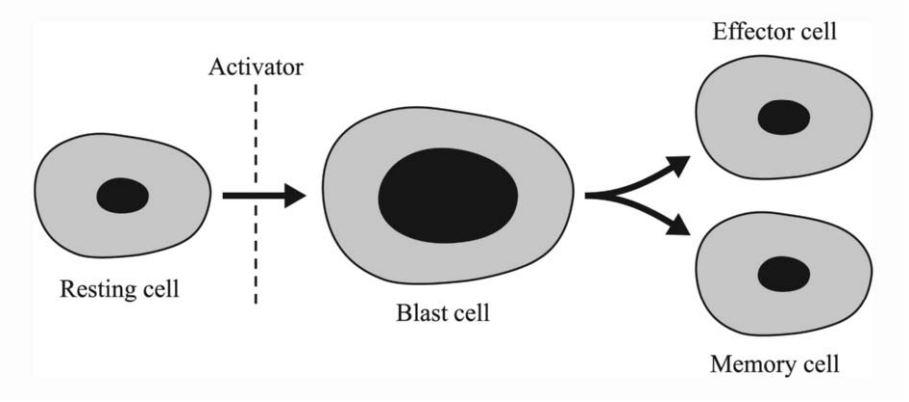

Figure 4-18. Activation of T-lymphocytes. Resting cells in the G_0 phase are activated in vitro with a mitogen thus triggering the events occurring in vivo during antigenic activation, e.g., with a virus or a bacterium. Within two days the cells increase in volume and enter mitosis on the third day of activation.

T-lymphocytes from human peripheral blood may be activated *in vitro* by several substances of different origin, called *mitogens*, which are able to trigger the events occurring *in vivo* following exposure to an antigen (Figure 4-18). The activation of T-cells with mitogens is, therefore, a good model to simulate *in vitro* this key aspect of the immune response. Concanavalin A (Con A) is a widely used mitogen. Its binding to the cell membrane mimics the first signal required for T-lymphocyte activation, which usually is transmitted to the "resting" T-cells by the antigen, or a

fragment of it, bound to the "antigen-presenting cell" by means of the major histocompatibility complex. Resting lymphocytes are in the G_0 phase and after recognition of the first signal the complex activation process is started. The cells enter the G_1 phase, a number of cytokines such as interleukines and interferon-gamma are produced, cell division leads to the appearance of T-effector and T-memory cells. While antigen recognition is limited to one clone of a small number of cells, mitogen activation is polyclonal and involves a large number of T-cell clones.

The activation process consists of three phases:

a. Recognition via *T-Cell Receptor*, TCR (of which CD3 is the main constituent) (Figure 4-19), either of the antigen "presented" by the antigen presenting cell or of the mitogen;
b. Cell-cell interaction and exchange of signals between T-cells and "accessory" cells (in general monocytes via CD28 on the T-cell and B7 on the monocyte) respectively;
c. Expression and secretion of interleukin-2 (as autocrine signal) as well as expression of interleukin-2 receptor and recognition of interleukin-2 by T-cells.

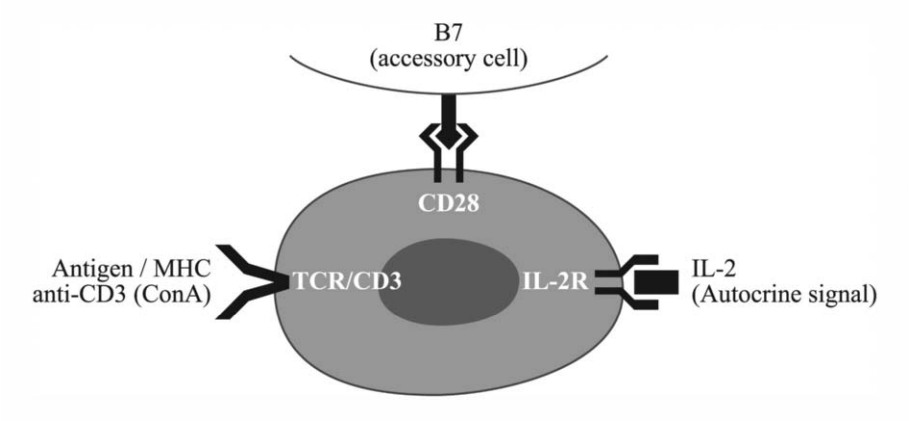

Figure 4-19. Three signals are required for full T-cell activation. The first signal is delivered in vivo by the antigen "presented" by the major histocompatibility complex to the T-cell receptor TCR/CD3 complex on the membrane of the cell. Such interaction can be mimicked in vitro with a mitogen (e.g., Con A) or by anti-CD3 antibody. The second signal is delivered by an "accessory" cell, usually a monocyte, carrying a B7 ligand that is recognized by the CD28 receptor on the T-cell. During the activation process, T-cells produce α and β subunits of the IL-2 receptor that combine with the γ subunit present on the membrane and secrete IL-2 as autocrine third signal.

The endpoint of the activation is mitosis of the T-cell, which is maximal 72 h after addition of the mitogen. The *mitotic index*, an indicator of

the proliferation rate triggered by the mitogen, is determined by treating the cells either with a pulse of a radioactive marker (e.g., trititated thymidine) or of a marker that can be detected via enzyme-linked immunosorbent assay (e.g., bromodeoxyuridine).

Ex Vivo* and *In Vivo

Ex vivo experiments are based on blood samples drawn from space crewmembers prior to, during, and after flight, which are diluted with culture medium and incubated in the presence of concanavalin A. *In vivo* studies consist of the application of antigens to the skin of space crewmembers in order to determine the delayed hypersensitivity, i.e., the specific response of T-lymphocytes, prior to, during, and after flight to a number of antigens (delayed-type hypersensitivity test or skin test). *In vitro* experiments are based on immune cells isolated from the peripheral blood of healthy donors (not necessarily astronauts) a few hours before the experiment is started, either in the space or in the ground laboratory, and cultured in a standard culture medium in the presence of a mitogen.

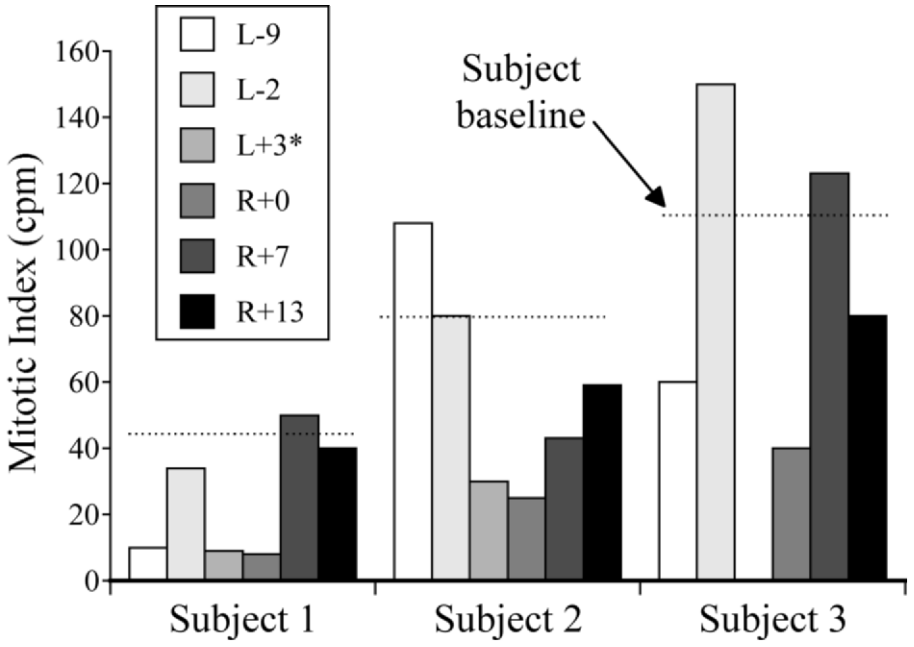

Figure 4-20. Mitotic index of T-cells from three space crewmembers exposed in vitro to Con A. Whole-blood samples taken 9 and 2 days before launch (L-), during flight on the 3rd mission day (L+), and 0, 7, and 13 days after landing (R+) were cultured for three days in the presence of the mitogen. The inflight samples were incubated in a centrifuge at 1 g. The data are expressed as counts per minute (cpm) of tritiated thymidine incorporated into DNA.

The objective of *in vitro* studies during spaceflight is to investigate the biological mechanism of T-cell activation under the influence of gravitational changes. The experiments *in vitro* in microgravity have contributed to understand certain aspects of signal transduction in T-cells. Studies *ex vivo* and *in vivo* on the immune cells and on the delayed hypersensitivity of astronauts on board Spacelab and the Mir space station, respectively, have helped to distinguish between the effects of gravity and those of physical and psychological stress. Briefly, the *in vivo* and the *ex vivo* studies permitted to establish that the depression of the T-cell-dependent immune response is due to the psychological and physical stress of spaceflight on the neuroendocrine system of the astronaut rather than to weightlessness *per se* (Figure 4-20).

In an experiment performed with blood samples from four astronauts in a multi-g centrifuge on Spacelab SLS-1 we were able to see that the threshold of sensitivity in T-cells ranges between 0 g and 0.6 g (Figure 4-21). An experiment designed to narrow the sensitivity gap by using a 0.2 g centrifuge was lost with Columbia STS-107. A new attempt will be undertaken on board the ISS.

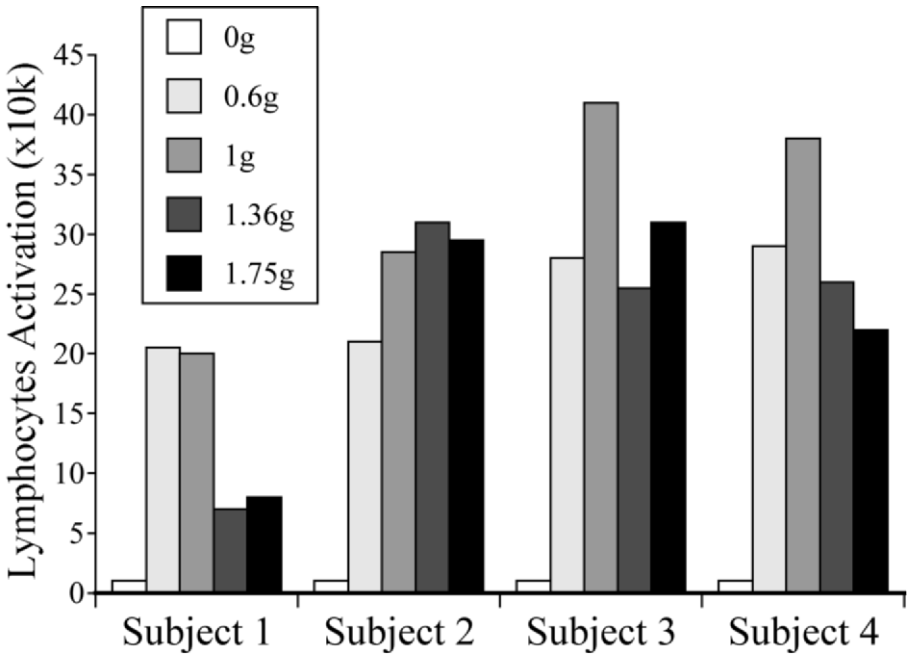

Figure 4-21. Mitotic index of T-cells from four space crewmembers exposed in vitro to Con A. Whole-blood samples taken during flight from four crewmembers and incubated for three days in the presence of the mitogen at 0 g and in a centrifuge providing 0.6, 1.0, 1.36, or 1.75 g. The data are expressed as counts per minute of tritiated thymidine incorporated into DNA.

Table 4-02 gives an overview on the most important findings on the effect of spaceflight on mammalian cell proliferation. Besides lymphocytes, hybridoma cells and macrophages also showed remarkable proliferation rate changes, whereas no changes were observed with embryonic lung cells, hamster kidney cells, rat myoblasts, or rat osteoblasts. However, in embryonic lung cells, the glucose consumption from the medium was 20% higher in the flight cultures than in the ground control, thus pointing to important metabolic changes that were not further investigated.

Cell type	***Effect***	***Remarks***
T lymphocytes with monocytes as accessory cells, human	*60-90% reduction of mitotic index upon activation by Con A of resuspended cells (6 independent experiments); 100% increase of activation of cells attached to microcarrier beads*	*Experiments in Spacelab 1, D-1, SLS-1, IML-2–all, except the first one, with a onboard 1-g control*
7E3 hybridoma cells	*40% increase of cell number after 4 d in space*	*Spacelab IML-1–onboard 1-g control*
Bone marrow derived macrophages, mice femora and tibiae	*Up to 60% increase in cell number after 6 d in space*	*Space shuttle STS-57, -60, and -62–no onboard 1-g control; incubation temperature between 23 and 27°C*
WI38 embryonic lung cells, human	*No effect of growth rate during 28 d in space*	*Skylab–automatic medium supply; no onboard 1-g control*
Kidney cells, hamster	*No alteration of cell number in cells attached to microcarrier beads after 7 d in space*	*Spacelab IML-1–onboard 1-g control*
L8 myoblast cells, rat	*No change of proliferation rate in cells attached to collagen-coated microcarriers beads*	*Space shuttle–no onboard 1-g control*
Osteoblasts, rat	*No change in cell growth rate*	*Spacelab IML-2–no onboard 1-g control*

Table 4-02. Effects of spaceflight on cell proliferation in mammalian cells.

As said above, proliferation is the end-point of a biological process. To understand the mechanism of such effects, it is necessary to analyze intermediate signal transduction pathways. Such studies are outlined below.

Interestingly enough, the mitotic index is increased significantly in a number of cell types cultured at 10 g in a centrifuge. In a study conducted on HeLa cells[11] it could be seen that proliferation rate is increased at 10 g while motility tracked on colloidal gold was strongly reduced compared to 1-g controls. In analogy with the considerations made with *Paramecium*, it was

[11] *HeLa cells* are an established line of human epithelial cells derived from a cervical cancer.

speculated that at 10 g the cells switch their energy turnover from motion towards mitosis.

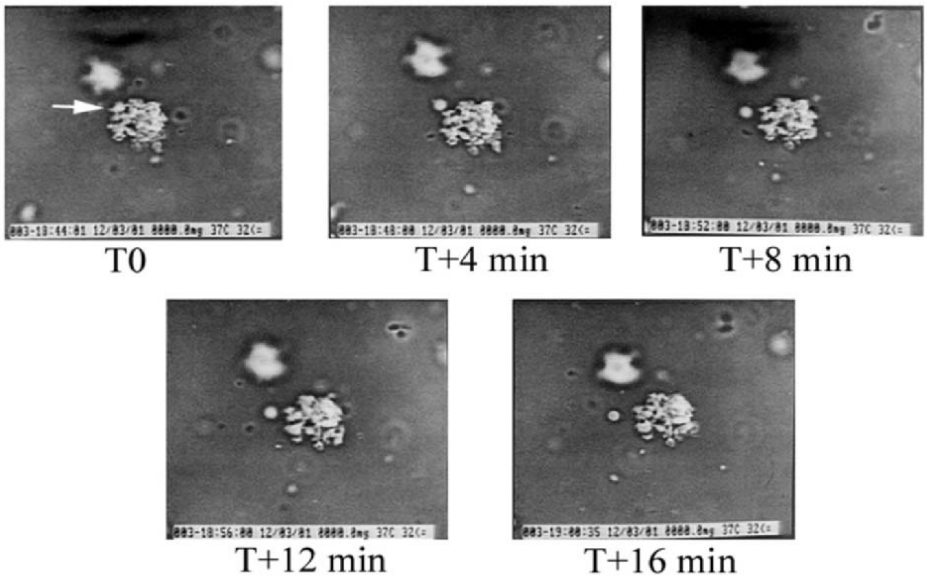

Figure 4-22. Aggregates of lymphocytes incubated for 78 h in the presence of Con A in the NIZEMI facility. The arrow indicates a cell moving out of the aggregate. Courtesy of M. Cogoli-Greuter, Zero-g Life Tec GmbH, Zurich, Switzerland.

3.2.2 Morphology and Motility

Important cellular functions are regulated by cell-cell interactions. This is particularly important in the activation of T-lymphocytes. It was possible to show that white blood cells are capable of autonomous movements and interactions in microgravity (Figure 4-22). Again, this is a surprising and unpredictable finding. It was thought that mammalian cells can move only on a substratum, and that gravity is somehow driving the motion. Moreover, it was also seen that the cytoskeleton undergoes structural changes few seconds after exposure to 0 g (Figure 4-23). The cytoskeleton plays an important role during signal transduction, in particular, in the interaction of the cytoskeleton with G-proteins. Alteration of microtubules and increased apoptosis in space were detected in Jurkat cells, a T-cell derived cell line. As shown in Table 4-03, leukocytes are again the cells showing the most remarkable effects of microgravity on the cytoskeleton.

The attachment to a substratum of adhesion-dependent cells was tested in microgravity with human embryonic kidney cells in an experiment carried out in an incubator installed in the flight deck of Space Shuttle mission

STS-8. Microcarriers were added inflight to the cells in culture at 37°C. Scanning electron microscopy showed that attachment took place qualitatively and quantitatively as in the ground controls, thus confirming that the related membrane functions are not altered at 0 g. Similar conclusions have been arrived at from clinostat experiments with human colorectal carcinoma cells.

Cell type	***Effect***	***Remarks***
T lymphocytes with monocytes as accessory cells, human	*Normal attachment of Con A to the cell membrane; slightly retarded patching and capping; cell motion in the presence of Con A is higher at 0 g than at 1 g; elongated cell shape and contraction waves.* *In the presence of Con A: cell motion as above; formation of cell aggregates smaller than at 1 g; cells move out/in of aggregates; Apoptotic cells in suspension at 0xg, normal morphology at 0 g in microcarrier-attached cells*	*Sounding rockets* *NIZEMI rotating microscope, on board Spacelab IML-2; Spacelab D-1, SLS-1–onboard 1-g control*
A431 epidermoid cells, human	*No change in clustering of the receptors of epidermal growth factor*	*Sounding rocket*
Embryonic kidney cells, hamster	*Normal attachment to microcarrier beads*	*Space Shuttle–onboard 1-g control*
WI38 embryonic lung cells, human	*No changes of ultrastructure, no effect on cell migration*	*28d culture in Skylab; onboard time-lapse cine cameras–no onboard 1-g control*
Erythrocytes, human	*Dramatic decrease of cell aggregation*	*2 experiments in Space Shuttle, no 1 g control*
L8 myoblasts, rat	*Cells fail to fuse and differentiate into myoblasts and show atypical morphology in culture after exposure to 0 g*	*Space Shuttle–no onboard 1-g control*
Jurkat cells, human T-cell line	*Significant changes of the cytoskeleton: large bundles of vimentin are formed after 30 sec in 0 g* *Alteration of the microtubules and increased apoptosis*	*Sounding rockets* *Shuttle flight–onboard 1-g control*
Friend leukemia-virus transformed cells, murine	*No changes in the ultrastructure of the cell*	*Spacelab–onboard 1-g control*
Cerebellum cells, murine	*At 0xg Cells form aggregates that are larger in number but smaller in size than in the inflight 1-g controls*	*New instrument in Spacelab IML-2–onboard 1-g control*
Osteoblast cell line MC2T3-E1	*Changes in cell shape and extracellular matrix*	*Space Shuttle–onboard 1-g control*
Tubulin/microtu-bules	*Almost no self organization of tubulin into microtubuli*	*Sounding rocket–onboard 1-g control*

Table 4-03. Effect of spaceflight on morphology and motility of mammalian cells.

In an experiment with Friend cells* conducted in *Biorack* during IML-1, extensive analysis (scanning, transmission, volume measurements) of the ultrastructure of cells cultured for six days in the presence of DMSO did not reveal differences between the cell cultures at 0 g and in the onboard 1-g centrifuge. As mentioned above, in an experiment with WI38 human embryonic lung cells carried out in Skylab, cinematographic recording, phase, electron and scanning microscopy produced no observable differences in ultrastructure and in cell migration between flight and ground controls.

Several permanent phenotypic alterations were recorded in cell cultures of rat myoblasts, which were recultured on Earth after return from a 10-day Space Shuttle flight (STS-45). The differences included altered morphology and failure to fuse and differentiate into myotubes. Unfortunately, the spaceflight cultures were accommodated in an automated cell culture apparatus in a middeck locker of the Space Shuttle, and there was no onboard 1-g control. Consequently, the cause of the altered phenotype is unknown.

Changes in bone extracellular matrix and osteoblast shape were detected in cell cultures in real microgravity by Hughes-Fulford et al. (2002). Such changes were not caused by an alteration of the transcription determined with the *Reverse Transcriptase-Polymerase Chain Reaction* (RT-PCR) technology, translation of fibronectin, nor by altered matrix formation.

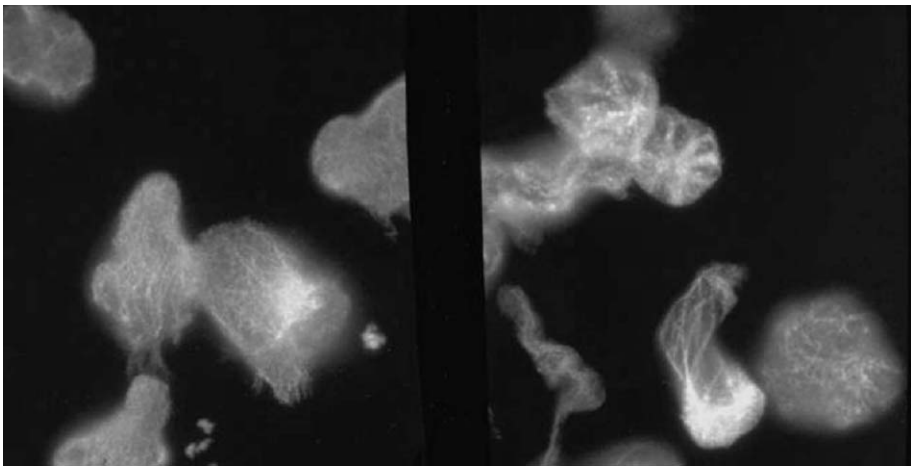

Figure 4-23. Vimentin filaments in Jurkat cells (a derived T-cell line) flown on the sounding rocket Maxus detected with fluorescent antibodies. Courtesy of G. Sciola (see color insert).

* In 1956, Charlotte Friend described a new "virus-like" agent that caused a malignant disease of the hematopoietic system in mice. These cell lines came to be known as *Friend Erythroleukemia Cells* (FELC).

3.2.3 Signal Transduction and Gene Expression

In this section, I discuss in some detail how exposure to microgravity may change the production and secretion of specific cell products. Such changes usually reflect important alterations of the signal transduction pathway that may be located at different steps such as the recognition of an activator or the genetic expression of a cytokine. Several techniques have been used in space experiments like the simple determination of proteins in the cell culture medium, the measurement of gene expression with RT-PCR technology, or with the modern and sophisticated microarray technology. Signal transduction is an extremely complicated process involving membrane receptors, G-proteins, the cytoskeleton, several protein kinases, transcription factors, and oncogenes. Many aspects of such process are still obscure and extensively studied worldwide. Microgravity may offer a new tool to study this subject from a new perspective.

Table 4-04 presents an overview of the most important data on genetic expression and metabolism of mammalian cells in space. Thereby it is important to distinguish between genetic expression determined as the amount of a protein (generally a cytokine) newly produced and secreted in response to a specific signal and, more properly, as the specific mRNA determined quanti- and qualitatively, with either the RT-PCR or the microarray technology.

It has been possible to identify the failure of the expression of the interleukin-2 receptor in T-lymphocytes as one of the possible causes of the loss of activation in microgravity. In a ground-based study conducted in the random positioning machine in our laboratory Walther could show, with the PCR-RT technology, that genetic expression of IL-2 is depressed at early time of mitogenic activation of T cells. Moreover, the genetic expression of the alpha subunit of the IL-2 receptor (IL-2Rα) is depressed in the random positioning machine while that of the beta subunit remains unchanged. A differential effect of simulated microgravity on the genetic expression of two strictly related components of the IL-2 receptor points to a direct effect of gravity on the activation mechanisms of T cells.

Investigations conducted by Hashemi et al. (1999) on human peripheral mononuclear cells in conditions of simulated microgravity (by means of a fast rotating clinostat) as well as in space have confirmed that the expression of IL-2Rα is inhibited at 0 g in T-cells exposed to anti-CD3 (a component of the TCR). However, such inhibition disappears in the clinostat and is partially restored in actual microgravity when activation is carried out with phorbol ester and calcium ionophore thus bypassing the TCR and *Protein Kinase C* (PKC). These data point to PKC as a possible key element of the sensitivity to gravity of T-cells and are in agreement with the findings on Jurkat cells described below.

Cell type	Effect	Remarks
T lymphocytes with monocytes as accessory cells, human	500% increase of interferon-α secretion induced by various agents Con A activation of cells attached to microcarrier beads: 2.5 fold increase in interferon-γ production and 2 fold increase in production of IL-2. Depression of genetic expression of IL-2 and IL-2 receptor α-unit, but not of β-unit. Depression of genetic expression of IL-2 receptor α-unit and of CD69; depression restored bypassing TCR activation by phorbol ester and calcium ionophore	Salyut 6–incubator switched off during crew-sleep period; Spacelab–onboard 1- g control Random positioning machine Shuttle flight–onboard 1-g control
Monocytes as accessory cells in T-lymphocyte culture, human	Contradictory results: Nearly total inhibition of IL-1 production in resuspended cells; normal IL-1 secretion	Spacelab SLS-1 and IML-2, respectively—onboard 1-g control
Jurkat cells, human T cell line	Normal production of IL-2 after induction with anti-CD3 monoclonal antibodies in the presence of THP-1 cells; 100% inhibition of IL-2 production induced by calcium ionophor and phorbolester. The distribution of PKC is altered in 0 g	Russian biosatellite Spacelab IML-2–onboard 1-g control
THP-1, myelomonocytic cell line	Normal production of IL-1β after induction with anti-CD3 monoclonal antibodies in the presence of Jurkat cells; 85% inhibition of IL-1β production induced by phorbolester	Russian biosatellite
7E3 hybridoma cells	Production of monoclonal antibodies, consumption of glucose and glutamin as well as secretion of lactate and ammonia decreased	Spacelab IML-1–onboard 1-g control
Spleen cells, murine	Increased secretion of interferon-α upon stimulation with polyinosini-polycytidylic acid	Space Shuttle middeck, ambient temperature
B6MP102 macrophage cell line	Increased secretion of IL-1 and interferon-γ induced by lipolysaccharide	Space Shuttle middeck, ambient temperature–no onboard 1-g control
Bone-marrow-derived macrophages, mice femora and tibiae	150% increase of IL-6 secretion, up to 100% decrease of phenotypic marker expression of MHC-II and MAC-2	Space shuttle STS-57, -60, and -62–no onboard 1-g control–incubation between 23 and 27°C
Friend leukemia-virus transformed cells, murine	No changes in metabolic behavior: glucose and glutamin consumption, production of haemoglobin, lactate and ammonia in dimethylsulfoxide-induced production of hemoglobin.	Spacelab–onboard 1-g control

Table 4-04. Effect of spaceflight on signal transduction, genetic expression, and metabolism in mammalian cells.

A number of interesting effects were observed by Chapes et al. (1992) in space cultures of three types of immune cells. However, the cultures were kept in the middeck of the Space Shuttle at ambient temperature throughout the incubation time instead of in an incubator at 37°C (an obvious requirement in work with mammalian cells), so the results must be interpreted with caution. The anchorage-dependent bone marrow-derived macrophage cell line B6MP102 secreted, upon activation with lipopolysaccharide, significantly more IL-1 and TNF-a in space than on the ground. Murine spleen cells, stimulated with poly I:C released significantly more IFN-a in space than on Earth. Also, human lymphocytes as well as murine lymph node T-cells activated with Con A produced significantly more IFN-g in space than on Earth. Experiments on Shuttle flight STS-50 found that cellular cytotoxicity caused by TNF-α was inhibited. This was confirmed in experiments on later flights (STS-54 and STS-57) and it was found that TNF mediated cytotoxicity was restored to levels observed in the ground controls in the presence of inhibitors of PKC. The authors conclude that spaceflight ameliorates the action of TNF by affecting PKC in target cells, but none of these experiments were accompanied by onboard 1-g controls (only ground controls were available) so what aspect of spaceflight is effective has not been established.

The metabolic data of an experiment with *Hybridoma* 7E3-N cells[12] in Spacelab IML-1 revealed another interesting behavior pattern: the production per cell of monoclonal antibodies, the glucose and glutamine consumption per cell, as well as the secretion per cell of waste products like lactate and ammonia were lower at 0 g than at 1 g. In fact, the lack of significant differences of metabolite concentrations in the supernatants at 0 g and 1 g is only apparent since approximately 40% more cells were present in the cultures at 0 g than in those at 1 g. Although there is not yet an explanation, the data show that gravitational unloading had significant effect on hybridoma cell metabolism. It appears that the transition from a two-dimensional configuration, as in the case of cells sedimented to the flat bottom of the culture flask at 1 g, to a three-dimensional configuration, as for free-floating cells at 0 g, increased cell proliferation despite a lower metabolic turnover. It appears also that the biosynthesis of a specific cell product was coupled to the glucose/glutamine consumption and to the lactate/ammonia secretion rather than to the proliferation rate.

[12] *Hybridoma* is a type of hybrid cell produced by fusing a normal cell with a tumor cell. When lymphocytes (antibody-producing cells) are fused to the tumor cells, the resulting hybridomas produce antibodies and maintain rapid, sustained growth, producing large amounts of an antibody. Hybridomas are the source of monoclonal antibodies.

In an experiment with Friend cells during the IML-1 mission, the amount of hemoglobin produced upon induction with *Dimethyl Sulfoxide* (DMSO) was the same in the flight 0-g and ground 1-g samples. The counts of haemoglobin-positive cells show that 60 to 70% of the cells were induced to express haemoglobin upon exposure to DMSO. Again, there were no significant differences between cultures at 1 g and 0 g. The metabolic analyses on glucose and glutamine consumption, as well as on lactate and ammonia production, clearly reflected the fact that Friend cells do not change their behavior in microgravity.

Production of *tissue plasminogen activator* (t-PA) by hamster kidney cells was determined during the IML-1 mission. Tissue plasminogen activator is a substance of high pharmaceutical value since it is used to prevent the formation of blood clots where there is risk of thrombosis. There was no difference in metabolic data on t-PA production, data on the consumption of glucose and glutamine from the medium, nor on the secretion of waste products like ammonium and lactate between the cultures kept at 0 g and those kept at 1 g in the onboard centrifuge or in the ground laboratory.

Limouse et al. (1991) and deGroot et al. (1990, 1991) were the first researchers to investigate intermediate steps of signal transduction in space. The former suggested that the function of *Protein Kinase C* (PKC) is altered in Jurkat cells exposed to 0 g. Hatton and Schmitt (1999) continued these studies, and showed that the intracellular distribution of PKC was changed in microgravity. The use of the RT-PCR technology was introduced for the first time in space experiments to study the activation of epidermoid cells by epidermal growth factor. A significant depression of the expression of the early oncogenes c-fos, c-jun was detected (deGroot et al. 1990, 1991). With the same technology, Kumei et al. (1996) were able to show that the amount of mRNA of the enzyme prostaglandin G/H synthase-2 is remarkably enhanced in rat osteaoblasts cultured in space. Akiyama et al. (1996) have developed a RT-PCR procedure tailored to the peculiar constraints of spaceflight, in particular to very low amounts of biological material. Thanks to this progress, the RT-PCR technology will certainly contribute to important investigations on future space missions.

Hammond et al. (1999) grew primary human renal cell cultures in a steady state 0-g environment onboard the STS-90 Neurolab mission for six days. Gene expression analysis using microarray technology was used to monitor gene expression. More than 1,632 genes changed at steady state.

The identification of clock genes in mammalian cells raises a new and exciting question: Does gravity and spaceflight in general interfere with the expression of clock genes? It is conceivable that altered gravitational conditions may have an influence on the mechanisms regulating the circadian rhythms. Experiments in such direction are planned on future missions.

3.3 Conclusions

Based on the experimental data outlined in the previous sections, we can answer a number of questions and draw some conclusions on the sensitivity to gravity at the cellular level:

a. The function of receptors (like the TCR) seems not to be influenced as shown by the normal binding of Con A to the T-cell membrane;
b. The membrane function is not affected as well as shown by the normal patching and capping of the membrane proteins interacting with Con A;
c. Cell-cell interactions and autonomous movements are occurring under 0 g conditions as shown by the experiments conducted with NIZEMI;
d. There are changes in the cytoskeleton and in cell shape as shown in several experiments. This may have an important impact on signal transduction as G-proteins, a pivotal element in the signal transduction pathway between receptor and protein lipase C are interacting with the cytoskeleton;
e. PKC is probably one of the key elements affected by altered gravity;
f. The consequence of all this is the depression of the genetic expression of IL-2 and IL-2Rα in T-cells and probably also of the oncogenes c-fos and c-myc in epidermoid cells (depression of oncogenes expression may also occur in T-cells, such experiments will be conducted soon in space);
g. The differential genetic expression under simulated microgravity in the random positioning machine of IL-2Rα (depressed) and of IL-2Rα (unchanged) is a strong argument in favor of direct effects of gravity at the cellular level;
h. It seems that cells undergoing differentiation processes are more sensitive to gravitational changes than cells, like cancer cells, that have reached the endpoint of their development.

4 SPACE RESEARCH IN CELL BIOLOGY: ISSUES

The results and impact of the findings in space biology are not well known to the majority of the scientific community. Main reasons are the limited access to space laboratories and the difficulty to repeat the experiments to confirm their results and to increase their statistical significance. Nevertheless, the data collected so far confirm the scientific, technological, and biomedical relevance of space biology. Some of the problems preventing a large community of scientists from conducting experiments in space are outlined here.

First, the access to space is limited. Only a small number of projects can be accommodated on board a Spacelab or SpaceHab mission. The consequence is that the statistical significance of the data is sometimes questionable and the reproducibility of important results is difficult to verify by independent team. For instance, less than 20 experiments were hosted in each of the seven Biorak flights. In addition, the number of flight opportunities in Spacelab, Mir, ISS, automated satellites, and sounding rockets is very low compared to the number of investigations proposed.

Second, the resources available in a space laboratory are very limited. Energy, weight and volume of the payload, as well as crew time have to be shared among several users from different disciplines, such as material and fluid sciences, medicine and biology. The incubation temperatures usually available are 22°C and 37°C. While the last value is adequate for all mammalian cells, 22°C is often offered as a compromise for "ambient temperature". Freezing conditions are limited to -10°C or -20°C, a large difference from the standard preservation conditions on ground, which usually include -80°C and -180°C. This means significant restriction of the manipulations, analytical procedures such as microscopic and biochemical determination, and controlled storage and stowage of biological samples in orbit. Another disturbing limitation is the so-called late access time, i.e., the latest time at which biological samples can be delivered for installation on board. This time ranges between 15 and 25 h before launch (See Chapter 3, Section 2.2). Also, several living probes must undergo special treatment in order to be viable for the processing in orbit. The consequence is that the flight experiment protocols are less sophisticated and comprehensive than those of equivalent investigations on Earth. ESA has supported programs of investigations to assess condition for optimum preservations of biological samples before and after experimentation in space. Another important issue is that of the proper controls. There is a consensus in the scientific community today that centrifuges providing 1-g in flight are necessary to control for all the other spaceflight environment factors, such as vibrations, accelerations, temperature fluctuations, and cosmic radiation typical. While *Biorack* was fitted with an onboard 1-g centrifuge, most of the experiments performed in other Shuttle flights lacked such control. Newer facilities like *Biopack*, *Kubik*, *Biolab*, and the *Modular Cultivation System* are equipped with centrifuges providing centrifugal accelerations ranging from 0 g to 1 g.

Third, the safety of the astronauts requires severe acceptance criteria for instruments and biological materials on board. For example, the tolerance limits for out-gassing of toxic or bad-smelling gases, and for electromagnetic contamination are extremely low; sharp edges must be avoided; and biological/chemical contamination's derived from viruses and bacteria or biological fluids must be prevented by independent triple containment

(Figure 4-24). Moreover, instruments shall not interfere, both electrically and acoustically, with each other.

Figure 4-24. ISS Expedition-6 Astronaut Kenneth D. Bowersox works with an experiment in a portable glovebox facility in the Destiny laboratory on the International Space Station. Photo courtesy of NASA.

Fourth, the period between the acceptance of a proposal and the flight of the experiment flight can span over several years. This is without counting the delay due to technical problems with the flight vehicles. For example, the first flight of the Space Shuttle took place in 1981 instead of 1978, as originally planned when the call for experiment proposals was issued. Another long delay followed the loss of Challenger in 1996, and the same holds true now after the loss of Columbia. The consequence is that many science proposals are obsolete at the time of flight. Requests of updates of flight protocols or new requirements during the preparation of the experiments are very difficult to have approved by the space agencies.

Fifth, failures due to instrument malfunctions, break-down of resources, and crew errors may even cause the total loss of an investigation prepared for years, often without an opportunity for a reflight.

5 CELL BIOLOGY IN SPACE: OUTLOOK

The Mir space station and Spacelab eras delivered invaluable lessons on how to carry out life and physical science research in space and on the management of emergency situations during spaceflight. Such know-how will

be very useful during ISS operation in the next 20 years. ISS will be dedicated mainly to microgravity experimentation and technology. Cell and plant biology will play a prominent role. The *European Modular Cultivation System* (mainly for plants) and *Biolab* (mainly for cells) will host dozens of experiments. Whole experiment cycles will be repeated. Cells and plants will be cultivated over generations. Bioprocesses with interesting commercial return may develop. One biotechnological application will certainly consist of closed ecological life support systems aimed at the recycling of anthropogenetic water, carbon anhydride and other biological waste, and the production of fresh food (vegetables as well as animal) in space. A new ESA facility for cell biology experiments, called *Biopack* and designed to bridge the gap between Spacelab and *Biolab* on ISS, was lost with Columbia STS-107. Another instrument, called *Kubik*, will be used on ISS (see Figure 3-10), while the experiments will be transported to orbit by the Russian Soyuz spaceships.

In the meantime, several investigations are being carried out on the ground with the random positioning machine, or three-dimensional clinostat, invented by Hoson. Such a machine shall be used first to select biological systems suitable for basic investigations or for profitable bioprocesses in space and, second, to optimize experimental protocols of investigations selected for spaceflight.

The problems encountered in the preparation and execution of experiments in space shall not, however, discourage those scientists who might be interested to carry out experiments during spaceflight. The question these scientists should ask is the following: Am I ready to accept all the kind of hurdles to perform a space investigation that has great chances to fail, when the same resources used in a ground laboratories would allow the conducting of other interesting studies on the most challenging questions of today's biology?

Nevertheless, there are at least four good reasons motivating the efforts and the patience of space biologists. One is the scientific curiosity to expose living systems to conditions that have never been experienced before throughout evolution, such as microgravity and cosmic radiation. The unexpected and important results of several experiments show that even very simple organisms display drastic changes in microgravity. In this context, microgravity can be considered as a new tool to study complex biological mechanisms from a new perspective. For example, in cell cultures the transition from 1 g to 0 g changes the geometry of the system from a two-dimension to a three-dimension environment. Most living systems are thermodynamically very complicated non-equilibrium systems. Therefore, they may follow interesting bifurcations. Microgravity may favor a new path that is not even suspected in normal gravity. The study of such a path contributes to the clarification of unknown biological processes. As

microgravity and cosmic radiation are not reproducible on Earth, the only way to perform this research is to go to space. Simulations in devices like clinostats are a useful and necessary complement, but not a replacement for space. In general, the data from ground-based simulations are qualitatively but not quantitatively similar to those obtained in space.

Another reason is to study specific physiological functions at the cellular level, either *in vitro*, i.e., in a test tube, or *ex vivo*, i.e., in cells drawn from tests subjects exposed to the conditions of spaceflight. Examples are the studies of the immune system with peripheral blood lymphocytes, or the bone system with condrocytes and osteoblasts. Such studies have shown evidence, for instance, of the effect of physical and psychological stress on the human immune system. This is a very interesting topic of neuroimmunology, a young discipline of growing importance in today's hectic life. Another intriguing problem is to determine the impact of gravity on the cell. Such question is obviously clearly answered in the case of the plants with their geotropism. It is also true that all major discoveries in cellular biology never took gravity into account.

A third reason is the technological return of space cell biology. The constraints of spaceflight result in high-tech challenges in the application of analytical techniques and in the development of flight instrumentation. Examples are the adaptation of the RT-PCR technology to the very small amounts of biological material available. In fact, the limits of weight and volume in orbit do not permit the use of the same samples or aliquots that biologists are accustomed to working with on ground. An example of sophisticated instrumentation is the development of the space bioreactor installed in *Biorack*. The introduction of microsensors, a new pH control system based on the electrolysis of water instead of the traditional neutralisation of acidity with NaOH, and piezoelectric micropump for fresh medium supply, opened new ways to the bioreactor technology.

In addition, basic research with single cells in space may show new perspectives in biotechnology and biochemical bioengineering. The fact that mammalian single cells undergo profound alterations in microgravity has nourished hypotheses and speculations on their possible commercial and medical applications. Bioprocessing in space is one promising theme for the commercial exploitation of the ISS. Several pharmaceutical companies have manifested their interest in joint application research programs with national and international space agencies. In Europe, ESA has started a *Microgravity Application Program* (MAP) to support application-oriented projects with participation of non-aerospace industries. An example of such activity is the first MAP project that started in May 2000 and that is aimed at the development of instruments, such as bioreactors, and technologies for tissue engineering. The objectives of the project are: to develop procedures of in vitro organogenesis of pancreatic islets, thyroid tissue, liver, vessels and

cartilage; to study the mechanism of organogenesis in low-g; to define the requirements of a modular space bioreactor for medically relevant organ-like structures; and to set up procedures for the production of implants for medical applications. Experiments will begin in the random positioning machine on the ground and continue on board the ISS. There is also a strong support by NASA in the U.S. An example is the tissue-engineering project dedicated to cartilage conducted by Freed and Vunjak-Novakovic (2002) at MIT in Boston (Figure 4-25).

Finally, the last but not least reason is the exploration of space. This includes trips to an Earth orbit as well to the planets of the Solar System and, in a far future, to other planetary systems. It is important that the adaptation of the physiological functions of humans and other mammals, as well as other organisms like plants, invertebrates, and microbes are investigated and clarified. It was and it will be an irresistible drive of our mankind to explore first all continents of the planet Earth and, later, any accessible site of the Universe as soon as the required technology becomes available. Space exploration includes also the search for extraterrestrial life. The study of terrestrial life out of the terrestrial environment will contribute to the identification and understanding of alien forms of life.

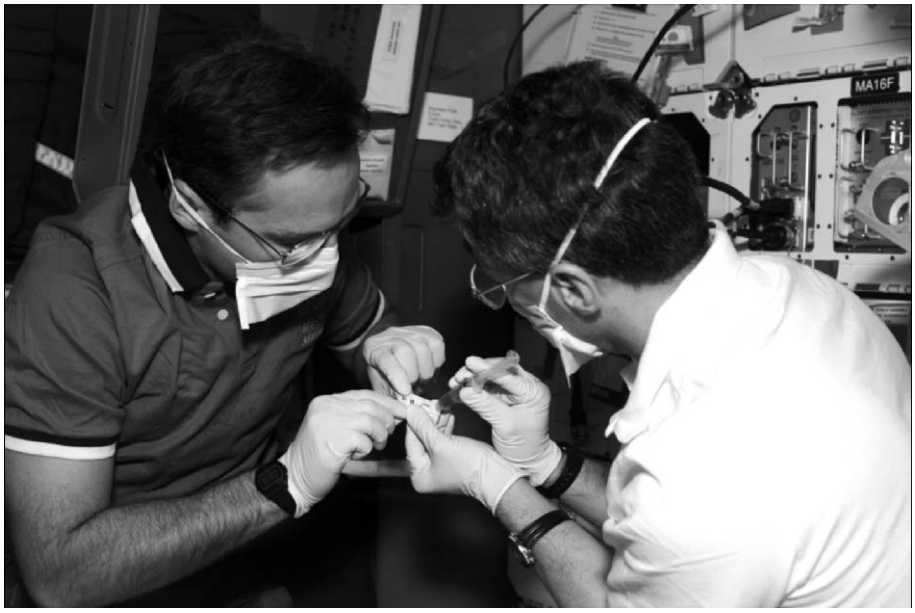

Figure 4-25. On board the SpaceHab module in the Space Shuttle Atlantis, astronauts Carl Walz and Jerome Apt analyze a bovine cartilage for its pH, CO_2, and O_2 content. Photo courtesy of NASA.

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Chapter 5

ANIMAL DEVELOPMENT IN MICROGRAVITY

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Life on Earth has developed under a constant gravitational field. It is therefore reasonable to assume that living organisms incorporate the ever-present gravitational signal in some or even all mechanisms of their development. Microgravity provides a research environment allowing to identify those developmental processes for which the underlying mechanisms depend, at least in part, on the gravitational signals for their initiation or successful completion. This chapter reviews both pioneer and recent results from space experiments on developmental processes and their behavioral consequences in insects, fish, amphibians, birds, and mammals. A number of these experiments were performed under the responsibility of the author[1]. Also, development cannot be discussed without remarks on aging. Finally, this chapter concludes with perspectives of necessary research strategies in the area of developmental animal biology for the future.

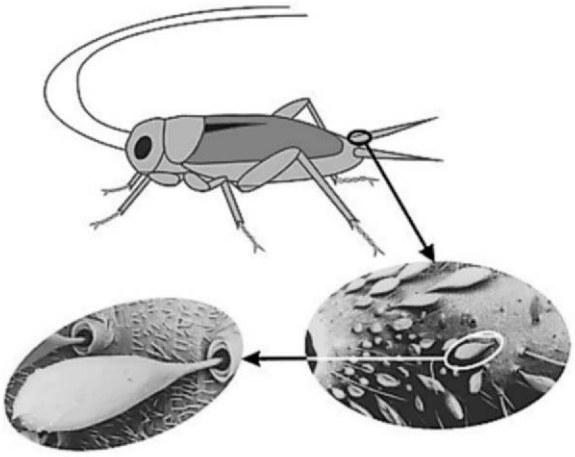

Figure 5-01. The house cricket (Acheta domesticus) has club-shaped gravity sensitive structures on appendages, called cerci, at the end of its body. Space studies have investigated the development and regeneration of these gravity sensors in microgravity and their consequences on the animal's posture during passive body tilt after return to Earth.

[1] The German Space Agency (DLR) supports studies from the author since 1989.

1 INTRODUCTION

The development of a fertilized egg to an adult organism requires an excellent tuning of molecular, morphological, and physiological mechanisms, which are under the control of genetic programs and environmental factors. All organisms and their coordinated functions are affected by this concerted interplay because the aim of development is the formation of a stable organism that can survive despite of disturbing genetic and environmental factors. The principles of these mechanisms as well as the susceptibility to modified environmental conditions can be studied in the complete system as well as in isolated components such as cell cultures or isolated organs.

The success of developmental processes including fertilization, cleavage, and organogenesis, as well as function and behavior, nursery and regeneration determines the ability of a species to survive in a certain environment. The spaceflight environment includes several potential hazards such as radiation, alterations in atmospheric pressure, prolonged toxic exposure, and weightlessness that may affect developmental processes.

The last step in development is maturation, i.e., the formation of a complex adult organism able to reproduce for maintaining survival of the species. Under normal health and environmental conditions, the adult organism is characterized by a complete harmony between morphological, physiological, molecular, and genetic features. They guarantee beating of the heart, circulation of blood, or goal-directed behavior, and, as the ultimate step, self-consciousness. However, each developmental stage, from the fertilized egg to the embryo and larva, is a step along the way to the adult stage and possesses its own functional stability.

To explain the changes in behavior, physiology, anatomy, or biochemistry induced by micro- and hyper-gravity, it is necessary to know the developmental characteristics of each specific structure and function. This knowledge allows distinguishing between developmental acceleration or retardation on one hand, and activation of neuroplastic or adaptive processes on the other hand, in response to modification of environment or genetic influences. Aquatic animals have been largely used in space studies on development biology. This is because the characteristics of anatomical, physiological, biochemical, and behavioral changes during development and maturation from embryonic stages to the adult are relatively well known, in particular for the sensory systems. For example, space biologists have studied the development of brain in fish (Slenzka et al. 1993), vestibulo-ocular reflex in fish (Sebastian and Horn 1999) and amphibians (Horn et al. 1986a, 1986b), otolith organs (Koike et al. 1995, Wiederhold et al. 1995) and the efficiency of vestibular compensation in lower vertebrates (Rayer et al. 1983). Similar characteristics with a comparable high resolution are rare in mammals, which makes it difficult to use them as models for space studies.

2 FERTILIZATION AND EMBRYONIC DEVELOPMENT

Survival of organisms over the generations depends on successful reproduction. This process is critical for plants and animals including humans. Its aim is the production of viable progeny. In organisms affected by altered gravity, reproduction is not a self-evident fact, as shown by the following two examples:

a. Male rats mated 5 days after flight to non-space experienced females produced offspring with growth retardation and many abnormalities such as hydrocephaly, ectopic (out of place) kidneys, and enlargement of the bladder. Mating 2.5 to 3 months after the spaceflight produced healthy and viable offspring. Mating in hypergravity at 2 g revealed a reduction of pregnancy, while no successful pregnancy was reported after mating during exposure to 4.7 g (Tou et al. 2002).
b. Mating activity of orbited males of the parasitic wasp *Habrobracon* was severely disrupted, hatchability decreased, and fecundity of eggs was enhanced (von Borstel et al. 1970).

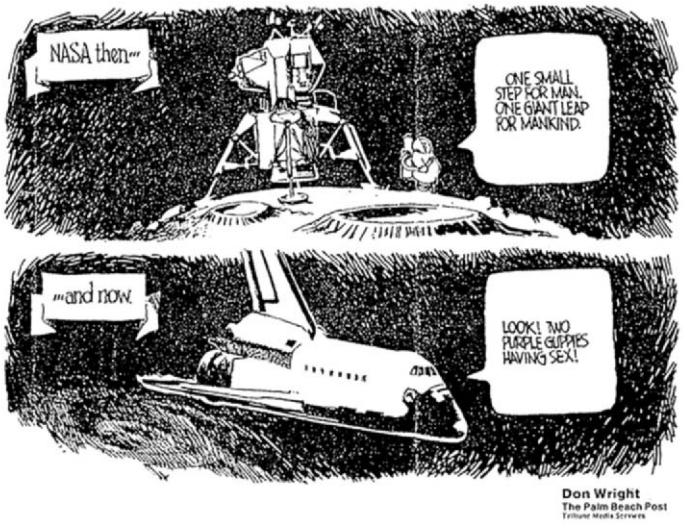

Figure 5-02. This cartoon has been published in the New York Times (24 July 1994) during the IML-2 Spacelab mission. This flight carried out an experiment entitled "Mating behavior of the Medaka fish and development of their eggs in space". Source Ijiri (1995).

Fertilization might also be affected by mobility changes in sperm. In fact, it is known that bull sperm swim with higher velocity in microgravity. This increased velocity is coupled to changes in phosphorilization of specific flagellar proteins (Tash and Bracho 1999).

Altered gravity changes mammalian male and female reproductive systems in a rather complex manner. For example, a transient but dramatic reduction in testis weight and testosterone has been reported in male rats.

However, the pituitary[2] responded in a physiological manner to changes in plasma testosterone, indicating that the hypothalamic-pituitary-gonadal axis was not impaired by spaceflight. So, spermatogenesis was not reduced. Examination of the ovaries of postpartum[3] rats flown in space during 9 to 20 days of gestation showed no effect on ovarian weight or number of pre-ovulatory or atretic follicles[4] (Tou et al. 2002). Observations about hormonal changes revealed reduced pituitary *Luteinizing Hormone* (LH), but no change in plasma LH. Pituitary *Follicle Stimulating Hormone* (FSH) was not affected while plasma FSH was elevated (Burden et al. 1997). Thus, the physiological mechanisms for reproduction are obviously intact in microgravity, despite of modifications of some components of the complete system.

2.1 Fertilization in Microgravity

During the 15 days of the second International Microgravity Laboratory mission (IML-2, STS-65), natural mating of male and female vertebrates occurred in microgravity and was videotaped for the first time. The Japanese Medaka fish *Oryzias latipes* mated successfully in space and young fish hatched during the flight (Figure 5-02). Back on the ground, the offspring produced healthy second-generation (F2) animals (Ijiri 1998).

Years before this video-based demonstration of successful fish mating in microgravity, several observations pointed to successful natural in-flight mating in other species. For example, the effect of spaceflight on fertilization, growth, and development of the fruit fly *Drosophila melanogaster* was observed during a 4-day Vostok mission. It was likely that in-flight mating occurred because embryos were recovered after landing (Antipov et al. 1965). Later, a colony of nematodes *Caenorhabditis elegans* flew on board the European facility Biorack (see Figure 4-03) during the Spacelab IML-1 mission. The animals successfully reproduced twice in space and generated thousands of offspring (Nelson et al. 1995). Natural mating in microgravity by rats was tried during an 18.5-day spaceflight on board the Russian Cosmos-1129 biosatellite flight. Post flight investigations on the flight dams revealed that ovulation, copulation, and fertilization had occurred in microgravity, but due to some unclear events, females were prevented from delivery of young. This experiment was never repeated so that until today, no rat or other mammal has undergone birth in space. It is worthwhile to note, however, that in this early experiment, the ground-control females, too, failed to deliver pups (Ronca 2003b).

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[2] The *pituitary* is an endocrine gland located at the base of the brain, which secretes important hormones including growth hormone and sexual hormones.

[3] The period of time after giving birth.

[4] Each month an egg develops inside the ovary in a fluid filled pocket called a *follicle*.

In 1998, the tropical freshwater snail *Biomphalaria glabrata* flew on board Space Shuttle STS-89 and STS-90 missions. Videotaping of *Biomphalaria* in orbit revealed that the snails were easily dislodged from the aquarium wall, while on Earth they spent most of their time attached to the walls. Once separated from the wall they floated through the water, which gave them the chance to contact another snail in orbit. As these snails are hermaphrodites, mating pairs were often seen floating attached to one another. Therefore, after landing of the spacecraft, embryos of all developmental stages were present (Marxen et al. 2001).

In frog *Xenopus laevis*, the first successful fertilization in space was done during a ballistic rocket flight in 1988, using a fully automated hardware. The experiment was successfully repeated on another sounding rocket flight in 1989, and then on board the manned IML-1 and IML-2 Spacelab missions (Ubbels 1995), and the Japanese Spacelab-J mission, where eggs were fertilized *in vitro* by manipulations of the crew (Souza et al. 1995). Successful automatic fertilizations in microgravity were also obtained in sea urchins (Marthy et al. 1994) on board a sounding rocket and during Space Shuttle flights (Schatten et al. 1999a).

Another strategy for natural in-flight fertilization is possible in those animal species that require no in-flight mating. In these species, mating and insemination is performed on the ground before launch. Inseminated females store the sperm in a compartment of the body called *spermatotheca* and use the sperm cells at the moment of egg deposition. The advantage of this approach is that the time of fertilization, and therefore the age of embryos can precisely be determined by the experimenter.

This type of fertilization was successfully performed in two urodele species, the salamander *Pleurodeles waltl* (experiments "Fertile" on the Russian space station Mir in 1996 and 1998; Aimar et al. 2000) and the newt *Cynops pyrrhogaster* (experiment "Astronewt" on board IML-2 in 1994 with a repetition in 1995; see Izumi-Kurotani and Kiyomoto 2003 for review). The female newts keep spermatozoa in their cloacae ready to fertilize eggs after hormonal stimulation of ovulation. Thereafter, egg laying occurs within 24 to 48 hours. Presence of spermatozoa in the perivitelline space and of spermatic spots on the surface of the eggs in microgravity can be considered as a proof that the development of embryos is not based on parthenogenesis[5]. During the two "Fertile" projects on board Mir, about 56% of eggs were successfully fertilized, as calculated from the number of living embryos at the two-cell stage, or at later stages from the number of eggs in the spawning. By comparison, the ground experiments revealed a ratio of 51%, suggesting that

[5] *Parthenogenesis* is a form of reproduction in which the egg develops into a new individual without fertilization by sperm.

occurrence of egg fertilization was not affected by microgravity (Aimar et al. 2000).

Crickets use a fertilization strategy similar to that of salamanders. After insemination by a male, the female cricket keeps the sperm in its spermatotheca. Fertilization occurs during the process of egg laying, which is activated by offering a suitable substrate to the female. By means of this technique, we were able to obtain successful in-flight fertilization in the house crickets *Acheta domesticus* during the Italian Soyuz Taxi flight "Eneïde" to the ISS in 2005. After the flight, embryos were recovered, suggesting that eggs could develop for eight days in microgravity.

2.2 Cleavage, Gastrulation, and Neurulation

Since the beginning of the 20th century, authors hypothesized that gravity played a role on early embryonic development, in particular on the orientation of first cleavage, the formation of the antero-posterior axis, and the subsequent morphogenesis and organogenesis that are very often characterized by the regular patterning of morphological structures. The basic principles of embryonic development are described in Chapter 1, Section 3.1.

Experiments were done with frog eggs during centrifugation that increases gravitational forces (hypergravity), clinostat rotation that produces a vector-free gravitational environment (simulated microgravity), and true microgravity during orbital flights. All these experiments revealed that gravity was involved in the early developmental stages of embryonic processes.

In the radial-symmetrical mature egg of *Xenopus laevis*, the polar animal-vegetal axis indicates roughly the embryo's main body axis. Pigment concentrating around the sperm entry point marks the meridian that foreshadows the prospective ventral side. Because in most eggs the blastopore[6] forms at the meridian about 180 deg away from the sperm entry point, the embryo's general body pattern is established from that time on. However, the dorsoanterior and ventroposterior polarities can still be altered by the rearrangement of yolk's components due to the influence of gravity and centrifugal forces. This suggests that gravity, in conjunction with the sperm entry point, establishes the dorsoventral polarity.

Another typical feature of early development is the rotation of the egg inside the fertilization membrane by which the animal-vegetal axis aligns itself with gravity. This rotation is not a requirement for normal development,

[6] A *blastopore* is an opening into a developing blastula. *The blastula* is an early stage of embryonic development in animals. It is produced by cleavage of a fertilized egg and consisting of a spherical layer of cells surrounding a fluid-filled cavity. The blastula follows the *morula* and precedes the *gastrula* in the development sequence. A blastula has around 128 cells, with a large central cavity called the *blastocoel* (see Chapter 1, Section 3.1).

because eggs prevented from rotating can develop normally. Generally, the direction of rotation determines the polarity of the embryonic axis. Eggs inclined with respect to gravity form the dorsal structures on the side of the eggs uppermost in the gravitational field. These observations make it obvious that gravity is used during the early steps of the development of an embryo. However, the answer to the question of whether the presence of gravity is *necessary* for normal morphogenesis in early development could only be given by conditions of gravity deprivation during spaceflight. Aquatic vertebrate (fish, frogs, salamanders, and newts) and invertebrate species (sea urchins) were first-choice species to answer this question. Experiments with simulated gravity using the fast-rotating clinostat also gave valuable hints to spaceflight experiments (Yokota et al. 1994).

2.2.1 *Xenopus laevis*

In *Xenopus*, development under simulated microgravity in a clinostat revealed no change in the cleavage rhythm. At the eight-cell stage, however, the location of the first horizontal cleavage furrow was shifted towards the vegetal pole and completed earlier. Further modifications include:

a. A more centered position of the blastocoels and an increase in the number of cell layers in the blastocoel roof at the blastula stage;
b. A significant smaller blastocoel (Figure 5-03);
c. A dorsal lip that appeared closer to the vegetal pole at the gastrula stage;
d. And head and eye dimensions that were enlarged at the hatching tadpole stage.

Despite of these morphological changes, tadpoles at the feeding stage were largely indistinguishable from controls (Yokota et al. 1994). Similar observations were obtained from studies in *Rana dybowskii* by exposure to simulated microgravity (Neff et al. 1993).

After successful fertilization of *Xenopus* eggs in real microgravity during sounding rocket and spaceflights, subsequent embryonic development revealed the same features as seen in simulated microgravity. The cleavage rhythm was normal, but the numbers of cell layers of the blastocoel roof increased from two to three and the blastocoel became smaller (Figure 5-03). Further development in microgravity continued as observed during the Spacelab-J mission. In particular, neurulation[7] was not impaired and the

[7] A *neurula* is an embryo at the early stage of development in which neurulation occurs. *Neurulation* is the development of the nervous system in the embryo. The neural plate will fold to produce the neural tube that will develop into the brain. Remaining tissue will develop into the spinal cord (see Chapter 1, Section 3.1).

neurula at stage 20 appeared normal. After this particular spaceflight with the in-flight fertilization, normal tadpoles were retrieved (Souza et al. 1995).

This observation contrasted somehow the observations following earlier sounding rocket flights. In embryos raised in 1 g after the MASER-3 flight, further development was slightly retarded compared to the ground embryos. In addition, microcephalization and reduced tail formation were observed, while after the MASER-6 flight, embryos developed normally including axis formation (Ubbels 1997, Ubbels et al. 1995).

Simulated microgravity by means of clinostats allows a more detailed analysis of the individual periods of development. In fact, anuran embryos revealed that, in addition to the above mentioned modifications, the dorsal lip approached the vegetal pole at the gastrula, and there was enlarged head and eye dimensions at hatching (Neff et al. 1993).

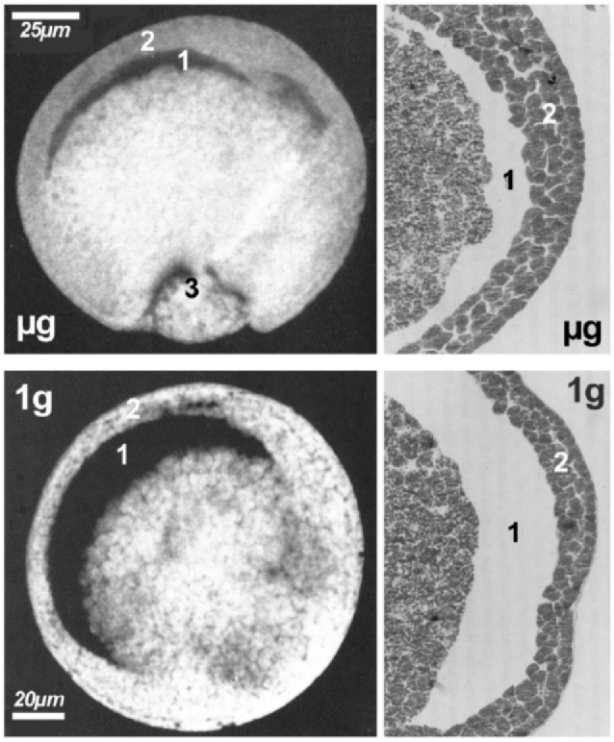

Figure 5-03. Gastrulae from Xenopus laevis fixed in microgravity (µg) and on the ground (1g) showing the thickening of the blastocoel roof in microgravity. Note: 1, blastocoel; 2, blastocoel roof; 3, blastopore. Adapted from Ubbels et al. (1995)

2.2.2 Pleurodeles

Some other aquatic animals gave hints to the extent of modifications in the embryo caused by spaceflight. Some were similar to those found in *Xenopus*, while other were absent or not detectable due to the analysis methods used. In *Pleurodeles*, 24 out of 25 eggs fertilized in 0 g exhibited

normal location of the first furrow. However, subsequent cleavages were irregular and 3, 5, or 7 cells were observed in the animal hemisphere (Figures 5-04 and 1-18). About 35% of microgravity eggs exhibited large unpigmented areas in the animal pole, and movements of the pigment towards the animal pole were amplified up to the morula stage. As in *Xenopus*, the blastocoel roof in gastrulae was thicker in the 0-g eggs than in the 1-g controls, but the blastocoel was still composed of two cell layers. In contrast to *Xenopus*, however, neurulation was strongly affected by microgravity (Gualandris-Parisot et al. 2002).

2.2.3 Fish and Newts

Poor or even absent sensitivities to microgravity were observed in the Medaka fish (*Oryzias latipes*) during the IML-2 mission (STS-65 in 1995) and in the newt *Cynops*. After the successful mating of Medaka fish in microgravity (Ijiri 1998), the subsequent developmental steps were similar in flight and ground-control fish. Newly laid eggs formed a cluster on the belly of the female fish. After detachment from the female's body, young fish hatched in microgravity (Ijiri 2003) (see Figure 2-12).

This lack of microgravity effects contrasts with the changes in the plane of bilateral symmetry and the orientation of the microtubules in the vegetal pole region of zygotes induced by tilting or centrifugation (5 g) (Fluck et al. 1998).

In-flight video-recordings of early *Cynops* stages also revealed normal morphological shapes of the late morula, early blastula, gastrula, neurula and tail bud stage up to the stage shortly before the first gill (respiratory organ) ramification appeared (Yamashita et al. 2001).

2.2.4 Conclusion

All these modifications seem to occur only transiently, because after spaceflights or simulated microgravity hatched *Xenopus* tadpoles at the feeding stage are largely indistinguishable from controls (Souza et al. 1995, Yokota et al. 1994). Long-term microgravity exposure revealed that *Pleurodeles* larvae were able also regulate the morphological changes of the gastrula and neurula stages while being in microgravity. Even the time of hatching in microgravity was identical to that in the ground controls. Histological and immunohistochemical studies with larvae fixed within five hours after landing showed no microgravity specific effects in their central nervous system, eyes, somites, pronephros[8], and gut (Dournon 2003).

These facts demonstrate the efficiency of self-regulatory genetic mechanisms during development in altered gravitational environment (see Horn 2005 for review). The reasons for the observed developmental

[8] *Pronephros* is the first temporary stage of kidney development.

modifications during early development are not yet understood. In both gastrulae and cultures of presumptive ectoderm cells[9] of *Cynops pyrrhogaster*, TUNEL staining[10] and electron microscopy revealed apoptotic cells, but the number of these cells was always smaller in clinostat-treated samples than in the controls (Komazaki 2004).

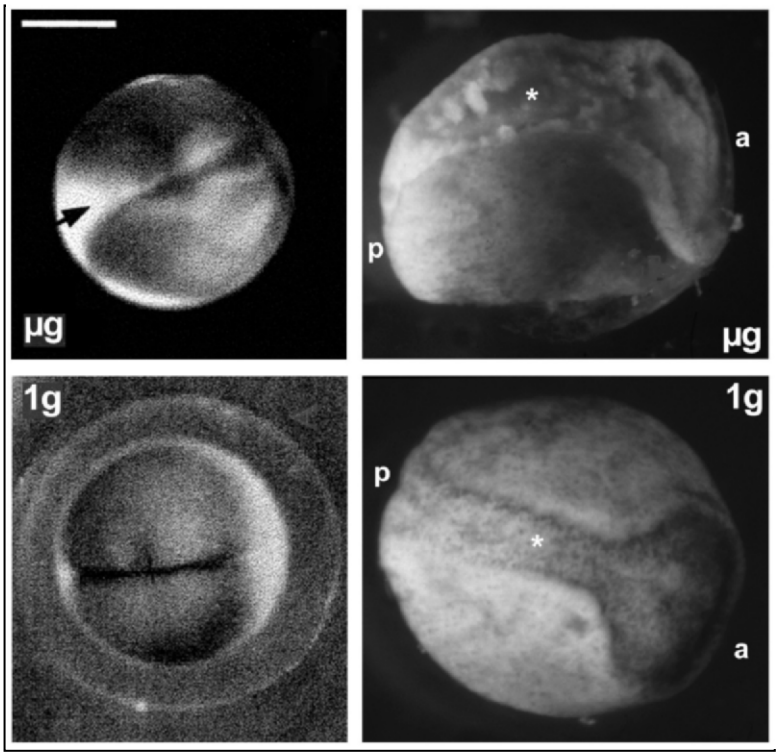

Figure 5-04. Morphological effects during early embryogenesis. Left: Light micrographs of Pleurodeles eggs fertilized in space (µg) and on the ground (1g). In microgravity, the pigmentation concentrated around the animal pole and an unpigmented area covered a large part of the animal hemisphere (arrow). Bar = 600 µm. Right: Note the incomplete closure of the neural tube (*) in an embryo of salamander Pleurodeles waltl fixed in microgravity (µg) compared to the ground control (1g). a and p, anterior and posterior pole of the embryo, respectively (see also Figure 1-18). Courtesy of C. Dournon, Henri Poincare University-Nancy 1, Vandoeuvre-les-Nancy, France.

[9] The *ectoderm* is the outer most of the three primary germ layers of the embryo, from which the skin, nerve tissue and sensory organs develop

[10] *TUNEL staining* is a procedure for detecting apoptotic cells. Because DNA fragmentation is a hallmark of apoptosis, the TdT-mediated UTP-biotin nick end-labeling (TUNEL) uses the enzyme deoxynucleotidyl transferase (TdT) to directly label the fragmented DNA ends. The apoptotic cells can then either be quantified using flow cytometry or visualized in tissue sections by using colorimetric reagents.

2.3 Comparative Aspects of Embryonic Development

Successful embryonic development was observed in several other species from many animal orders. The most obvious observation was that, independently of pre- or in-flight fertilization, embryogenesis proceeds in the space environment both with and without modifications in morphology. An exception occurred in chicken eggs fertilized shortly before launch. These eggs did not survive a 7-day spaceflight, probably because the slight differences in the specific gravity of yolk (1.029) and albumen (1.040) prevented a normal separation during spaceflight (Suda 1998).

Normal embryogenesis, hatching, and larval development in microgravity were observed in the brine shrimp *Artemia franciscana* following activation of dehydrated cysts in orbit during the Space Shuttle STS-37 and STS-43 missions. Hatching of larvae and further larval development continued normally, as shown by formation of the single naupliar eye, subsequent formation of the lateral pair of eyes, and differentiation of skeletal muscle and gut ectoderm (Spooner et al. 1994).

Similarly, the development of the freshwater snail *Biomphalaria glabrata* including reproduction, development, and growth proceeded normally in the absence of gravity. In particular, the spiral cleavage of the primary eggs appeared normal and the yolk remained distributed randomly in microgravity. The embryos developed quickly within their egg capsules, i.e., in about one week. Due to the short embryonic phase of this hermaphroditic snail, which lasts about one week, the entire growth took place under microgravity conditions, and normal young snails were recovered after landing of the Space Shuttle STS-89 mission (Marxen et al. 2001).

Normal development was also obtained in chickens that developed from preflight fertilized eggs pre-incubated for 7 and 10 days on Earth. All the tissues, including cartilage and bone, were formed normally during spaceflight Spacelab-J. Also, post flight hatching was in the precise time scheme (Suda 1998).

Development of sea urchins was sensitive to long-term exposure to microgravity during development, while short-term microgravity exposure during parabolic flight had no significant influence (see Izumi-Kurotani and Kiyomoto 2003 for review). After in-flight fertilization, several developmental processes of the sea urchin *Lytechinus pictus*, such as fusion of cortical granule membranes with the plasma membrane, elongation of microvilli, and elevation of the fertilization coat, were reduced in the absence of gravity (Schatten et al. 1999b).

The most dominant mechanism of multiplication in jellyfish *Aurelia* is *strobilation*, i.e., polyps segment transversely and each segment develops into a jellyfish larva, the ephyra (see Figure 1-07). In-flight induction of strobilation during the Spacelab SLS-1 mission caused a slightly higher

number of ephyra compared to induction performed on ground one or two days before launch. However, scanning and transmission electron microscopic and light microscopic inspection of ground- and space-reared ephyra revealed similar morphological development as shown by the number of arms (Spangenberg et al. 1994).

Thus, there is no uniform picture of how gravity deprivation affects the early periods of development. Unfortunately, it is likely that the finding of common basic principles for the effects of microgravity on development will remain a scientists' dream because spaceflight opportunities are too few to allow for the systematic study of morphogenesis in a large number of animal species. It also for this reason that the following Section only presents some examples of organs development in a few well-investigated species.

3 ORGAN DEVELOPMENT

3.1 Nervous System and Sensory Organs

Neurulation is the first step in the formation of the nervous system. It starts in the median part of the antero-posterior axis of the neurula, spreads simultaneously along this axis towards the rostral and caudal parts of the embryo, and finally forms a closed tube.

Neurulation was not affected by microgravity in *Xenopus laevis* embryos (Duprat et al. 1998). In contrast, experiments on board Mir in 1996, 1998, and 1999 showed retardation in the closure of the neural tube in *Pleurodeles waltl* (Figure 5-04). Also, microcephaly developed more frequently in 0-g embryos than in 1-g control embryos. Despite of these modifications, epidermal ciliated cells functioned normally, and each 0-g embryo rotated randomly clockwise or counter-clockwise around its antero-posterior axis as in 1-g controls. The five brain subdivisions were morphologically normal, and sense organs such as eye and ear developed normally (Gualandris-Parisot et al. 2001).

The cytological differentiation of neuronal and glial structures was investigated in neural precursor cells from *Pleurodeles*, isolated in culture immediately after neuronal induction at the early neurula stage. During microgravity exposure on a 16-day Foton biosatellite flight, they differentiated without significant abnormalities. They developed long neurites and normal networks. Some slight modifications were related to a faster differentiation of cells and to the formation of varicosities along neurites (Duprat et al. 1998).

3.1.1 Axonal Growth and Dendritic Morphology

Further development includes the outgrowth of neurites, the formation of neuronal networks, and the establishment of the neuromuscular synapses.

As for the early period of development, the effects of microgravity on nervous system development were considered in only a few animal species and specific tracts. While these effects on the early formation of the nervous system were mainly based on studies in the aquatic animals, axonal growth and dendritic morphology was also studied in rats. Studies in developing rats considered model tracts such as the projections from the vestibular system, or the retino-hypothalamic tract which connects the retina with the suprachiasmatic nucleus (SCN). These pathways are related to functions such as equilibrium control and control of circadian activity, respectively.

Rat embryos exposed to microgravity from the gestation day G9 to G19, which is the period when the vestibular system starts to become functional, showed that afferents from the posterior canal projecting to the medial vestibular nucleus developed similarly in microgravity, in hypergravity at 1.5 g, and in normal gravity. However, afferents from the saccule showed delayed development in microgravity compared to development in hypergravity and in controls (Bruce 2003). In particular, three hours after Shuttle landing, peripheral vestibular branches had developed similarly in the flight and control rat embryos. Central projections of semicircular canal receptors to the vertical vestibular nuclei reached similar states of development in the flight and control animals. However, central projections from the gravisensing organs receptors to the medial vestibular nucleus were more immature than in the controls (Bruce and Fritzsch 1997). This result suggests that gravity is required for appropriate synaptic development and fine-tuning of the projections from the gravity sensing receptors to the central nervous system.

These observations were supplemented by studies in the vestibular nuclei of neonate rats launched at postnatal day P8. Several tests during the 16-day Neurolab STS-90 mission revealed an absence of connections into the vestibular nuclei from the cerebellum, the main control center for balance and coordination of movement (Raymond et al. 2003).

3.1.2 Synapse Formation *In Vivo* and in Cell Cultures

The transfer of information between nerves and muscles and among nerve cells occurs mainly at the synaptic level. The establishment of synaptic contacts is one primary goal of development. For example, in vertebrates, *motoneurons* (also called motor neurons) are efferent neurons that originate in the spinal cord and synapse with muscle fibers to facilitate muscle contraction and with muscle spindles to modify proprioceptive sensitivity. During development of the neuromuscular system, outgrowing motoneurons find their muscle fiber to form the motor *endplates*.

During normal development, the number of synapses undergoes a period of overproduction followed by a significant reduction to a standard level. In mature neurons, synaptic proteins are highly concentrated in axon

endings where they help to regulate neurotransmitter release. Their distribution experiences significant modifications during development. At early stages, they are distributed throughout the neuron, but with increasing maturity they concentrate in the axon endings. Two of the most understood synaptic proteins are synaptophysin found at the synaptic vesicles and SNAP-25, a protein that probably functions in synaptic vesicle exocytose. Adaptive processes to altered gravitational conditions have to consider modifications at the synaptic level, including the formation of contacts between neurons and muscles, as well as the formation of proteins such as synaptophysin or SNAP-25 involved in the information transmission.

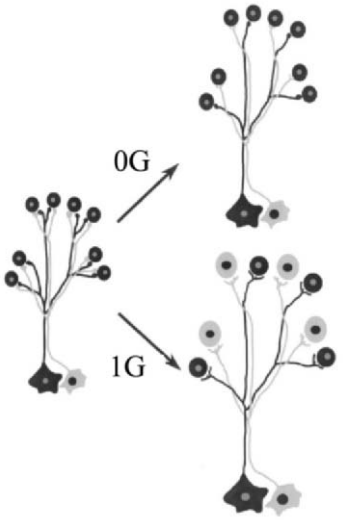

Figure 5-05. Morphological development of neuromuscular connections during gravity deprivation. Spaceflight animals (0G) revealed a depressed development of axonal terminals compared to ground controls (1G). At onset of the mission, more than 75% of motor endplates had a multiple innervation of the immature muscle fiber. Further development of the ground control (1G) was normal; motoneurons and muscle fibers increased their size, the multiple innervations disappeared. Animals from the microgravity group (0G) revealed disappearance of multiple innervations but a depressed growth of neurons and muscle fibers. Adapted from Riley and Wong-Riley (2003).

This assumption of a g-sensitivity of synapse formation was revealed to be true, but the effects were related to some sites within the brain and showed a time window of sensitivity. In P8 rat neonates that developed for 16 days in microgravity during Neurolab, a reduced growth of motor neuron terminals was observed (Figure 5-05). At launch, more than 75% of motor endplates were innervated by multiple motor nerve terminals. During spaceflight, reduction of terminal numbers proceeded as on ground, so that after landing all but one terminal per endplate was eliminated. However, the frequency of complex branching patterns, which is a marker for advanced developmental progress, was significantly higher in ground (44 ± 3%) than in flight (16 ± 1%) neonate rats (Riley and Wong-Riley 2003).

In the P8 neonate group of the same flight, expression of proteins linked in the synaptic transmission was determined for the hippocampus as well as for the vestibular and cochlear nuclei. During and after the STS-90 flight, the cellular distribution of synaptophysin and SNAP-25 in the

vestibular and cochlear nuclei differed significantly from those of the 1-g control neonate rats. The ground animals revealed a more developed type of distribution, whereas synaptic proteins were more distributed throughout the neurons in the flight neonate rats, characterizing a more immature status (Raymond et al. 2003). In contrast, the hippocampus of these neonate rats orbiting in space between postnatal stage P8 and P24 revealed no significant difference in the staining of synaptophysin and SNAP-25 (Temple et al. 2003).

In co-cultures of spinal neurons and myocytes (muscle fibers) isolated from *Xenopus laevis* embryos that were exposed to simulated microgravity, the formation of ACh receptor patches[11] was strongly affected depending on the level of maturity of this system at onset of microgravity. Inhibition of incidence and area of these patches was obvious if nerve contact took place during or shortly before onset of simulated microgravity (Gruener and Hoeger 1990) (Figure 5-06).

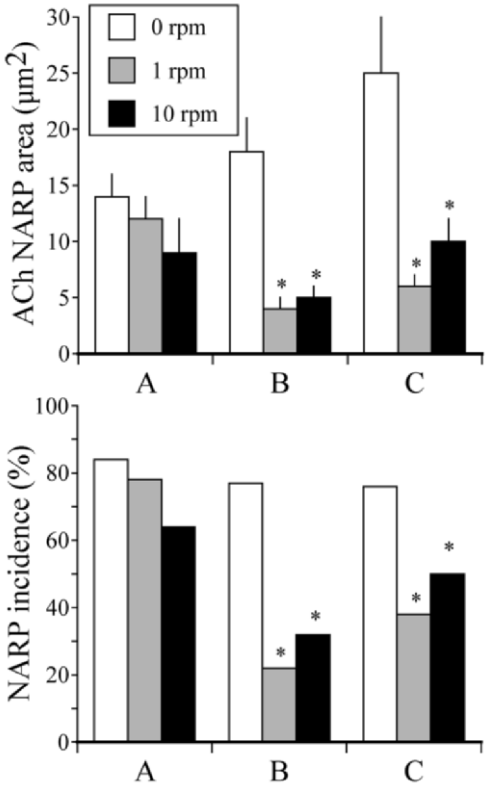

Figure 5-06. Physiological development of neuromuscular connections during gravity deprivation. Effects of clinostat rotation on the area (top diagram) and incidence (bottom diagram) of nerve-induced acetylcholine receptor patches, or ACh NARP, in myocytes in mature (A: maturity before clinostat rotation onset), immature (B: synaptic contacts developed just before onset of rotation) and de-novo formed synapses (C: synapses formed during clinostat rotation). Clinostat rotation was performed at 1 or 10 revolutions per minute (rpm); 0 rpm indicates no rotation. Note the significant effects of simulated microgravity in sets B and C and their absence if maturation occurred before onset of clinostat rotation. Adapted from Gruener and Hoeger (1990).

[11] *Acetylcholine* (ACh) is one of the neurotransmitters. After being released into the nerve terminal, ACh binds to the post-synaptic ACh receptor, resulting in a transient increase in membrane permeability to Na, K, Ca, and Mg, leading to an endplate potential (EPP).

These observations were confirmed in space-flown cell cultures (Gruener et al. 1994). Surprisingly, the changes in the receptor's cellular organization by clinostat rotation did not alter the ACh receptor single channel properties. Indeed, the mean open-time and conductance of the ACh receptor channel were statistically not different from control values (Reitstetter and Gruener 1994).

3.1.3 Vestibular Apparatus

At the beginning of the era of space biology, many experiments studied the effects of microgravity on the vestibular apparatus, and in particular the *otoliths* or *otoconia*. Otoliths, or "ear stones", are calcium carbonate crystals found in the inner ear of most fish and vertebrates (see Figure 1-13). Pressure or shear motion of the otoliths on the hair cells of the macula (the most sensitive area of the inner ear) provides sensory inputs about the orientation of the head relative to gravity. Bony fish were the first choice of species for developmental studies of the vestibular apparatus because they possess species-specific solid otoliths of constant shape that grow in layers. This specific feature allows for a clear-cut quantification of microgravity effects on the developing otolith.

After experiments on board Salyut-5, Russian scientists claimed that the development of the vestibular apparatus of *Brachyodanio rerio* was not affected by spaceflight. The fine structure of the receptor epithelium and the otolith apparatus, as well as the ional composition of the intravestibular fluid, remained unchanged. Studies in *Fundulus heteroclitus* developed on board Skylab and the Cosmos-782 biosatellite confirmed these observations. Also, no changes were observed in young fish launched before the earliest stage of development of the vestibular apparatus had appeared (Vinnikov et al. 1983). In the swordtail fish *Xiphophorus helleri*, however, otolith growth in slowly growing embryos was retarded, but growth was augmented in fast growing embryos. The otoliths of juveniles developed in microgravity in the same way as on the ground (Wiederhold et al. 2003). Retarded otolith development was also observed in *Danio rerio* during exposure to simulated weightlessness in a rotating bioreactor (Moorman et al. 1999).

Studies in aquatic amphibians are more difficult to perform because they possess many otoconia. In *Xenopus laevis,* tadpoles launched at the embryonic stage before hatching or shortly thereafter, the expression of CalBindin, a marker for maturity, was similar in vestibular cells compared to ground controls. Furthermore, morphometric investigations of cell size and number in the otolith maculae revealed no difference between flight and ground tadpoles (Horn et al. 2006). These recent results confirmed earlier observations on tadpoles (Ross 1993).

Otoconia from 0-g exposed *Xenopus* tadpoles were reported as 30% larger than those from 1-g controls (Lychakov 1991), while their basic shapes

remained unaffected after a 10-day microgravity exposure on board the ISS (Horn et al. 2003). During the IML-2 mission, embryos of newt *Cynops pyrrhogaster* were sent in orbit before any stones were formed. After the flight, otoliths and otoconia from the utricle and saccule were found to be larger compared to those from ground animals (Wiederhold et al. 1997). This increased size of otoconia might be the basis of a sensitization of the developing vestibular system by spaceflight, which was observed in *Xenopus* tadpoles (see below) and young fish *Oreochromis* (Sebastian et al. 2001).

3.1.4 Other Sensory Organs

During the STS-72 flight, microgravity affected the retina of neonatal rats, probably by degeneration of cells or parts of individual cell types. In the age- and weight-matched test animals, the most obvious defects observed in all the three test populations launched when they had reached postnatal days P5, P8, and P15 were the absence of the outer segments of rods, a decreased thickness of the inner plexiform layer, and a reduced number of retinal ganglion cells (Tombran-Tink and Barnstable 2005). As the affected sites of the retina are involved in visual transduction and first steps of visual information processing, it is likely that vision would have been strongly disturbed in these animals.

This rather discouraging report is completely opposite to studies on the embryonic eye of the Japanese quail *Coturnix japonica*. Fertilized eggs were launched on STS-76, incubated at 39-40°C on board Mir, and embryos were fixed in microgravity on specific days, ranging from embryonic days E0 to E16. Their eyes were less affected by microgravity than those of the animals during the STS-72 mission described above. Indeed, eye weight, eye, corneal, and scleral ring diameters, numbers of bones in scleral rings, transparency of corneas, and corneal innervation were indistinguishable from the ground controls except for the corneal diameter of E16 eyes (Barrett et al. 2000).

3.2 Muscle and Bone Development

The impact of microgravity on muscle and skeleton development was mainly studied in rats. These organs are formed from the mesoderm and its cellblocks, the *somites*. Main features of somite formation are periodicity, epithelialization, specification, and differentiation.

Cells of the somites are precursors for:

a. The cartilage of the vertebrates and ribs;
b. The muscles of the rib cage, limbs, abdominal wall, back, and tongue;
c. The dermis of the dorsal skin depending on the site within the somites.

The ventral-medial mesenchymal cells become chondrocytes that form the cartilage and part of each rib. Laterally located mesenchymatic cells form the *myoblasts*. Myoblasts from the region closest to the neural tube form the *epaxial* muscles (deep muscles of the back), while those myoblasts formed in the region farthest from the neural tube produce the *hypaxial* muscles of the body wall, limbs and tongue (see Gilbert 2003 for review).

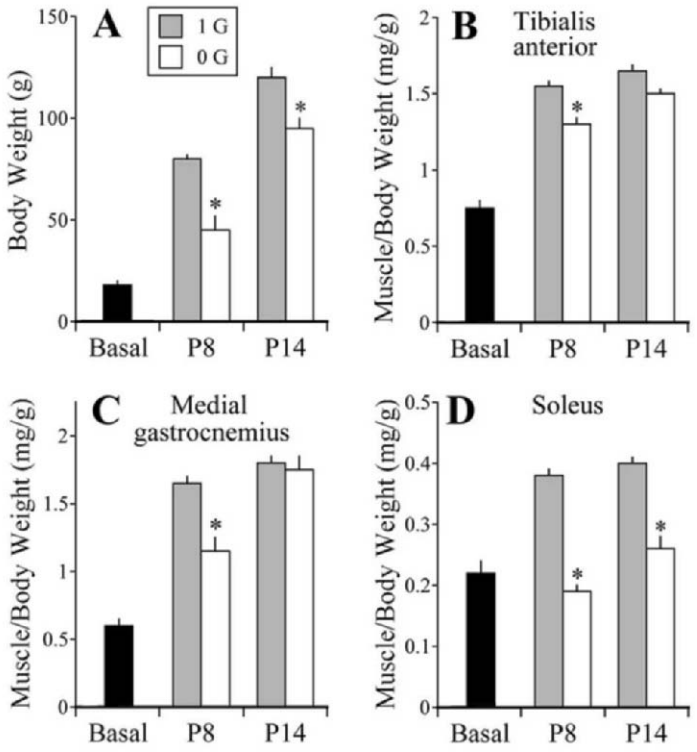

Figure 5-07. Effects of microgravity on postnatal development of rat muscles. Neonate rats body weight (in A) and muscle weight normalized to body weight for a non-weight-bearing locomotor muscle, the tibia anterior (in B), a weight-bearing locomotor muscle, the medial gastrocnemius (in C), and a weight-bearing postural and locomotor muscle, the soleus (in D). Note: asterisk = $p<0,05$ relative to respective controls. At launch, pups were at postnatal stages P8 and P14. Basal: measurements at the day of launch. Note the differences between the effects of microgravity for these two stages: the P14 group was less sensitive than the P8 group, in particular for the non-weight-bearing (tibialis anterior) and the weight-bearing and locomotor (gastrocnemius) muscle. Adapted from Adams et al. (2003).

3.2.1 Muscle Development

Further development and maturation of muscles depends on growth factors and hormones, such as growth hormone, *Insulin-like Growth Factor* I (IGF-I) and thyroid hormone T3. As long as growth factors are present, myoblasts proliferate without differentiating. When growth factors are depleted, myoblasts stop dividing, secrete fibronectin onto their extracellular membrane, and attach to other myoblasts. After alignment of the myoblasts into chains mediated by membrane glycoproteins, fusion occurs, even between myoblasts from different species (Gilbert 2003).

At birth, rat muscles are in an undifferentiated state in terms of their relative size and functional properties. At this stage, various motor units express immature forms of *Myosin Heavy Chains* (MHC), a family of proteins important for muscle contraction. Different types of motor units, such as slow motor units, fast oxidative motor units, or fast glycolytic motor units express different MHC phenotypes, which can be related to the extent of function in weight-bearing, i.e., antigravity action (Adams et al. 2003). This feature of immaturity at birth makes it possible to study developmental processes during postnatal life.

The effect of microgravity on muscle mass and function occurs within less than one week (Tischler et al. 1993). In developing animals, species-, muscle- and age-related dependencies are known, in particular during the first period of postnatal life, which involves remarkable dynamics of muscle growth. In rats, the period from 7 to 30 days after birth is crucial for the development of hind limb locomotive pattern. Loss of weight-bearing function in microgravity during the early part of this period resulted in a decrease in muscle growth not only in weight-bearing muscles but also in non-weight-bearing muscles (Figure 5-07).

Postnatal P14 and older rats lose the 0-g susceptibility, even in the non- or slightly weight-bearing muscles. On the other hand, muscles with dominant weight-bearing properties, such as the soleus, keep their 0-g sensitivity even during late periods of postnatal development, as observed in mature adult animals and humans (Tischler et al. 1993, Adams et al. 2000). These observations were in concurrence with the changes in plasma and muscle IGF-I levels (Figure 5-08). So, spaceflight not only depresses slow, type-I MHC gene expression in the developing soleus muscle, it also creates a profile typically seen in most fast muscles, in which the MHC isoforms dominate the MHC protein pool (Adams et al. 2003).

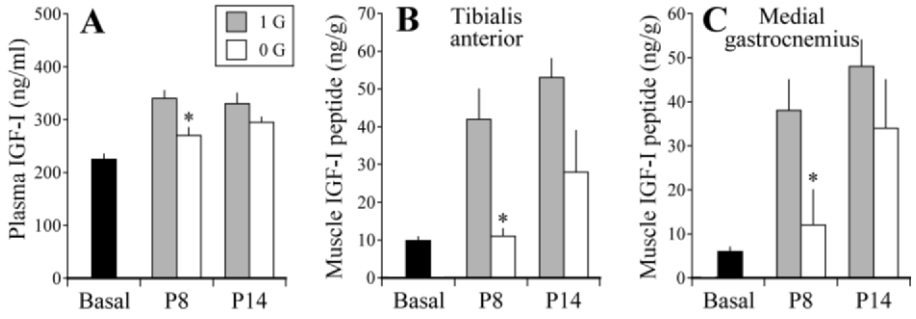

Figure 5-08. Effect of microgravity on the Insulin-like Growth Factor I (IGF-I) in the plasma (in A), the tibia anterior (in B), and the medial gastrocnemius (in C). Note the clear stage-related microgravity susceptibility, with the older stage P14 being less sensitive or even insensitive to a 16-day microgravity exposure. Adapted from Adams et al. (2003).

Muscle development of the salamander *Pleurodeles* was insensitive to microgravity. Typical markers in somites differentiation, such as their position and the appearance of striated structures, i.e., organized myofibrils, did not differ from normal development (Gualandris-Parisot et al. 2001). In contrast, axial muscles of *Xenopus laevis* tadpoles exposed to microgravity exhibited a variety of abnormalities associated with muscle degeneration. Their weight-bearing muscles became abnormally infolded and widely spaced, and the number of fibers was about 48% less compared to the controls. Non-postural muscles of tadpoles, such as the muscle orbitohyoideus that is involved in respiration and feeding, showed no sign of degeneration (Snetkova et al. 1995).

3.2.2 Mineralization and Bone Development

Some of the most obvious structures derived from the mesoderm are bones. There are two major ways of bone formation, or *osteogenesis*. The first way is a direct conversion of mesenchymal tissue into bone, called *dermal ossification*. The second way is the replacement of a cartilage intermediate by bone cells, called *endochondral ossification*. The replacement is characterized by the death of hypertrophic chondrocytes[12] and the subsequent differentiation of cells surrounding the cartilage into *osteoblasts*. The replacement of chondrocytes by bone cells is dependent on the mineralization of the extracellular matrix. This remodeling releases the angiogenesis factor VEGF, which can transform mesodermal mesenchym cells into blood vessels. By this developmental change, more blood can flow around the dying cartilage enabling chondroclasts to "eat" the debris of apoptotic chondrocytes and osteoblasts to enter the forming bone. At the same time, there is a hollowing out of the internal region of the bone to form the bone marrow cavity. This destruction is carried out by *osteoclasts* (see Gilbert 2003 and Clément 2005 for review).

Bones are targets for microgravity. In adults, bone loss or *osteoporosis* is extremely pronounced, and developing animals experience retardation probably because osteoblasts exposed to microgravity follow a slower progression toward a differentiated function (Landis et al. 2000). The impact of microgravity was observed very early during the period of development. An example was given by studies on ossification of skeletal bones of fetuses of white female rats flown on the Russian Cosmos-1514 biosatellite during their pregnancy days G13 to G18. The effects were described as a 13-17% arresting of the development of nearly every area of the fetal skeleton. Signs of the developmental arrest were more pronounced in less mature skeletal structures. During the 1-g re-adaptation period between pregnancy days G18 to G23, the reduced ossification of the embryos was

[12] A *chondrocyte* is a cell that produces cartilage.

over-compensated, and newborns from this mission were ahead of the controls (Denisova 1986). Also, hypergravity affected skeletal development and caused suppression of morphogenesis in mouse developing limbs, advanced fusion stages in mouse neural plates, and smaller crown-rump lengths[13] (Duke et al. 1994).

Exposure of bone and bone cell cultures originating from mammals and chicken to microgravity is a widely used tool for understanding the underlying mechanisms of bone formation. Nevertheless, the basic mechanisms of the modifications in developing bones in microgravity are poorly understood. One of the reasons is that there is no clear-cut effect of microgravity at neither the morphological, biochemical, nor functional levels (Table 5-01). There is no doubt that impaired mineralization and increased calcium resorption cause bone loss during spaceflight. In fact, isolated fetal mouse long bones experience no change in relative length increase and collagen synthesis induced by microgravity, but instead a decreased mineralization (Figure 5-09), as well as a decrease in glucose consumption and an increase in calcium release (Velthuijzen and van Loon 1995, van Loon et al. 1995).

Some specific biochemical mechanisms were tested for their susceptibility to microgravity, with the goal to understand these reasons for bone loss and impaired bone formation. For example, mRNA levels of osteocalcin, type 1 collagen, and *Transforming Growth Factor* ß (TGF-ß) in rat bone periosteum[14] was decreased during spaceflight (Burger and Klein-Nulend 1999). Thyroid hormone and *Parathyroid Hormone-related Protein* (PTHrP) are essential for development, maturation and hypertrophy program of the epiphyseal growth plate, as well as for homeostasis of bone. Analysis of PTHrP expression in femurs and tibias from rats flown during the STS-58 mission revealed a 60% depression compared to ground controls, whereas no difference was obtained for parietal bone, indicating that the effect is specifically due to unloading (Torday 2003).

Osteoblasts reveal a high sensitivity to microgravity but not for all of their actions. In cell cultures, they show a reduced growth and hormone responsiveness during spaceflight (Burger and Klein-Nulend 1999). But their ability to produce neutral proteinases, including collagenase and *Tissue Plasminogen Activator* (tPA), which are thought to be important in bone development, is not affected by spaceflight.

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[13] When a pregnant woman goes in for a fetal ultrasound, the person doing the test is taking several measurements of the baby on the ultrasound screen. One of these measurements is taken from the top of the baby's head, or *crown*, to his bottom, or *rump*. This measurement is considered to be the most accurate method for determining fetal age early in pregnancy.

[14] *Periosteum* is a connective tissue membrane covering a bone.

Experimental Model	***Duration (Ref)***	***Effects of Microgravity***	***Lack of Effects***
*Cultures of fetal **mouse** cartilaginous long bones*	*4 days (van Loon et al. 1995)*	*Glucose utilization and mineralization decreased; mineral resorption increased*	*Normal length and collagen synthesis*
*Bone growth in rapidly growing **rats***	*4 days (Turner 1995)*	*Site- and bone specific increased (periostreal) and decreased (endocortical) formation of cortical bone*	*Normal bone growth and rate of bone elongation*
*Tibiae of 12-13 weeks old male **rats**; cultures*	*7 days (Vico et al. 1991)*	*Inhibition of longitudinal growth in the proximal tibial metaphysis*	
*In-utero development of calvariae of **rat** pups*	*9 days (Davis et al. 1998)*		*Normal presence and expression of collagenase and tissue plasminogen activator; normal thickness of calvariae*
*Fertilized **chicken** eggs pre-incubated pre-flight for 0, 7, and 10 days on Earth*	*7 days (Suda 1998, Kawashima et al. 1995)*	*0-day-eggs: High mortality*	*7- and 10-day-eggs: Normal cartilage and bone development; normal hatching time; normal calcium and phosphorus contents*
*Skeletal bones of fetuses of white **rats** during G13 and G18*	*5 days (Denisova 1986)*	*Stop of development of nearly every area of the fetal skeleton; **but** larger ossified areas in the skeleton of the flight newborns*	*Normal calcium content*
*Cultures of primary osteoblast cell derived from normal embryonic **chicken** calvaria*	*11 days (Landis et al. 2000)*	*Less extensive extracellular matrix; reduced collagen expression and collagen protein appearance; reduced osteocalcin expression*	*Normal metabolic activity (glucose uptake and lactate production)*
*Metaphyseal and cortical bone in **3-month old rats***	*13 days (Doty et al. 1990)*	*More pronounced changes in cortical than in metaphyseal bone*	*Normal cortical cross-sectional and perimeter*
*Cultures of **mouse** pre-metatarsals*	*6 days (Klement and Spooner 1994)*	*Increase in cartilage rod size and maintenance of rod shape if pre-metatarsals had initiated chondrogenesis and morphometric patterning prior to launch*	*Normal rod structure and cartilage phenotype if, premeta-tarsal had terminally differentiated to hypertrophied cartilage prior to launch*

Table 5-01. Effects of microgravity during spaceflight on the development of bone and cartilage. These selected examples demonstrate the variability of effects as well as the impact of flight duration and type of bones on extent of bone modification.

Indeed, these substances are probably not responsible for bone loss and impaired bone development, as shown by the following observations. After a 9-day exposure of rat embryos and pups (age between G20 and P35) to microgravity, collagenase and tPA were present at all ages in the calvariae[15], but with the greatest amount in P14 neonate rats. The location of collagenase was associated with bone-lining cells, osteoblasts, osteocytes, and in the matrix along cement lines. The location of tPA was associated with endothelial cells lining the blood vessels entering bones. All these observations were similar to ground results. The thickness of the calvariae was also not affected by microgravity (Davis et al. 1998). *Fibronectin* (FN) plays a crucial role in the growth and differentiation of osteoblasts, but its participation in the establishment of osteoporosis can be neglected. In the MC3T3-E1 osteoblast line that is clonally derived from embryonic mouse calvaria, synthesis of FN-mRNA as well as FN protein were significantly reduced by the spaceflight. However, this reduction was present only for some hours after activation (Hughes-Fulford and Gilbertson 1999).

Unlike observations in young growing mammals, fertilized chicken eggs pre-incubated before launch for 7 and 10 days on ground and flown for 7 days on board the Spacelab-J mission, showed no effect of microgravity on bone formation. Morphology of osteoblasts, osteoclasts, and osteocytes of humerus and tibia, bone-resorbing and -forming activities of the femur, calcium and phosphorus contents of the femora, and alkaline phosphatase activity determined for three regions of the tibia (i.e., resting cartilage, growth cartilage, and cortical bone) were similar between flight and control groups. In addition, no significant difference of gene expressions of hepatocyte growth factor and receptors of fibroblast growth factor were observed in perichondrium, trabecula, and skeletal muscles and tendons of hind limbs between control and flight groups. These morphological and biochemical results indicate that bone metabolism was at control level (Kawashima et al. 1995). On the other hand, studies in primary osteoblast cell cultures derived from normal embryonic chicken calvaria suggested that microgravity exposure affects bone cells by down-regulation of type I collagen and osteocalcin gene expression, thereby inhibiting expression of the osteogenic phenotype notably by committed osteoblasts (Landis et al. 2000).

In contrast to higher vertebrates, there is currently no evidence for an effect of microgravity exposure on bone development and bone formation in fish and amphibians. There are promising efforts to establish Medaka fish *Oryzias* as a model organism to study molecular mechanisms underlying gravity dependent bone loss using *osteoprotegerin* (OPG) as a marker. The use of OPG is justified as it seems to control the balance between the bone forming osteoblasts and bone resorbing osteoclasts, i.e., bone mass. In

[15] *Calvaria* is the domelike superior portion of the cranium.

addition, the sequence and expression domains of OPG genes and the entire genetic network for bone formation are highly comparable between Medaka fish and higher vertebrates (Wagner et al. 2003).

To which extent the development of mineralization *per se* is directly affected by microgravity was also studied in some invertebrate animal models, such as snails and sea urchins. Observations in sea urchins after the IML-2 Spacelab mission revealed that the biomineralization process, a cascade of developmental events leading from the micromeres at the 16-cell stage via the *Primary Mesenchymatic Cells* (PMC) to well-defined skeletal structures, occurred even in the absence of perceived gravity. It also turned out that in this animal species, the demineralization phenomenon in the pluteus larvae was negligible (Marthy et al. 1996). Similarly, mineralization of the shell of old snail embryos *Biomphalaria glabrata* flown during the STS-89 and STS-90 missions proceeded normally in microgravity (Marxen et al. 2001).

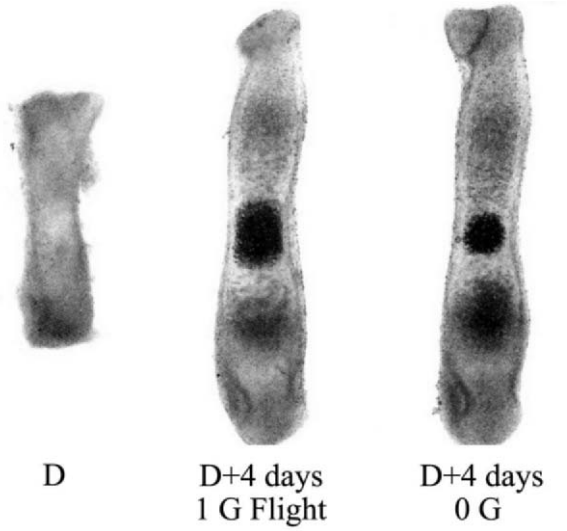

Figure 5-09. Bone mineralization during mouse embryogenesis. Photograph of a 16-day-old bone cultured for 4 days in space in microgravity (D+4 days, 0 G) or in an onboard 1-g centrifuge (D+4 days; 1 G Flight). The photograph on the left shows a bone rudiment immediately after dissection (D). Note that in microgravity the center of the bone is less mineralized (dark area) than in 1 g. Adapted from Velthuijzen and van Loon (1995).

3.3 Respiratory Organ

A study of cultured lung rudiments during the Space Shuttle STS-54 mission suggested that development and differentiation of mammalian lung continues in microgravity (Spooner et al. 1994). *Parathyroid Hormone-related Protein* (PTHrP) has been shown to be essential for the development and homeostasis of lung. It is a highly evolutionarily conserved stretch-regulated gene that is necessary for the embryonic transition from branching morphogenesis to alveolization of the lung. The differentiation of the alveolar epithelium and the surrounding mesoderm are critical for preparing the

newborn for extra-uterine life because the alveoli must be prepared for the transition from 3% to 21% oxygen, and for maintaining their potency by producing surfactant[16]. PTHrP is expressed throughout vertebrate phylogeny, beginning with its expression in the fish swim bladder, an organ that is obviously used for coping with gravity.

The expression of PTHrP by alveolar type-II cells was down regulated by 80-90% within 8 hours of rotation in a bioreactor on Earth (Figure 5-10). This lower level lasted for at least three days, suggesting that PTHrP signaling had been exploited for adaptation to 1-g conditions. Similarly, the production of PTHrP declined over the first 24 hours and maintained this level for the following 48 hours. After return to 1 g, both parameters returned to normal levels (Torday 2003).

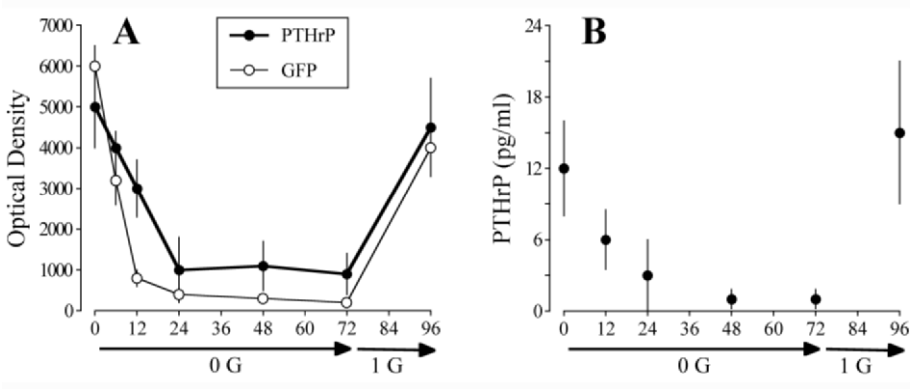

Figure 5-10. Effect of simulated microgravity on PTHrP in fetal rat epithelial type II cells. Lung cells were maintained in a rotating wall vessel for up to 72 hours (0 G), and then put back in normal gravity (1 G). Cells were analyzed for PTHrP expression using both RT-PCR and GFP florescence (in A) and for PTHrP content by means of a PTHrP protein assay (in B). Note the strong depression of PTHrP expression during rotation and the complete recovery after the samples were returned back in normal gravity. Adapted from Torday (2003).

Studies on the development of the lung in amphibians in microgravity revealed reduced lung size after spaceflight. However, this could be an indirect effect. Indeed, *Xenopus* tadpoles reared on the ground usually come up to the water surface to fill their lungs within 2-3 days after hatching. This finding of the water surface is supported by a negative *geotactic behavior*, i.e., the animals swim in the direction opposite to gravity. In space, such geotactic behavior cannot be performed. In addition, in microgravity there is no such thing as a water surface in aquatic habitats, except around air bubbles. Consequently, filling of lungs with air is prevented in aquatic animals reared

[16] *Surfactant* is the fluid in the lungs that helps to keep them open and expanded.

at microgravity, and they have smaller lungs compared to 1-g controls when they return on Earth (Pronynch and Wassersug 1994, Souza et al. 1995).

3.4 Other Organs

Cultured embryonic mouse pancreas developing in microgravity during the STS-54 Space Shuttle mission underwent characteristic exocrine acinar tissue and endocrine islet tissue differentiation. The tissue appeared to grow larger than in the ground controls. Differentiation was accelerated, as judged by the presence of exocrine zymogen granule, the digestion enzymes that are produced by the pancreatic cells and released into the intestine where they are activated (Spooner et al. 1994).

In neonate rats that had reached stage P7 at launch, several organs revealed remarkable differences in size compared to their ground siblings after the Neurolab 16-day spaceflight. Lung, heart, kidney, and adrenal glands became significantly larger by microgravity while thymus, spleen mesentery, and pancreas were smaller. Furthermore, the aortic nerve had a smaller number of un-myelinated fibers in flight neonates (Miyake et al 2004).

4 FUNCTIONAL DEVELOPMENT

Like morphology, all physiological functions in organisms, as well as their behavior, experience modifications during development. One of the most impressive examples are flies and bees that change their body shape from a worm-like to a beautiful looking animal with legs, wings, and a highly developed nervous system.

The study of physiological development is an important tool to understand the mechanisms in adults. Unfortunately, it is also a very time-consuming approach. The most reliable way to describe physiological development is to apply all those techniques that are used to understand the physiology of adults *at each developmental stage*. This is because each development stage has its own physiological mechanisms, which might be completely different from those found in adults. It is, therefore, not surprising that the number of detailed physiological characteristics describing the relationship between age and physiology with a high developmental resolution are rarely found in the literature. But since the discovery of the so-called *sensitive* or *critical* periods (Wiesel 1982), this knowledge became extremely important. Indeed, it is fundamental to find out not only the extent to which environmental and genetic factors determine the central processes and behavior, but also those periods of life during which these factors have a determining influence.

Most research in the physiology of development related to microgravity was performed in the field of *neurobiology*, i.e., the study of the nervous system. For this reason, this section exclusively considers space

neurobiological experiments. Short descriptions of these developmental characteristics, as well as the effects of microgravity on neuronal activity and behavior in the immature animal models, are presented. To some extent, the results of biochemical investigations that are related to the metabolism and the energy demand during adaptive processes to microgravity are also included.

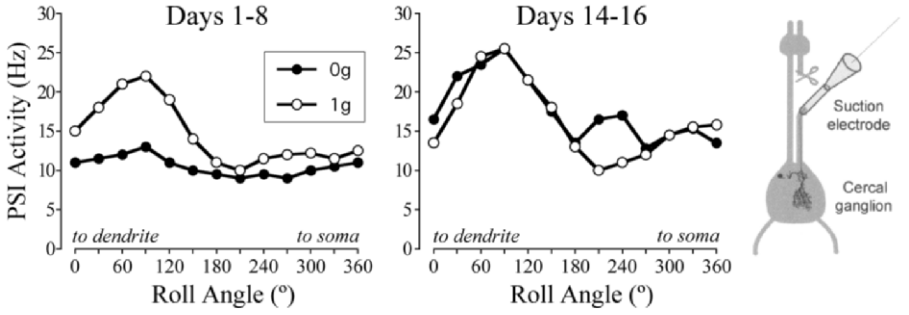

Figure 5-11. Postembryonic development of a neurophysiological response in microgravity in the cricket Acheta domesticus. The activity of the posture sensitive interneuron (PSI) was recorded extracellularly during a 360-deg lateral rotation of the animal. The PSI revealed desensitization immediately after microgravity exposure (Postflight Days 1-8), as shown by the marked depression of the activity modulation during body roll. This desensitization disappeared two weeks after return (Postflight Days 14-16). Adapted from Horn et al. (2003).

4.1 Neuronal Activity

Neuronal activity is composed of individual action potentials that strongly depend on potential shifts across the cellular membranes. These can be recorded by single cell techniques or by means of summed action potentials.

Insects offer the possibility to study the development of individual neurons because they possess very large neurons with long axons and widely spread dendritic arborization. These features offer access to individual neurons even in the case of extracellular recordings. In vertebrates, this approach is limited, particularly in developing animals. In fact, the only example in which the development of an individual neuron, its physiological properties, and its importance for behavior were described was in the Mauthner cell in fish and in larval amphibians (Eaton et al. 2001, Hatta and Korn 1998). But this excellent model was, unfortunately, never considered for physiological adaptation studies in altered gravity at the cellular level.

The development of neuronal activity was determined after the spaceflight of two species, the house cricket *Acheta domesticus* and the clawed toad, *Xenopus laevis*. In both species, larval stages were exposed to microgravity and then returned to Earth to investigate their changes in

neuronal activity. In both species, the neuronal activity was clearly related to development. Furthermore, in both species there was evidence that microgravity exposure caused transient but pronounced desensitization or developmental retardation after return to normal gravity.

Crickets possess a neuron that changes its activity in relation to the creature's posture (Sakaguchi and Murphey 1983). This neuron is called the *Posture Sensitive Interneuron* (PSI). In each developmental stage up to adulthood, there is only one PSI on each side of the nervous system. The cell body (or soma) of this neuron lies on the contralateral side with respect to its dendritic arborization, and its long axon ascends from the terminal ganglion towards the brain passing the thoracic ganglia ipsilateral to the location of the dendritic tree. The PSI receives its input from the cercal gravity receptors (see Figure 5-01).

This peculiar anatomy allows for recording its activity in an extracelluar manner at each stage. This activity is modulated by a 360-degree lateral roll tilt of the animal. The development of the mean maximal frequency modulation increases steadily between the 4th larval stage and the adult stage (Riewe 2000).

The effect of microgravity on the modulation of this activity in *Acheta domesticus* was investigated in two stages: one had reached the 4th stage, the other the 6th stage at the beginning of a 16-day spaceflight. Post flight recordings revealed a significant depression of the PSI's activity modulation. However, the modulation returned to normal baseline values about two weeks after the flight (Figure 5-11).

In *Xenopus*, the development of physiological activity at the synaptic level was performed in cultures of myocytes and embryonic neurons. This study revealed a lack of significant sensitivity to simulated microgravity at the level of ion ACh-channels (Reitstetter and Gruener 1994), despite significant maturation-related modifications in the morphology neuro-muscular synapses (Gruener and Hoeger 1990, Gruener et al. 1994) (Figure 5-06).

The development of neuronal activity was also analyzed for spinal motoneurons using the model of *fictive swimming* (Figure 5-12). Fictive swimming is a regular occurring rhythmic activity that can be recorded from the ventral roots during early embryonic and tadpole periods of life up to the hind limb bud stage, i.e., between stages 38 and 47 according to the standard atlas of development (Nieuwkoop and Faber 1967).

In older tadpoles, fictive swimming disappears and is substituted by struggling activity, an irregular activity in contrast to the regular occurring burst activity during fictive swimming. During normal maturation of embryos to tadpoles the rostrocaudal delay, burst duration, and cycle length increased while episode duration decreased.

After a 10-day spaceflight, this rhythmic motor activity was considerably affected. The episodes of fictive swimming became longer,

while the rostrocaudal delay was significantly depressed. Burst duration was slightly decreased. However, cycle length was not affected by development under microgravity compared to controls. For this period of life, these modifications can be defined as developmental retardation. The increase in episode duration corresponds to the increase in freely swimming duration after the flight. Normalization of fictive swimming occurred during the post flight days 3 to 6 (Böser 2003) (Figure 5-13).

Hypergravity exposure also modified this activity but in an age-related manner: young stages were sensitive after 10 days at a 3-g exposure, whereas older stages were insensitive (Böser and Horn 2006).

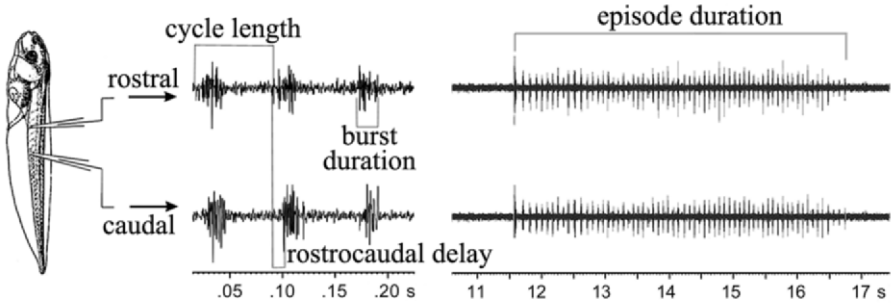

Figure 5-12. Swimming pattern is generated by a central oscillator. It is possible to observe the rhythmical, burst-like activity of motoneurons by extracellular recordings from ventral roots of the spinal cord in paralyzed animals. This figure shows the method used by the author for recording this fictive swimming in Xenopus laevis young tadpoles. An episode of fictive swimming induced by a mechanical stimulus is shown on the right. Three bursts from this episode, with the relevant parameters used for analysis (cycle length, burst duration, and rostrocaudal delay), are shown in the middle.

4.2 Metabolic Activity

The cellular mechanisms underlying the adaptation to altered gravity require energy consumption. This consumption can directly be determined by glucose utilization. Marker enzymes can also be used to study the energy demands during adaptation. For example, *Glucose-6-Phosphate Dehydrogenase* (G6P-DH) and *Succinate Dehydrogenase* (SDH), which are the limiting enzymes of the Krebs' cycle, are important to maintain energy availability in the cells. There is also the *creatine kinase* involved in the mechanism of ATP-regeneration, or the *cytochrome oxidase* that characterizes basic metabolic activity.

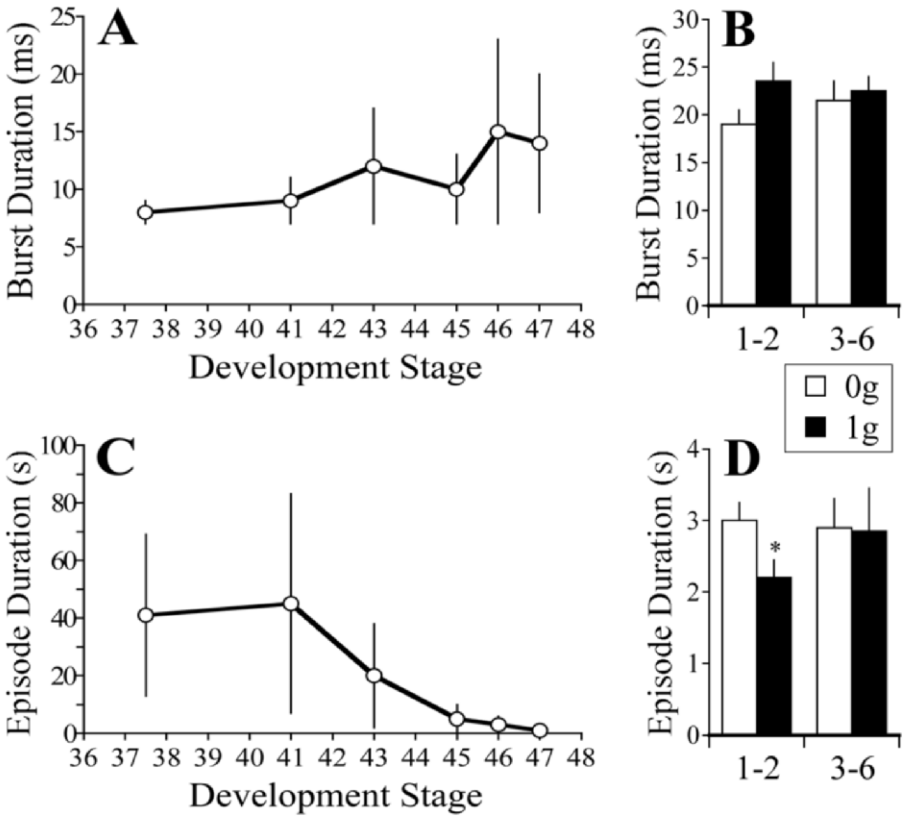

Figure 5-13. Developmental characteristics of fictive swimming demonstrated for the parameters "burst duration" and "episode duration". Fictive swimming in Xenopus laevis can only be induced up to developmental stage 47. Note the increase in burst duration (A) and the decrease in episode duration (C) up to this stage. Similar characteristics exist for cycle length and rostrocaudal delay. The effects of microgravity on burst and episode durations are shown in B and D, respectively. Recordings started 1-2 days after landing of the Soyuz capsule, or several days later (3-6). The embryos were at stage 24/27 at launch of the spacecraft. Adapted from Böser (2003) and Böser et al. (2002).

Using these markers, space studies revealed that brain metabolism was strongly affected by a change in gravity level. In immature and adult aquatic animals, modifications occurred mostly in vestibular related structures of the developing brain or in the sense organs. After the 20-day Cosmos-782 biosatellite mission, creatine kinase activity was increased in the cortex of the vestibular cerebellum of killifish *Fundulus heteroclitus* that hatched during the flight, with respect to the ground controls (Krasnov 1977). Cytochrome oxidase activity was decreased in the sensory epithelia of the utricle but not in the saccule after spaceflight exposure (Anken et al. 1998).

In some instances, hypergravity can induce an effect opposite to that of microgravity. In fact, hypergravity increases and microgravity decreases energy consumption, or vice versa. For example, mammary metabolic activity in pregnant rats was significantly increased in response to spaceflight (STS-70) but decreased under conditions of hypergravity (Plaut et al. 1999, 2003) (Figure 5-14, right). The reactivity of G6P-DH in the whole brain of young fish *Oreochromis* was increased after development in 3 g and decreased after development in simulated microgravity. Similar observations were made for the reactivity of SDH in brain nuclei connected to the vestibular system, such as the nucleus magnocellularis of larval fish.

On the other hand, altered gravity was ineffective at this period of development in nuclei that were not connected with the vestibular input, such as the pretectal nucleus (Figure 5-14, left). In the nucleus magnocellularis, cytochrome oxidase was also positively correlated with gravity and followed the relation: 0 g in orbit < 1 g in orbit and 1 g on the ground < 1.4 g < 3 g (Anken et al. 1996, 1998).

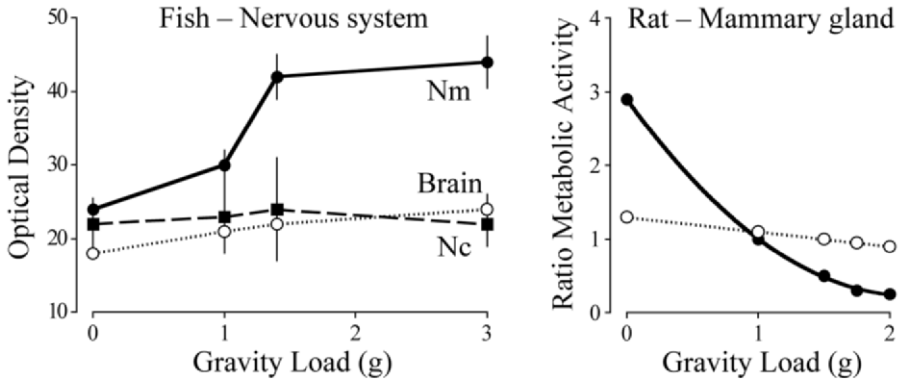

Figure 5-14. Sensitivity of metabolic activity to microgravity exposure. Left: SDH reactivity in the brain of young cichlid fish after 10-day exposure to microgravity, in normal gravity, and after exposure to 1.4 g and 3 g. The optical density of brain sections was used as a measure of SDH reactivity. Total brain sections (Brain) were compared with the area of the N. magnocellularis (Nm) that receives input from the vestibular (especially utricular) endorgan and the pretectal N. corticalis (Nc) of the retinohypothalamic system that is not a primary vestibular center of the brain. Note the strong effects of altered gravity in the Nm and the absence of any effect in the non-vestibular nucleus, and the opposite efficiency of microgravity and hypergravity (modified from Anken et al. 1996, 1998). Right: Mammary metabolic activity in relation to altered gravity. Rats were exposed to 9-day of microgravity during spaceflight (STS-70) or hypergravity up to 2 g on a centrifuge from day 11 to 20 of gestation. Within 2 h after the centrifuge was stopped, the abdominal mammary glands were removed, and metabolic activity was measured as oxidation of [U-14C] glucose to CO_2 (open symbols) or incorporation into lipid (closed symbols). The ratio between data from animals exposed to altered gravity and the 1-g controls was plotted against g-load. Note the opposite effects of microgravity and hypergravity. Adapted from K. Plaut et al. (2003).

4.3 Behavior

Gravity plays an important role on behavioral responses such as maintenance of posture, swimming, walking, and the control of eye or head movements. It is, therefore, not surprising that both microgravity and hypergravity affect these types of behavior significantly in adults as well as in developing animals.

Eye and head movements offer the best possibility for a high-resolution quantification of the standard developmental behavioral characteristics in vertebrates, in particular fish and amphibians. Reflexive eye movements are induced by stimulation of the vestibular sense organs, the so-called *Vestibulo-Ocular Reflex* (VOR). In some insects, compensatory head movements are induced by a change in body position, measured either by proprioceptors of the legs, or by special gravity sensing organs at the end of the abdomen (see Figure 5-01). The extent of these compensatory eye or head movements is clearly related to the animal's position relative to gravity and can, therefore, be measured as relative angular displacements (Horn 1985). Measurements include the reflex amplitude, i.e., the maximum displacement during a 360° lateral roll tilt of the animal, or the reflex gain, i.e., the ratio between eye angle and body angle. These measurements can be expressed as a function of the age of the animal. For this reason, the effects of altered gravity on eye and head movements can be detected with a higher stage-related resolution than by recording swimming, walking, or maintenance of upright posture.

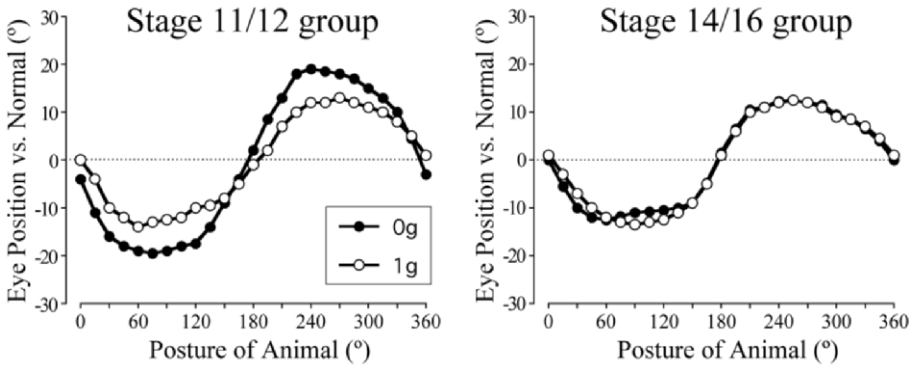

Figure 5-15. Development of the static roll-induced vestibulo-ocular reflex in a fish (Oreochromis mossambicus) during spaceflight. When the animal is tilted to one side, its eyes roll in the other direction. The postflight sine-like response characteristics from young fish flown in 0 g are compared with those of fish raised in 1 g. The developmental stage at onset of the microgravity period is indicated in each plot. Note that the young stage was sensitized by microgravity (STS-84 mission), as shown by the increased amplitude of eye movements. The older group (flown on the STS-55 mission) was not affected by microgravity.

4.3.1 Compensatory Eye and Head Movements

Eye and head movements with respect to the gravitational vector that are induced by stimulation of the gravity sensing organs can be observed in many animals including vertebrates, cephalopods, crustaceans, or insects. The basic shape of the response characteristics is sine-like. This response is very reproducible: repeated recordings have revealed that short-term adaptation of the roll-induced response is weak or absent in the clawed toad *Xenopus* (Horn 2004).

Developmental characteristics of this response have been determined with a high stage-related resolution for three species: the fish *Oreochromis mossambicus*, the amphibian *Xenopus laevis*, and the house cricket *Acheta domesticus*. Their developmental characteristics differ in some respect. In *Xenopus*, gain and amplitude of the *roll-induced Vestibulo-Ocular Reflex* (rVOR) are the largest in young tadpoles during the period of limb development. After that period, in particular during the maturation of the cerebellum, the rVOR decreases towards a final constant level (Horn et al. 1986).

In *Oreochromis*, rVOR gain and amplitude also increase during early life. After reaching a maximum, it decreases during the formation of visual-vestibular connections, and later on it increases until maturity of the fish (Sebastian and Horn 1999). In crickets the gain of roll-induced head movement increases continuously after each molt until the final molt to an adult cricket (Horn and Föller 2001).

Microgravity modified the rVOR of young immature *Oreochromis* and *Xenopus* tadpoles. In very young fish that had not yet developed a functional rVOR response before entering into microgravity, the rVOR was augmented after a 10-day spaceflight. In older fish that were able to perform the rVOR at launch, no difference in the rVOR was found with respect to the ground control animals (Sebastian et al. 2001) (Figure 5-15).

In *Xenopus*, the effects were more complex. During the spaceflight, some of the tadpoles developed a *dorsalization* of the tail, a so-called *tail lordosis*, while other tadpoles developed normally. The dorsalized tadpoles that did not have a functional rVOR at launch showed a depressed rVOR after landing, while normal, “undorsalized” tadpoles were unaffected with respect to the ground controls. Older tadpoles that could perform the rVOR at launch and developed a dorsalized tail behaved like the younger group, i.e., their reflex was depressed (Figure 5-16). But those tadpoles from that older group with normal tails showed an augmented rVOR after the flight (Sebastian et al. 1996, Sebastian and Horn 1998, Sebastian and Horn 2001).

However, microgravity did not affect the compensatory head response of *Acheta domesticus* (Figure 5-17), although the activity of its PSI was strongly affected by microgravity (see Figure 5-11).

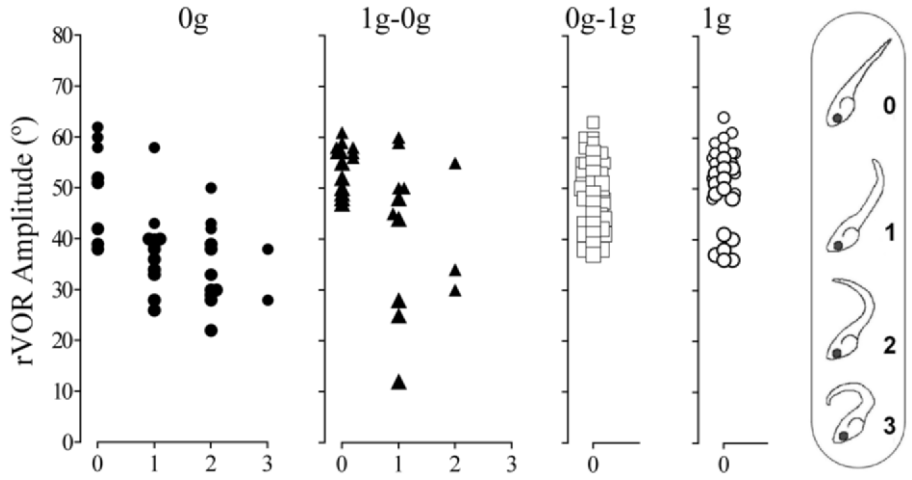

Figure 5-16. In the experiment with the amphibian Xenopus laevis, the duration of 0 g exposure was modified during the spaceflight (STS-84 mission) by means of an onboard centrifuge. Tadpoles were exposed to 0 g throughout the mission (0g), during the second half of the mission only (1g-0g), during the first half of the mission (0g-1g), or they were exposed to 1 g throughout the mission (1g). The rVOR amplitude represents the maximal extent of eye movement during a 360 deg lateral roll body tilt. Only the 0g and the 1g-0g groups show dorsalized tails after landing of the spacecraft. The extent of this dorsalization is ranked from 0 (normal) to 3 (extreme). The 1g-0g and the 1g groups did not develop a dorsalization. Note that the rVOR amplitude is clearly related to the extent of the dorsalization (Sebastian and Horn 2002).

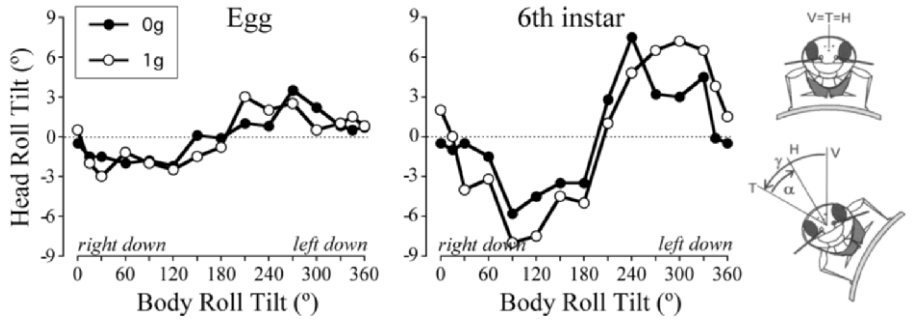

Figure 5-17. Compensatory head tilt induced by a body roll tilt of crickets Acheta domesticus after a 16-day spaceflight. The sketch on the lower right shows the compensatory head response. V, vertical axis; H, dorsoventral axis of the head; T, dorsoventral axis of the thorax. These axes are colinear in the normal position of the cricket, but differ if the animal is tilted in roll. α, compensatory head roll tilt; γ, roll body tilt. At launch, the animals were either at an embryonic stage shortly before hatching (egg) or they had reached the 6th instar stage. Note that, for both developmental stages, microgravity exposure had no effect on the response characteristics, i.e. this behavior was insensitive to an exposure to microgravity. Adapted from Horn et al. (2003).

Nevertheless, the increased response of the young fish and tadpoles after microgravity exposure can be considered as an increase in sensitivity of the vestibular response. It is interesting to note that such increase in sensitivity of the vestibular system was also obtained in some adult fish (Boyle et al. 2000) and in astronauts (Clément et al. 2001) after exposure to microgravity.

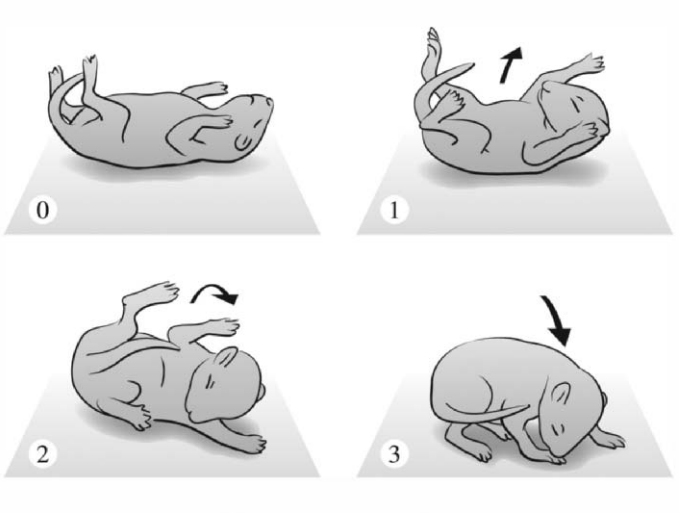

Figure 5-18. This cartoon shows the sequence (from 0 to 3) of body movements made by neonatal rats (P0) during righting after being placed on their back on a surface.

4.3.2 Righting Response

Righting responses from a supine posture to a prone posture are common in animals. Beside the vestibular system, tactile cues from the contact with the solid surface, as well as proprioceptive cues from muscle spindles and tendons contribute to a successful righting response.

It is well know that the strategy of this type of response changes during development. For example, newborn rats assume a U-shaped posture (ventroflexion) followed by a rotation of the head, neck, and shoulders, with forepaw support (Ronca and Alberts 2000). The last step is a rapid axial rotation, a response known as the corkscrew behavior (Figure 5-18). By contrast, adult rats execute a complete lateral (axial) roll without any U-shape of the body (Kalb et al. 2003). For this reason, this righting response is a good experimental model to test maturation of vestibular function.

In addition, to separate the contribution of vestibular from other sensory inputs, such as touch, the righting response can be studied during water immersion, i.e., the animal is positioned in the supine position in a water-filled container and then released (Figure 5-19).

Testing this type of righting behavior, both on land and in water, can start on the day of birth (P0).

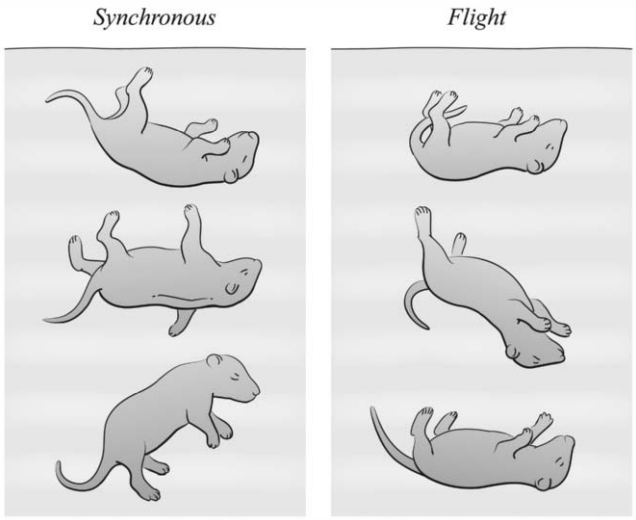

Figure 5-19. This cartoon shows the sequence of body movements during the righting response in water by neonatal rats raised on Earth (Synchronous) or exposed to microgravity (Flight). Left: Pups raised in 1 g. Right: Pups raised in microgravity.

Prenatal animals were exposed to microgravity during the beginning of morphological and physiological development of the vestibular apparatus. This exposure significantly altered postnatal maturation of righting.

On a solid surface, attempts for righting were achieved by all neonates at the day of birth. Usually, 50% of these attempts were successful and the animal reached the prone position with both forelimbs in contact with the surface, independent of whether they were born from microgravity-exposed dams or from 1-g ground (vivarium and synchronous) controls (Figure 5-20).

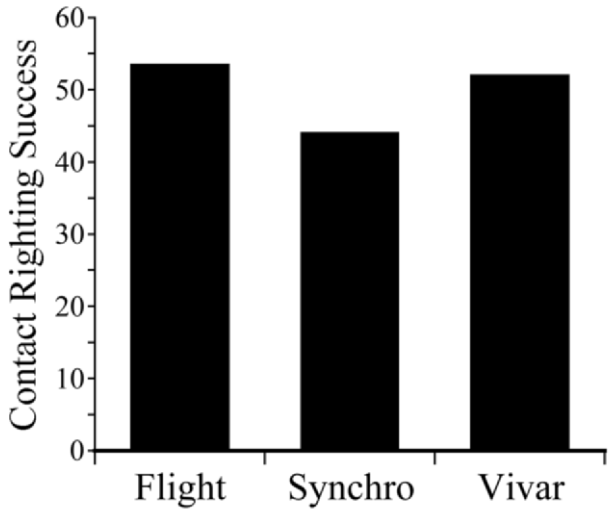

Figure 5-20. Percentage of neonatal rats showing successful contact righting response after exposure to microgravity (Flight) and in the control groups. The vivarium group (Vivar) includes animals in standard laboratory conditions. The synchronous group (Synchro) is considered as the real ground-control group, because the animals were reared under the same conditions, including feeding and caging, and schedule as the flight animals.

However, righting behavior from the supine to prone position in the water immersion test, i.e., in the absence of tactile cues, revealed clear response deficits in neonates that underwent prenatal development in space (Ronca and Alberts 2000). This deficit persisted until postnatal day P3. Normalization of righting took place at P5 (Figures 5-19 and 5-21).

Exposure to microgravity (STS-90) during postnatal periods of life significantly retarded the development of this righting behavior. Indeed, rats launched at postnatal day P14 and tested on the day of landing showed a U-shape posture, which is typical of immature behavior. In contrast, the axial rotation clearly dominated in the ground control animals (Kalb et al. 2003).

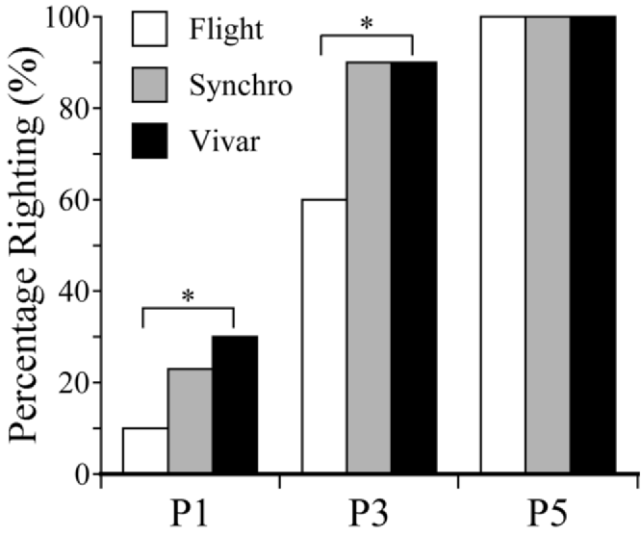

*Figure 5-21. Only flown P5 neonatal rats showed successful righting response during water immersion. By contrast, flown P1 and P3 neonates (Flight) showed abnormal behavior compared to the vivarium group (Vivar). * p<0,05. Adapted from Ronca and Alberts (2000) and Plaut et al. (2000).*

4.3.3 Locomotion

Locomotion is strongly affected by the gravity load because the legs are regularly moved with and against the gravitational force. Thus, it was assumed that gravity-related information contributed to the patterns of leg movements. To test the impact of load deprivation on locomotion, young animals were exposed to tail suspension or to microgravity. During suspension, the forelimbs wear the weight of the animal, while the hind limbs are unloaded (Figure 5-22, left). Suspended animals move their forelegs similar to non-suspended animals while hind limbs provide a torque such that the young rats walk in circles.

The studies revealed that a suspension applied to the animals from postnatal days P13 to P31 induced an increase in the ankle angle during walking. This increase persisted for more than one month thereafter. After exposure to real microgravity for 9 days during the NIH.R3 mission, the analysis of free walking showed differences in hind limb and forelimb joint

angles. The most characteristic effect was the extension of the hind limb joints, in particular during the stance phase (Walton 1998).

Also, balance control during swimming was impaired in suspended animals. Stroke duration of the hind limbs can be used as a measure for swimming ability. Measurements indicated that the swimming impairment was more pronounced in rats suspended from P8 to P13 than in rats suspended from P2 to P7. In particular, on postnatal day P11, most pups from the P8-P13 group were unable to swim at all, and in the most severe cases, 180-deg body rotations occurred. After termination of suspension, swimming ability was indistinguishable between normal and suspended pups. When suspension began after P13, swimming was largely unaffected (Figure 5-22, right).

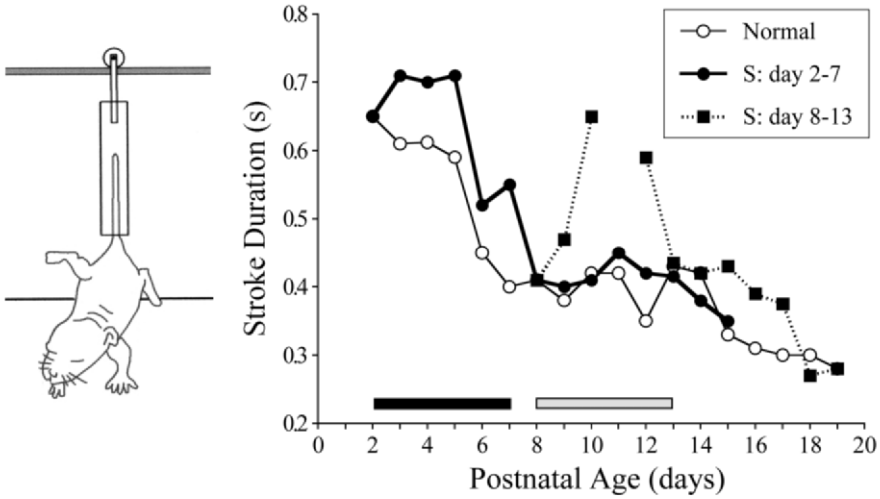

Figure 5-22. Left: Tail suspension technique used to unload the gravity load on the hind limb of the animals during various phases of their development. Right: Postnatal development of hind limb movements during swimming in neonate rats following tail suspension (S) during postnatal days P2 to P7 or P8 to P13. Swimming abilities defined by the stroke duration of the hind limb was slightly impaired after periods of suspension between postnatal days P2 to P7, while suspension between postnatal days P8 to P13 caused a dramatic impairment of swimming. When tested on P11, they were unable to swim. This demonstrates a sensitive period in the development of motor patterns in the rat. Adapted from Walton (1998).

4.4 Age-Related Microgravity Effects and Critical Periods

4.4.1 Critical Period

One interesting feature of sensory, neuronal, and motor systems is the existence of critical periods during their development. The concept of critical period during development goes back to studies performed by Nobel prizes

laureates Huber and Wiesel (1982) on the visual system in kitten. Deprivation is the preferred scientific method to study the existence and duration of critical periods. Consequently, every long-lasting change in the environment may have its specific critical period.

In general, three criteria have to be fulfilled to define a development period as "critical":

a. The developing system must be susceptible to a specific environmental modification;
b. The extent of modification must be related to age, and in particular to a well-defined period of development;
c. The modification must persist for long periods of postnatal life or even permanently.

In space studies, the first two criteria were observed. However, long-duration effects of irreversibity were rarely noted.

4.4.2 Development of Organs

Exposure to gravity deprivation using microgravity or weight unloading techniques gave evidence for the existence of age-related susceptibilities for morphological as well as for physiological and behavioral development. During the 16-day Neurolab mission, the development of various organs in flown neonate rats was strongly modified in the group launched at postnatal day P7. However, the modifications were smaller or absent in pups launched at postnatal day P14. In particular, after the flight, lung, heart, kidney, and adrenal glands of the P7 group were larger than ground controls. Thymus, spleen mesentery, and pancreas were smaller, and the aortic nerve had less unmyelinated fibers. In contrast, in the flight rats from the P14 group, only the kidney was heavier and the ovary lighter than in the ground controls (Miyake et al 2004). These observations clearly identified the second week of life as sensitive to gravity deprivation for morphological organ development.

4.4.3 Cell Cultures

Age-related effects of microgravity exposure became also obvious in cell cultures. Flown isolated embryonic mouse pretarsal mesenchym differentiated to cartilage as in the ground controls. The extent of this differentiation, however, depended on the state at launch. If pre-metatarsals had initiated chondrogenesis and morphometric patterning prior to launch, then cartilage rod size increased and rod shape was maintained. By contrast, older pre-metatarsal tissue, which had already terminally differentiated to hypertrophied cartilage, maintained rod structure and cartilage phenotype during the spaceflight (Klement and Spooner 1994).

Another example for age-related susceptibilities of cell cultures came from the development of neuron and myoblast synapses, as revealed by the

ACh receptor patches (Figure 5-06). Clinostat rotation on Earth inhibited the formation of nerve-associated ACh receptor patches if nerve contact took place during or shortly before onset of microgravity simulation, but not if this contact took place long before microgravity stimulation (Gruener and Hoeger 1990).

4.4.4 Motor and Sensory Systems

An age-related susceptibility to actual and simulated microgravity has been extensively described for motor and sensory systems. These studies mainly included observations in rats, amphibians, and fish.

In rats, motor development concerning the ability of swimming revealed a high susceptibility to weight unloading if tail suspension was performed between P8 and P13 (Figure 5-22). Tail suspension during other periods was either ineffective or only slightly impaired swimming (Walton 1998).

The existence of a critical period for the development of the rVOR in zebrafish *Danio rerio* was unequivocally shown. The study by Moorman and collaborators (1999, 2002) is, so far, the only study for which the duration of the critical period was clearly determined. Zebrafish embryos were placed in a bioreactor developed by NASA at different periods of embryonic development. The bioreactor rotation started either at 3, 24, 30, 36, 48, or 72 hours after fertilization. The animals were then tested for their rVOR at 96 hours after fertilization. In other animals, rotation was started immediately after fertilization and measurements were done at different ages (24, 36, 48, 60, 66, 72, or 96 hours) after fertilization. Modifications of the rVOR were classified as normal, weakly depressed of short persistence, or depressed during a period of 5 days. Based on this classification, it was found that the critical period for rVOR lasted from 30 to 66 hours after fertilization.

As mentioned above, an age-related sensitivity of the rVOR with respect to microgravity also exists in fish *Oreochromis mossambicus* and in tadpoles *Xenopus laevis* (see Figures 5-15 and 5-16, respectively). In both species, the age at which the rVOR appeared for the first time revealed to be critical concerning the effects of microgravity (Sebastian et al. 1996, Sebastian and Horn 2002, Horn 2004).

Age-related susceptibilities also exist for exposure to hypergravity. For example, the rVOR of *Xenopus laevis* was not modified after a 12-day exposure to hypergravity starting about 12 hours after egg fertilization, but its further development in 1-g conditions was blocked. Older stages including the hind limb bud stage exposed to 3 g for 10 days revealed a significant decrease in the rVOR gain after return to normal gravity. In these groups, however, development continued normally after return to 1-g conditions, and normalization took place after several weeks depending on the stage at the onset of hypergravity (Horn and Sebastian 1996). Another example of a

stage-related susceptibility to hypergravity is the development of the size of specific inhibitory GABAergic neurons (the common inhibitors CI1, CI2 and CI3) within the thoracic ganglia of house crickets *Acheta domesticus* after a 16-day exposure to 3-g centrifugation (Horn et al. 2001).

4.5 Pregnancy

Numerous aspects of pregnancy, birth, and early mammalian development have been shown to proceed normally under altered gravity conditions (Ronca 2003a). The development of an animal requires a strong connection to its parents, in particular in vertebrate animals. Nursery is found in all vertebrate groups, and also in many invertebrates. In particular, the arthropods, including insects and spiders, are known for this behavior. Thus, the loss of contact of the neonates with its parents, amplified by the stressful conditions of spaceflight, might alter their development.

Studies in rats support the view that biologically meaningful interactions between mothers and offspring are changed in space. Whereas studies of mid-pregnant rats in space have been extraordinarily successful, studies of young rat litters launched at 9 days of postnatal or earlier age have been faced with problems related to, at least in part, bilateral linkages between mothers and offspring. The most important contributions to these data came from the Cosmos-1514 biosatellite flight, and the Space Shuttle NIH.R1 (STS-66) and NIH.R2 (STS-70) missions during which the microgravity exposure was entirely during prenatal periods. During the subsequent NIH.R3 (STS-72) and Neurolab (STS-90) missions, microgravity exposure took place during postnatal periods of life (Ronca 2003b).

In December 1983, pregnant rats stayed in orbit from *Gestational Days* (GD) 13 to 18 during the Cosmos-1514 biosatellite flight. After recovery, they gave birth to viable litters and the females reared their offspring to weaning. Follow-up experiments on Space Shuttle NIH.R2 and NIH.R1 missions carried pregnant female rats from GD 11-20 for 9 days and from GD 9-20 for 11 days in space, respectively. Before the flight, the rats were screened for a sufficient number of embryos. The most fundamental finding of these experiments was that dams displayed twice more labor contractions as the controls. The flown rats had typically 134 contractions, including a mean of 84 lordosis contractions. However, after landing, flight dams had uncomplicated vaginal deliveries, and number and size of litters were similar to that of controls (Alberts and Ronca 1997).

Spaceflights of females with pups revealed some difficulties. In particular, nursery of pups that were launched at stage P5 revealed to be extremely difficult probably because of disturbances in the mother-offspring relation in the microgravity environment. Only 10% of these pups survived the flight, whereas 90% of the P8 pups and all P14 pups survived the 9-day flight on board NIH.R3. During the 16-day Neurolab STS-90 flight, P15 pups

survived the mission in good health, but more than 50% of P8 neonates died during flight. Survivors had severe deficits in weight compared to the synchronous and vivarium ground controls (Figure 5-23). It is likely that the mother-offspring relation is a critical factor for postnatal development in microgravity (Ronca 2003).

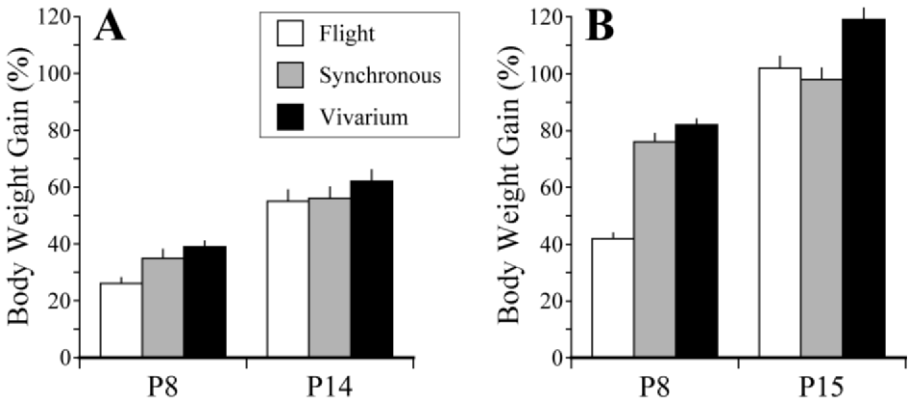

Figure 5-23. Percentage of body weight gain in rat pups flown during the 9-day NIH.R3 mission at postnatal day P8 and P14 at launch (in A), and in rat pups flown during the 16-day Neurolab mission at postnatal day P8 and P15 at launch (in B), compared with ground controls. Note the significant decrease in body weight in P8 rats after exposure to microgravity. This decrease is presumably caused by a disturbed mother-child relationship in the flight group and, as a consequence, extreme mal-nutrition. Adapted from Ronca (2003b).

4.6 Developmental Velocity

In laboratory cultures, embryos and postembryonic larval stages develop and grow with different progress, i.e., some animals stay at a specific stage for a longer time than others. Nutrition might be one reason. But it is also likely that the naturally occurring genetic variability is responsible for different growth and maturation velocities, particularly in the case of standard rearing procedures under laboratory conditions. In some cases, it was found that the growth rate of the animal determined the extent of the effects induced by altered gravity.

For example, during the 16-day Neurolab mission, larvae of the house cricket *Acheta* molted twice or three times, independently of the developmental stage at the onset of microgravity. In a group in which one of the two cercal gravity sensing organs (see Figure 5-01) was removed before launch, the so-called regeneration group, some animals molted in space only twice. Their compensatory head righting response was significantly smaller

than that recorded in the ground controls. The difference did not exist for animals that molted three times in space (Horn et al. 2003).

Experiments from the same flight and the preceding STS-89 flight on the growth of otoliths in the absence of gravity in the swordtail fish *Xiphophorus helleri* pointed into the same direction. No significant effect of gravity deprivation on otolith growth was found in juvenile swordtails, while otolith growth in the embryos was strongly affected. Embryos from the Neurolab flight had larger otoliths, whereas those from the STS-89 had smaller otoliths compared to their respective ground controls. This contradictory observation correlates with the fact that embryos from STS-89 were smaller than those from Neurolab (Wiederhold et al. 2003).

Another example came from a hypergravity experiment. After 9 days spent at 3 g, the rVOR of *Xenopus laevis* tadpoles was depressed (Horn 2004). Within 11 days after termination of the 3-g exposure, the depression was still present in slowly developing tadpoles that had reached only stage 46. By contrast, a complete re-adaptation to standard rVOR development was observed in fast developing tadpoles that had reached stage 47 within the same period (Sebastian et al. 1996).

4.7 Longevity and Aging

After about 50% of standard lifetime, the aging process follows development. Well before that period, however, during very early periods of postnatal life, degenerative processes occur, such as degeneration of neurons and synaptic contacts. It was possible to study the effects of microgravity on a few aged individuals, including animals and humans. Observations showed that basically spaceflight was tolerated by all ages. In humans, a 76-year old astronaut flew on STS-95 and three 55-65 year old "tourist" cosmonauts flew on board Soyuz flights to the ISS. No signs of decreased physiological and mental capacity were observed in these older astronauts/cosmonauts compared to younger crewmembers.

In animals, data on aging comes from studies in fruit flies *Drosophila melanogaster* and nematodes *Caenorhabditis elegans*, which were reared for at least one generation in microgravity. In *Drosophila*, the morphological development proceeded normally under microgravity (STS-55, STS-65), but after landing, aging was accelerated in flown males but not in flown females as compared to ground controls (Marco et al. 1986, Benguria et al. 1996). This conclusion was based on observations about the performance of geotactic behavior and lifespan. By contrast, aging seems to be insensitive to microgravity in *Caenohabiditis*. Many morphological parameters, such as number and distribution of cells, nuclear morphology, karyotype and symmetry relations, cell division planes and gonad symmetry, and fertility, revealed no difference with ground controls (Nelson et al. 1995).

Some post flight observations concerning longevity were obtained from houseflies *Musca domestica* after 7 days in space and from the Chinese silkworm *Bombyx mori L.* that flew for 12 days on board Bion-10. Compared to ground controls, houseflies exhibited similar longevity but their reproductive output was reduced, probably due to failures in ovarian development. Offspring of the flown flies revealed normal reproduction, i.e., the immediate post flight effect was not genetically transmitted (Lee et al. 1985). Two out of seven silkworm varieties had a shorter life span after return to Earth, despite of normal completion of spinning, cocooning, mating, oviposition, larval hatching, pupation, and moth emergence in space. However, times of hatching and oviposition in the flight group were two to three days earlier than in the control group, and the hatching rate of diapause eggs[17] seemed higher during spaceflight than on the ground (Shi et al. 1998).

4.8 Regeneration

Morphological regeneration is a reactivation of development in postembryonic life to restore missing tissue. Its most spectacular aspects are the demonstration of multipotent properties of specific tissue and that the correct positional information is re-specified, so that normal body structures such as complete extremities or retinas are formed. Only a few aquatic vertebrates such as salamanders and newts possess the potency for regeneration. Among invertebrates, species of much lower order, such as insects (crickets, cockroaches), flatworms (planaria), and coelenterates (*Hydra*), are able to regenerate lost or lesioned organs.

Despite this large number of species with regeneration potencies, microgravity investigations were limited to studies on the regeneration of lens, forelimb, and tail in *Pleurodeles waltl* during the Bion-10 and -11 flights (Grinfeld et al. 1994; Grigoryan et al. 2002), and of abdominal appendages or *cerci* in crickets (Horn et al. 2001) (see Figure 5-01). The main observation was that lost or lesioned parts of the body could regenerate in microgravity. In some instances, cell proliferation in the flight animals was increased. Differences became less pronounced the longer the time span was between lesion and onset of microgravity. Microgravity experience persists in intact animals for some time because newts flown intact and operated after the flight regenerated faster than 1-g ground controls (Grigoryan et al. 2002).

Retina regeneration can be induced by several lesion techniques, such as by removal of neural retina using microsurgery or by optic nerve transsection. An experiment performed on the two-week Bion-11 flight revealed an intensification of regenerative processes. In particular, the

[17] *Diapause* is a period in the life cycle of an insect during which development is temporarily suspended. Diapause is usually induced by environmental signals or extreme conditions like winter or summer.

proliferative activity as shown by the number of [^{3}H]-thymidine-labeled cells in the retinal pigmented epithelium, eye growth zone, and other retinal areas was 1.2 to 1.5 times higher in flown salamanders compared to ground controls (Grigoryan et al. 2002) (Figure 5-24).

Many features of tail regeneration in the salamander, including blastema elongation, neuronal tube, cartilage of future vertebrate, muscles and connective tissue, proceeds in microgravity at the same pace as in normal gravity. The only condition is that, at launch, the tail blastemas must have just formed a 1-mm thick translucent, convex layer. Similarly, some molecular markers of central nervous system activity such as *Glial Fibrillary Acidic Protein* (GFAP), specific intermediate filaments NF150, and *Tyrosine Hydrolase* (TOH), were found in both space and ground groups in similar amounts. However, other features of tail regeneration were modified, including the connective tissue of the blastema of salamanders exposed to 0-g that developed more GABA-positive cells than ground controls (Grinfeld et al. 1994).

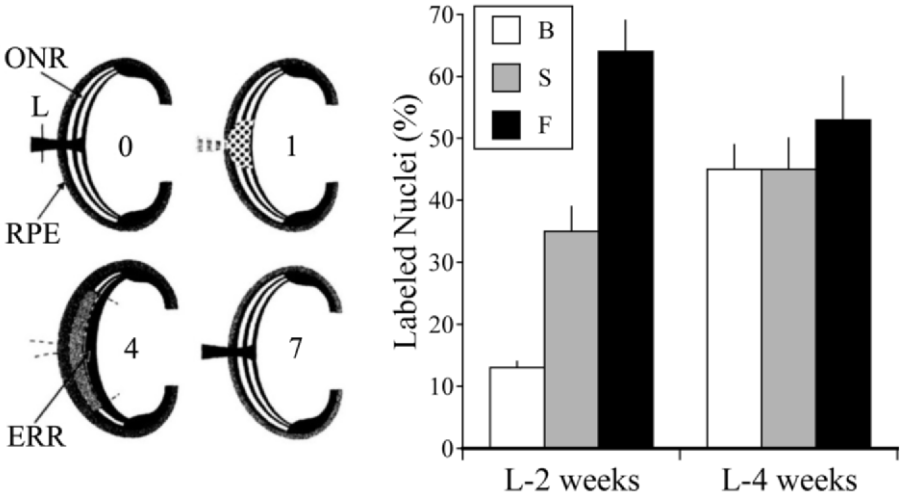

Figure 5-24. Regeneration of the retina under spaceflight conditions in the newt Pleurodeles waltl. Left: Selected stages of neuronal regeneration after lesioning of the optic nerve. Stage 0: Operation; Stage 1: Degeneration of original neural retina (ONR); Stages 4: Formation of early retinal regenerate (ERR) by trans-differentiating cells of the retinal pigmented epithelium (RPE) and cells of eye growth zone; Stage 7: Morphogenesis of newly formed retina and regeneration of optic nerve. Right: Percentage of [^{3}T]-thymidine-labeled nuclei in the central part of the neural retina when lesion was performed two (L-2 weeks) and four weeks (L-4 weeks) before launch in space-flown newts (F) compared to the basal (B) and synchronous (S) control groups. The "basal" measurements describe the status of regeneration at the day of launch. The synchronous group is the ground control group kept under the same conditions and timeline as the flight animals and fixed for histological studies on the same day after landing as the flight animals. Adapted from Grigoryan et al. (2002).

5 RESEARCH PERSPECTIVES

The actual knowledge of how microgravity affects embryonic and postembryonic development makes clear that:

a. Development continues in the absence of perceived gravity;
b. The impact of spaceflight seems to be weak;
c. Existing modifications are compensated for, up to a level that a stable organism is finally created with a good prognosis for a life in microgravity;
d. The final stable level looks identical to the standard organism;
e. It is likely that significant modifications occur, for example, in the formation of the skeleton, which is of little use for supporting the whole body in microgravity.

Because of the low number of spaceflight opportunities for basic and applied research in space life sciences, it is very likely that many of the possible developmental modifications induced by spaceflight will not be studied, and consequently will remain unknown. Some adaptive mechanisms, however, could to be clarified by future research. These questions include mainly two directions of research: the determination of fertility modifications during long-term exposure to microgravity, and the clarification of whether and where the gravitational condition is converted into genetic information.

5.1 Fertility during Long-Term Exposure

A research program on the issues of fertility during long-term exposure to microgravity is clearly related to multi-generation studies. Prerequisites for multi-generation experiments in orbit are successful fertilization and delivery of young animals under microgravity. As described above, viable progenies were obtained from natural in-flight fertilization in fish, crickets, fruit flies, and nematodes, as well as from artificial in-flight fertilization in amphibians. A fertilization experiment with rats on board the Cosmos-1129 biosatellite failed, although initial phases of reproduction were demonstrated after this particular flight. Also, experiments with rats, mice, and hamsters revealed that fertilization in hypergravity were successful, provided the g-load was less than 3 g (Ronca 2003b).

Other prerequisites for this research are the production of F1- and further generations. So far, the number of experiments in which F1-generations have been produced in microgravity is extremely low. *Drosophila* was reared for one generation in orbit (Marco et al. 1986), but no information about the fertility in the F1-generation is available from this model animal. *Caenorhabditis* worms reproduced twice in space and generated thousands of offspring (Nelson et al. 1995), demonstrating that fertility was maintained in F1-animals in this species.

Multi-generation experiments will give answers about delayed impact of microgravity on development. Hypergravity studies revealed that bone development in the F3-generation of mice was strongly affected if reared under permanent 2-g conditions. Concentrations of Ca and P in the cervical vertebrates were increased compared to controls, while Ca and P concentration in the thigh bone and lumbar vertebra were depressed (Kita et al. 2003).

It is also clear that multi-generation experiments demand extensive work in the design and development of suitable habitats (see Chapter 3, Section 3.2). To a large extent, these habitats are species specific, in particular in those model animals in which there is a strong mother-offspring relation during nursery.

5.2 Is Gravity Genetically Coded?

The objective of this research is the analysis of molecular and physiological interactions that generate the proper organism. It relies on the use of mutant mice to delineate the molecular basis of development, including differentiation to their final destination, and the use of physiological methods to understand the meaning of related modifications.

This research program is related to ground-based analyses on the impact of genetic modifications (for example by knock-down approaches) on specific gravity-related systems and functions of the body. So far, these questions preferably have been related to the development of neuronal and sensory systems. It is likely that almost everything from formation and path-finding to specific targets and survival is regulated by sophisticated genetic programs that perform most of their function independently of external (epigenetic) inputs such as altered gravity. It can be therefore assumed that the formation of the most initial connections and synapses will occur normally regardless of the vestibular stimulation. However, following this initial "hard wired" molecular phase, a second phase will presumably concentrate on the fine-tuning of these early connections via the physiological factor activity. So, the task ahead is to unravel the molecular conditions that produce the most striking and most lasting effect of connectivity modifications driven by altered gravity (see Fritzsch 2003 for review).

A step into this type of research was done using the fish model *Oryzias*. Its otolith-deficient mutants were compared with other strains, and different strains of this species were crossed. One of the most intriguing results was the formation of fish that were less dependent on gravity and therefore less sensitive to microgravity, as shown by less frequent loop swimming during the flight (Ijiri et al. 2003).

Future studies in developmental biology will consider to which extent gravitational forces affect gene expression. In periods of limited access to space, microgravity simulation methods such as clinostats or bioreactors will

give insights into the extent of gene expression, and its relationship with the periods of embryonic and postembryonic life. An example of this approach is the study in transgenic zebrafish, which were exposed to simulated microgravity for different durations at various developmental times, in an attempt to determine the susceptibility periods for a large number of developing organs, including the heart, notochord[18], eye, somites, and Rohon-Beard neurons[19]. The observations revealed changes in gene expression with periods of maximum susceptibilities characteristic for each organ. They also indicated a complete recovery of gene expression, despite of continuous exposure to simulated microgravity (Shimada et al. 2005). These observations support the idea of a self-organized intrinsic normalization of the development during continuous exposure to altered gravity, despite of the often-observed deviations from normal development.

Figure 5-25. Behavioral studies in parabolic flight.

[18] *Notochord* is a rudimentary of embryonic spinal column in the fish.
[19] *Rohon-Beard neurons* are primary mechanosensory neurons that differentiate in the dorsal spinal cord and are found in most lower vertebrates.

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Chapter 6

PLANT DEVELOPMENT IN MICROGRAVITY

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This chapter examines how the microgravity environment of spaceflight can affect plants. Results from studies of the mechanism of gravity perception and the role of microgravity in determining the development and growth of plants during various stages in their life cycle, at the organ, cellular, and subcellular level, are presented and discussed. The influence on plant development of other factors of spaceflight, such as the absence of 24-h cycles, changes in magnetic or electrical field, were reviewed in *Fundamentals of Space Medicine* (Clément 2005).

Figure 6-01. A close-up view of a bloom on the Rasteniya-2/Lada-2 (Plants-2) plant growth experiment photographed by the astronauts on board the International Space Station. Photo courtesy of NASA.

1 INTRODUCTION

Plants on Earth are subjected to a constant gravitational field, which has played a major role in their evolution. The actions of gravity on plants have been studied for more than a century (reviews by Larsen 1962, Sack 1991) and it is now well known that this physical factor has a great impact on

the orientation of plant organs (gravitropism) and on the development of plants (gravimorphism).

Gravitropism is a response of bending due to a change in the orientation of plant organs or to an inadequate orientation of their extremity with respect to gravity. For instance, when a seedling root germinates on the ground, its extremity can be oriented in any direction but must penetrate into the soil quickly to survive. The final orientation of the root tip is the direction of gravity (even if it is reached only after one day of stimulation). The primary root therefore has a positive gravitropism. Shoots, on the contrary, have a negative gravitropism since their extremity curves in the opposite direction. The optimal orientation of growth of an organ can be parallel to gravity (*orthogravitropism*) or oblique (*plagiotropism*) with respect to the g vector. Thus, most of the plant organs have an optimal angle of orientation with respect to gravity that is called the *Gravitropic Set-Point Angle* (GSPA) (Firn et al. 1999).

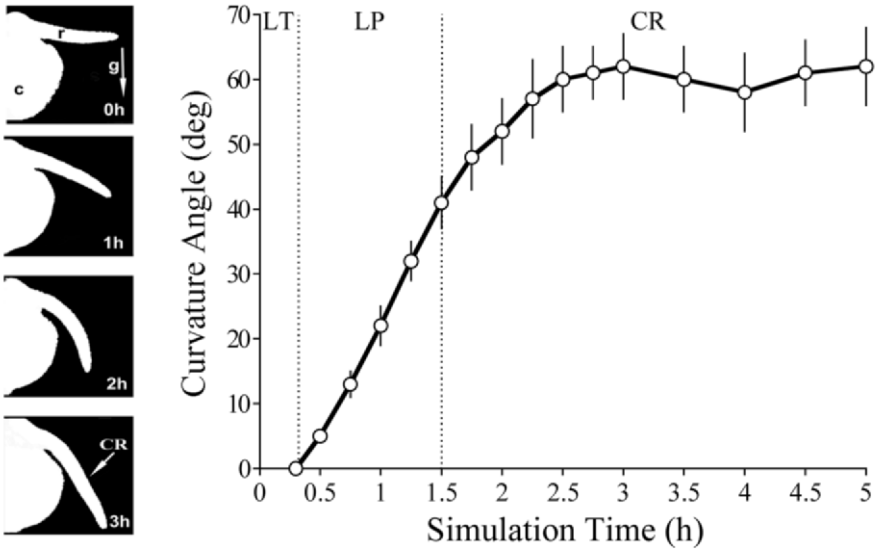

Figure 6-02. Gravitropic bending of lentil roots. Left: This lentil seedling was grown in the vertical position for 27 h. Then, it was placed in the horizontal position for 3 h and photographed every hour. The counter-reaction (CR), which occurred after 3 h, led to a reduction of the curvature. c, cotyledon; r, root; g, direction of gravity. Right: Kinetics of the gravitropic response of 60 lentil roots grown in the vertical position and stimulated in the horizontal position as in A. The angle (α) of curvature (see insert) is reported as a function of time of stimulation in the horizontal position. After a latent time (LT) of about 20 min, there is a phase (LP) during which the response is linear as a function of time. After a strong slowing down of the rate of curvature a counter-reaction (CR) occurs which reduces the angle of curvature. The vertical bars represent the interval of confidence at the 5% level.

Gravimorphism is the result of the effects of gravity on plant development. The actions of gravity on plant growth can be either quantitative (growth rate of plant organs) or qualitative (formation of plant organs). Some of these actions have been discovered by forcing a stem for instance to remain in an abnormal horizontal position by attaching its extremity. In this case the axillary buds (which are inhibited when the stem is in the upright position) begin to grow since the apical dominance of the apex over these buds is cancelled. This type of experiment shows that an inadequate orientation with respect to gravity provokes some changes in plant morphogenesis and not only on the orientation of the organs. However, the effect of this factor on a plant growing in the vertical position is not known, since the study of gravimorphism should include a comparison of plant growth on the ground and in microgravity. In that way, gravimorphism is more difficult to analyze than gravitropism. This is the reason why plant physiologists have used for more than a century special devices called *clinostats* in order to simulate microgravity.

The principle of these devices is simple: the clinostat prevents the unilateral effect of gravity by rotating the plant about a horizontal axis or about a point at 1 to 2 rpm (see Figure 1-19). These clinostats could actually simulate microgravity if the perception of gravity were too slow to induce a gravitropic signal. But, they can also induce slight omnilateral gravitropic stimulation if the perception time is short, i.e., in the order of 1 sec.

Plant physiologists have also carried out experiments with centrifuges assuming that there could a kind of continuum of the effect of gravity from 0 g to thousands g and by extrapolating the results obtained in the range of 1 g to thousands g. It is clear that works with clinostats or centrifuges can only give some information about what could be the action of microgravity on plant growth, and that space will remain a unique tool to study the effect of gravity on plant development (Figure 6-01).

2 THE RESPONSE OF PLANTS TO A CHANGE IN THE DIRECTION OF GRAVITY

2.1 Perception of Gravity in Plants

Experimentally, the gravitropic response can be studied by growing a seedling in the vertical position (the root tip down) and then placing the root in the horizontal position. The root is then subjected to gravistimulation. In this case, its extremity bends downward in order to recover its normal direction of growth (Figure 6-02). The curvature is due to a differential growth in the upper and lower halves of this organ.

It is generally accepted (Perbal and Driss-Ecole 2003) that the gravitropic response is composed of four different phases (Figure 6-03), which correspond to:

a. The perception of the stimulus (physical phase);
b. The transduction of this stimulus (change of the mechanical effect into a biochemical factor);
c. The transmission of the signal (from the gravisensing cells to the responding cells);
d. The differential growth of the upper and lower sides of the organ.

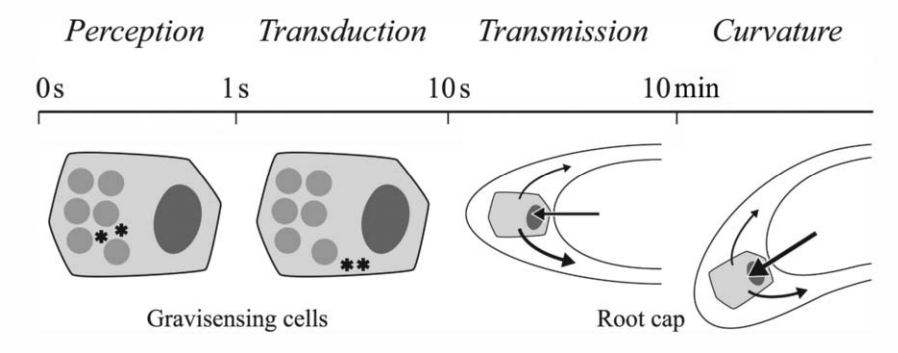

Figure 6-03. The different phases of the gravitropic curvature of the root. Four phases are generally distinguished. The perception is the physical phase of the gravitropic reaction and corresponds to the movement of the statoliths in the gravisensing cells located in the root cap. It is followed by the transduction of the stimulus, i.e., the transformation of the mechanical effect of gravity into a biochemical factor. Both phases occur within the gravisensing cells. The transmission of gravistimulus to the reaction zone consists in an asymmetrical hormonal message (downward transport of auxin). It is responsible for a differential growth (curvature) that occurs far away from the perception zone. Note the time scale.

At the beginning of the 20th century, Haberlandt and Nemec (see Larsen 1962) have shown that special tissues called *statenchyma* of shoots and roots contain movable organelles, the *amyloplasts*, in their cells, which sediment under the influence of gravity (Figure 6-04). Their sedimentation is due to the density of starch (1.44 g x cm^{-3}) contained in these organelles (Sack 1991). These authors hypothesized that the amyloplasts were responsible for gravisensing and called them *statoliths* in reference to those observed in invertebrates. The great difference in the gravisensing of plants and animals is that the former possess statoliths that are inside specialized cells, the *statocytes*, whereas in the latter they are outside a group of specialized cells. It must be added that mosses, which are gravitropic, show a special sub terminal zone were amyloplasts can sediment under the influence of gravity (Schwuchow et al. 2002a, 2002b). In the rhizoid of the characean green alga

Chara, gravisensing is due to BaSO4-cristal-filled statoliths (Sievers et al. 1996).

In roots, where the statenchyma has been more intensively studied, this tissue is located in the center of the cap (Volkmann and Sievers 1979, Boonsirichai et al. 2002, Perbal and Driss-Ecole 2003) (Figure 6-05A). When the root is placed in the horizontal position, these organelles move toward and sediment along the lower longitudinal wall. One of the best evidence of the involvement of the statenchyma in gravitropism was provided by Juniper et al. (1966) who showed that removing the cap of the maize root without damaging the root tip suppressed their ability to respond to a *gravistimulus*, i.e., a change in orientation in the gravitational field. This experiment demonstrated that at least one step of the gravitropic curvature occurred in the cap.

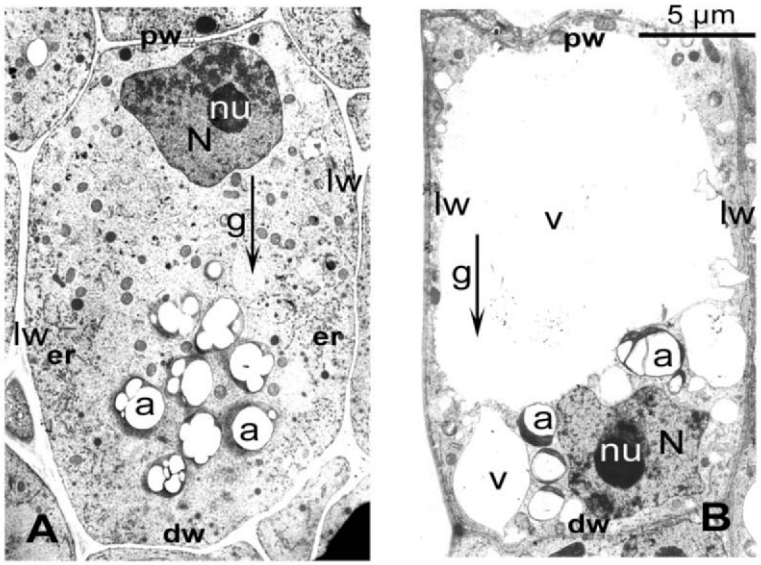

Figure 6-04. Gravisensing cells (statocytes) in a lentil root (in A) and in the Asparagus shoot or epicotyl (in B). The shoot statocyte possess a large vacuole (v), whereas only very small vacuoles can be seen in the root statocyte. Both statocytes show a structural polarity. In both cases, the amyloplasts (a) are located close to the distal wall (dw, at the bottom of the cell). The nucleus (N) is situated near the proximal wall (pw) in the root statocyte and near the distal wall (dw) in the shoot statocytes. g: direction of gravity; lw: longitudinal wall; nu: nucleolus; er: endoplasmic reticulum.

Taken together with the Picard's experiment (see Larsen 1962), which proved at the beginning of the 20th century that sensitivity to gravity was greater in the root tip than in any other region of the root, these results indicated that the perception of gravity mainly took place in the central root

cap cells or statocytes. However, it is possible that other cells could be gravisensitive to a lesser extend (Wolverton et al. 2002). More recently, it has been demonstrated (Blancaflor et al. 1998) by using a method of cell ablation with a laser beam that statocytes of *Arabidopsis thaliana* have a sensitivity that depends upon their state of differentiation, and therefore their location in the cap. Thus, it is well accepted that roots statocytes (Figure 6-04A) are responsible for gravisensing (Rosen et al. 1999, Boonsirichai et al. 2002, Blancaflor and Masson 2003).

In shoots, the role of statocytes was demonstrated only recently (Perbal and Rivière 1980). In these organs, the statocytes are located in a cellular layer which surrounds the vascular bundle (Figure 6-04B). Fukaki et al. (1998) have shown that two *agravitropic* (i.e., which do not respond to a gravistimulus) mutants (sgr 1, sgr 7) of *Arabidopsis thaliana* did not possess this tissue, which confirmed the role of statocytes in shoot gravitropism, even if it does not prove that the perception of gravity occurs in this cell layer.

Although it is well accepted that statocytes are involved in gravisensing, the role of amyloplasts in graviperception is less clear (Barlow 1995, Sack 1997). Studies on starch-depleted mutants (Kiss et al. 1989, Caspar and Pickard 1989) as well as experiments leading to experimentally reducing the volume of starch by various treatments have not demonstrated that the amyloplasts are the unique graviperceptors (Sack 1997). Some authors have therefore proposed that the whole protoplasm[1] could play this role (Wayne et al. 1992), and it has been demonstrated that this possibility cannot be discarded if amyloplasts act by exerting pressure on structures lining the plasma membrane (Perbal 1999). In this case, the pressure exerted by the amyloplasts on these structures should be greater than that of the protoplasm. The hypothesis of the protoplasm playing the role of graviperceptor could explain the reason why starch-depleted mutants (Kiss et al. 1989, Caspar and Pickard 1989) can still respond to gravistimulus (Figure 6-05). It implies that gravireceptors are very sensitive to pressure and that the amyloplasts do not need a large amount of starch to be efficient. The results obtained by Kiss et al. (1996) on root gravitropism in intermediate-starch mutants of *Arabidopsis thaliana* showed that 51-60% of the level of starch is near the threshold amount needed for full sensitivity. The statolith apparatus (the amyloplast bulk) could be therefore overbuilt in the sense that it could be larger than necessary (Aarrouf and Perbal 1996).

It is well known that the phase of perception occurs even at low temperature (4°C), although no gravitropic response takes place for hours (Wyatt et al. 2002). If *Arabidopsis* plants stimulated in the horizontal position at 4°C are placed in the vertical position at room temperature their

[1] *Protoplasm* is the substance inside the membrane of a living cell. At the simplest level, it is divisible into *cytoplasm* and *nucleoplasm*.

inflorescence curves, which means that there is a persistence of the signal. At 4°C, the amyloplasts sediment but the stimulus is not transmitted.

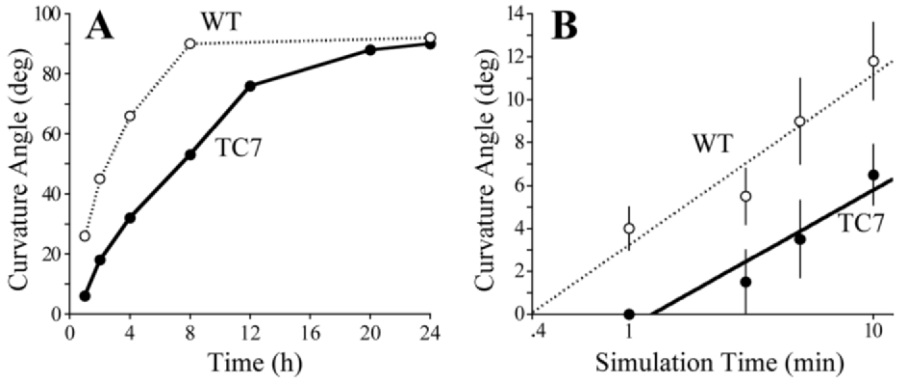

Figure 6-05. Root gravitropism of the starch-depleted (TC7) and the wild type (WT) mutants of Arabidopsis thaliana. In A, the angle of curvature is graphed as a function of time in h. In B, the response is graphed as a function of the logarithm of the stimulation time (in min). The presentation time (minimal time of stimulation to induce a visible response) is estimated by extrapolating the regression line down to zero curvature. The presentation time is approximately 24 s for the WT and 78 s for the TC7 mutants. Adapted from Kiss et al. (1989).

2.2 Transduction of Gravistimulus

The nature of the gravisensors being not yet known, it is difficult to determine which cellular structure contains the gravireceptors. These receptors are able to transform the mechanical effect of graviperceptors into a biochemical factor. In 1972, Sievers and Volkmann suggested that the endoplasmic reticulum, which is always located at the basal pole of the statocyte, could be involved in the first steps of the transduction of gravistimulus. For these authors, after gravistimulation, an asymmetrical message could be created in the root cap because the amyloplasts sediment on the endoplasmic reticulum in statocytes of the lower half of this organ, whereas almost no contact are seen in the upper half. This hypothesis was consistent with the fact that it is well established that the concentration of cytosolic calcium in the cytoplasm is very low (Sinclair and Trewavas 1997), whereas its concentration in the endoplasmic reticulum is much greater. It was therefore proposed that the amyloplasts exerting a pressure on the endoplasmic reticulum tubules could provoke an efflux of calcium and a locally increase in calcium concentration which could activate some calcium-dependent proteins as calmodulin (Evans et al. 1986).

Another hypothesis was proposed by Iversen and Larsen (1971) and Perbal and Perbal (1976), who have observed that the gravitropic reaction was

stronger when the amyloplasts moved along the longitudinal wall. According to these authors, the pressure and the movement on a cellular structure located along the longitudinal wall could be responsible for graviperception. An analysis of the distance from the amyloplasts to the plasma membrane showed that these organelles could exert a pressure on the cytoskeleton located along the longitudinal wall (Perbal et al. 2004).

The actin network of root and shoot statocytes has been intensively studied (White and Sack 1990, Collings et al. 2001, Driss-Ecole et al. 2003). It has been shown that this network is thin which allows the sedimentation of statoliths. The involvement in of the actin filaments in the transduction of gravistimulus is disputed since treatments with cytochalasins or Latrunculin B, which perturb the polymerization of actin, does not suppress the gravitropic response and can even enhance gravisensitivity (Yamamoto and Kiss 2002, Hou et al. 2004).

It could be advanced that the mechanical effect of the amyloplasts can be the cause of the activation of stretch-activated ion channels (Ca channels probably) as proposed by Pickard and Ping Ding (1992). This hypothesis implies that there is a local enhancement of cytoplasmic calcium, which has never been demonstrated in roots (Legué et al. 1997). However, the changes in calcium within the cell could be subtle which could explain that despite some preliminary trials no calcium change has been detected yet on single organ (Sinclair and Trewavas 1997). To demonstrate that cytosolic calcium transients are indeed induced by gravitropic stimulation (Plieth and Trewavas 2002), measurements have been made in groups of young seedlings of *Arabidopsis thaliana* producing aequorin in the cytoplasm and reconstructed *in vivo* with cp-coelenterazine, a synthetic high-affinity luminophore. In such transgenic plants, cytoplasmic increase of calcium can be followed by fluorescence. After gravistimulation, there is an initial calcium spike (20-30 sec) in the cytoplasm followed by a much longer shoulder that peaks about 90 sec after the change in orientation of the seedlings (Figure 6-06). The spike could be related to the early steps of gravisensing, whereas the shoulder could be related to the movement of amyloplasts (which can take minutes). The calcium signature is peculiar since it is biphasic and lasts much longer than those provoked by the mechanical effect of wind for instance (Plieth and Trewavas 2002).

Calcium is the most widespread ionic second messenger in plants, but changes in cytoplasmic pH are also known to have regulatory effects on cell function (Scott and Allen 1999, Blancaflor and Masson 2003). The cytosolic[2]

[2] The *cytosol* (as opposed to cytoplasm, which also includes the organelles) is the internal fluid of the cell.

concentration of protons is tightly controlled and the columella[3] cytoplasmic pH of 7.2 corresponds to a concentration that is very close to that of calcium. However, protons diffuse rapidly within the cell, unlike calcium, which is trapped by calcium-binding proteins. Therefore, without any structure in the cytoplasm to slow their movement, protons should not regulate localized changes in cellular microdomains. However, the pH changes could serve as a regulatory system for the whole statocyte (Fasano et al. 2001).

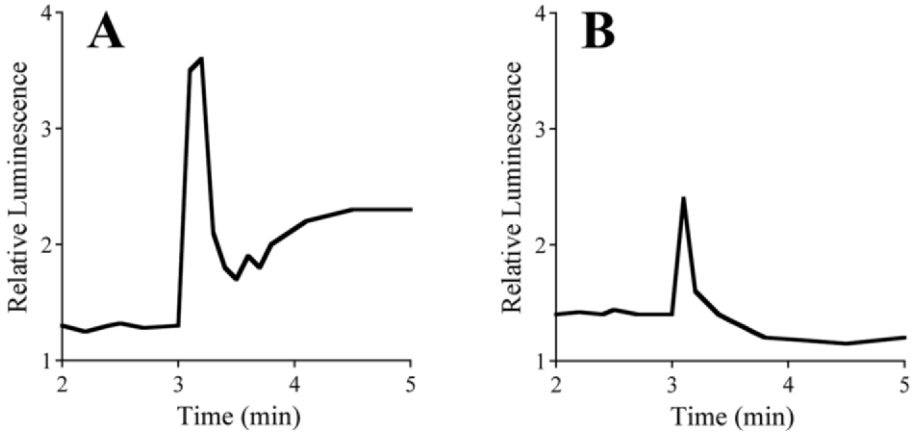

Figure 6-06. Effect of the gravitropic stimulus on the calcium response of a group (hundreds) of transgenic Arabidopsis seedlings. These seedlings are producing apoaequorin in their cytoplasm. Aequorin is reconstructed in vivo with cp-coelenterazine, a synthetic high-affinity luminophore. Aequorin fluoresces in the presence of cytoplasmic calcium. In A, gravistimulation: the plate with seedlings was turned through 135 deg (the root tip being slightly upward) at time 3 min. In B, control: the plate was turned 135 deg and immediately placed back to vertical position. Adapted from Plieth and Trewavas (2002).

Several researchers have indicated that proton flux from the root cap shifts after stimulation. When the root is vertical, fluxes are low and variable but, after gravistimulation, substantial proton efflux can be detected from the upper flank of the cap (Fasano et al. 2002, Monshausen and Sievers 2002). This asymmetry could be associated with both a sustained apoplastic[4] acidification and transient (10 min) cytoplasmic changes in the statocytes (Fasano et al. 2001, Scott and Allen 1999). Acidification of the statocyte apoplast is induced upon reorientation but the wall pH only returns to pre-stimulation levels as the root tip reaches the vertical position. In maize

[3] The *columella* is the central part of a rootcap in which the parenchyma cells are arranged in a series of column.

[4] *Apoplast* is the cell wall continuum of a plant or organ; the movement of substances via cell walls is called apoplastic movement or transport.

pulvinus (a large swollen region at the base of the petiole composed predominantly of parenchyma cells), gravistimulation induces an alkalinization of the base of the statocytes where the plastids[5] accumulate (Johannes et al. 2001), which indicates that gravisensors might create restricted zones where pH changes can occur. The link between pH changes and calcium increase is not yet known, and the chain of events leading to the transduction of gravistimulus remains to be determined.

2.3 Transmission of the Stimulus to the Reaction Zone

Plasma membrane potentials were measured in lateral statocytes of vertically and horizontally stimulated roots of *Lepidium sativum* using glass microelectrode techniques (Behrens et al. 1985). Upon tilting the root 45 deg from the vertical position an electrical asymmetry was observed. Statocytes of the lower side of the root depolarized by approximately 35 mV. This depolarization occurred after a latent period of 8 sec, reaching a minimum after 130 sec. Statocytes of the upper flank showed a low hyper-polarization ($t_{1/2}$ = half time for hyper-polarization = 168 sec), reaching a final stable potential at a level 10 mV more negative. To determine whether or not the gravielectrical response recorded in the statenchyma reflects an electrical excitation that is propagated as a signal for differential growth in the form of action potentials (from the site of perception to the responding tissue), measurements were made in the rhizodermal and sub-rhizodermal layers of the elongation zone. They showed only insignificant membrane potential changes. Behrens et al. (1985) therefore discarded the mediation of excitatory voltage changes in transmission of gravistimulus in the *Lepidium* roots.

However, it has been proposed that calcium efflux can be responsible for the transmission of gravistimulus. Calcium gradient under gravistimulation was reported in shoots and it has been shown that calcium moved through the parenchyma to the upper side of gravistimulated hypocotyls and coleoptiles (Goswami and Audus 1976). The redistribution of calcium was shown to occur just before bending started, and greater concentration of calcium was observed in cell walls of the upper side of coleoptiles (Slocum and Roux 1983). In some primary roots of higher plants, gravistimulation caused an asymmetrical movement of calcium toward the lower side (Lee et al. 1983, Bjorkman and Cleland 1991). However, recently Legué et al. (1997) were unable to show this lateral movement of calcium in *Arabidopsis* roots loaded with a fluorescent probe, whereas they demonstrated a clear movement of calcium due to touch stimulus.

The involvement of a hormone in root gravitropism was shown by a series of classical microsurgery experiments carried out on maize roots. It has been demonstrated that removing one half of the cap led the root to curve

[5] *Plastids* are structures within cells that perform photosynthesis or store starch.

toward the remaining half cap whatever the orientation of this organ was (Shaw and Wilkins 1973). Thus, the presence of the remaining half cap inhibited the elongation of the root side. Various experiments dealing with barriers inserted between the cap and the reaction zone indicated that the root curved because of the lateral (downward) transport of an inhibitor in the root apex and the increase of an inhibitor concentration in the elongation zone of the lower side of the roots (Pilet 1976).

The nature of the hormone involved in the gravitropic curvature of these organs was studied in the 1960's and 1970's. It was generally accepted that auxin redistribution was responsible for gravitropic curvature (Evans 1991). The *Cholodny-Went Theory* in its simplest form stated that a lateral (downward) transport of auxin is the cause of the gravitropic bending by inducing greater elongation of the lower side of the shoot (or of the coleoptile) and an inhibition of growth of the lower side in the roots (see Figure 6-02). However, this hypothesis does not completely account for the patterns of the gravitropic bending.

Recently, this hypothesis has been confirmed in roots by the analysis of AUX1 and PINs mutants. It has been shown that AUX1 gene family mediate auxin influx (Marchant et al. 1999, Muday and DeLong 2001, Boonsirichai et al. 2002), whereas members of the PIN family contribute to its efflux (Friml 2002, Noh et al. 2003). This finding lead to the so-called *Fountain Model* of auxin transport in roots. AUX1 ensures auxin uptake by the statocytes, whereas PIN3 ensures its efflux. When the root is stimulated in the horizontal position, a lateral movement of auxin (see Figure 6-02) occurs (Ottenschläger et al. 2003, Rashotte et al. 2000). The subcellular localization of PIN3 is dependent upon the root orientation within the gravitational field. When the root is orientated vertically downward, the PIN3 protein is located symmetrically at the plasma membrane of the statocytes. However upon gravistimulation PIN3 relocalizes within 2 min, accumulating in the plasma membrane at the bottom side of statocytes (Friml et al. 2002). A relocation of PIN3 within the statocytes could therefore be the initial step of the establishment of the lateral auxin gradient upon gravistimulation.

2.4 Differential Growth

In the negatively responding organs, differential growth is due to greater elongation rate of the lower side and slower elongation rate on the upper side, whereas gravitropic curvature in roots is due to a greater elongation in the upper part than in the lower part.

Detailed studies on the *Relative Elemental Rates of Elongation* (RELEL) at different points along the main axis of the maize root stimulated in the horizontal position or growing in the vertical position have been carried out by Barlow and Rathfelder (1985). These authors showed that the lower side of the stimulated root was inhibited, but only in the distal part of the

reaction zone, whereas the whole upper part of this zone was stimulated (Figure 6-07A).

Thus, the spatial distribution of growth during gravitropic curvature was important. This was confirmed by anatomical study at the level of the curvature of the lentil root stimulated for 2 h (Darbelley et al. 1986). The comparison of the average cortical cell length at the level of the distal zone of the meristem and the proximal zone of the cell elongation region (i.e., at the level of the curvature) demonstrated that there was a greater cell elongation in the upper side of the distal meristem and an inhibition of cell growth in the lower part of the cell elongation zone.

It has been concluded that there were two different types of cells that responded to gravistimulus: a) those which were located in the root meristem; and b) and those located at the beginning of the cell elongation zone (Darbelley et al. 1986). This difference in cell growth between the lower and the upper part of the lentil root started very close to the root cap junction. The heterogeneous nature of the zone of curvature could explain, at least partly, the controversial results published on differential growth.

Selker and Sievers (1987) have also analyzed the temporal component of the gravitropic bending in the *Lepidium sativum* root. They showed that for the first hour after tilting the root to the horizontal position, the relative extension rate of the upper side of the root was higher than that during straight growth. On the contrary, the relative growth rate of the lower side fell to near zero during this period. For the second hour, the two sides had approximately equal growth rate. At the end of the second hour, the rate of the lower side increased suddenly. For these authors, a reversal in the extension gradient is necessary to prevent the root from continuing to curve and to overshoot the direction of gravity.

In 1990, Baluska et al. pointed out the special status of cells toward the distal end of the elongation zone in maize roots and note that although most of these cells have ceased dividing they have not entered the phase of rapid elongation. They proposed the term of "postmitotic isodiametric growth zone" in reference to the shape of the cells. However, cell expansion is not isodiametric except in a very narrow region. Ishikawa and Evans (1993) proposed to refer to this region of the root as the *Distal Elongation Zone* (DEZ). When primary roots of maize are gravistimulated, a major factor causing downward curvature is the induction of very fast elongation in the DEZ on the top side of the root (Figure 6-07B) (Ishikawa and Evans 1995).

In *Arabidopsis* roots, Mullen et al. (1998) showed that after gravistimulation, the growth patterns of the root changed comparatively to vertical straight growth. Within the first hour of graviresponse, the basal limit of the DEZ and the position of the peak of *Relative Elemental Growth Rate* (REGR) shifted apically on the upper flank of the root. This was due to a combination of increased growth in the DEZ and growth inhibition in the

central elongation zone. On the lower flank the basal limit of the DEZ shifted basipetally as the REGR decreased.

The results obtained showed that a large proportion of the initial curvature originated in the DEZ. Some evidence indicated that auxin may not be responsible for the induction of differential growth in the DEZ of gravistimulated roots. Muday and Hayworth (1994) on tomato roots and Ishikawa and Evans (1993) on maize roots found that a treatment by auxin at strong inhibiting concentrations did not suppress gravitropic response whose kinetics was similar to that of untreated roots. These results have been considered as an argument against an involvement of auxin in the differential growth. However, it has been proven recently that the lateral movement of auxin occurs in the lateral cells of the root cap even after treatments by auxins (Ottenschläger et al. 2003). Thus, the Cholodny-Went hypothesis has been nicely confirmed in the recent years and its mechanism, which is dependent upon auxin influx and efflux carriers, is now better understood.

The gravitropic signal is an asymmetrical transport of auxin, which leads to a differential growth. It is well known that this hormone induces cell wall acidification and a cell wall loosening (see review by Cosgrove 1997). Thus, gravitropic bending should be, at least in part, mediated by differential control of wall pH on the lower and upper sides of a gravistimulated organ.

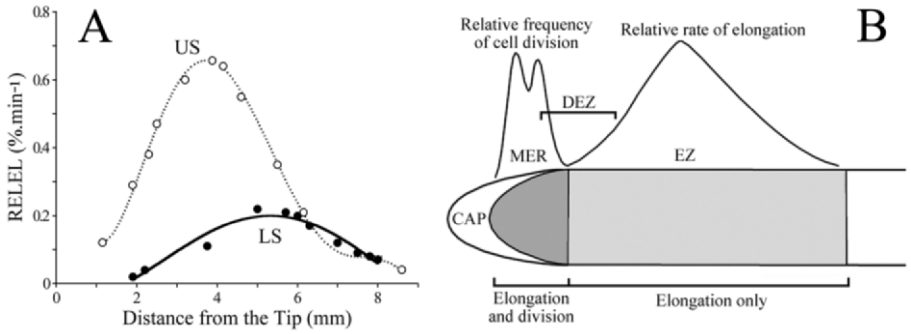

Figure 6-07. Differential growth in the upper side (US) and lower side (LS) of maize roots. In A, the curve shows the relative growth (RELEL, in % x min^{-1}) of small root segments as a function of the distance of this segment from the root tip. Adapted from Barlow and Rathfelder (1985). In B, the graphic shows the different functional zones of the maize root. The curves show the level of the mitotic activity within the meristem (MER) and the relative rate of elongation in the elongation zone (EZ). The distal elongation zone (DEZ) is mainly responsible for the curvature of the root. Adapted from Ishikawa and Evans (1995).

3 GRAVITROPISM IN ACTUAL AND SIMULATED MICROGRAVITY

3.1 Estimate of Gravisensitivity

Gravisensitivity represents the ability to respond to a gravistimulus and can be estimated by different stimulation thresholds (Volkmann and Sievers 1979):

a. The minimal angle (*rm*) with respect to gravity that induces a reorientation of the extremity of the organ;
b. The presentation time (*tp*), which is the minimum duration of gravistimulus at 1 g to provoke a slight but significant gravitropic response;
c. The threshold acceleration (*at*) that can be sensed by the plant organs.

The first parameter (*rm*) can be determined on the ground by stimulating the organs at small angles with respect to gravity and by following their gravitropic response. Although simple in its principle, this experiment was not carried out very often (see Volkmann and Sievers 1979) since the growth of the plant organs is not straight, their tip being subjected to oscillations (Johnsson 1997). A displacement of a few degrees from the vertical position seems to be perceived (see Volkmann and Sievers 1979). However, it has recently been shown that 15 deg is the minimal angle which induces a response in *Arabidopsis* roots (Mullen et al. 2000).

For determining the two other parameters (*tp*, *am*) experiments should be carried out in the absence of sensed Earth's gravity. Plant physiologists have therefore used clinostats for decades in order to simulate weightlessness by rotating the plant about a horizontal axis at about 1-2 rpm (Figure 6-08A). The unilateral effect of gravity is thus compensated by the rotation on the one axis clinostat and the plants do not show any gravitropic response. However, it is clear that clinorotating does not nullify gravity, but can induce a slight and continuous stimulation (Aarrouf et al. 1999).

The presentation time is determined by stopping the clinostat for various periods and clinorotating the plants again to follow the gravitropic response resulting from gravistimulus (stop of the clinostat). It is thus possible to draw a dose-response curve of the gravitropic reaction. By hypothesizing that the gravitropic response varies as a function of the logarithm of the dose of stimulus:

$$\text{Response} = a \times \log(\text{Stimulation time}) + b$$

where a and b are constants. One can estimate the presentation time, which corresponds to the intercept of the curve with the x-axis (see Figure 6-05B). In theory, it should be possible to submit plants to very short periods of stimulation and to analyze their gravitropic response after clinorotation in

order to determine directly the presentation time without any extrapolation. However, this is made difficult because the extremity of the organs oscillates. Direct measurements often lead to longer presentation times (see Larsen 1962), whereas by extrapolation they were estimated in general at about 10-30 sec for roots and 20-80 sec for shoots.

The presentation dose (*dp*, expressed in g x s) can be measured when stimulus varies as a function of time and as a function of the acceleration. The presentation dose *dp*, equals the acceleration (*a*) times the presentation time for a given acceleration (*tpa*). Thus, $dp = a \times tpa$.

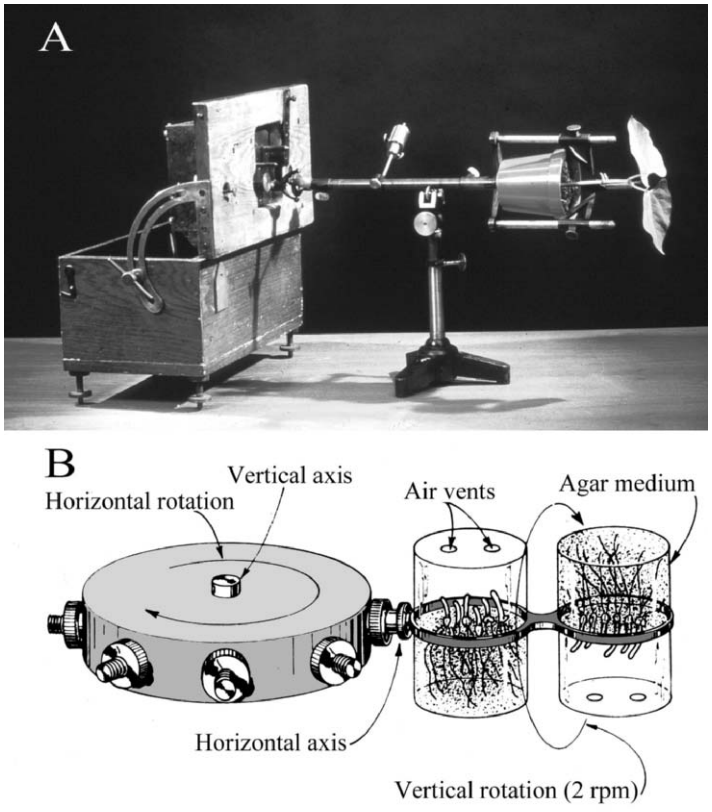

Figure 6-08. One-axis (in A) and two-axes (in B) clinostats. In A, Pfeffer's clinostat. The plant is rotated about a horizontal axis with a rotation speed of 1-2 rpm. Thus, the unilateral effect of gravity on plant in the vertical position becomes an omnilateral action of gravity. In no case gravity is nullified. The one-axis clinostat is a simulation of weightlessness in the way that no gravitropic curvature is observed although the main axis of the plant is horizontal. In B, the plants are rotated about a horizontal axis (1-2 rpm) to simulate weightlessness. They are also centrifuged about a vertical axis to subject them to gravistimulus. The dose of stimulus depends upon the amplitude of the centrifugal acceleration and the duration of the centrifugation. The two-axes clinostat is very useful to determine the threshold of gravisensing. Adapted from Shen-Miller et al. (1968).

To analyze the presentation dose, a two-axis clinostat is needed on the ground. The plants are rotated about a horizontal axis to simulate weightlessness and centrifuged about a vertical axis (Figure 6-08B). This device permits to determine the threshold acceleration by exposing the plants to very low centrifugal acceleration for long periods. It has been demonstrated that plant organs are able to sense about 10^{-3} g, but roots seem to be more sensitive than shoots, since *tpa* is always lower for roots than for shoots (Shen-Miller et al. 1968).

It is well known that gravitropic response can be induced by stimulation periods shorter than the presentation time if gravistimulus is repeated intermittently (Volkmann and Sievers 1979). The minimal duration of stimulation which, repeated, leads to a gravitropic response is called *perception time* and should be about or even less than 1 sec (0.5 sec for *Avena* coleoptiles, according to Pickard 1973; or 1 sec for *Cress* roots, according to Hejnowicz et al. 1998).

Authors	***Species***	***Organ***	t_p	d_p	a_t
Merkys and Laurinavicius (1990)	*Lactuca*	*R* *H*			*1.5 x 10^{-4}g* *2.9 x 10^{-3}g*
Perbal and Driss-Ecole (1994)	*Lens culinaris*	*R*	*27 sec*		
Johnsson et al. (1995)	*Avena sativa* *1 g tall* *1 g short*	 *C* *C*		 *55 g x s* *120 g x s*	
Volkman and Tewinkel (1996a)	*Lepidium sat.* *0 g grown* *1 g grown*	 *R* *R*		 *20-30 g x s* *50-60 g x s*	
Perbal et al. (1999)	*Lens culinaris* *0 g grown* *1 g grown*	 *R* *R*		 *10-24 g x s* *18-31 g x s*	

Table 6-01. Presentation time (t_p, in sec), presentation dose (d_p, in g x s) and threshold acceleration (a_t, in g) of the gravitropic reaction of roots (R), coleoptiles (C) or hypocotyls (H) estimated in microgravity.

Space represents a unique opportunity to study graviperception since it offers the possibility to carry out experiments on presentation time or threshold acceleration (Table 6-01). Although only few experiments have been done in space, the results obtained in microgravity confirmed to a certain extent those obtained with clinostats at least for roots. Thus, *tp* was found to be about 27 sec in lentil roots grown in microgravity (Perbal and Driss-Ecole 1994) and *dp* was 20-30 g x s in *Cress* seedling roots grown in the same condition (Volkmann and Tewinkel 1996a, 1996b). However, it was found with *Cress* roots grown on a 1-g centrifuge in space before stimulation, that *dp* was 50-60 g x s which indicated that the sensitivity was different when the roots were grown in 1 g or in microgravity. A similar result was observed by

Perbal et al. (1997) in lentil grown on a clinostat (in simulated microgravity) or in 1 g (in the vertical position) where *tpa* were 25 and 60 sec, respectively.

The fact that *tpa* seems to be almost equal when determined by the mean of a clinostat or in microgravity shows that a clinostat can to some extent simulate weightlessness. The difference in gravisensitivity between 0-g grown or 1-g grown seedlings roots was also observed recently on lentil seedlings (Perbal et al. 2004). This finding opened a new way of investigation for analyzing graviperception.

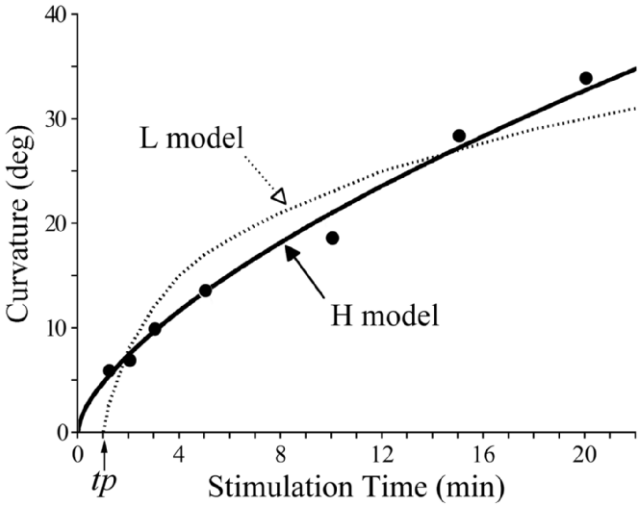

Figure 6-09. Dose response curve of the gravitropic reaction of lentil roots. The curvature observed after 90 min on the clinostat is reported as a function of the time of stimulation. In this example, the lentil seedlings were stimulated by gravity (1 g) from 1 to 20 min and placed on a clinostat (1 rpm). The two curves represent two different mathematical models to fit the experimental points. The L model (logarithmic model, dotted line) was commonly used (see Figure 6-05) until it has been shown that the H model (hyperbolic model, solid line) fitted better the experimental points (Perbal et al. 2002). The L model had the advantage to permit to determine the so-called presentation dose (or presentation time, tp), which corresponds to the intercept of the curve with the x-axis. In this particular example it is about 60 sec. This parameter was used to estimate gravisensitivity. This is not possible for the H model and gravisensitivity in this case can be measured as the slope of the curve at the origin.

These space experiments have led Perbal et al. (2002) to reconsider the way of determining the presentation time and presentation dose. The majority of the results concerning *tp* or *dp* were obtained by extrapolation assuming that the logarithmic model fitted the experimental points (see Figure 6-05B). Perbal et al. (2002) showed that very often the logarithmic model fitted correctly the data for stimulation time less than 10 min and greater than 1 min. They also demonstrated that the hyperbolic model corresponding to a ligand receptor system, (where response equals $= \frac{a * \text{dose}}{b + \text{dose}}$ with *a* and *b* being constants) was better for almost all data published in the literature since the 1960's (Figure 6-09).

This model implies that there is a slight response even for a very short stimulation time. Thus, the only parameter directly related to the perception phase should be the perception time. According to these authors, the presentation dose should correspond to a dose of stimulation that induces a sufficient signal to provoke a visible curvature. Below this presentation dose, the quantity of stimulus should be too low to induce a curvature, or the curvature should be too slight to be measured.

Thus, space experiments have given the opportunity to reconsider and better estimate the parameters used for measuring graviperception and have led to new methods for estimating gravisensitivity.

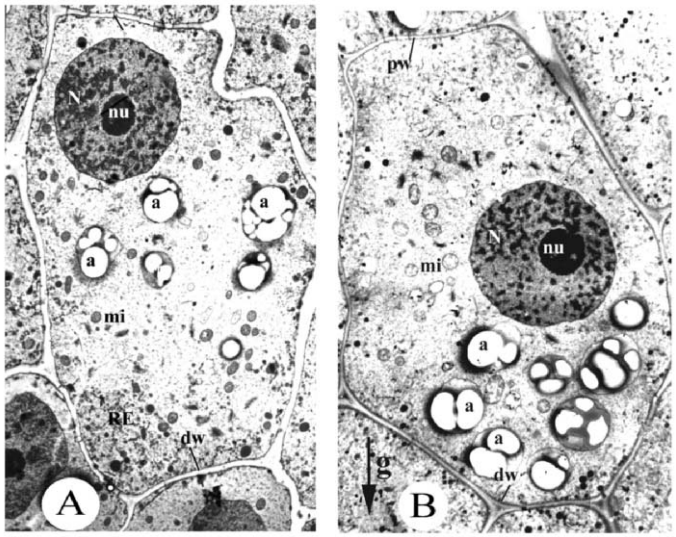

Figure 6-10. Statocyte polarity in microgravity (in A) and after a Cytochalasin B treatment (in B).
A. Lentil seedlings were grown in microgravity for 27 h and chemically fixed in space. They were treated on the ground for routine microscopy. Note that the amyloplasts (a) are located in majority close to the nucleus (N).
B. Lentil seedlings were hydrated with a solution containing Cytochalasin B and grown on the ground for 27 h in the vertical position and chemically fixed as in A. N: nucleus; nu: nucleolus; mi: mitochondria; dw: distal wall; RE: endoplasmic reticulum; pw, proximal wall. Courtesy of Driss-Ecole, University Pierre et Marie Curie, Paris, France.

3.2 Statocyte Polarity

The structural polarity of the root statocyte (see Figure 6-04A) has been intensively studied on the ground (Sievers and Volkmann 1972, Perbal and Driss-Ecole 1989) and can be considered to be similar from one species to another. The nucleus of gravisensing cells is always situated close to the proximal wall, whereas the amyloplasts are sedimented on large *Endoplasmic Reticulum* (ER) aggregates located along the distal wall. During differentiation of the statocyte, the endoplasmic reticulum migrates toward this wall, whereas the nucleus remains located very close to the plasma membrane lining the proximal wall (Sievers and Volkmann 1972).

Concomitantly, plastids synthesize starch and voluminous starch grains are formed in a dense stroma[6]. Amyloplasts have a high density (1.44 g/cm^3) and therefore sediment within the cytoplasm, the density of which is 1.03 g/cm^3 (for the density of the organelles, see review Sack 1991).

The development of the structural polarity of the root statocyte is genetically determined and does not depend upon gravity (Sievers and Braun 1996), since plants grown in microgravity have distal ER tubules and a proximal nucleus in their statocytes (Figure 6-10A). However, a precise analysis of these two organelles in the lentil statocyte showed that their location was slightly different in the microgravity-grown sample and in the 1-g flight control (Perbal and Driss-Ecole 1989). In microgravity, the majority of the ER aggregates were situated close to the distal wall of the statocytes, whereas some of these aggregates lined the longitudinal wall when root growth occurred on a 1-g centrifuge in space. This could well be linked to the sedimentation of the amyloplast, which could: a) exert a pressure on the ER aggregates and push them upward; or b) perturb the migration of the ER tubules.

One of the most intriguing phenomenon observed in microgravity dealt with the position of the nucleus in the statocyte. In microgravity, this organelle was closer to the longitudinal axis of the statocyte, in a more central position than in the statocytes differentiated on the 1-g centrifuge (Perbal and Driss-Ecole 1989). Many observations (Perbal et al. 1987) indicated that there was a denser region between the plasma membrane and the nuclear envelope. This suggested that the nucleus could be attached to the cell periphery by actin filaments. This hypothesis was confirmed by a treatment by cytochalasin (B or D), which provoked sedimentation of the nucleus in 1 g (Figure 6-10B) and a more central distribution of this organelle in microgravity-grown lentil seedlings (Driss-Ecole et al. 2000a).

In the lentil statocyte and in microgravity, the majority of the amyloplasts was located in the proximal part of the gravisensing cells around the nucleus. A 3D cell reconstruction done by Smith et al. (1997) has demonstrated that the distribution of the amyloplasts was not random in statocytes of *Trifolium repens* grown in microgravity (Figure 6-11). These organelles were grouped near the cell center as on the clinostat, but the grouping was less dense in the latter case.

On lentil roots, it was also shown that clinorotation led to different amyloplast distribution when the roots were rotated with their axis parallel or perpendicular to the horizontal axis of the clinostat. The amyloplast distribution in the horizontal clinorotated statocytes was close to that observed in microgravity (Lorenzi and Perbal 1990).

[6] The non-membranous matrix material of a chloroplast.

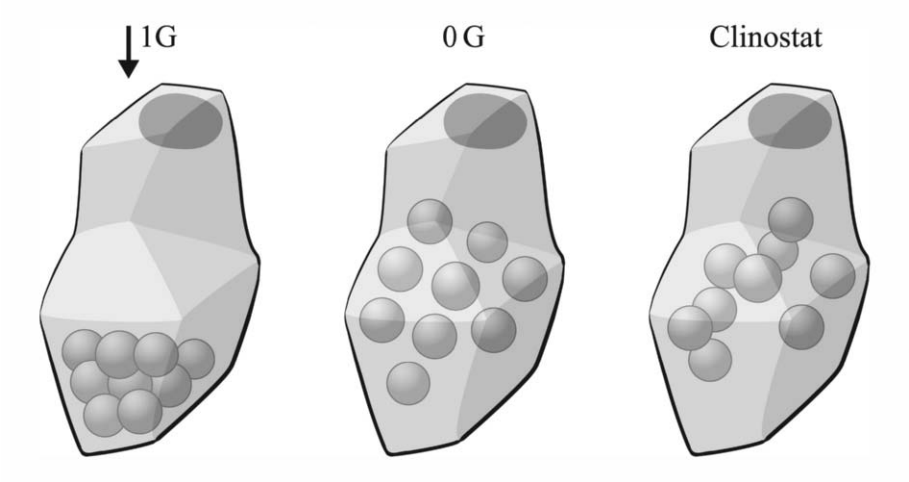

Figure 6-11. Comparison of the position of the amyloplasts in root statocytes of white clover grown in the vertical position (1G), in microgravity (0G) or on the clinostat. These drawings represent 3D reconstruction of the statocytes from longitudinal sections. The limits of the protoplasm, the amyloplasts and the nucleus are represented. Adapted from Smith et al. (1997).

3.3 Gravisensors: Starch Content and Volume

The volume of the amyloplasts, as well as their volume of starch, was studied in different species (*Lepidium sativum, Lens culinaris, Trifolium repens*). In *Lepidium sativum*, starch granules were much smaller in microgravity than on the ground (Volkman et al. 1986, Buchen et al. 1993). There were no quantitative data in these analyses, but the micrographs shown indicated that the amyloplasts could have the same volume. In *Lens culinaris* roots (Perbal et al. 1987), the number of amyloplasts was less in microgravity than in the flight control but the result obtained could be due to a different grouping of the amyloplasts, which could reduce the probability of observing one of these organelles on a section. However, their volume was greater in microgravity than in the 1-g space control. Smith et al. (1997) have shown in *Trifolium repens* that starch content (estimated on sections) was similar in plastids of roots grown in microgravity and in 1 g, whereas the amyloplasts volume was greater in microgravity than in 1 g. This result indicated (although this point was not discussed by the authors) that the stroma of the plastids could be more voluminous in microgravity.

However, starch content cannot be correctly estimated by measuring the volume of starch. Its density must also be taken into account, since it is known that a starch grain has not the same density on its border as in its center. It is the reason why starch content was examined on lentil roots by the

means of densitometry (Perbal et al. 2004). The Acid Periodic-Schiff technique was used to specifically stain polysaccharides and starch density was determined by image analysis by measuring the mean optical density (which estimated the density of starch) and the area of starch grains. It was found that the volume of starch as well as its density was greater in microgravity than on the ground (Perbal et al. 2004). It is clear that the differences observed between the species can be due to the stage of development and/or the culture conditions.

3.4 Movement of the Organelles in Microgravity

As said before, the nucleus occupies the proximal end of the statocyte in most cases examined (Perbal and Driss-Ecole 1989, Sievers and Volkmann 1972). Sievers and Heyder-Caspers (1983) observed sedimentation of the amyloplasts and of the nucleus toward the distal cell pole and a lateral displacement of the endoplasmic reticulum after centrifugation for 20 min at 50 g. Statocytes re-established the structural polarity within 1 h after cessation of centrifugation. In lentil roots, centrifugal forces were applied toward the tip to move the nucleus and it was shown (Lorenzi and Perbal 1990) that at 19 g (20 min) this organelle sedimented in the oldest statocytes, whereas at 40 g (20 min) all nuclei had settled down on the amyloplasts. These results showed that the displacement of the nucleus needed a very strong centrifugal force.

In microgravity, the location of the nucleus was different from that observed in 1 g on the ground (Perbal and Driss-Ecole 1989). But the 1-g control in space was similar to the microgravity sample. According to Lorenzi and Perbal (1990) this could be due to the fact that the 1-g space control was exposed to microgravity for about 15 min before fixation, because it was not possible to fix the seedlings on the centrifuge.

Experiments carried out on a slowly rotating clinostat demonstrated that simulation of microgravity also displaced the nucleus toward the cell center (Lorenzi and Perbal 1990). Cytochalasin B or D, which inhibits polymerization of actin filaments, caused sedimentation of the nucleus on the amyloplasts in 1 g (Figure 6-10B). This displacement of the nucleus in statocytes after cytochalasin B application indicated that an inhibition of polymerization of actin monomers affected the position of the nucleus (Lorenzi and Perbal 1990).

It is well known that the nucleus is surrounded by a kind of basket of actin filaments, which is involved in the positioning of this organelle. It has been suggested that actin filaments are associated with both the nucleus and the plasma membrane and generated tension between them (Figure 6-12). The results obtained by Lorenzi and Perbal (1990) were in good agreement with this hypothesis since the displacement of the nucleus in microgravity could be induced by a relaxation of the cytoskeleton.

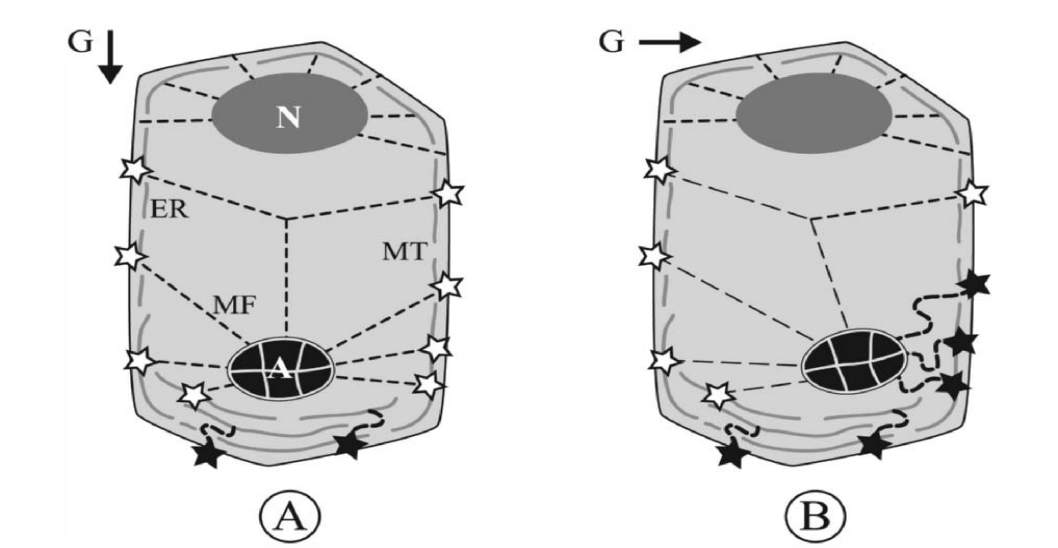

Figure 6-12. Cytoskeleton and statocyte polarity. A. Statocyte of a root placed in the vertical position. B. Statocyte of a root stimulated in the horizontal position. The amyloplasts (A) and the nucleus (N) are in contact with actin filaments (MF), which are attached to stretch activated ion channels (star symbols). Theses channels are open or closed depending upon the tension exerted by the actin filaments. In stimulated statocytes, the tension in the actin network increases in the upper half of these cells and decreases in the lower half which leads to an asymmetrical efflux or influx of ions in the cell. ER: endoplasmic reticulum; MT: microtubules; G: gravity. Adapted from Sievers and Braun (1996).

The amyloplasts within the statocytes of lentil roots grown in microgravity were located in the proximal part of these cells (Perbal et al. 1987). In 1 g on the ground, these organelles have settled down on the ER membranes close to the distal wall. The 1-g control during the Spacelab D-1 mission showed a distribution of the amyloplasts that was different from the 1-g ground control and had some similarities with the microgravity sample. As said above, this result could be due to the fact that the 1-g space control was exposed to a period of 15 min of microgravity before fixation.

The reason for this difference between the two controls has been given by Volkmann et al. (1991), who showed during a sounding rocket flight experiment that the transfer from 1 g to microgravity for about 6 min was sufficient to provoke a movement of amyloplasts toward the nucleus. These authors and Sievers and Braun (1996) hypothesized that the amyloplasts were in fact attached to microfilaments bound to the cell periphery. According to these authors, the gravitational force on the ground was greater than the basipetal force exerted by the cytoskeleton. However, in microgravity this force became prominent and the amyloplasts moved toward the nucleus.

Recently, Driss-Ecole et al. (2000a) have studied kinetics of the displacement of the amyloplasts as follows. First, vertical roots were inverted for various periods (tip up) in order to study the movement of the amyloplasts under the effect of the gravity force. Second, roots grown on an onboard 1-g centrifuge were placed in microgravity for various periods to analyze the movement of the amyloplasts that occurred in the same direction as in the inverted roots. The velocity of the displacement was seven times faster in inverted roots on the ground than in roots grown on a 1-g centrifuge in space and later placed in microgravity. In another experiment, the lentil seedlings were treated by cytochalasin D in order to show that the movement of statoliths in microgravity could be due to actin filaments. The displacement of these organelles was much slower in cytochalasin D treated roots, but still occurred. This was an important finding since it proved that in the condition used, the actin network was perhaps not completely destroyed. In this way, the fact that cytochalasin D treated roots are graviresponsive (Hou et al. 2004) does not discard the hypothesis that actin could be involved in graviperception (Driss-Ecole et al. 2000b).

The results obtained showed that actin filaments were surely responsible for the movement of the amyloplasts and interestingly myosin was found to be located around these organelles (Baluska and Hasenstein 1997, Driss-Ecole et al. 2002). The space experiments on statocytes have demonstrated that the cytoskeleton (at least actin filament) was sensitive to tensions created by the weight of organelles (Lorenzi and Perbal 1990, Volkmann et al. 1991, Volkmann et al. 1999), including the nucleus and the amyloplasts, which are the densest organelles in the gravisensing cell. The movement of these organelles that was observed in microgravity could be due to the action of motor proteins, such as myosin (Driss-Ecole et al. 2003). On the ground the force exerted by these motor proteins is less than that exerted by gravity, so that the amyloplasts can sediment.

3.5 Gravitropic Response in Microgravity

The gravitropic response of lentil seedling roots was first analyzed during the Spacelab S/MM05 mission (Perbal et al. 1999). The lentil seedlings were grown in microgravity for 26 h and then placed on the centrifuge for 22 min. The gravitropic response was followed by time-lapse photography. In microgravity, the tip of gravistimulated roots could overshoot the direction of the acceleration after 3 h (Figure 6-13), whereas roots stimulated on the ground or in space continuously did not (see Figure 6-02). On Earth, there must be a regulation (inhibition of gravistimulus) that is gravity dependent. This regulation is seen when in some roots there is a kind of counteraction that reduces the bending. The regulation of the gravitropic response appears to be linked to the actin filaments, since *Arabidopsis* roots treated with Latrunculin B (which perturbs actin polymerization) are more responsive than

control roots (Hou et al. 2004). According to Stankovic et al. (1998a), the straightening of the root is part of the gravitropic reaction chain.

Tewinkel and Volkmann (1996a) have shown that *Lepidium* roots were more sensitive to gravistimulus when the seedlings were grown in microgravity than when the seedlings were grown on a 1-g centrifuge. During the S/MM05 flight, Perbal et al. (2004) have also shown higher sensitivity of lentil roots grown in microgravity than grown in space on the 1-g centrifuge. The response for the same stimulation dose was in effect much greater for the former than for the latter. Perbal et al. (2004) have proposed that this should be due to the position of the amyloplasts within the statocyte and to the direction of their movement upon gravistimulus.

It must be noticed that in the case of lentil roots grown in 1 g as for roots grown in microgravity, no counteraction was observed even after a period of 6 h in microgravity at least for strong stimulations.

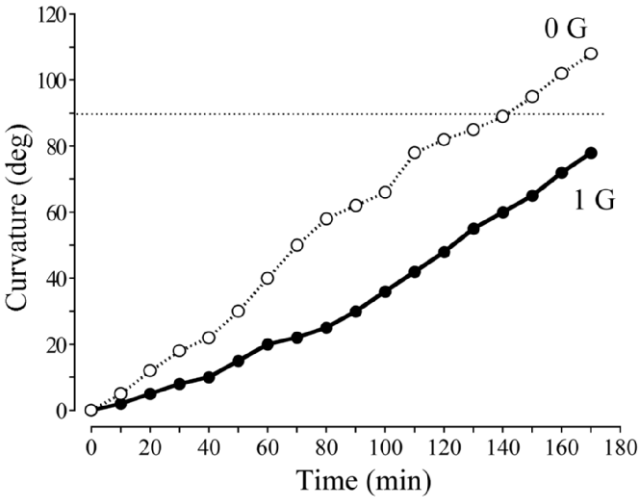

Figure 6-13. Gravitropic curvature of lentil roots grown for 26 h on a 1-g centrifuge (1G) or in microgravity (0G) and subjected to a dose of stimulation of 14.7 g x min (22 min at 0.67 g). The curvature rate is almost constant and the root tip overshoots the direction of the acceleration (90 deg) in the microgravity sample after 150 min. the curvature is slower in the 1-g sample so that it takes more times to observe the over-shooting (not shown).

This counteraction must not be mistaken for *autotropism,* which is a complete different phenomenon (Stankovic et al. 1998b). Autotropism, i.e., the fact that after curving the organs straighten out, has been studied by Chapman et al. (1994) on *Avena* coleoptiles. The oat seedlings were grown on a 1-g centrifuge in space and were then stimulated laterally by variable centrifugal forces in such a way that acceleration and duration of the stimulus varied. Stimulus doses were thus comprised from 1.8 to 25.2 g x min. This analysis showed that after gravitropic bending of the coleoptiles, these organs straightened and the curvature disappeared after about 5 h. Stankovic et al. (1998a) proposed to limit the use of autotropism to the straightening occurring in microgravity (or on the clinostat) after gravitropic stimulation. Recently,

Stankovic et al. (1998b) studied autotropism on *Lepidium* roots. In this species, autotropic straightening also occurred on the clinostat.

3.6 The Clinostat as a Tool for Studying Gravisensitivity

The clinostat has been used for a century to simulate microgravity. It could be considered as a good tool for the analysis of gravisensing if (at least) it had the same effects as microgravity on the statolith apparatus, i.e., if the volume of the statoliths, their density, and their distribution in the statocytes were similar in both culture conditions. Some analyses were performed to compare the effects of microgravity and clinostats. However, these studies were carried out on different species and with different devices. For instance, Lorenzi and Perbal (1990) used a 2D clinostat on which the seeds were rotated about a horizontal axis (1 rpm) and about a vertical axis (1 rpm). The roots were either perpendicular (vertical clinorotation) or parallel (horizontal clinorotation) to the horizontal axis of rotation. The two different conditions led to different positions of the amyloplasts (and of the nucleus) in the statocyte. Horizontal clinorotation led to the same distribution of the amyloplasts as in microgravity.

It must be pointed out that the slow rotating clinostat should not able to mimic microgravity since the rotation speed is about 1-2 rpm. The stimulus in a given direction should be therefore strong enough to be perceived at every rotation, even if no curvature is observed because the gravitropic stimulation is omnilateral. The fast rotating clinostat (at 50-100 rpm), however, should be able to better simulate microgravity since the perception time is greater than the short stimulus applied in a given direction at every rotation.

Hilaire et al. (1995) have used a slow and a fast rotating clinostat, but they found the same statocyte polarity and the same distribution of amyloplasts as in microgravity for both devices. However, these results were not quantitative.

Another clinostat has been developed recently, the 3D clinostat (see Figure 3-07). On this device the specimens are rotated around a point (and not about a horizontal axis) and gravity should thus be compensated in all directions. Using this device, Buchen et al. (1993) have found that the amyloplast distribution was the same as in microgravity in *Lepidium* roots, but the volume of starch was greater in simulated microgravity.

The amyloplasts were often considered to be distributed randomly in the statocyte in microgravity (Volkmann et al. 1986). A careful analysis performed by Smith et al. (1997) showed that this was not the case. The distribution of the amyloplasts of white clover was different in microgravity and on the 2D clinostat, and it was not random and in both conditions.

Thus, at present we have no clear-cut evidence that clinostats (2D, 3D, or fast rotating clinostat) can simulate microgravity for analyzing the

perception of gravity. The only direct indication we have comes from the study of gravisensitivity in lentil roots grown on a clinostat (horizontal clinostat) or in microgravity, which showed that presentation time was similar in simulated microgravity, i.e., 25 sec (Perbal et al. 1997) and in actual microgravity, i.e., 27 sec (Perbal and Driss-Ecole 1994). Thus, it seems that clinostat can simulate microgravity well enough to study gravisensitivity at least in the first approach.

4 THE ROLE OF GRAVITY IN PLANT DEVELOPMENT

4.1 Plants and their Environments

4.1.1 Role of Meristems in the Plant Development

Plants are unique multicellular organisms that possess the capacity for unlimited growth throughout their lives. This potentiality is due to stem cells (Laux 2003, Byrne et al. 2003) located within *meristems* (Figure 6-14, inserts). Stem cells perpetuate themselves by cell division and give rise to derivatives that differentiate to form tissues. This results in a repeated initiation of new organs throughout the plant life.

Plant meristems are classified on the basis of their location and function. A plant seedling typically starts out with two meristems. They are primary meristems located at the tip of the shoot and close to the tip of the root (Figure 6-14). They are responsible for primary growth of these organs, i.e. the elongation of the plant body.

Lateral meristems are located on the sides of roots or stems, and are involved in the secondary growth, i.e. the increase in diameter of these organs. These meristems or *cambiums* are the origin of cork and secondary vascular tissues. The vascular cambium form tissues that are involved in the conduction of water and salts (*xylem*) or sugars and organic compounds (*phloem*).

The secondary xylem is called *wood*. It is composed of several types of cells among which fibers and vessels are lignified which gives rise to the properties of resistance of wood to mechanical stresses. Wood is also greatly responsible for the rigidity of the plant body and allows a vertical growth of woody plant the mass of which can be enormous (commonly several tons). Its formation is dependent upon gravity and this tissue can have different properties in the trunk and in lateral branches. In seedlings that do not possess secondary xylem, structural support for the plant body is made possible by the presence of cell walls. Turgor pressure inside cells also contributes to the rigidity of the organs.

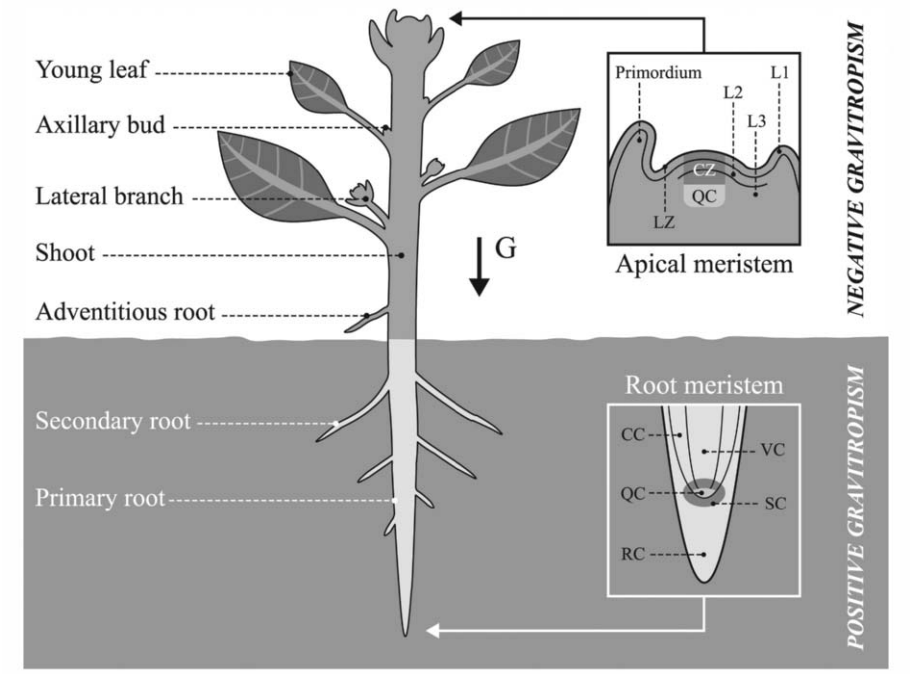

Figure 6-14. Plant development. The primary axis of a plant is formed by the primary root and shoot. These organs bear secondary roots and lateral branches respectively. Their growth is due to meristems where cells are dividing rapidly (see inserts). The apical meristem is formed of three layers of cells (L1, L2, L3). Its central zone (CZ) is composed of cells whose activity is regulated by the organizing center (QC) located just below. The fastest cell cycle is observed in the lateral zone (LZ) where the leaf primordia are formed. Stem cells are considered to be located in the central zone. The root meristem is covered by the cap (RC). The quiescent center (QC) is regulating the activity of the stem cells (SC) that are around it. CC: cortical cells; VC: vascular cylinder. Adapted from Laux (2003).

4.1.2 The Plant Body

The adult plant is composed of the shoot-root axis, i.e., the *stem* and the *primary root*. Both organs harbor lateral appendices, which are *leaves* (or branches with leaves) and *secondary roots*, respectively (Figure 6-14). The origin of these organs is completely different.

In the primary root, the apical meristem is sub terminal and is covered by the root cap, which is involved in the perception of gravity, but it also protects the meristem during growth through the soil. Cell division occurs throughout the apical meristem but is regulated by the quiescent center, which is situated close to the root cap. The outer layer of cells, the epidermis, also protects the root and gives rise to root hairs, which are mainly responsible for water and salt uptakes.

Above the meristem, two regions can be distinguished: the cortex and the vascular cylinder. The outermost cell layer of the vascular cylinder is the *pericycle* and is surrounded by another layer of cells, the *endodermis* (Figure 6-15). The former is responsible for a selective screening in the uptake of minerals and the latter is involved in the formation of lateral roots. These organs arise by local divisions in this tissue and have therefore an internal origin. The first divisions are *anticline* (perpendicular to the root surface) and the following divisions are *pericline* (parallel to the root surface). This activity is linked to a high concentration of the hormone auxin (3-indole acetic acid) at a certain level of the pericycle (Benkova et al. 2003, Blilou et al. 2005). The auxin gradient within the root primordium is due to the cellular efflux of this hormone mediated by specific carriers belonging to the class of the PIN proteins (Muday and DeLong 2001).

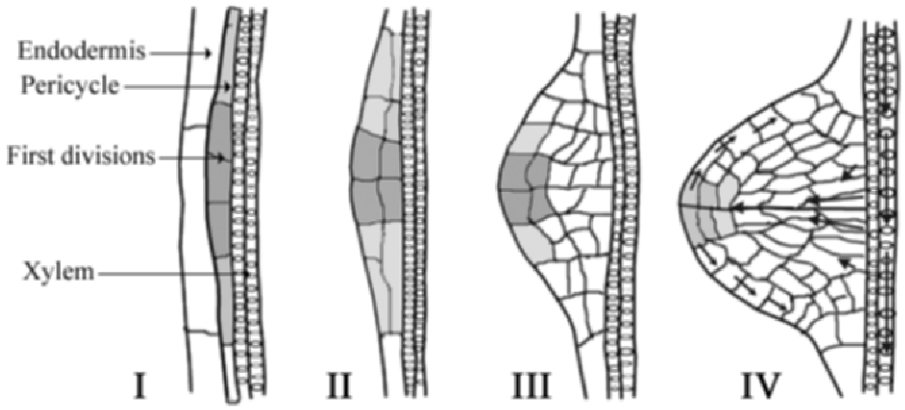

Figure 6-15. Lateral root formation, auxin distribution and auxin transport in Arabidopsis thaliana. I: The fist divisions, which occur in the pericycle, are anticline. They are adjacent to the vascular pole (first vessel formed). II: Pericline divisions take place to form the root primordium. III: The primordium grows within the cortex. IV: The arrows indicate the auxin transport within the root primordium. Grey areas show the expression of the reporter gene DR5::GUS. which indicates the zones where there is high concentration of the hormone auxin (the darker the area, the higher the concentrations). Adapted from Benkova et al. (2003).

The shoot apex is covered by a series of leaf *primordia* with the youngest being closest to the top (Figure 6-16A). The shoot apical meristem is restricted to the part of the shoot apex with is above the youngest primordia. As in the root apex, the central zone is a region of low mitotic activity. The cells of this zone divide slowly and produce cells below and on the sides, which give rise to the rib meristem and the peripheral zone, respectively. Cell division is intense in localized areas in the peripheral zone where lateral organs such as leaves are initiated and in the rib meristem that contributes to

internodal elongation. The surface and sub-epidermal layers are designated L1, L2, and L3 (Gross-Hardt and Laux 2003, Castellano and Sablowski 2005). L1 and L2 divide anticlinally, whereas L3 divides both anticlinally and periclinally (Figure 6-16B).

Leaves are highly plastic organs and show a great variety of form and size. Typical leaves are flat, dorso-ventral organs adapted for photosynthesis. It is now well established that a dorsiventrally flattened lamina requires adjacent abaxial and adaxial domains (Byrne 2005, Bowman et al. 2002). A loss of either the adaxial or abaxial domain results in a complete or partial radialisation of the leaf.

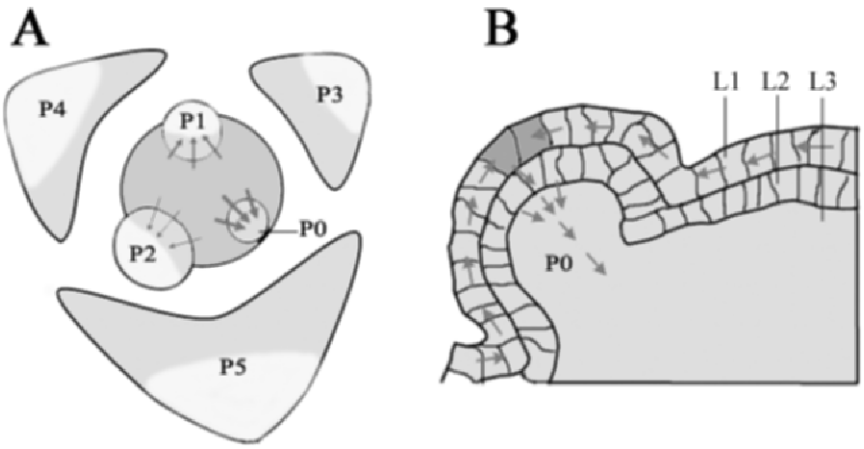

Figure 6-16. Leaf formation and auxin transport. A. Transverse section in the shoot meristem showing the formation of the primordia (P1 to P5) in Arabidopsis thaliana. P0 indicates the zone where the next primordium will be formed. The primordia present very early an abaxial and an adaxial domains (represented by different gray levels) which are characterized by a differential expression of some genes (PHB/PHV/REV). It is hypothesized that primordia represent a sink for auxin (they need auxin to develop). The youngest they are the more they need this hormone. Depletion of auxin in some areas should inhibit primordium formation, so that new leaf can be only initiated in a given area of the shoot meristem. Adapted from Castellano and Sablowski (2005), and Byrne (2005). B. Auxin transport in the young primordium. This hormone is transported in the peripheral layers toward the top of the primordium and then toward its center. Adapted from Benkova et al. (2003).

Recent work has revealed that one of the signals for the initiation of leaf primordia might be *auxin*, which is continuously transported to the shoot meristem and controls the position at which cells are recruited into organogenesis (Castellano and Sablowski 2005). Primordia emerge in regions of the meristem that have high auxin concentration. Once a primordium is established, it functions as a sink for auxin (this hormone is concentrated at

this level), depleting auxin in the surrounding cells (Figure 6-16A). The next primordium can only emerge at a certain distance, in a position at which auxin can accumulate. Auxin enters the primordia via the peripheral layers whereas it flows out of them via the internal tissues (Figure 6-16B).

Axillary buds are often initiated at the flank of the shoot meristem by cell division in the axil of leaves (see Figure 6-14). The axillary bud is formed by the shoot apex and leaf primordia.

4.1.3 Plasticity of the Plant Development

Being rooted, plants have evolved to perceive environmental factors, such as light, gravity, temperature, water, and touch. These environmental stimuli are sensed in some cases with exquisite precision and a response is affected in term of growth, differentiation, or reproduction. Plant development, as in all other organisms, is basically regulated by its genetic complement, but, in contrast to multicellular animals, it is characterized by extreme plasticity. Plants have the ability to change form or shape in response to a change in environment, no genetic change being involved. Except for light, the perception of environmental stimuli or the receptors for environmental signals are not well known, but the signaling pathway in many cases involves hormones.

Due to this plasticity, it was expected that plants should be able to grow in microgravity if proper nutrients, light and so on were provided. However, the way they could respond to this new environment was unknown.

4.2 The Role of Gravity in Plant Growth: Gravimorphism

4.2.1 Orientation of Plant Organs with Respect to Gravity

Most of the plant organs have an optimal angle of orientation with respect to gravity, which is called the *Gravitropic Set-Point Angle* or GSPA (Firn et al. 1999). The normal orientation of growth of a plant organ can be parallel to gravity (*orthogravitropism*) or oblique (*plagiotropism*) with respect to the g vector (see Figure 6-14). Thus, the primary root and the shoot are orthotropic, whereas lateral roots and branches are plagiotropic. A special case of plagiotropism is *diagravitropism,* which is observed when the organs grow perpendicularly to gravity (rhizomes, runners). Only a few organs do not show any preferential orientation with respect to gravity: they are called *agravitropic*.

4.2.2 Role of Gravity in the Formation of Organs

In herbaceous plants and shrubs, which show abundant branching, the axillary *buds* may grow into a branch. In plant with a strong dominant main shoot, lateral buds close to the apical meristem stay dormant for a long time.

However, if the dominant shoot is cut or injured, they start growing and one of them forms a new dominant shoot. It is well accepted that auxin is involved in the apical dominance by inhibiting the activity of the axillary buds. However, its mechanism of action remains to be clarified (Leyser 2003).

Seedlings of some species develop a protuberant tissue during embryogenesis or soon after germination (Kamada et al. 2000). This protuberance has been called *peg* and is considered to be useful to hold the seedling coat during hypocotyl elongation. In cucumber, the formation of peg is considered to be gravity dependent since it occurs on the lower side of the transition zone (located between the hypocotyl and the root) when the seedling is in the horizontal position. In addition, when cucumber seedlings are grown on a clinostat or in the vertical position with their radicle pointing downward, they develop one peg on each side of the transition zone or are pegless.

4.3 Formation of the Cell Wall and Differentiation of the Supporting Tissues

4.3.1 Role of Gravity in The Cell Wall

Plants have evolved under the constant gravity force after having went ashore more than 400 million years ago, and they had to develop a resistance to this force to grow in the upright position. The cell wall plays an important role in resisting to gravitational force and supporting the plant's body on Earth (Hoson and Soga 2003). Basically, the cell wall is composed of microfibrils of cellulose embedded in a complex matrix. The analysis of the effects of gravity on the cell wall has been limited, since clinostats are not able to suppress this factor by rotating plants about a horizontal axis. This device can only transform the unilateral effect of gravity into an omnilateral action. Alternatively, centrifugation was used to analyze the modification of the composition of the cell wall under hypergravity, assuming that there could be a continuum of the response in a wide range of g levels (from microgravity to thousands of g). It was found that hypergravity reduces growth rate of various organs (Waldron and Brett 1990, Hoson and Soga 2003). In parallel, cell wall extensibility was decreased in garden *Cress* (Hoson et al. 1996) and *Arabidopsis* (Soga et al. 2001) hypocotyls, as well as in *Maize* coleoptiles (Soga et al. 1999). This is due to an increase of the cell wall thickness and a polymerization of certain matrix polysaccharides related to a reduction of some degrading enzyme activities correlated with an increase of the cell wall pH (for review, see Hoson and Soga 2003). Some other experiments have been conducted by immersion of plants in water to counteract the action of gravity. This was possible only with aquatic or semiaquatic plants such rice.

Submergence induces a higher extensibility of the cell wall and causes diverse changes in the levels and structure of the cell wall (Masuda et al. 1994).

4.3.2 Secondary Growth and Vascular Cambium

In gymnosperms and woody dicots, a vascular cambium appears in the region of root or stem that has ceased elongating and produces secondary xylem and phloem. This induces an increase in diameter of these organs and provides the needed structural support to trees. In herbaceous plants, only a small amount of secondary vascular tissues is formed and the cambium activity is not extensive. In woody plants, these tissues constitute a complete ring. The vascular cambium is a layer of meristematic cells (called *initials*) that arises between primary xylem and phloem. With few exceptions, the cambium consists of two types of initials, the *fusiform* and *ray initials*. Fusiform initials are elongated cells that divide periclinally and give rise to axially elongated cells in the phloem and the xylem. Ray initials are more or less isodiametric and occur in clusters that appear spindle shaped in tangential sections. Ray initials produce xylem and phloem rays, which extend radially into the vascular tissue and are responsible for the radial transport of water, minerals and photoassimilates.

If a square block of tissues, including the cambium, is lifted off a tree trunk and rotated by 90 deg and replaced, the wound heals in time and the lifted block continues to produce xylem and phloem cells on the two sides, but the new cells which are produced are horizontally oriented (and not vertically oriented as in the trunk). It must be noticed that some pressure is needed for this orderly continuation of the cambial activity because, in its absence, fusiform initials divide up into numerous small cells and form an unorganized tissue mass, known as *callus*. Thus, forces applied on the cambium are necessary for its normal activity. However, they should be much greater than the gravity force, which also can't be responsible for orientation of the plane of division within the cambium.

Thus, gravity during evolution has surely played an important role in the formation of vascular tissues, which are responsible for transport of photoassimilates (phloem), water and minerals (xylem), and the rigidity of woody plants (xylem). But the activity of the vascular cambium appears not to be greatly influenced by this external factor.

4.3.3 Compression and Tension Woods

Mechanical stress in woody species results in the formation of reaction wood. This response creates physical strains in the wood that force the stem or branch back toward its original orientation in space (Scurfield 1973, Wilson and Archer 1977, Timell 1986). *Angiosperm* and *gymnosperm* trees differ in their nature of reaction wood. In angiosperm trees, reaction wood is called tension wood and forms on the upper side of stems that have

been displaced from their normal position. Tension wood characteristically has few, small vessels, and fibers with an inner gelatinous cell wall layer (the G-layer), which consists of almost pure cellulose with microfibrils that are parallel to the long cell axis (Kwon et al. 2001). In gymnosperm trees, such as pine, reaction wood is called compression wood and forms at the lower side of displaced stems. Compression wood is characterized by short, rounded tracheids that have thick walls with increased lignin content and increased microfibril angles. The formation of reaction wood is often (but not always) accompanied by a stimulation of cambial cell division, whereas the cell division at the opposite side is more or less inhibited. The physiology and development of reaction wood formation has been extensively explored (Timell 1986). The induction of reaction wood by gravistimuli rather than by mechanical stimulation has been deduced from a large number of bending, leaning, and clinostat experiments.

Numerous experiments involving applications of *Indole-3-Acetic Acid* (IAA) or IAA-transport inhibitors have suggested that reaction wood is induced by a redistribution of IAA around the stem. However, a recent analysis of endogenous IAA demonstrated that reaction wood was formed without any obvious alterations in IAA balance (Hellgren et al. 2004). Cambial growth on the tension wood side was stimulated without an increase in IAA. Taken together, these results suggest a role for signals other than IAA in the reaction wood response.

5 DEVELOPMENT OF PLANTS IN ACTUAL AND SIMULATED MICROGRAVITY

5.1 Vegetative Development of Plants

5.1.1 Germination and Root Orientation

Many species have been grown in microgravity or on clinostats and it appeared that the absence of gravity or simulation of weightlessness had no real effect on germination (Halstead and Dutcher 1987, Kordyum 1997). However, the orientation of growth of the radicle, which is strongly dependent upon gravity on Earth, is related to the position of the embryo in microgravity or in simulated microgravity (Volkmann et al. 1986). Thus, as it develops on a clinostat the primary root shows spontaneous curvatures, which have been studied extensively on maize by Hoson (1994). The maize root on the 3D clinostat did not grow straight (Figure 6-17A). Control roots grown in 1 g showed some degrees of curvature in three regions. The curvature around the basis (angle *K*) of the root was always prominent. The distribution of angle *A* and *M* of control roots was more concentrated around 0 deg than angle *K*. Clinorotation greatly enhanced the curvature and caused an increase in the

dispersion of the angle of bending. These curvatures in the segments of the root under simulated microgravity conditions may be derived from inherent properties of plants, which could be modified by the gravity vector on the ground. Such an automorphogenesis appears to play a major role in the regulation of plant development under a microgravity environment. The spontaneous curvatures were not due to osmotic concentrations within the cell or to mechanical properties of the cell wall between the two sides of the bending roots. According to Hoson (1994), the bending could be linked to *circumnutation* (regular oscillations of the tip), which have been observed by Volkmann et al. (1986).

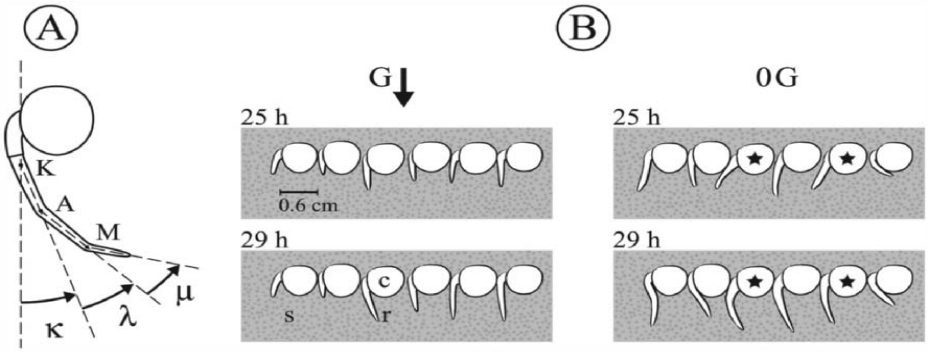

Figure 6-17. A. Root development in a Zea mays seedling on a 3D clinostat (simulation of microgravity). The angles K, A, and M indicate the angles of bending at the level of three points. Adapted from Hoson (1994). B. Root orientation in lentil seedlings grown in space after 25 h and 29 h spent inside an onboard 1-g centrifure or in microgravity. Asterisks indicate seedlings whose extremity was subjected to a strong change in orientation between 25 and 29 h. r: root; s: sponge. Adapted from Legué et al. (1996).

Such movements have also been observed in lentil roots by Perbal et al. (1987) and Legué et al. (1996). After a growth period of 25 h in microgravity, the emerging root was bent and its tip was most often pointing away from the cotyledons (Figure 6-17B). Although the mean angle of curvature was about the same after 25 and 29 h, some roots were subjected to a strong change in orientation during 4 h. This meant that the movement was not synchronous from one root to another.

Root growth in *Lepidium sativum* has been studied by Johnsson et al. (1996) in order to determine whether the root tip was subjected to random walk (growth in a random direction). The seedlings were grown between two agar slices and the deviation angle of the root tip (α_t) at time t was measured with respect to a fixed reference direction (α_0). Theoretically, random walk is characterized by a mean deviation equal to 0, $\alpha_t - \alpha_0 = 0$ and the variance of the deviation should be proportional to time: $(\alpha_t - \alpha_0)^2 = k \times t$ (k constant).

These authors showed that the displacement of the root tip could be considered to be random walk since it fulfilled the two criteria cited above, at least at the beginning of the root growth. These results could appear contradictory to those obtained by Volkmann et al. (1986) since these authors observed that the orientation of the root tip depended upon the position of the embryo and that it was subjected to nutations (movement of oscillation). This controversy can be eventually explained by the fact that these nutations are asynchronous even at the beginning of the root development (Figure 6-17B). It could be also due to different culture conditions since in the Johnsson's experiment the displacement of the root tip could occur only in a plane between two agar slices whereas in the Volkmann's experiment the root tip had the possibility of 3D movement. In any case, the experiments with clinostat or in microgravity have shown that without the unilateral effect of gravity, the root tip is subjected to various movements, its orientation during germination being strongly dependent upon the position of the embryo in the seed.

5.1.2 The Growth of the Primary Root in Microgravity or on the Clinostat

5.1.2.1 Root Elongation

Root length was sometimes found to be the same, and sometimes to be greater or less in microgravity as in normal gravity (for reviews, see Halstead and Dutcher 1987, Claassen and Spooner 1994, Kordyum 1997, Aarrouf et al. 1999, Hoson and Soga 2003). These results can be considered controversial if it is assumed that the effect of microgravity is constant (always inhibiting or stimulating) during the whole period of development of the primary root. However, as pointed out by Claassen and Spooner (1994) the action of microgravity can depend upon the species and/or the stage of development, i.e., the duration of exposure to microgravity. By a careful review of the literature, these authors have observed that root length was about the same in different species grown for 1-2 days, while it was greater for a growth period of 3-5 days and less for longer periods of culture.

This observation was at least partly confirmed by Aarrouf et al. (1999) on *Brassica napus* seedlings grown on a clinostat rotating at 1 rpm (Figure 6-18). The primary root elongated faster in simulated microgravity than in the vertical position during 5-15 days. After 15 days, clinorotation had an inhibitory effect, in such a way that after 25 days root length was less in simulated microgravity than in 1 g. Unfortunately, root growth was not studied during the first five days, but the results reported showed that the difference in root length was slight after five days. One could therefore assume that root growth should have been almost similar during this period. This hypothesis was confirmed by Hilaire et al. (1996), who examined the

growth of Soybean seedlings for seven days. A significant difference in root length on the clinostat and in 1 g was only detectable after five days.

According to Aarrouf et al. (1999) the effect of clinorotation should be slight and cumulative. Thus, during the first phase of development in simulated microgravity there should not be noticeable differences. However, after several days (period depending upon the species) a stimulating effect of microgravity could take place, which should be followed by an inhibiting effect.

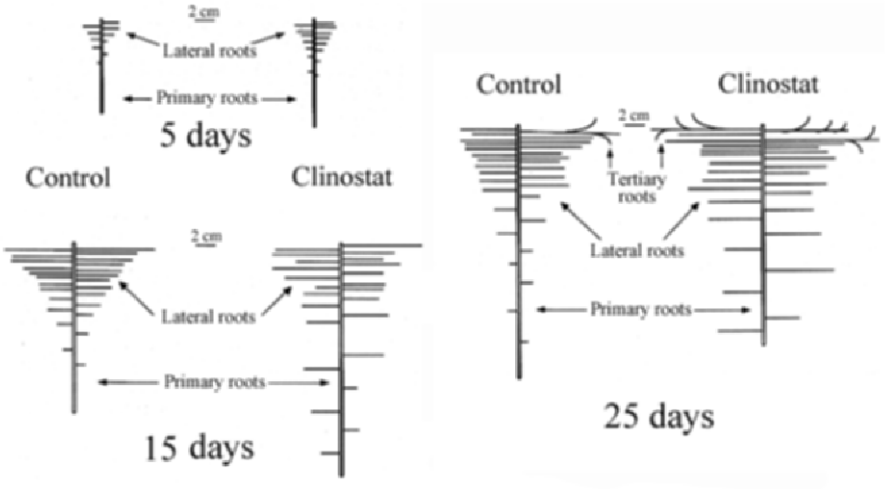

Figure 6-18. Architecture of the root system of Brassica napus after 5, 15, and 25 days of culture on a clinostat rotating at 1 rpm, or in the vertical position (Control). These models present the length of the primary root and the length and position of the secondary (lateral) roots, as well as those of the tertiary roots (only after 25 days). The primary root grows faster on the clinostat for about 15 days and stops elongating after so that root length is greater for the vertical control after 25 days. The secondary roots are more numerous and are formed closer to the primary root tip on the clinostat after 15 and 25 days.

5.1.2.2 Hormone Content

Plant hormones play a key role in developmental processes of the root system (see Figures 6-03 and 6-16), including control of cell cycle in the meristem, elongation (Muday and Hayworth 1994) and lateral root initiation (Benkova et al. 2003). Perturbation in the synthesis, transport, and distribution of the hormones could therefore be responsible for modifications of root growth in microgravity. As IAA (indole-3-acetic acid) and ABA (abscissic acid) were shown to be involved in the gravitropic reaction of the primary root (Pilet and Elliott 1981), it was obvious that these two hormones had to be

analyzed first since their distribution in the plants grown in microgravity had more chance to be perturbed.

In a space experiment, Schulze et al. (1992) have examined the IAA and ABA contents in *Zea mays* seedlings grown in microgravity. These seedlings were cultivated in darkness for five days and then frozen in a gaseous nitrogen freezer. The IAA and ABA content was studied in the shoot, the root and the kernel, but a significant difference was observed only for IAA in roots (there was less free IAA in space-grown plants). Interestingly, it must be noticed that in roots:

a. The auxin transport is dependent on the root cap and the statenchyma (see Figure 6-03), which is not the case in the other organs;
b. The period of growth in this experiment roughly corresponds to the beginning of a significant change in root elongation in microgravity. It is clear that this type of analysis should be done for longer periods of growth in microgravity.

Aarrouf et al. (1999) have carried out a similar experiment on rapeseed seedlings grown on a clinostat for 5, 15, and 25 days. IAA and ABA contents were greater in plants grown on the clinostat for 5 and 15 days, but they were similar after 25 days. This result showed once again that the effect of microgravity or simulated microgravity can be specific to certain stages of development since the greatest difference in IAA and ABA contents in 0 g or in 1 g was observed after 15 days.

Interestingly, the amount of zeatin (another hormone) was also analyzed by Aarrouf et al. (1999) and was found to be similar after 5 days and greater after 15 and 25 days on the clinostat than in the vertical control. These authors hypothesized that the variation in the zeatin content should have been a consequence of the modification of the IAA and ABA balance.

Although carried out in simulated microgravity, the work done by Aarrouf et al. (1999) showed that it is not possible to extrapolate data obtained on 5-day old seedlings to data on older plants since microgravity (or simulated microgravity) seems to have a slight but cumulative effect on plant growth and disturbances should appear only after a certain period of development.

5.1.2.3 Mitotic Disturbances in the Primary Root

Krikorian et al. (1996 and citations therein) had the opportunity of studying the mitotic activity and the chromosome disturbances in roots of several species. These experiments concerned roots of seedlings (oat, mung bean, and sunflower) grown in space. In the three species that were studied, the mitotic activity was substantially reduced after completing their first cell cycle after landing on Earth. In oat and sunflower roots, there were

chromosomal aberrations ranging from aneuploidy[7], breakage and bridge formation (Figure 6-19), but no chromosomal aberration was detected in *Mung* bean. The karyological disturbances were not questionable, but their cause had to be determined. Evaluation of the available facts indicated that indirect effects played a major role in these karyological disturbances and that plants grown in space were subjected to various stresses.

It must be pointed out that the culture conditions in space were not always satisfactory since the cabin atmosphere could contain gas, as ethylene for example, which could affect plant growth (Kiss et al. 1998). To some extent, this problem can be solved by using a 1-g centrifuge in space that can permit to discriminate between the effects of gravity and those due to other space factors, such as cosmic radiation or cabin atmosphere. It should be also necessary to chemically fix the samples in space and not after their retrieval on Earth. Only a few experiments were done in these conditions and using seeds that were less sensitive than seedlings to all kind of stresses both before and during launch. Unfortunately, when a 1-g centrifuge was available, the period of growth was most often very short.

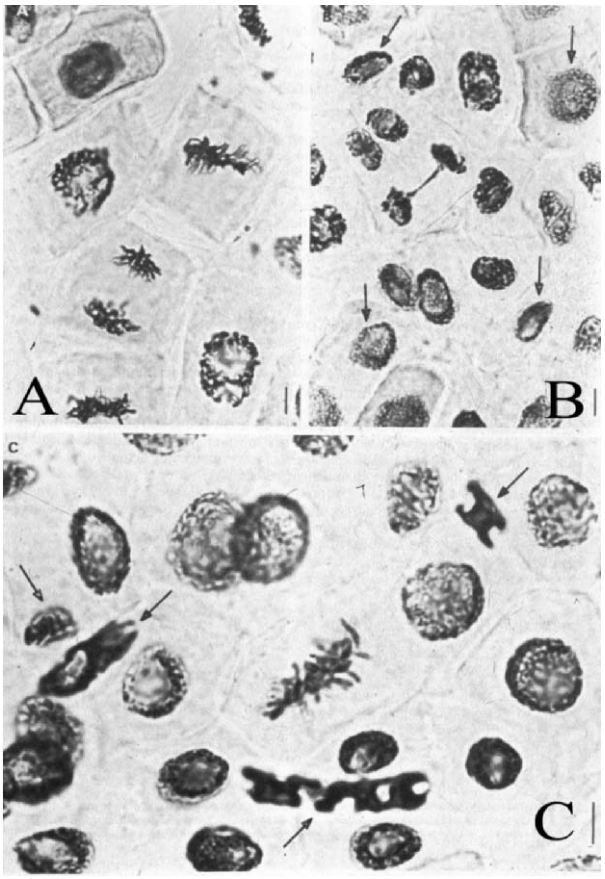

Figure 6-19. Cell divisions in sunflower root tips exposed to microgravity. A. Field showing various stages of division: interphase, prophase, meta-phase, anaphase are visible.
B. Field showing normal nucleus activity (arrows) and a chromosome bridge at telophase. C. Field with dislocated Feulgen staining nuclear components. The arrows show different damage to nuclei. Krikorian and O'Connor (1984), with permission.

[7] A chromosome problem that happens when one or more whole chromosomes either are missing or are present in more than the typical number of copies.

5.1.2.4 Cell Cycle in the Primary Root

For the majority of species investigated in space, a decrease in the *mitotic index* (MI = % of mitosis) was observed in many species (see Halstead and Dutcher 1987 for review). In some cases, no difference was found between spaceflight and control samples. A greater MI was shown in the lentil root meristem of young seedlings grown in microgravity than in the seedlings grown on a 1-g centrifuge in space (Darbelley et al. 1989, Driss-Ecole et al. 1994, Yu et al. 1999). It must be noticed that there were only a few experiments with a 1-g control in space and that very often the seedlings were fixed after retrieval on the ground, i.e., after having been subjected to gravitational forces during landing.

The results on MI seemed to be as controversial as those obtained on root growth. The same causes could be responsible for the discrepancy of the data of different authors: (a) the period of growth (which was very short in the case of lentil root, for example); (b) the different species which eventually could have the same behavior but not necessarily at the same time; (c) and sometimes the method used, since different results have been observed on the same material.

It must also be stressed that MI is a very poor indicator for studying cell cycle, because it can vary as a function of many different factors. Darbelley et al. (1989) have indicated that MI could be greater because the other phases of the cell cycle (or only one of them) became shorter or because mitosis was longer. It is the reason why Driss-Ecole et al. (1994) and then Yu et al. (1999) have studied the mitotic index and the proportion of the different phases of the cell cycle in a homogenous tissue: the cortical cells of the root meristem. The period of growth was 28 h and 29 h for the IML-1 and the IML-2 missions, respectively.

In a preliminary analysis, a comparison of the cell cycle in clinorotated lentil seedlings and vertical seedlings was done. But, to better understand the results obtained, some additional experiments were necessary on vertical roots on the ground. First, the mitotic index was followed as a function of time after hydration and it was shown that no cell division was observable before 13 h and that there was a clear peak of mitoses at 25 h. Taking into account the duration of the mitosis itself (1-2 h) it could be assumed that the first cell cycle was about 25-26 h, which meant that during the IML-1 and the IML-2 missions, cortical cells in the lentil root should have completed at the most one cycle. In principle, the majority of the cells should be at the beginning of the second cell cycle after 28 or 29 h (Table 6-02).

This also meant that the cells were at least partially synchronized. In order to demonstrate on the ground that all cells have left the G_1 phase of the first cycle, Yu et al. (1999) have analyzed DNA synthesis by using IUdR (iododesoxyuridine), which is an analogue of thymidine and is therefore incorporated in DNA. By using a flash labeling it was shown that there was a

peak of labeled nuclei at 17 h, whereas a continuous labeling with IUdR indicated that all nuclei were labeled after 25 h, which demonstrated that all cells have left the G_1 phase of the first cell cycle. As the percentage of labeled nuclei decreased after the flash labeling after 25 h but increased again after 29 h, it has been assumed that at this time most of these labeled nuclei were in the S phase of the second cell cycle.

Cycle		***First***		***Second***	
Phase		***G_2***	***M***	***G_1***	***S***
Horizontal Clinostat–27h	*G1g*	*14.9*	*6.4*	*57.9*	*20.8*
Legué et al. (1992)	*Cl*	*15.4*	*6.1*	*58.6*	*19.9*
IML-1–28 h	*G1g*	*11.2*	*7.2*	*55.4*	*26.2*
Driss-Ecole et al. (1994)	*F1g*	*11.9*	*8.9*	*58.7*	*20.5*
	F0g	*27.9*	*6.8*	*55.5*	*9.8*
IML-2–29 h	*G1g*	*10.6*	*4.0*	*52.8*	*32.6*
Yu et al. (1999)	*F1g*	*19.7*	*6.0*	*48.2*	*26.1*
	F0g	*17.8*	*3.9*	*61.1*	*17.1*

Table 6-02. Percentages of the various phases of the cell cycle (G_2, M, G_1, S) in the primary root meristem of lentil seedlings grown on the clinostat in 1992, and in space during the IML-1 and IML-2 missions of Spacelab in 1992 and 1994, respectively (G1g: ground 1-g control; F1g: in-flight 1-g centrifuge control; F0g: in flight microgravity specimen). The analyses were done on the meristematic region of 1 mm (from the root cap junction) for the experiment with the clinostat. The percentages of the various phases were determined by flow cytometry, whereas they were calculated by image analysis after Feulgen treatment for the space experiments.

From the data of Table 6-02, it can be concluded that in 1 g on the ground, there are more cells further along in the process of the second cell cycle (more cells in the S phase of the second cell cycle), which means that the first cell cycle was faster on the ground or on the onboard 1-g centrifuge than in microgravity[8]. It must be noticed that the 1-g control in space was continuously on the centrifuge during the entire growth period for the IML-1 experiment, whereas it was subjected to 30 min (after 25 h) of microgravity in the second space experiment. This could explain why the percentage of the various phases was similar in the two controls (F1g, G1g) in the IML-1 mission, whereas they were different in the IML-2 mission. This also indicated that a short period of microgravity could lead to a change in the cell cycle. Since the percentage of G_2 nuclei was high in the F1g sample of IML-2,

[8] The *M phase* or *mitosis* is the process of chromosome segregation and nuclear division that follows replication of the genetic material; the *S phase* is the phase of the cell cycle in which DNA synthesis occurs; The *G_1 phase* is an interphase period in the cell cycle between mitosis and the S phase; The *G_2 phase* is the final stage of interphase: it follows the S phase and is considered a "gap" phase in which the cell continues to grow and duplicates in preparation for mitosis.

it can be hypothesized that the transition G_2/M was lengthened because of the short period (30 min) of microgravity at about 25 h.

5.1.3 Development of the Root System

It has been shown that in the primary root the elongation zone was modified in microgravity. For instance, in lettuce seedling roots the length of the cell elongation zone was shorter in microgravity than in the onboard 1-g centrifuge (Merkys and Laurinavicius 1990). The presence of cells elongating closer to the quiescent center[9] indicated that there was an acceleration of the cell differentiation process. For example, the formation of root hairs occurred at a shorter distance from the root tip. The meristematic activity slowed down earlier in microgravity than in the ground controls, and root apices of the same age in orbital flight contained only differentiated cells (review by Kordyum 1997). An early decrease or a removal of the *apical dominance* (i.e., the fact that the primary root tip inhibits the formation of secondary root primordia) led to the abundant formation of lateral roots described in many investigated species (Halstead and Dutcher 1987, Kordyum 1997).

Interestingly, Driss-Ecole et al. (1994) have shown that there was an increased biomass of roots in *Veronica arvensis* seedlings grown for 45 days on a slowly rotating clinostat (Figure 6-20). Dry weight of the hypocotyl and roots was 40% greater on the clinostat than in the controls due to a higher production of secondary roots.

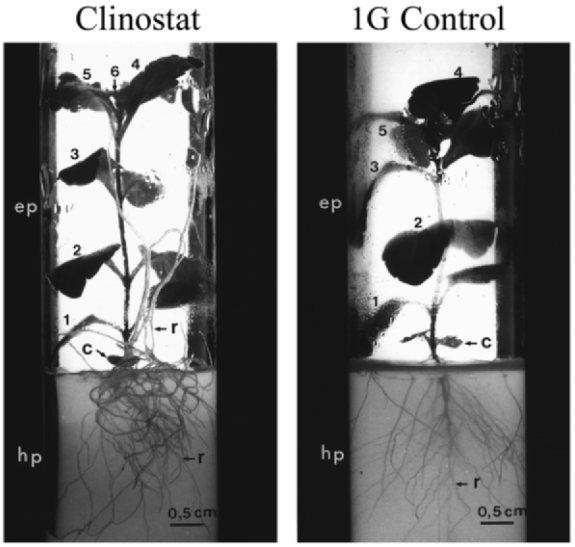

Figure 6-20. Veronica arvensis plants grown on a slowly rotating clinostat (on the left) or in the vertical position (on the right). The seedlings were cultivated on agar containing Heller medium (half-strength) for 45 days. ep: epicotyl part; hp: hypocotyl part; c: cotyledons; r: roots; numbers represent the order of the successive pairs of leaves. Note the greater development of the root system on the clinostat and the presence of adventitious roots growing at the level of the hypocotyl in simulated microgravity. Adapted from Driss-Ecole et al. (1994).

[9] The *quiescent center* is a region of apical meristems in roots in which there is relatively little or no mitotic activity.

Aarrouf et al. (1999) have also observed similar results on rapeseed seedlings grown on the clinostat (see Figure 6-18). They have demonstrated that during the first 10 days of culture there was a faster development of the root system (primary root and secondary roots). However, after 15 days the apical dominance of the primary root was perturbed on the clinostat and the formation of secondary roots could occur very close to the tip of the primary root. Later, the primary root stopped growing on the clinostat whereas the secondary roots continued to elongate. The loss of apical dominance that was observed in microgravity on several species was also seen on the clinostat. It could be linked to a perturbation of the cell cycle in the primary root meristem since Aarrouf et al (1999) have shown that there were more cells in the G_2 phase in the control than on the clinostat after five days of culture. But, after 25 days, there were more cells in the G_1 phase on the clinostat. According to Aarrouf et al. (1999) the slowing down of the cell cycle could be due to a slight but continuous stimulation, which could occur on the clinostat and to the modification of the hormonal balance (IAA and ABA, at least).

If the results on the root system grown in microgravity are compared to those obtained on the clinostat, it can be concluded that at least during the first steps of development clinorotation has not the same effect as microgravity. In this way, clinostat could not be a good tool for simulating weightlessness. However, there is some similarity of the development of the root system in both conditions for longer period of growth. It is well known that the sensitivity of the primary root to gravistimulus is much greater during the first steps of its development. It is therefore possible that during this particular period (two days) there could be slight gravitropic stimulation on the clinostat that increases the growth of the primary root. Then after this period, the gravitropic sensitivity being lower, clinorotation could have the same effects as microgravity and could be a better simulation of microgravity.

5.1.4 Development of the Shoot System

A careful analysis of the results obtained on shoot development in microgravity has been done by Claassen and Spooner (1994). They have remarked that in general, the growth of the shoot was slower or faster in microgravity than on the ground. However, slower growth rate was most often observed when the plants were subjected to accelerations or gravity during launch and/or during re-entry of the vehicle in the Earth's atmosphere. When the plants were grown in microgravity, the growth of the shoots was greater than on the ground.

The most impressive experiments were conducted on board Salyut-6 and Salyut-7 flights (Merkys and Laurinavicius 1990). *Arabidopsis*, cress and lettuce seedlings were grown in microgravity or on a centrifuge at 0.01, 0.1, and 1 g. However, in the Salyut-7 experiments, the centrifuge ran continuously, whereas it was stopped during the night period on Salyut-6. The

results obtained in Salyut-7 on lettuce were therefore more reliable since the 1-g sample in space represents a true 1-g control. A 1-g centrifuge control was also carried out on the ground in order to discriminate between the effects of the centrifugation and those due to microgravity. The comparison of the 1-g stationary sample and the 1-g centrifuge sample on the ground showed that there was no significant difference in the growth of the hypocotyl due to centrifugation. When considering the length of the hypocotyl growing on the 1-g centrifuge as 100%, a decrease of the g level led to an increase of hypocotyl length by 8-16%.

It must be stressed that the growth of the shoot as well as that of the root in space were much slower than that on the ground for this particular experiment. If no 1-g controls were available, the conclusion of this experiment should have been that microgravity inhibited seedling growth. But, surely other space factors were responsible for the slowing down of growth, including changes in atmosphere and gas exchanges (see this Chapter, Section 6.2). It could be also the case for numerous experiments that did not have any space 1-g control.

A recent analysis of the hypocotyl growth of *Arabidopsi*s seedlings grown in microgravity and various g levels showed an accelerated growth of these organs in space (Soga et al. 2002). This experiment indicated that as expected there is a continuum from microgravity to thousands of g of the action of acceleration on growth. It is also interesting to compare the results obtained on clinostat and those obtained in space. The data provided by these experiments indicated that for short periods of growth no significant difference was found in various species grown on the clinostat. An increase in shoot growth was observed very often after two to three days. This was well documented by Hilaire et al. (1996) who followed shoot growth as a function of time over a large period of time. The only exception seemed to be the study of *Avena* coleoptile (Shen-Miller et al. 1968) in which growth was slower on a two-axis clinostat (growth period was 70 h).

If we compare the results on primary roots and shoots, one can conclude that both types of organs should have the same growth on the ground and in microgravity during one to three days. Then, there is an increase in growth of the shoots and the primary roots for less than one week. For longer periods, it seems that the growth of the primary roots decreases in space, with a loss of apical dominance. If we take into account the results obtained on clinostat, it appears that the development of the root system is more perturbed than that of the shoot system. This could be due to the fact that auxin flux goes mainly through the root statenchyma and that its efflux should depend upon the gravisensing cells (see Figure 6-03), whereas statenchyma does not appear to have a main role in the movement of auxin in shoots.

5.1.5 The Formation of Peg in Microgravity

Cucumber seedlings grown in microgravity developed a peg on each side of the transition zone between the root and the hypocotyl (Kamada et al. 2000), whereas seedlings grown in the horizontal position on the ground developed a peg on the concave side of the gravitropically bending transition zone. In order to determine the role of auxin in the formation of the peg, the in-situ hybridization of an auxin- inducible gene, CS-IAA1, has been studied in space and on the ground (Kamada et al. 2000). The analysis showed that its mRNA accumulated on the lower side of the transition zone in the seedlings placed in the horizontal position, while this mRNA was distributed in the whole transition zone when the seedlings were grown either in microgravity or in the vertical position on the ground. These results demonstrate that gravity regulates the formation of the peg via the distribution of auxin.

5.2 Cell Wall in Microgravity

The study on cell wall changes in microgravity has shown that the amounts of cellulose and lignin decreased in microgravity (Cowles et al. 1984, Nedukha 1996). More recently, the physical properties of cell walls in *Arabidopsis* hypocotyls and *Rice* coleoptiles were studied by Hoson et al. (2002) and Soga et al. (2002). An increase of irreversible extensibility was observed. Both materials had lower levels of cell wall polysaccharides per unit growth than the controls, which indicated that microgravity could decrease cell wall thickness. The matrix of the cell wall in rice is mainly composed of (1→3)(1→4)-β-Glucans, whereas in *Arabidopsis* it mainly contains Xyloglucans. The amount of both polysaccharides diminished in microgravity. For Hoson and Soga (2003), both polymers may be involved in the response to microgravity since they are key players in the response to environmental factors and growth regulation of plants.

The formation of compression wood induced by harnessing 1-year old *Douglas fir* at a 45-deg angle (forcing the stem to grow in a oblique direction) in microgravity or on the ground has been studied by Kwon et al. (2001). Compression wood was identical in both culture conditions, showing that the mechanical loading (harnessing) could override the gravity effects since the force exerted by harnessing is much stronger that the gravity force.

This kind of study should also be done on the compression wood formed in the lateral branches growing obliquely in presence of the gravity force alone. However, this requires long-term flights since the growth of woody plants is slow.

5.3 Plant Protoplasts and Embryogenesis

Plant protoplasts have been used as a model system and flew several times (Iversen et al. 1999). A study by Rasmussen et al. (1994) on normal

rapeseed hypocotyl protoplasts showed a retardation of protoplast regeneration on board the Russian Cosmos biosatellites during a 14-day period in orbit. During the IML-1 mission, Rasmussen et al. (1992) also obtained evidence that cell formation and division in plant protoplasts exposed to microgravity for eight days were significantly delayed compared with development on a 1-g centrifuge. A few small cell aggregates were formed under microgravity while the 1-g control samples both on board the Shuttle and on ground regenerated rapeseed plants. These results were unexpected. However, under microgravity the distribution of protoplasts in the liquid medium was random, whereas in 1 g the cells were concentrated in a monolayer perpendicular to the direction of the gravity force. There were two hypotheses on the effect of microgravity on protoplasts. The first one is that microgravity affected each single cell. The second one dealt with the sedimentation of groups of cells permitting cell-to-cell contacts and interactions.

Results obtained using free floating protoplasts on the S/MM-03 mission of the Shuttle have demonstrated that the small calli (cell aggregates) in orbit develop either shoots or roots but were not capable of regenerating to new whole plants.

In 1986, Theimer et al. have used cell aggregates of anise *Pimpinella anisum* to follow their somatic embryogenesis in space. The transfer of the cell aggregates from the callogenic to the embryogenic medium was done in microgravity. The number of cell clumps showing polarity (development of roots or primordia leaves) was studied as a function of time, and it was shown that a faster embryogenesis occurred in microgravity than in the ground control.

These results are only preliminary and it is difficult now to draw any conclusion on the effect of microgravity on somatic embryogenesis. It should be necessary to make sure that in these experiments no physical effects is responsible for the problems observed in space.

5.4 Conclusion on the Vegetative Phase of Plant Development in Microgravity

The orientation of the primary axes that is vertical on the ground (see Figure 6-14) is variable in microgravity and these axes could be subjected to random walk (Johnsson et al. 1996) eventually after some nastic movements (due to asymmetrical growth) at the beginning of their development. The apical dominance of the primary root is reduced in actual and simulated microgravity in such a way that secondary roots elongate faster and are initiated very close to the primary root tip. According to Aarrouf et al. (1999), this could be due to the cessation of the activity of the root meristem provoked by a change in the hormonal balance (at least for IAA, ABA, and

zeatin). Such analyses should be performed on shoots since it seems that apical dominance of the shoot meristem is also weakened.

In space experiments the environment was most often not controlled and the atmosphere was not monitored in plant growth chambers. As apparently microgravity has a slight but continuous effect on plant growth, it may happen that other space factors could become prominent in microgravity. That is the reason why experiments without an onboard 1-g centrifuge should be questioned.

Another problem deals with the data that must be numerous enough to be analyzed by statistics, which is obviously in opposition with the need of using very small volumes or masses in space. New hardware is being developed like EMCS, for *European Multi-Cultivation System*, which will provide gas control and the availability of growing a (small) plant from seed to seed. This instrument should be helpful for determining the mechanism of the action of gravity on Earth (Figure 6-21).

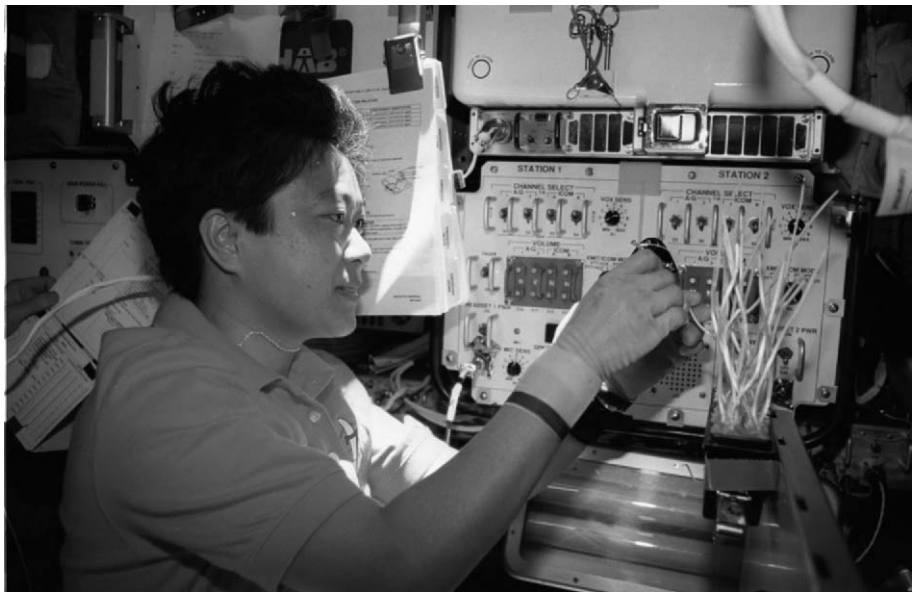

Figure 6-21. Astronaut Chiaki Mukai works with plant sprouts on board the Space Shuttle Discovery. Photo courtesy of NASA.

6 PLANTS AND THE SPACE ENVIRONMENT

6.1 Space Environment and the Organs Formation

Krikorian and O'Connor (1984) have studied first the emergence of seminal roots in space on sunflower, oat and mung bean, but on seedlings it was impossible to study the early stages of development of the root. They later used clonal tissue culture derived propagules of *Daylily* and *Haplopappus*. The micropropagated shoots have either no roots or roots were trimmed. During the flight, roots were formed and grew (Levine and Krikorian 1996). Krikorian et al. (1995) used also embryoids, which developed from so-called somatic embryo *initials* that have been multiplied in a liquid medium and subsequently inoculated onto semi solid media Petri dishes. These embryoids can mature and yields plantlets. Karyological studies were performed on these different types of biological samples grown in space and the results can be summarized in the following way (Krikorian 1998).

Sampling from intact, well-defined meristem like root tips of *Haplopappus gracilis* (2n = 4) derived from germinated seedlings shows fewer aberrations than those from de novo-generated root initials produced from aseptically generated propagules or stem cuttings. In the case of embryoids, the younger they are in their developmental progression, the more sensitive they are. The more advanced developmentally, the less damage.

It seems also that the least growth the least damage, but species with a fast rate of cell division like wheat tends toward higher resistance to mutation. The results suggest also that the more polyploid the system, the more resistant to perturbation it appears to be.

Thus, the more developed a system is, viewed by Krikorian (1996) as the more pre-stressed, the less likely it is to suffer stress effect in the space environment; the lesser developed, the greater the vulnerability. Intact seedlings and whole plants are predictably less vulnerable, whereas systems growing *de novo*, like tissue and cell cultures, are more vulnerable.

These results indicate that the developmental stage reached by the plants when launched in space is an important parameter. However, they did not prove that the effects of microgravity are direct or indirect (Krikorian 1996). For instance, convection does not occur in space orbiters since there is no gravity. Diffusion can provide a mixing of gases, for example, but much slower than convection does. In the case of intact plants in contact with the atmosphere, gas exchanges can be faster than in a liquid medium or on a solid medium.

6.2 Gas Composition of the Atmosphere in the Satellite

6.2.1 Ethylene

An experiment carried out by Kiss et al. (1998) has demonstrated the action of ethylene in the development of *Arabidopsis thaliana* grown in space. They observed that flight seedlings (both microgravity and 1-g control) were smaller (60% in total length) compared to the ground controls and to plants rotated on a clinostat. Seedlings grown in space had two structural features that distinguished them from the control, i.e., a greater density of root hairs and an anomalous hypocotyl hook structure. Kiss et al. (1998) have shown that slower growth and morphological changes observed in the flight seedlings may be due to ethylene present in the spacecraft since plant treated by 10-ppm ethylene on the ground presented the same features as in space.

A series of experiments have confirmed the involvement of ethylene in the disturbance of plant growth in microgravity. Super dwarf wheat was grown on board the Mir space station (Levinskikh et al. 1999). The height of the shoots was reduced by half in microgravity, and the number of headed shoots were 2.7 times less. No seed was found in the heads formed in space. The analyses showed that the most profound changes observed in the reproductive stage of this plant were caused by the phytotoxic effect of ethylene rather than spaceflight factors as its concentration in the Mir station amounted to 0.3-1.8 mg x m^{-3} which was high enough to account for the modifications observed in space grown wheat plants.

6.2.2 Oxygen

The analysis of plant growth in space indicated that microgravity exposure induces a metabolic response in the roots of *Arabidopsis* that was consistent with the hypoxia (Porterfield 2002). In two separate experiments, measurements of root *Alcohol Dehydrogenase* (Adh) showed that this enzyme had higher activity in microgravity-grown plants, and that the ADH gene was more expressed.

The transgenic *Arabidopsis* harboring the Adh linked to the *β-Glucuronidase* (GUS) reporter gene has been constructed to address whether hypoxia-induced responses occur in space (Paul et al. 2001). The Adh/GUS reporter was expressed only in roots during flight. However, on the ground when Arabidopsis roots were subjected to hypoxia, the reporter gene was always expressed in shoots even if these organs did not experience hypoxia. According to Paul and Ferl (2002), in space the normal hypoxia response signaling from the root to the shoot could be impaired or Adh/GUS activity is induced for unknown reasons. A 1-g centrifuge in space should be necessary to determine whether a gravity vector could restore the apparent disruption of signaling from the root to the shoot.

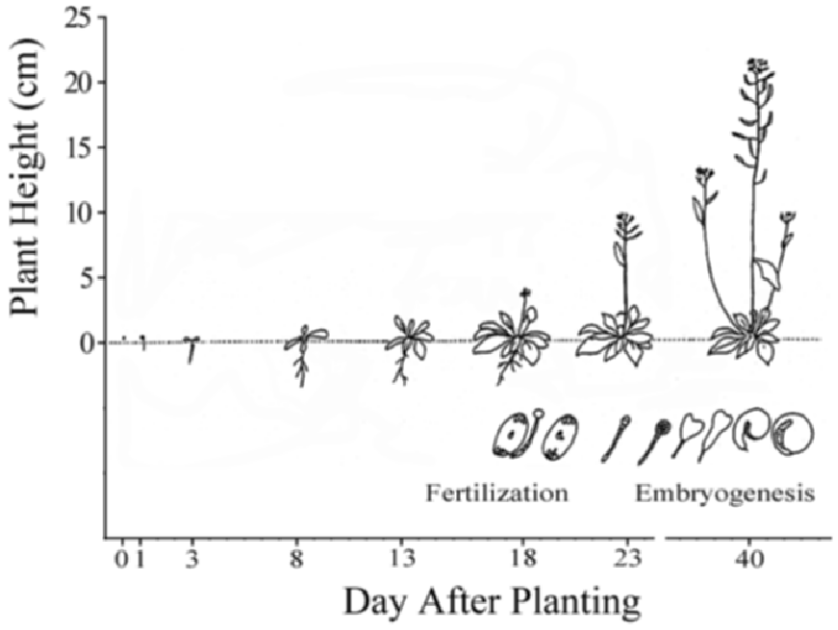

Figure 6-22. Development of Arabidopsis thaliana. This plant model presents a rosette of leaves and a reduced stem. In the culture conditions provided, the vegetative phase ends after 13 days and an inflorescence harboring the flowers grows in the following days. Fertilization occurs after 18 days and is followed by embryogenesis, which lasts about 20 days. Seeds are formed in siliques. A seed-to-seed cycle takes about 6 weeks. Adapted from Musgrave et al. (1998).

6.3 Gas Exchanges and the Reproductive Phase

Several attempts to grow plants through a complete cell cycle in space were unsuccessful because of delayed development and death of the plants (Halstead and Dutcher 1987, Kordyum 1997). *Arabidopsis thaliana* has been the most successfully studied species because of its small size and short life cycle (Figure 6-22). However, a partial or total sterility has been observed in this species (Merkys and Laurinavicius 1990).

By a detailed study of the different hardware used in space, Musgrave et al. (1997) were able to show that before 1997, about eight different hardware setups were used for studies on plant reproduction and that those having some kind of ventilation permitted seed formation. These authors performed three different experiments with the hardware called *Plant Growth Unit* (PGU) (see Figure 3-20). The differences in success of subsequent reproductive development in microgravity were related to variations in the gas phase of the plant growth chambers. In their first experiment (Kuang et al.

1996) no viable pollen was observed and young megaspores were deformed and empty. The authors hypothesized that during the experiment there was a limitation of carbohydrate synthesis, which could be due to a lack of carbodioxyde in the atmosphere. In their second experiment, they supplemented the gas phase with CO_2 and the plants had mature pollen and normal embryo sacs. However, no fertilization occurred because pollen was not released from the anthers.

In their following experiment (Musgrave et al. 1997), they provided a flow of filtered air through the plant growth chambers and development proceeded normally on orbit through the stage of immature seeds. The space-grown plants were similar to the ground control. In these three experiments, the plants were launched after a period of 13 days of growth and have completed their vegetative phase on the ground (Figure 6-22). It is not thus possible to determine if there was an effect of microgravity on their development before the reproductive stage.

Whether or not a seedling growing from the beginning in microgravity can flower and produce seeds is no more a matter of debate, since Musgrave et al. (2000) were able to obtained a seed-to-seed cycle with *Brassica napus*, as Merkys and Laurinavicius (1990) did with *Arabidopsis*. Seed quality in *Brassica* was also compromised by development in microgravity. The most surprising result of this experiment (Musgrave et al. 2000) deals with seed storage. Cytochemical analysis showed that starch was retained in the spaceflight material, whereas proteins and lipids were the primary reserves in the ground control seeds.

7 CONCLUSIONS

7.1 Plant Gravitropism: What is Known and What is to be Done

Microgravity has represented a very useful tool to analyze gravitropism since it was the only opportunity to clearly measure parameters such as the presentation time, the presentation dose, and the threshold acceleration for the gravitropic reaction. Although clinostats have been used for a century, their action remains not clear. Clinostat works (Aarrouf et al. 1999) have shown that the rotation about a horizontal axis on a clinostat could eventually produce a slight but continuous stimulation, so that the estimate of presentation time and dose calculated with this device should be questionable. However, the values obtained in space for these parameters are of the same order as those obtained on the clinostat, which validates to a certain extent the use of this device for studying these parameters. The re-examination of these parameters in the frame of the space experiments led Perbal et al. (2002) to demonstrate that the way of estimating the presentation dose (which was used

for decades) was not the best one. A new model for fitting the data has been proposed and it has been shown that the presentation dose did not measure gravisensitivity but dealt with the minimal stimulus to provoke a differential growth in the upper and lower halves of the organs. For stimulation doses less than the presentation dose, the stimulus is transduced but does not provoke a curvature. This can be due to a kind of resistance to curve of the growing organ (Pickard 1973). The *perception* dose (or perception time) should be the only parameter directly linked to the phase of perception and should be measured in space.

This result is important since it implies that the *perception* and the *transduction* phases can be very short, less than 1 sec at 1 g. In such a short period of time, the sedimentation of the amyloplasts should be very limited (for a rate of displacement equals to 1 μm x min^{-1} it should be 1/60 μm). In this case the potential energy dissipated by one amyloplast should not be greater than the thermal noise.

The level of acceleration that can be perceived by the organs is about 5 x 10^{-4} g for roots and 10^{-3} g for shoots (Shen-Miller et al. 1968), but these values were obtained on clinostat with a background of 1 g. The estimates obtained in space by Merkys and Laurinavicius (1990) were extrapolated from data obtained on plants placed on a centrifuge and subjected to 0.1 g or 0.01 g. These estimates are too high if the threshold acceleration (5 x 10^{-4} g) is about 20 times less than the lowest value (0.01 g) of acceleration the organs were subjected to. To confirm this, an experiment on the threshold acceleration will be performed on the International Space Station in the frame of the European microgravity program.

The main result obtained in space deals with the role of the endoplasmic reticulum (ER). Volkmann and Sievers (1979) have proposed that the pressure of the amyloplasts on the ER membrane should lead to the stimulus and to an asymmetrical signal coming from the cap. The fact that the statocytes are more sensitive in 0-g grown plants than in 1-g grown plants does not confirm this hypothesis (Perbal et al. 2004). In microgravity, the amyloplasts are situated near the nucleus, whereas in 1 g they are sedimented on the ER tubules. When a centrifugal force is applied to the organs, the probability of having contacts between amyloplasts and the ER tubules is therefore much less in 0 g than in 1 g, although the response is greater in 0 g than in 1 g. Thus, experiments performed in space brought a strong argument against the hypothesis based on a role of ER in the transduction of gravity stimulus. This conclusion is also supported by experiments carried out on the ground in which the ER tubules were displaced by centrifugal forces (Wendt et al. 1987).

The analysis of the statocyte polarity in space showed that the amyloplasts were in majority located in the center of the statocyte close to the nucleus (Perbal et al. 1987). The transfer from gravity to microgravity induces

a movement of the amyloplasts toward the nucleus (Volkmann et al. 1991, 1999, Lorenzi and Perbal 1990, Driss et al. 2000a), which shows that these organelles are not free in the statocyte. Treatment by cytochalasin B or D can strongly slow down this movement, which indicates that actin filaments could be responsible for the movement of the amyloplasts in microgravity (Buchen et al. 1993, Driss et al. 2000a). This result obtained in space led to a new hypothesis about the signal transduction of gravity stimulus. According to Volkmann et al. (1991), the amyloplasts could exert tension in the actin network, which becomes asymmetrical when the organ is placed horizontally (see Figure 6-11). However, there is a controversy between the authors who think that the amyloplasts are the gravisensors and those who think that the whole cell is the gravisensor (see Sack 1997). It may happen that both can play this role, the amyloplasts being more efficient than the protoplast. If the nature of gravisensor is still disputed (Barlow 1995), one must recognize that space experiments have brought new data about gravisensing, which forced plant physiologists to change their view on how plants sense gravity (Perbal and Driss-Ecole 2003).

Another finding concerns the regulation of root curvature by gravity. After a slight stimulation on a centrifuge, the roots show autotropism, i.e., straightening after several hours. On the opposite, a strong stimulation induces a curvature, which can lead the root to overshoot the direction of the stimulus. This overshooting does not occur on the ground or on a centrifuge, which demonstrates that gravity regulates the curvature. The mechanism of this regulation is not yet known but could depend on the amyloplast sedimentation (Perbal et al. 2004). On the ground, these organelles can move along the longitudinal wall in gravistimulated roots during the bending of the root, whereas when the root are placed in microgravity after stimulation on a centrifuge the amyloplasts are pulled away from the longitudinal wall, i.e., away from the mechanoreceptors.

To some extend the transduction pathway of gravistimulation could be analyzed in space by using transgenic plant expressing the aequorin gene in order to observe calcium responses under different stimulus conditions (see Figure 6-06). One experiment will be performed soon to examine calcium redistribution by the means of a special chemical fixation: the glutaraldehyde contains potassium antimonite, which reacts with calcium to form a precipitate that can be observed in electron microscopy. Such a technique was already used in space (Hilaire et al. 1995), but only on plants grown in microgravity and not subjected to gravistimulus.

With new tools that have been developed recently as *Arabidopsis* plants harboring a DR5::GUS construct (see Figure 6-16), it should be possible to analyze auxin distribution during and after gravistimulation on a 1-g centrifuge in space. The analysis of the distribution of the PIN and AUX proteins, which are auxin transporters, should also be investigated.

Figure 6-23. Astronaut Carl E. Walz holds a plant in the Russian Zvezda Module on board the International Space Station. Photo courtesy of NASA.

7.2 Contribution of Space Experiments to our Knowledge of Plant Development

The analysis of the development in space has shown that germination is normal in microgravity. However, even during the first steps of root growth, some differences can be observed between plants grown in microgravity or in 1 g. The orientation of the root tip during germination in space depends upon the orientation of the embryo within the seed (Volkmann et al. 1986). In lentil seedlings, there is a nastic movement, due probably to the fact that the embryo is curved in the dry seed. When the root germinates, its extremity bends first away from the cotyledon and then straightens out. This nastic movement is not clearly displayed on the ground because gravitropism is stronger than the nastic movement in orienting the root tip.

After germination in microgravity, roots can grow straight if they are in humid air, but if they are growing on agar or between agar plates, the orientation of their tip is random (Johnsson et al. 1996), at least for several days. After stimulation on a centrifuge in space, a gravitropic curvature occurs in microgravity for several hours, but it tends to disappear afterward (Stankovic et al. 1998a, 1988b). This phenomenon is called *autotropism* and cannot be observed on the ground because of the presence of gravity.

Although the morphology of the primary root is not strongly modified during the first two days of growth, there is a change in the cell cycle in the root meristem. In lentil roots, the first cell cycle appears to be longer in microgravity than in 1 g. After several cycles the delay seems to increase because the mitotic index in roots grown in microgravity is lower than in 1 g. In lentils, cell cycle is not changed when the roots are grown on a clinostat with exactly the same conditions of growth (same containers and so on). In this particular case, it is clear that clinostat cannot simulate the effects of microgravity, which shows the limits of the simulation.

The reproductive phase is completed in microgravity when the culture conditions are correct (Figure 6-23). A lot of problems encountered in growing plants in space are related to the fact that the physical environment is different in microgravity (Porterfield 2002), like the absence of convection and it is clear that the limitation of gas exchanges greatly influences plant growth (Musgrave et al. 1997).

Thus, it is clear that microgravity has a great impact on the development of plants. However, it remains to demonstrate whether it is due to (a) indirect effects on plant growth (for instance, lack of convection); or (b) direct effects (for instance, on cell cycle). It is now necessary to analyze these effects at the molecular level and in a well-monitored environment to remove the indirect effects of microgravity. EMCS (*European Multi-Cultivation System*) is a facility that will be used for plant growth in microgravity on board the ISS. It will have the advantage of monitoring gases (O_2, CO_2, ethylene) and to carry out experiment on a onboard 1-g centrifuge.

In microgravity, like on the clinostat, the apical dominance of the primary root over the secondary roots is reduced (Kordyum 1997, Aarrouf et al. 1999). The morphology of the root system is different from that observed in the vertical controls. In particular, there are a greater number of secondary roots and these roots grow faster.

The loss of apical dominance has been well documented with clinostat experiments (Driss-Ecole et al. 1994, Aarrouf et al. 1999). It is linked to the modification of the hormonal balance in the primary root. Experiments in space should be done to confirm that the reduced apical dominance results from the hormonal content in roots. Once again *Arabidopsis* harboring the DR5::GUS construct should be used to analyze auxin distribution in space grown seedlings.

Cell cycle has been intensively studied in plants in the last decade (for review, see Inzé 2005) and plant molecular biologists have the opportunity of using many molecular tools (Paul and Ferl 2002) to analyze plant growth in space. It should be important to confirm that gravity has an influence on the G_2/M transition, as hypothesized by Yu et al. (1999). This transition corresponds to a phase of checking which takes place just before the mitosis.

It is clear that we are far from understanding the causes of the changes in the development of plants in space. Many pioneering experiments have been done in space without monitoring gas composition, temperature and so on, so that the conclusion of their authors must be questioned since plants are very sensitive to external factors. More clear-cut results have been obtained on board Space Shuttle flights or the International Space Station, since dedicated facilities providing onboard 1-g controls and better culture conditions have been developed. The experience gained from the past studies will be useful for the future. Undoubtedly, future research on board the International Space Station will provide new insights on the role of gravity on plant growth and development (Figure 6-24).

Figure 6-24. This photograph shows a close-up view of sprouts on the Russian plant growth experiment performed by Expedition-6 crewmembers during their stay on board the International Space Station. Photo courtesy of NASA.

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Chapter 7

RADIATION BIOLOGY

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This chapter describes the radiation field in space and basic radiobiological mechanisms, and then concentrates on the specific biological responses resulting from exposure of biological systems to the space radiation environment. These responses include the biological effects of the heavy ions of cosmic radiation, and interactions of radiation with the other parameters of spaceflight, above all weightlessness. Comparisons are made with terrestrial findings using particle accelerators on Earth. The chapter concludes with concerns for radiation safety during human space missions and future research projects in radiation biology, particularly related to the planetary exploration program.

Figure 7-01. The Matroshka experiment, here shown on an outside wall of the ISS, measures radiation levels around and in a simulated (phantom) human torso made of natural bone and materials that resemble human tissue. Photo courtesy of ESA.

1 INTRODUCTION

1.1 Radiation on Earth

Planets and moons of our solar systems are exposed to a complex radiation field of galactic and solar origin (Figure 7-02). *Galactic Cosmic Radiations* (GCR) originate outside of our solar system in previous

cataclysmic events such as supernovae explosions. When they enter our solar system, their energies must be high enough to overcome the deflection by the magnetic fields of the solar wind. *Solar Cosmic Radiations* (SCR) consist of two components, the low energy solar wind particles that flow constantly from the sun, and the highly energetic *Solar Particle Events* (SPE) that are emitted from magnetically disturbed regions of the Sun in sporadic bursts.

The surface of the Earth is largely spared from this cosmic radiation due to the deflecting effect of the Earth's magnetic field and the huge shield of 1000 g/m² provided by the atmosphere. The terrestrial average annual effective dose equivalent from cosmic rays amounts to 0.30 mSv (for definition of units, see Section 3.3 in this Chapter), which is about 100 times lower than that experienced in interplanetary space. Natural radiations from terrestrial radioactive elements and diagnostic medical exposures to radiation increase the total annual effective dose equivalent to about 2.4 mSv. In areas of high concentrations of natural radionuclides, such as Kerala in India, annual dose values up to 13 mSv are reached. The maximum allowed annual dose for radiation workers amounts to 20 mSv. Ordinarily, aggregate background and diagnostic medical levels of radiation as well as the limits for occupational radiation exposure pose little risk to human health. Under this clement level of background radiation our biosphere has flourished since its beginnings about 4 billion years ago.

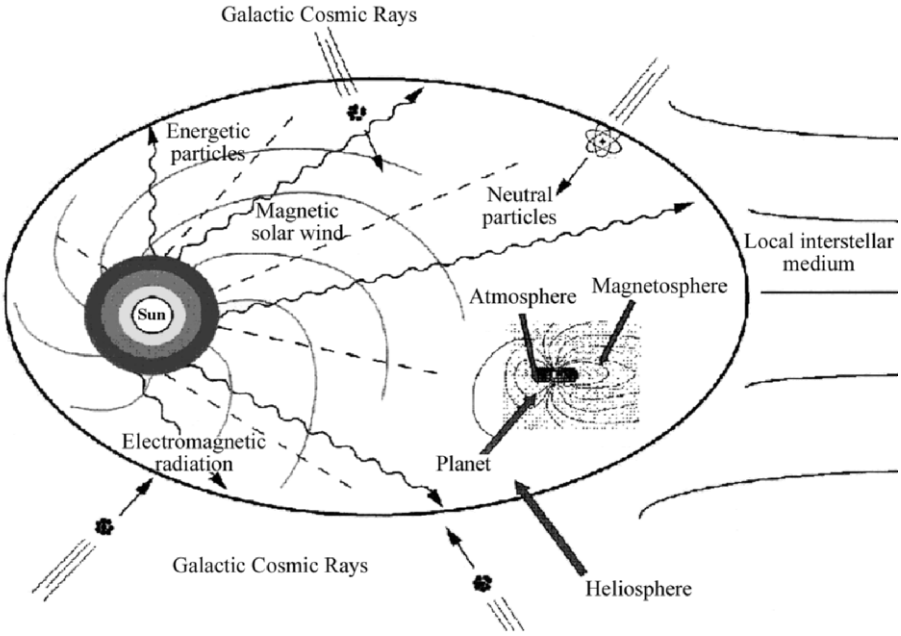

Figure 7-02. The radiation environment in our solar system.

1.2 Radiation in Low Earth Orbit

Since the advent of space flight and the establishment of long-duration space stations in Earth orbit, such as Skylab, Salyut, Mir, and the *International Space Station* (ISS), the upper boundary of our biosphere has extended into space. Such space missions expose humans and any other biological system to a radiation environment of a composition and intensity not encountered on Earth. In *low Earth orbit* (LEO), in addition to the GCR and SCR, the radiation field comprises a third source of radiation, the *Van Allen belts*, which are a result of the interaction of the GCR and the SCR with the Earth's magnetic field and the atmosphere. Above all, electrons and protons and some heavier ions are trapped in the geomagnetic field. Depending on the orbit parameters and flight data, radiation doses in the range of 20 mSv/month are received (Reitz et al. 1995). Of special importance for low Earth orbit is the so-called *South Atlantic Anomaly* (SAA), where the fringes of the inner proton radiation belt reach down to altitudes of 400 km. This behavior reflects the displacement of the axis of the geomagnetic (dipole) field by about 450 km with respect to the axis of the geoid[1] with a corresponding distortion of the magnetic field. This region accounts for up to 90% of the total exposure in low Earth orbit.

Inside the spacecraft the radiation field is modified by interaction processes with the shielding material, including activation, fission, and bremsstrahlung[2]. Secondary radiations, both charged and uncharged, are created during these processes. Radioactivity may also be induced, being slowly built up in the spacecraft. Only a very rough description of the radiation field inside the spacecraft can be made, especially in cases where the shielding is not constant, e.g., because of consumption of fuel or water, or of different orientations of the spacecraft relative to the Earth.

To prevent detrimental health effects caused by the radiation environment of space, radiation protection guidelines have been elaborated for humans in space. These guidelines are based on:

a. Dosimetry and modeling of the radiation field in space;
b. Studies on the biological effects of the heavy ions of cosmic radiation encountered in space or produced at heavy ion accelerators on ground;
c. Studies on potential interactions of cosmic radiation and other parameters of spaceflight, above all microgravity.

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[1] The *geoid* is a undulating surface of constant gravitational force that approximates the shape of the Earth.

[2] *Bremsstrahlung* ("breaking radiation" in German) is the electromagnetic radiation produced by the acceleration of a charged particle, such as an electron, when deflected by another charged particle, such as an atomic nucleus. The term is also used to refer to the process of producing the radiation.

1.3 Radiation Beyond Low Earth Orbit

Human missions beyond LEO, such as to the Moon, Mars, asteroids, or even to the moons of Jupiter and Saturn are considered as a natural extension of the current human activities in space. Such long journeys outside the protective umbrella of the geomagnetic field will expose both astronauts and equipment to the radiation environment found in the deep space (Horneck et al. 2003a). The lunar surface radiation environment is characterized by the GCR and SCR in the near Earth environment that impact the lunar surface thereby producing secondary particles which diffuse from the surface into the local environment. The mass shielding effect of the Moon itself is nearly a factor of two. On Mars which, like the Moon, lacks a magnetic field, GCR and SCR interact directly with the Martian atmosphere, whereby low energy charged particles are stopped and the composition of the particle fields penetrating to the surface of Mars is modified (Horneck et al. 2003b, Horneck et al. 2005). In the vicinity of Jupiter, the solar wind produces less deflecting effects on the GCR relative to the Earth. In addition, Jupiter's huge magnetic field traps electrons in a radiation belt extending up to large distances from Jupiter. The mechanism of magnetic trapping of radiation at Jupiter is the same that operates in the Earth's Van Allen belts. For a human mission to, e.g., Jupiter's moon Callisto, when crossing the jovian electron belts, peak radiation doses up to 3 mSv/day have been calculated for the habitat behind a 4 g/cm² shielding of the spacecraft (De Angelis et al. 2004).

1.4 Radiation and Life

It might also be possible for life to be confronted with the radiation of space by natural processes. Our atmosphere teems with viruses, bacteria, algae, microfungi, fungal spores, spores of mosses and ferns, pollen, minute seeds, and protozoan cysts. These are found at concentrations of possibly hundreds to thousands per cubic meter. Viable microorganisms, predominantly black conidia and fungal spores, have even been found at altitudes as high as 77 kilometers. In these instances, pigmentation protects the cells against the intense solar UV radiation prevalent at these high altitudes.

Since the discovery of a certain group of meteorites of probable Martian origin it has become obvious that matter can be exchanged between the planets of our solar system, e.g., from Mars to Earth. Especially during the early phase of heavy bombardment, which lasted until approximately 3.8 billion years ago, up to kilometer size bodies have struck the planets of our solar system. Such gigantic impacts lead to the ejection of a considerable amount of soil and rocks that are thrown up at high velocities, some fraction reaching escape velocity. These ejecta leave the planet and orbit around the sun, usually for time scales of a few hundred thousand or several million

years until they either impact another celestial body or are expelled out of the solar system. The question arises whether such rock or soil ejecta could also be the vehicle for life to leave its planet of origin. Soil microorganisms or endolithic microbial communities are candidate terrestrial microbial systems that might be ejected by such large impacts. If so, they will be exposed to space radiation during their interplanetary journey. Radiation effects and potential protection and repair mechanisms clearly could have profoundly affected the chances for a viable transfer of microbes within our solar system (Mileikowsky et al. 2000).

Among the planets of our solar system, Mars and probably the Jovian moon Europa are considered as best candidates for providing the prerequisites for the support of life, either in the past or present (Horneck and Baumstark-Khan 2002). A putative Martian biota would currently be exposed to much higher radiation dose levels than life on Earth. This is the consequence of the fact that Mars presently does not possess either an effective magnetic field or a thick atmosphere. Therefore, the biologically effective dose at the surface of Mars caused by ionizing radiation from space is about 100 times higher than that at the surface of the Earth. However, this might have been different in the past, when Mars possessed a denser atmosphere and even a magnetosphere.

The Jovian moon Europa lies deep within the strong magnetosphere of Jupiter which is filled with ionizing, magnetically trapped particle radiation of galactic and solar origin. This results in radiation doses of 500,000 Gy/year at its surface (for definition of units, see Section 3.3 in this Chapter). However, the thick crust of water ice at the surface of Europa effectively shields the lower layers against radiation. At a depth of 10 cm, the dose is decreased by 3 orders of magnitude to 600 Gy/year. At greater depths, the radiation environment continues to decrease, reaching values similar to those in the Earth's biosphere below an ice layer of 20 to 40 m. Hence, for putative indigenous life below a shallow depth or in the ocean below the kilometer thick ice crust, radiation is not a significant environmental hazard factor.

2 THE RADIATION FIELD IN SPACE

In the interplanetary space, the radiation field is composed mainly of the SCR and the GCR. In the vicinity of the Earth, a third radiation component, trapped by the Earth's magnetosphere, is present, the so-called Van Allen belts (McCormack et al. 1988, Reitz et al. 1995). Typical integral energy spectra for these radiation components in the vicinity of the Earth are shown in Figure 7-03.

SCR consist of the low energy solar wind particles that flow constantly from the sun and the SPEs that originate from magnetically disturbed regions of the sun which sporadically emit bursts of charged particles with high energies. These events are composed primarily of protons with a minor component (5-10%) being helium nuclei (alpha particles) and an

even smaller part (1%) heavy ions and electrons. SPEs develop rapidly and generally last for no more than some hours, however some proton events observed near Earth may continue over several days. The emitted particles can reach energies up to several GeV (Figure 7-03). In a worse case scenario, doses as high as 10 Gy could be received within a short time. Such strong events are very rare, typically about one event during the 11-year solar cycle. Concerning the less energetic, though still quite intensive events, e.g., in cycle 22 (1986-1996), there were at least eight events for proton energies greater than 30 MeV. For LEO, the Earth's magnetic field provides a latitude dependent shielding against SPE particles. Only in high inclination orbits and in interplanetary missions, SPEs create a hazard to humans in space, especially during extravehicular activities.

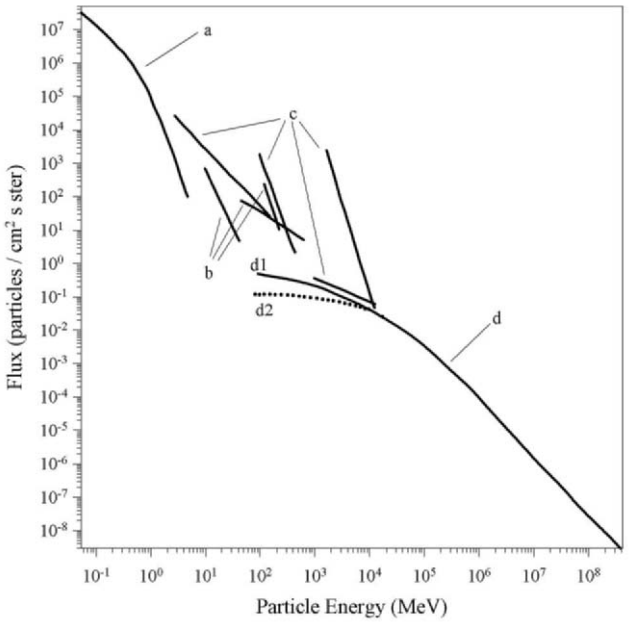

Figure 7-03. The energy spectra of the components of the radiation field in space in the vicinity of the Earth: (a) electrons (belts); (b) protons (belts); (c) solar particle events; (d) heavy ions of galactic cosmic radiation; (d1) during solar minimum; (d2) during solar maximum.

GCR originate outside the solar system in cataclysmic astronomical events, such as supernova explosions. Detected particles consist of 98% baryons and 2% electrons. The baryonic component is composed of 85% protons (hydrogen nuclei), with the remainder being alpha particles (helium nuclei) (14%) and heavier nuclei (about 1%). The latter component comprises particles of *High charge Z and high Energy* (HZE), which are defined as cosmic ray primaries of charges Z>2 and of energies high enough to penetrate at least 1 mm of spacecraft or of spacesuit shielding.

Although they only contribute to roughly 1% of the flux of GCR, they are considered as a potential major concern to living beings in space,

especially for long-term missions at high altitudes or in high inclination orbits, or for missions beyond the Earth's magnetosphere. Reasons for this concern are based on one hand on the inefficiency of adequate shielding and, on the other hand, on the special nature of lesions produced by HZE particles (see Section 4.1). If the particle flux (flow rate) is weighted according to the energy deposition, iron nuclei will become the most important component although their relative abundance is comparatively small.

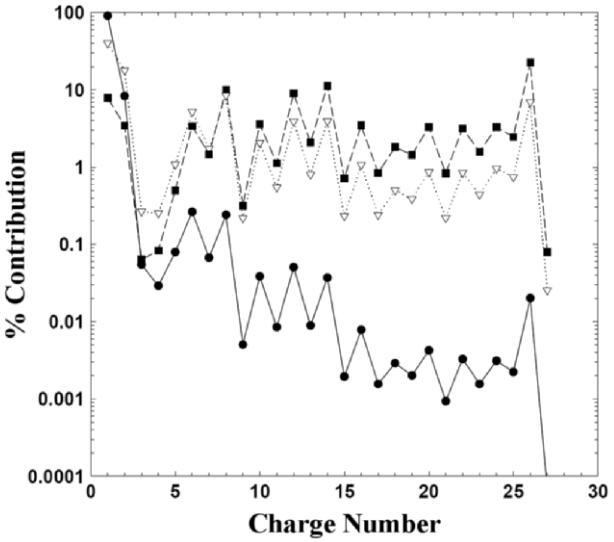

Figure 7-04. Filled circles: Percent contributions from individual GCR nuclei to the particle flux. Open triangles: Radiation dose, weighted by the square of the charge Z of the particle. Filled squares: Dose equivalent at solar minimum. Adapted from Cucinotta et al. (2003).

The fluence[3] of GCR is isotropic and energies up to 10^{20} eV can be present (Figure 7-03). When GCR enter our solar system, they must overcome the magnetic fields carried along with the outward-flowing solar wind, the intensity of which varies according to the about 11-year cycle of solar activity. With increasing solar activity the interplanetary magnetic field increases, resulting in a decrease of the intensity of GCR of low energies. This modulation is effective for particles below some GeV per nucleon. Hence the GCR fluxes vary with the solar cycle and differ by a factor of approximately five between solar minimum and solar maximum with a peak level during minimum solar activity and the lowest level during maximal solar activity (Figure 7-03). At peak energies of about 200-700 MeV/u during solar minimum, particle fluxes reach $2x10^3$ protons per 100 μm^2 per year[4] and 0.6 Fe-ions per 100 μm^2 per year.

[3] The *fluence* is the product of particle flux and time, expressed in units of particles or energy per square centimeter.

[4] 100 μm^2 is the typical cross-section of a mammalian cell nucleus.

Figure 7-04 shows the frequency distribution of the GCR nuclei. Although iron ions are one-tenth as abundant as carbon or oxygen, their contribution to the GCR dose is substantial, since dose is proportional to the square of the charge. This is visualized by the curve with the open triangles in the figure where the abundances of the GCR nuclei are weighted by the square of the charge of the particle to give a measure of the "ionizing power", the radiation dose.

The fluxes of GCR are further modified by the geomagnetic field. Only particles of very high energy have access to low inclination orbits. Towards higher inclination particles of lower energies are allowed. At the pole, particles of all energies can impinge in the direction of the magnetic field axes. Due to this inclination dependent shielding, the number of particles increases from lower to higher inclination.

In the vicinity of the Earth, the Van Allen belts are a result of the interaction of GCR and SCR with the Earth's magnetic field and the atmosphere. Two belts of radiation are formed, comprising electrons and protons, and some heavier particles trapped in closed orbits by the Earth's magnetic field. The main production process for the inner belt particles is the decay of neutrons produced in cosmic particle interactions with the atmosphere. The outer belt consists mainly of trapped solar particles. In each zone, the charged particles spiral around the geomagnetic field lines and are reflected back between the magnetic poles, acting as mirrors. Electrons reach energies of up to 7 MeV and protons up to about 200 MeV. The energy of trapped heavy ions is less than 50 MeV although their radiobiological impact is very small (Figure 7-03). The trapped radiation is modulated by the solar cycle: proton intensity decreases with high solar activity, while electron intensity increases, and vice versa.

3 BASIC RADIATION BIOLOGY

Throughout almost 4 billion years, life on Earth has been shaped by interactions of the organisms with their environment and by numerous adaptive responses to environmental stressors. Among those stressors, radiations, both of terrestrial and of cosmic origin, are a persistent stress factor that life has to cope with. Radiation interacts with matter primarily through the ionization and excitation of electrons in atoms and molecules. These matter-energy-interactions have been decisively involved in the creation and maintenance of living systems on Earth. Because it is a strong mutagen, radiation is considered a powerful promoter of biological evolution on the one hand, and an account of deleterious consequences to individual cells and organisms, e.g., by causing inactivation or mutation induction, on the other. In response to the harmful effects of environmental radiation, life has developed a variety of defense mechanisms, including the increase in the

production of stress proteins, the activation of the immune defense system, and a variety of efficient repair systems for radiation-induced DNA injury.

There are two alternative ways of radiation damage to the biological key substances, such as proteins, RNA, and DNA: either by direct energy absorption (*direct radiation effect*), or via interactions with radicals, e.g., produced by radiolysis of cellular water molecules (*indirect radiation effect*) (Figure 7-05).

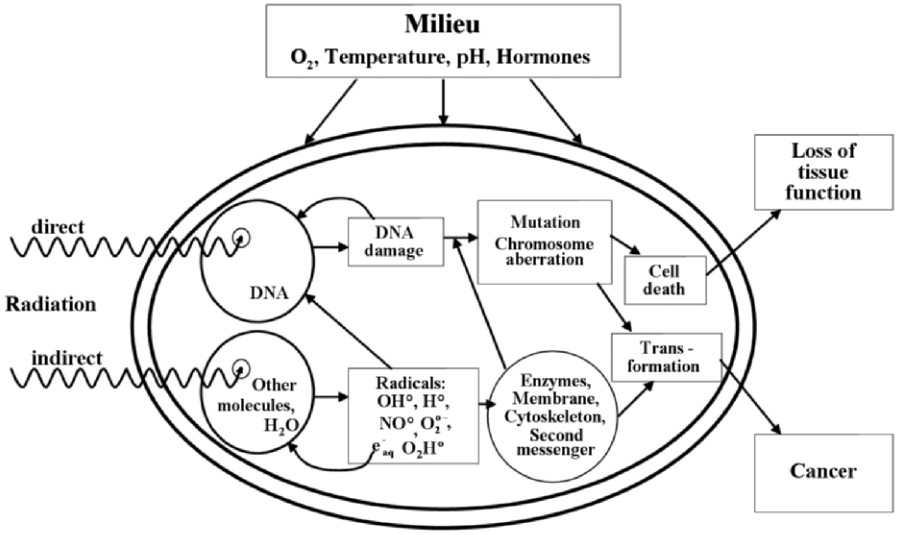

Figure 7-05. Radiobiological chain of events within a biological cell with the two alternative pathways of radiation damage, resulting in either direct or indirect radiation effects.

3.1 Indirect Radiation Effects

Water is the main constituent of all living systems. In somatic and vegetative cells its fraction lies between 40 and 70%, even in bacterial spores it amounts still around 20%. Therefore, in irradiated cells, most of the energy is absorbed by water molecules, which are either excited or ionized (Equation 1). Excitation of a water molecule is often followed by splitting of the molecule (Equation 2).

$$H_2O \xrightarrow{h\cdot\nu} H_2O^+ + e^-_{aq} \qquad \text{(Ionization)} \qquad [1]$$

$$H_2O \xrightarrow{h\cdot\nu} H^\bullet + {}^\bullet OH \qquad \text{(Splitting)} \qquad [2]$$

Hence, the primary products are H•, •OH, H_2O+ and electrons. All these species possess unpaired electrons, thus being highly reactive free

radicals. The electrons are particularly reactive and capture another water molecule thus forming a negatively charged ion (Equation 3).

$$H_2O + e^-_{aq} \longrightarrow H_2O^- \qquad [3]$$

The ions H_2O^+ and H_2O^- are not stable and almost immediately (10^{-16} seconds) dissociate into H^+ ions and $^{\bullet}OH$ radicals as well as into ^-OH ions and $H^{\bullet}$ radicals (Equation 4).

$$\begin{aligned} H_2O^+ &\longrightarrow H^+ + {}^{\bullet}OH \\ H_2O^- &\longrightarrow H^{\bullet} + {}^-OH \end{aligned} \qquad [4]$$

There will be a number of reactions among the free radicals themselves, thereby either reconstituting water (Equation 5) or forming molecular hydrogen and hydrogen peroxide (Equation 6). The interactions of free radicals both among themselves and with their own reaction products are dependent primarily on how closely they have been formed. After they are formed, they must diffuse through the medium until they encounter something with which they may interact. The probabilities of these reactions are favored within spurs, blobs and tracks. Interactions with other solute molecules are only possible, if the primary species are able to escape these zones.

$$e^-_{aq} + H^+ \longrightarrow H_2O \qquad \text{(Recombination)} \qquad [5]$$

$$H^{\bullet} + {}^{\bullet}OH \longrightarrow H_2O$$

$$e^-_{aq} + H_2O^- \longrightarrow H_2 + 2\ {}^-OH \qquad [6]$$

$$^{\bullet}OH + {}^{\bullet}OH \longrightarrow H_2O_2$$

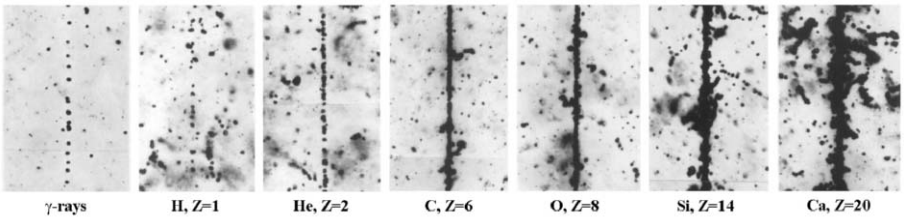

Figure 7-06. Tracks in photo-emulsions of electrons produced by γ-rays and tracks of different nuclei of the primary cosmic radiation moving at relativistic velocities. For biological radiation effects, the efficiency of a radiation type increases as the ion density along the tracks increases.

With increasing density of ionization, i.e., with increasing *Linear Energy Transfer* (LET), the number of changed molecules increases leading

to an increase of radiation effects in cells. Densely ionizing radiations, such as the heavy ions and α particles of radiations in space, produce clusters of ions and radicals that are very close together (Figure 7-06). Consequently, there will be a high probability of interactions between free radicals as well as with the key molecules of the cell (e.g., proteins and nucleic acids) leading to a broad spectrum of DNA lesions including damage to nucleotide bases, cross-linking, and DNA single- and double-strand breaks (Figure 7-07). In summary, for the indirect radiation effects the number of inactivated molecules depends on the dose and on the concentration of the water molecules.

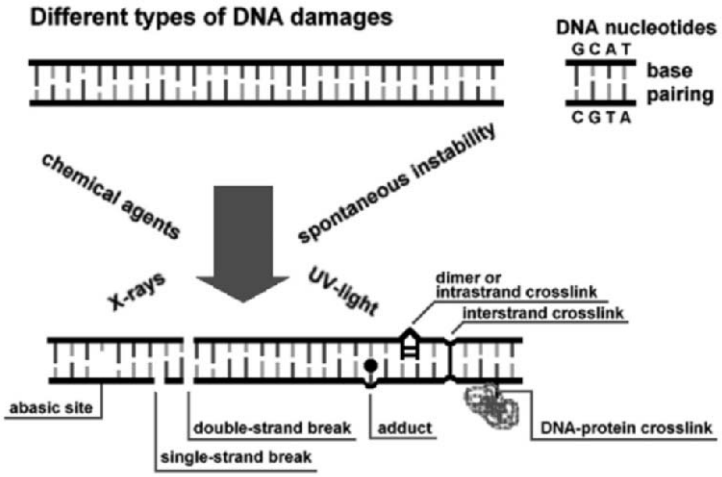

Figure 7-07. Different types of DNA damage induced by ionizing radiation and other genotoxic agents.

3.2 Direct Radiation Effects

For the direct radiation effect, the mean number of inactivated molecules of, e.g., DNA, is directly proportional to the dose. Despite all of these lesions (Figure 7-07), the DNA is functionally more stable than the two other cellular macromolecules, RNA and protein. This stability can be attributed to the following three factors:

a. The primary structure of DNA is all that is needed for transfer of information;
b. Because of the double-helical structure, DNA carries the information in duplicate;
c. There are molecular mechanisms of different complexity to undo the DNA damage thus maintaining cellular survival as well as genetic integrity.

DNA repair encompasses the molecular reactions which eliminate damaged or mismatched nucleotides from DNA. There are a variety of repair mechanisms, each catalyzed by a different set of enzymes. Nearly all of these mechanisms depend on the existence of two copies of the genetic information, one in each strand of the DNA double helix. If the sequence in one strand is accidentally changed, information is not lost irretrievably, because a complementary copy of the altered strand remains in the sequence of nucleotides in the other strand. However, incomplete or erroneous DNA repair may also lead to mutations and consequently to cancer or cell death (see Figure 7-05).

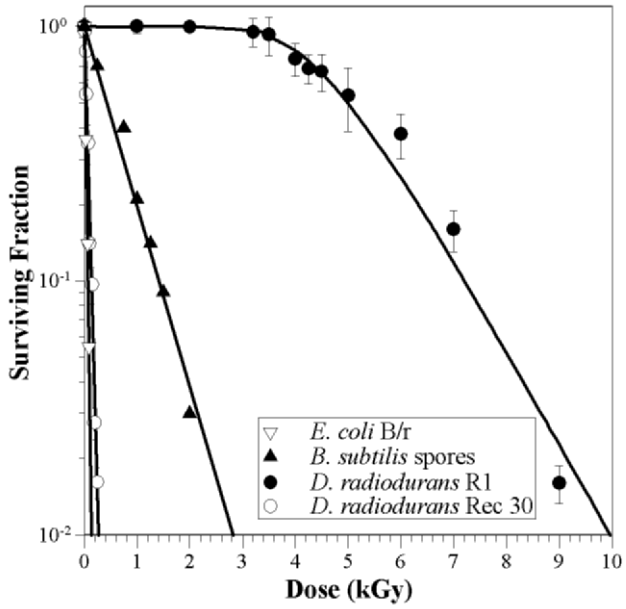

Figure 7-08. Representative dose-effect curves for survival of the bacteria Deinococcus radiodurans R1 (filled circles) and its recombination deficient mutant Rec30 (open circles), compared to survival curves of spores of Bacillus subtilis (filled triangles) and cells of Escherichia coli B/r (open triangles) following exposure to X-rays.

3.3 Radiation Units

Cellular reproducibility and conservation of genetic stability are the two cellular functions most important for the development and maintenance of life. However, both tasks can be disturbed by ionizing radiation with the result of cell death or induction of mutations. Cell survival, in radiobiological terms, is understood as the ability for indefinite reproduction. In dose-effect curves, the surviving fraction of irradiated cells relative to that of non-irradiated cells is plotted on the ordinate logarithmically versus dose on a linear abscissa scale (Figure 7-08).

With increasing dose the number of survivors decreases. The corresponding dose-effect curve declines continuously and can be simply characterized by two parameters. One is the D_0, which is defined as the dose

necessary to reduce survival to e^{-1} (=0.37), it can be calculated from the slope of the terminal straight part of the curve (–1/slope). The other parameter is the extrapolation number *n*, which is calculated from the backward extrapolation of the straight proportion of the effect curve.

Radiation sensitivity of different organisms can be compared on the basis of these parameters. Their sensitivity is related to the amount of genetic material per cell and to their DNA repair capacity. The most resistant organisms are exclusively single-stranded viruses, followed by double-stranded viruses, bacteria, algae, and yeast. For simple eukaryotes, it could be shown that haploid cells are about twice as sensitive as diploid cells. The most radiation resistant bacterium known is *Deinococcus radiodurans*. It was originally isolated from samples of canned meat that were thought to be sterilized by high doses of γ-radiation. Typically, it is found in locations where most other bacteria have died under extreme conditions, ranging from the shielding pond of a radioactive cesium source to the surfaces of Arctic rocks. *D. radiodurans* can tolerate doses up to 4 kGy without remarkable cell death (Figure 7-08).

Ionizing radiation is measured in the S.I. unit of *absorbed dose per mass unit*, the *Gray* (Gy), with 1 Gy equal to the net absorption of 1 J in 1 kg of water. Compared to the previously used unit *rad*: 1 Gy = 100 rad. However, the biological effectiveness of radiation largely depends on the local energy distribution, the *Linear Energy Transfer* (LET). Therefore, different qualities of radiation can have different biological effectiveness, even at the same physical dose. The *Relative Biological Effectiveness* (RBE) describes this dependence of the biological effectiveness on LET. RBE is the ratio of the physical doses of the test radiation and e.g., X-rays, leading to the same biological effect. The RBE value can be different for different biological systems, depending on their stage in the growth cycle and other environmental factors, such as the oxygen content.

In order to assess the effectiveness posed by radiation to humans and also the whole biosphere, estimates must be made of both the amount and type of radiation under consideration as well as the radiobiological effectiveness of the different components of the radiation. For this purpose, the *Quality Factor* (Q) has been introduced. Q is the biological weighting function of ionizing radiation and has been obtained by averaging over a variety of RBE values for the same LET value. Its relation to the LET of the radiation is shown in Figure 7-09.

It should be stressed that Q is an estimate of maximum RBE for the biological endpoint cancerogenesis only. For X-rays and γ rays, Q is equal to unity. For a given dose of high-LET radiation, the *dose equivalent* H is the product of the quality factor Q and the absorbed dose D (Equation 7):

$$H = QD \qquad [7]$$

The S.I. unit for the *dose equivalent* is *Sievert* (Sv)[5]. For a mixed radiation field composed of ionizing radiations of different *radiation qualities i* (as encountered in space), the dose equivalent H is given by (Equation 8).

$$H = \sum N_i Q_i D_i (Sv) \qquad [8]$$

with D_i = absorbed dose, deposited in biological matter by the radiation *i* (Gy), Q_i = radiation quality which is described as a function of LET, and N_i = a special factor which accounts for specific exposure conditions (e.g., dose rate, fractionated exposure, microgravity) or special physiological properties. For terrestrial radiation protection applications, N is set equal to unity.

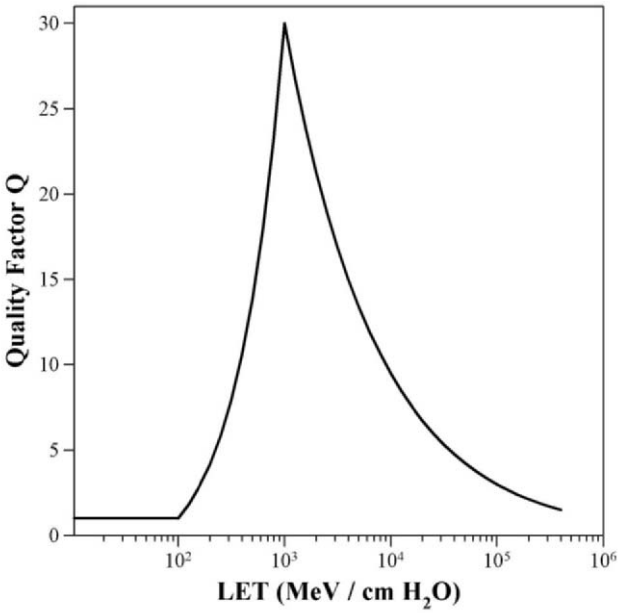

Figure 7-09. The quality factor Q is the biological weighting function of ionizing radiation and is dependent on the linear energy transfer (LET) of the radiation under consideration.

3.4 Effects of Radiation Exposure on Humans

The quality factor has originally been developed for radiation protection purposes. Therefore it is mainly based on radiation risks for cancer induction in mammals. The effects of radiation exposure on humans can be grouped into two basic categories: acute effects or delayed effects. Acute effects usually appear quite soon after exposure when people receive high

[5] Figure 7-04 shows the percent contribution from the different ions of GCR to the dose equivalent.

doses in a short period of time (minutes to a few hours). Delayed effects, such as cancer, can occur when the combined dose and dose rate are too small to cause acute effects leading to death or early morbidity[6].

The *Acute Radiation Syndrome* (ARS) is a sequence of phased symptoms which vary with individual radiation sensitivity, type of radiation, and the radiation dose absorbed. After radiation exposure with doses well above 1 Sv, the ARS is characterized by the rapid onset of nausea, vomiting, and malaise, which is followed by a nearly symptom free phase of weeks to days, depending on dose. Humans who have received doses of radiation between 0.7 and 4 Sv will have depression of bone-marrow function, known as the hematopoietic syndrome. This syndrome leads to decreased resistance to infections from lymphocyte deprivation and anemia within 2-6 weeks and death from sepsis. Death rate for this syndrome peaks at 30 days after exposure, but continues out to 60 days. Higher single doses of ionizing radiation (6–8 Sv) will result in a gastrointestinal syndrome, including severe fluid losses, hemorrhage, and diarrhea, starting after a short latent period of a few days to a week. Derangement of the luminal epithelium and injury to the fine vasculature of the sub mucosa lead to loss of intestinal mucosa. Without treatment, radiation enteropathy consequently results in an inflammatory response upon infection by bacterial transmigration. Deaths from sepsis may occur between 3 and 10 days post exposure. After radiation with very high acute doses (20–40 Sv) and a very short latent period from several hours to 1 to 3 days, the clinical picture is of a steadily deteriorating state of consciousness with eventual coma and death (neurovascular syndrome). Symptoms include loss of coordination, confusion, convulsions, shock, and the symptoms of the blood forming organ and gastrointestinal tract syndromes, survivors cannot be expected.

The *Chronic Radiation Syndrome* (CRS) was defined as a complex clinical syndrome occurring as a result of the long-term exposure to total radiation doses that regularly exceed the permissible occupational dose by far (2-4 Sv/year). Clinical symptoms are diffuse and may include sleep and/or appetite disturbances, generalized weakness and easy fatigability, increased excitability, loss of concentration, impaired memory, mood changes, headaches, bone pain, and hot flashes. The severity of delayed effects depends on dose. These delayed effects may include cancer, cataracts, non-malignant skin damage, death of non-regenerative cells/tissue, genetic damage, impact on fertility, and suppression of immune functions.

For radiation doses <1 Sv per year the induction of tumors is the most important long-term secondary disorder. Tumor induction with low doses is considered to occur stochastically, that means as a consequence based on statistical probability. Nevertheless, most of the data used to construct risk

[6] *Morbidity* is a disease, condition or state.

estimates are taken from radiation doses greater than 1 Sv and then extrapolated down for low-dose probability estimates. Significant direct data are not available for absolute risk determination of doses less than 0.1 Sv. In the case of the various radiation-induced cancers seen in humans, the latency period may be several years up to 2-3 decades. It is difficult to address the radiation-induced cancer risk on Earth of an individual person due to the already high background risk of developing cancer. Even less is known on cancer risk from complex space radiation.

4 RESULTS OF RADIO-BIOLOGICAL STUDIES IN SPACE

The accessibility of the unique radiation environment in space and the increasing involvement of human beings in space missions have initiated space activities in fields of radiobiological research as follows:

a. Biological mechanisms of *cosmic ray heavy ions*, the so-called HZE particles;
b. Impact of spaceflight environment on biological radiation response;
c. Radiation dosimetry;
d. Radiation protection issues.

The results of these studies are discussed in the following sections.

4.1 Biological Effects of HZE Particles

To understand the ways by which single particles of cosmic radiation interact with biological systems, methods have been developed to precisely localize the trajectory of an HZE particle relative to the biological object and to correlate the physical data of the particle relative to the observed biological effects along its path.

Such effect-particle correlations were accomplished in spaceflight experiments in different ways:

a. By use of visual track detectors that were sandwiched between layers of either biological objects in resting state, like viruses, bacterial spores, plant seeds or shrimp cysts, or embryonic systems, like insect eggs, realized in the so-called *Biostack* concept (Figure 7-10) (Bücker and Horneck 1975);
b. By use of nuclear track detectors that were in fixed orientation to biological targets of interest, like implantations beneath the scalp of animals or helmet devices for astronauts (see Kiefer et al. 1996 for review);
c. By correlating the occurrence of radiation effects, like the light flash phenomenon, with orbital parameters, such as passages through the SAA of the radiation belts.

The results from experiments in space investigating the radiobiological importance of the HZE particles of cosmic radiation are summarized by Horneck (1992), Swenberg et al. (1993), and Kiefer et al. (1996). The major findings are discussed below.

4.1.1 Effects on Biological Systems in Resting State

The need for experimental methods to localize each penetrating HZE particle and to determine its relationship to potential biological effects along its path, so far, has been accomplished in experiments on biological systems in resting state. For that purpose, monolayers of selected biological objects, fixed in position, were sandwiched between visual nuclear track detectors (Figure 7-10). Post flight analysis comprised steps as follows:

a. Localization of each HZE particle's trajectory in relation to the biological specimens;
b. Separate investigation of the response of each biological individual hit, in regard to radiation effects;
c. Determination of the impact parameter (i.e., the distance between particle track and sensitive target);
d. Determination of the physical parameters (Z, E, LET) of the relevant HZE particles;
e. Correlation of the biological effect with the HZE particle parameters.

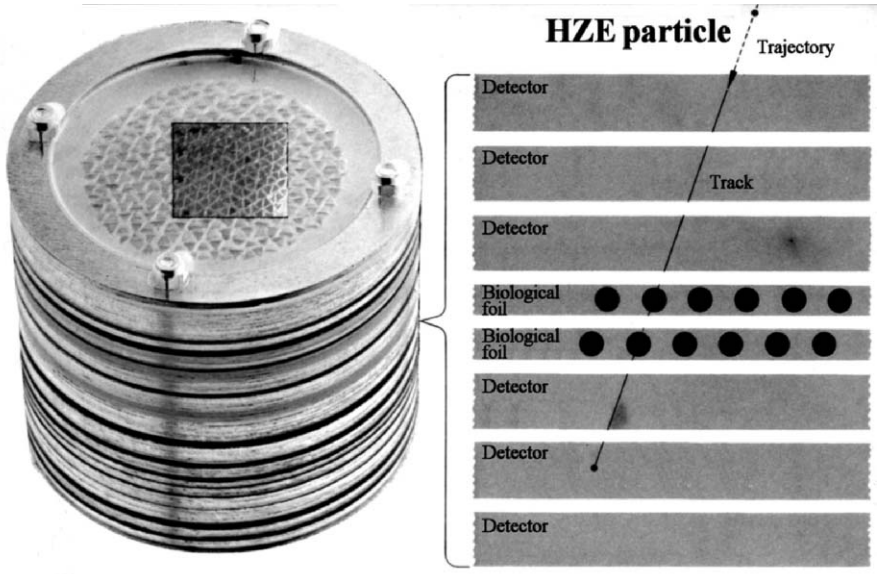

Figure 7-10. The Biostack concept to localize biological effects produced by single HZE particles of cosmic radiation. Biostack experiments were flown on board Apollo-16, -17, ASTP, Spacelab-1, D1, IML-1, IML-2, LDEF, Cosmos-1887 and -2004, and EURECA missions.

The biological systems investigated, all of them being in resting state, were characterized by a long shelf life. They had to endure fixed arrangement between the track detectors and relatively long pre- and post flight storage periods. A large variety of biological specimens, such as bacteriophages, bacterial spores, plant seeds, and animal cysts allowed the evaluation of radiation effects at different levels of biological organization. These specimens possessed different radiation sensitivities (as known from radiobiological experiments with X-rays, γ-rays, or electrons), and they consisted of either replaceable or non-replaceable cells, or embryonic tissue, respectively. Sandwiches of this type of combination of biological layers and nuclear track detectors were flown on several space missions (see Horneck 1992 for review).

In bacteriophage T4, placed in thin films between plastic track detectors during the ASTP mission, the mutation frequency was increased by a factor of 14 in areas traversed by an HZE particle compared to ground controls. The majority of genetic changes (65%) consisted of small deletions, insertions, inversions, elongated deletions, or multiple lesions, respectively, which let suggest DNA strand breaks to be the primary radiation damage.

The responses of a single microbial cell to the passage of a single HZE particle of cosmic radiation were studied on spores of the bacterium *Bacillus subtilis* in the Biostack experiments (reviewed in Nicholson et al. 2000). Figure 7-11 shows the frequency of inactivated spores as a function of the distance from the particle track, the impact parameter *b*. About 1000 individual spores were analyzed. Spores within $b \leq 0.25$ μm were inactivated by 73%. The frequency of inactivated spores dropped abruptly at $b > 0.25$ μm. However, 15-30% of spores located within $0.25 < b < 3.8$ μm were still inactivated. Hence, spores were inactivated well beyond 1 μm, which distance would roughly correspond to the dimensions of a spore. At the distance of 1 μm, the mean δ-ray (secondary electrons) dose ranged between 0.1 Gy and 1 Gy, depending on the particle, and declines rapidly with increasing b (Facius et al. 1978, 1994). This value of 0.1 to 1 Gy is by several orders of magnitude below the D_0 (dose reducing survival by e^{-1}) of electrons, which amounts to 550 Gy (see also Figure 7-08). Therefore, the radial long-ranging effect around the trajectory of an HZE particle (up to $b = 3.8$ μm) cannot merely be explained by the δ-ray dose.

These results were largely confirmed by experiments at heavy ion accelerators using single ions (Weisbrod et al. 1992). Taking the results from the experiments in space as well as those obtained at accelerators, one can draw the following general conclusions:

a. The inactivation probability for spores, centrally hit, is always substantially less than one;
b. The effective range of inactivation extends far beyond the range of impact parameter where inactivation of spores by δ-rays can be

expected. This far-reaching effect is less pronounced for ions of low energies (1.4 MeV/u), a phenomenon which might reflect the "thindown effect" at the end of the ion's path;

c. The dependence of inactivated spores from impact parameter points to a superposition of two different inactivation mechanisms: a short ranged component reaching up to about 1 μm may be traced back to the δ-ray dose and a long-ranged one that extends at least to somewhere between 4 and 5 μm off the particle's trajectory, for which additional mechanisms are conjectured, such as shock waves, UV radiation, or thermophysical events (Facius et al. 1978).

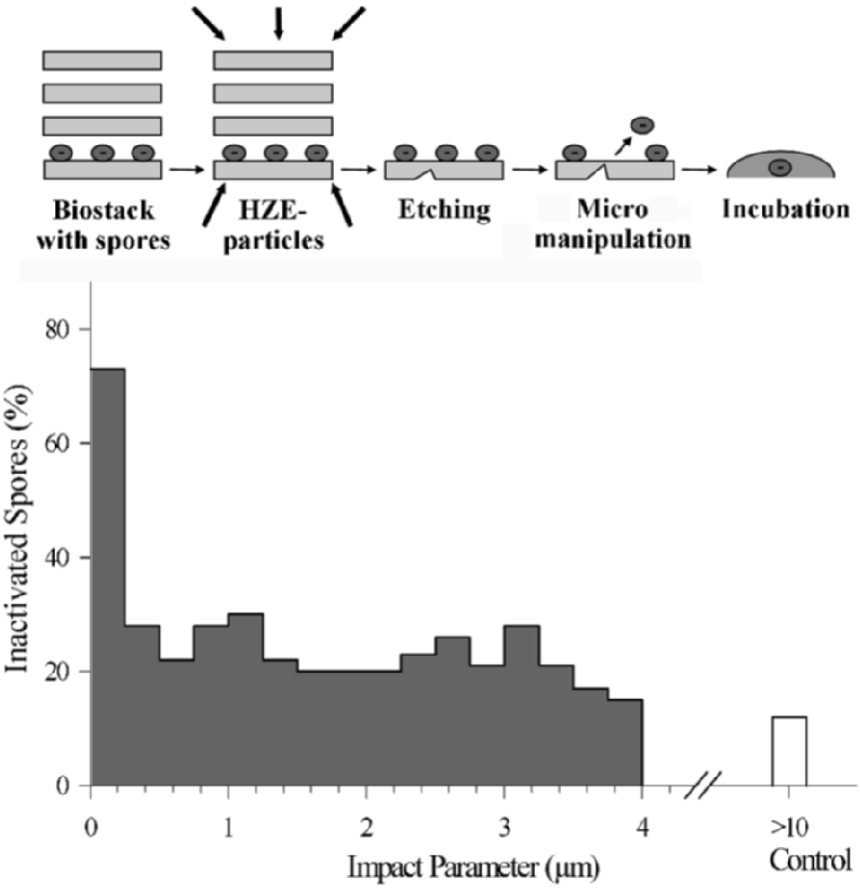

Figure 7-11. Percentage of inactivation of Bacillus subtilis spores by single HZE particles (Z>12, LET>200 keV/μm) of cosmic radiation as a function of the impact parameter. Results of the Biostack experiment flown on board the Apollo-Soyuz Test Project (ASTP). Adapted from Horneck (1992).

With plant seeds that were exposed to cosmic HZE particles when in fixed contact with track detectors, methods were developed to determine the impact parameter of the most sensitive target, i.e., the meristem of root or shoot (Figure 7-12).

In seeds of *Arabidopsis thaliana* or *Nicotiana tabaccum*, hit by an HZE particle, development is significantly disturbed, as demonstrated by loss of germination (early lethality) or embryo lethality. Seeds of impact parameters b<120 μm related to their shoot meristem were inactivated to 90%. In addition, seedling abnormalities, such as hypertrophy or deformation of cotyledones, hypocotyls, or root, or chlorophyll deficiency occurred with high frequency as a consequence of a passage of a single HZE particle close to the shoot or root meristem. Evidently, these severe impairments were based on irreparable damage to the genetic apparatus, as demonstrated by the high frequency of multiple chromosomal aberrations developed in *Lactuce sativa* seeds hit by an HZE particle.

Among *Zea mays* seeds flown on the Apollo-Soyuz Test Project (ASTP), one seed that received 2 hits by HZE particles (Z>20, LET = 100-150 keV/μm) in the central region of the embryo developed a somatic mutation, as evidenced by large yellow strips in all leaves. The extent of this mutation had never been observed before, neither in flight nor in ground experiments.

Among animal resting systems, the mosaic egg of the brine shrimp *Artemia salina* resting in encysted blastula or gastrula state represents an investigative system that, during further development, proceeds to the larval state, the free swimming nauplius, without any further cell division. Therefore, injury to single cells of the cyst will be manifested in the larva. A wealth of data has been compiled on the response of this encysted embryonic system to single HZE particle hits from a series of spaceflight experiments outside the geomagnetic shielding (Apollo-16 and -17) or in LEO (Biostack on ASTP, Biobloc on Cosmos-782, -1129, -1887 or Salyut-7). It was clearly demonstrated that the passage of a single HZE particle through a shrimp cyst damages a cellular area large enough to disturb either embryogenesis or further development or integrity of the adult.

Emergence, characterized by bursting of the eggshell and appearance of the nauplius larva still enclosed in a membrane, was slightly disturbed by an HZE particle hit. The subsequent step of *hatching*, characterized by release of a free-swimming nauplius, was severely inhibited by an HZE particle hit. From the lunar and ASTP missions, an approximately 90% loss of hatching was reported (Bücker, 1975). Whereas, after the ASTP mission, a high lethality was noticed during the days following hatching, this effect was less expressed after the Cosmos or Salyut missions. Additional late effects, due to a hit of a single HZE particle, were delay of growth and of sexual maturity, and reduced fertility. In the Biostack experiments, not a single nauplius larva

that developed from a cyst hit was normal in further growth and behaviour. Anomalies of the body or extremities appeared approximately ten times more frequently than in the ground controls.

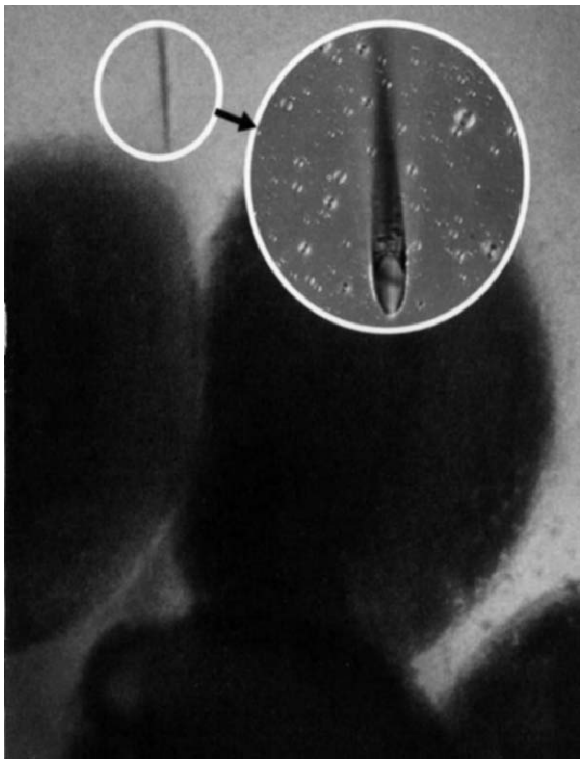

Figure 7-12. Biostack method used to determine the impact parameter for the most sensitive target in plant seeds after exposure to HZE particles of cosmic radiation (see color insert).

4.1.2 Effects on Developing Embryonic Systems

As animal embryonic systems, eggs of the beetle *Tribolium confusum* and of the stick insect *Carausius morosus* were studied in relation to a hit by a cosmic HZE particle. The development of larvae of *Tribolium confusum* up to the pupal state was severely hampered. The frequency of malformations, such as curved abdomen, fused segments of the abdomen or antenna, split or shortened elytra, was approximately 20 times higher than in the ground control. Likewise, hatching of the Indian stick insect *Carausius morosus* from eggs hit by a cosmic HZE particle was significantly reduced. Malformations were increased in individuals having developed from eggs hit. They are characterized by curved abdomina, fused segments, or shortened legs.

In summary, evaluation of the effects observed in bacterial spores, in plant seeds, and animal embryos demonstrated that single HZE particles induce significant biological perturbations in all these test organisms, although with varying efficiency. The observed effects comprise gross somatic mutations, severe morphological anomalies, disturbance of

development, or complete inactivation. From biophysical analysis of some of these results, it was concluded that the magnitude of these effects could not be explained in terms of established mechanisms and, in particular, that the lateral extension of effectiveness around the trajectories of single particles exceeds the range, where secondary electrons could be considered to be effective.

With most embryonic systems investigated so far, a reduced vitality was also observed in the flight non-hit specimens compared to controls on ground. This effect might be caused by additional spaceflight parameters, such as cosmic ray events which are microdosimetric, similar to HZE particles, such as stars of nuclear disintegration. Other parameters include cosmic background radiation and microgravity, which affect the integrity of the biological organisms in space individually or in combination. This phenomenon will be discussed in Section 4.2.

4.1.3 Effects in Mammals

First qualitative evidence of tissue damage produced by cosmic ray HZE particles in the skin of mice was given in 1956 by Chase and Post (cited in Horneck 1992). After high altitude balloon flight exposures, the animals developed segments of white hair. The number of white areas could roughly be related to the number of HZE particle hits.

In the retina of rats, exposed for 19 days to cosmic ray HZE particles during the high inclination (62.8 deg) flight of Cosmos-782, necrotic nuclei and channels of lengths up to 26 μm were detected. Their number was in agreement with the number of HZE particles received during that flight. Comparable lesions were produced by Ne or Ar ions in accelerators. During the subsequent Cosmos-936 biosatellite mission equipped with an onboard 1-g reference centrifuge, rats in 1 g in space developed morphological alterations in their retina that were comparable in number, type, and size to those from animals kept in microgravity. Although tracking of the HZE particles in the tissue were not studied in these experiments, all observations point to HZE particles as the cause of the channels and cellular alterations observed in the rats' retina (Philpott et al. 1980).

In rats flown on board Spacelab-3, the loss of spermatogonia in the testes was used as a biological dosimeter. Whereas only 0.5% loss of spermatogonia was expected from the radiation dose received, a 7% loss was detected. This increased loss of spermatogonia might be caused by a combined action of HZE particles and of other factors prevailing during spaceflight, such as background radiation, microgravity, or stress.

4.1.4 Light Flash Phenomenon

The problem of potential hazard to astronauts from cosmic ray HZE particles became "visible" when the astronauts of the Apollo-11 mission

reported *light flashes*, i.e., faint spots and flashes of light at a frequency of 1 or 2 per minute after some period of dark adaptation. These events were observed during translunar coast, in lunar orbit, on the lunar surface, and during transearth coast. Evidently, these light flashes that were predicted by Tobias in 1952, result from HZE particles of cosmic radiation penetrating the spacecraft structure and the astronaut's eyes, and producing visual sensations through interaction with the retina.

Systematic investigations were then performed during the following six Apollo missions that carried the spacecraft outside the magnetic shielding of the Earth, during the *Apollo-Soyuz Test Project* (ASTP) in LEO, as well as inside ground-based accelerators. These studies demonstrated a variety of different types of flashes, such as thin short or long streaks, double streaks, star like flashes or diffuse clouds, respectively, that were white in general. However, the pattern of types of flashes was different in LEO, in lunar missions, or in accelerators.

A helmet-like device with nuclear emulsions was used by the crew of Apollo-16 and -17 in order to record the passage of HZE particles through the astronaut's head and eyes and to correlate them with observed light flashes. This *Apollo Light Flash Moving Emulsion Detector* (ALFMED) consisted of two sets of glass plates coated with nuclear emulsions. One set was fixed in position, whereas the second parallel located set was moved at a constant rate of 10 μm/s for a total translation time of 60 min. Only in a few cases the passage of an HZE particle through the astronaut's eyes coincided with a light flash event. However, the number of HZE particles traversing the eyes of the astronaut during the translation period agreed with the total number of flashes observed during this period.

Investigations on the frequency of visual light flashes in LEO and its dependence on orbital parameters were performed on Skylab-4, ASTP, and Mir. The highest light flash rates were recorded when passing through the SAA. In this part of the orbit, the inner fringes of the inner radiation belts come down to the altitude of LEO, which results in a 1000 times higher proton flux than in other parts of the orbit. These high light flash event rates during the SAA passages can be deduced either to the high proton fluxes or to the occurrence of some particles heavier than protons in the inner belts of trapped radiation. Casolino et al. (2003) identified two separate mechanisms for the induction of light flashes with the SILEY experiments on board Mir. The first mechanism is a direct interaction of heavy ions with the retina causing excitation or ionization. The second mechanism results from proton-induced nuclear interactions in the eye (with a lower interaction probability) producing knock-out particles. Stimulation of the retina could be caused be electronic excitation resulting in UV radiation in the vicinity of the retina, ionization in a confined region associated with δ-rays around the track, or shock wave phenomena when HZE particles pass through the tissue matrix.

4.1.5 Effects on the Central Nervous System

The light flash phenomenon gives an example that HZE particle hits are "seen" by the astronaut. The question arises what happens to the other organs or tissues of the body exposed to cosmic radiation. Of special concern is the *Central Nervous System* (CNS) where the damage to relatively small groups of cells that cannot replace themselves may result in severe physiological effects.

Tracks of necrotic cells were detected in the brain of balloon-borne monkeys. These tracks were interpreted to be caused by the passage of single heavy ions of cosmic radiation. In order to correlate potential brain damage with the traversal of cosmic ray HZE particles, nuclear track detectors were implanted beneath the scalp of mice during the Apollo-17 mission in the experiment Biocore. Five pocket mice with subscalp dosimeters were exposed to cosmic radiation. Electron microscopic observations did not detect any lesions in the brain or retina that could be attributed to the passage of an HZE particle. This absence of demonstrable lesions might be due to the highly shielded location of the experiment inside the spacecraft resulting in a very low particle flux.

However, lesions were detected in the epidermis and in hair follicles on the scalp of the animals, characterized by necrotic epithelia cells and leukocytes. Only in one case, a coincidence between a lesion and a registered particle could be established. Since the tissue exhibited chronic inflammation attributable to the presence of the dosimeters, it remains uncertain whether the residual lesions were really produced by yet unregistered HZE particles, or whether they were just an experiment-dependent artifact. Hence, the issue whether cosmic ray HZE particles produce microscopically visible injury in the brain needs further consideration.

4.1.6 Chromosomal Aberrations

An elevation of the frequencies of chromosomal aberrations in peripheral lymphocytes has been reported in astronauts after long-term space flights. Obe et al. (1997) investigated lymphocytes of seven astronauts that had spent several months on board the Mir space station. They showed that the frequency of dicentric chromosomes increased by a factor of approximately 3.5 compared to preflight control.

The observed frequencies agreed quite well with the expected values based on the absorbed doses and particle fluxes encountered by individual astronauts during the mission. These data suggest the feasibility of using chromosomal aberrations as a biological dosimeter for monitoring radiation exposure of astronauts.

4.2 Cosmic Radiation and Spaceflight Factors

Besides the radiation environment, microgravity is another important source of potentially detrimental effects during spaceflight. In response to microgravity, several essential cellular functions are impaired, such as signal transduction, gene transfer, and immune response (Moore and Cogoli 1996) (see Chapter 4). Microgravity also affects the physiology of the cardiovascular, respiratory, interstitial, endocrine, immune, and muscular and bone systems in humans (see Hinghofer-Szalkay 1996 and Clément 2005 for review). In addition, spaceflight travelers as well as every organic or inorganic material are subjected to a multitude of factors of various kinds and intensities. These factors are both environmental (e.g., ambient gas medium, temperature, limited space, and cabin microflora) and body internal (e.g., physiological and health status, altered circadian rhythms, emotional stress, and drugs). These different factors rarely act individually. Spaceflight factors that act over extended periods of time, such as microgravity, radiation and those which depend on stay in a closed environment, are of particular interest with respect to combined influences. A potential interaction of radiation and microgravity has been observed in studies involving cell, plant (seeds as well as whole plants), and animal material (insect eggs, larvae, pupae and adults, and rats) (see Horneck 1999 for review).

4.2.1 Definitions

The interaction between two or more factors can be additive, synergistic, antagonistic, or independent. The terms may be more stringently defined in mathematical terms. For example, if *a* and *b* are doses of the two agents yielding the same effect if given separately, the effect x (a + b) of the combined action may be as follows (Equations 9 to12):

Additive: One agent is able to replace the other if the dose scales are appropriately adjusted

$$x\,(a + b) = x\,(b + a) \qquad [9]$$

Synergistic: One agent sensitizes the system to the other agent.

$$x\,(a + b) > x\,(2 \bullet b) \qquad [10]$$

Antagonistic: One agent reduces the sensitivity to the other agent.

$$x\,(a + b) < x\,(2 \bullet b) \qquad [11]$$

Independent: Both agents act independently of each other.

$$x\,(a + b) = x\,(a) \bullet x\,(b) \qquad [12]$$

4.2.2 Methods

Various methods have been applied to disentangle the complex interplay of the parameters of space encountered by humans or any other living being in space. In order to test the influence of microgravity on radiation response, an onboard 1-g centrifuge has been used in parallel, and in some cases in addition, with methods to localize the heavy ions hits in the biological system, e.g., the *Biostack* concept (see Figure 7-10).

In other experiments, the controlled application of additional radiation during spaceflight was used. This method was first used during Gemini missions, when chromosomal aberrations were studied in human blood cells irradiated with β-rays from ^{32}P. Later on, during the Biosatellite-II mission, plants and insects were irradiated in-flight with relatively high doses from a ^{85}Sr source. The biosatellite Cosmos-690 mission carried an onboard γ-radiation source (^{137}Cs) to irradiate rats with doses up to 8 Gy. Recently, yeast cells were irradiated in-flight during the Space Shuttle mission STS-84. Biological samples were also irradiated before or after spaceflight. This method was extensively applied during the Cosmos-368, -782 and Salyut missions and in DNA repair studies with cellular systems within the ESA *Biorack* during the Spacelab missions IML-1, IML-2, and SMM-03.

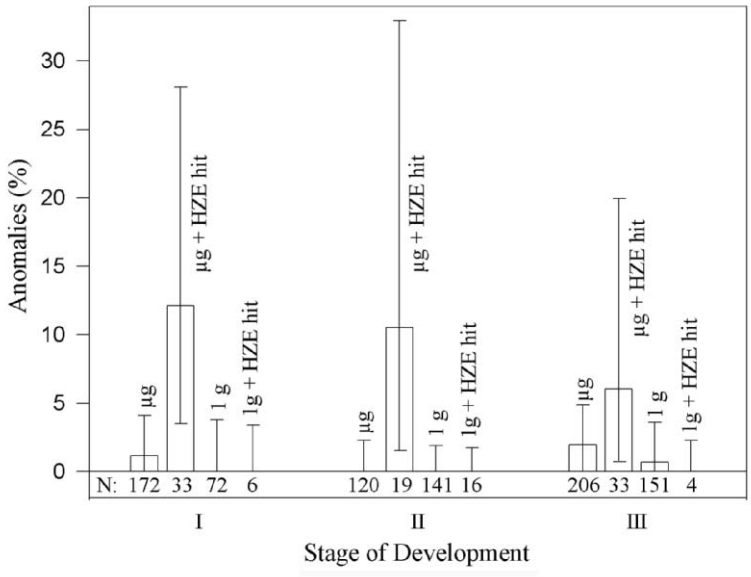

Figure 7-13. Frequency of developmental anomalies observed in larvae of Carausius morosus exposed at different embryonic stages to spaceflight conditions, either in microgravity (µg) or in the onboard centrifuge (1g), and analyzed with the Biostack method. Age of eggs during spaceflight: Stage I (16-23 days), Stage II (30-37 days), Stage III (45-52 days). N = number of larvae investigated.

4.2.3 Results

The combined effects of microgravity and individual cosmic ray HZE particles were investigated on embryogenesis and organogenesis of the stick insect *Carausius morosus* using *Biostack* and an onboard 1-g centrifuge. The combined influence of an HZE particle hit and microgravity acted synergistically on early embryonic stages of development. Evidences were reduced hatching rate, the presence of body anomalies, such as deformities of abdomen and antennae (Figure 7-13), and an increase in mortality. Malformations were observed in the early development stages of fruit fly *Drosophila melanogaster* exposed to ^{85}Sr γ-rays (up to 14.32 Gy) during spaceflight. In larvae and adults of *Drosophila*, genetic effects included lethal mutations, visible mutations at specific loci, chromosome translocations, and chromosome non-disjunctions. Synergism of spaceflight factors and radiation was also observed in chromosome translocations and thorax deformations. These effects have been suggested to be due to an increase in chromosome breakage followed by a loss or exchange of genetic information. It has further been suggested that, under conditions of spaceflight, some repair or recovery mechanisms, usually operating on Earth, may fail. From these results it can be concluded that embryonic systems appear to be especially susceptible to a synergistic interaction of radiation and microgravity.

Rats were γ-irradiated from an onboard ^{137}Cs source with doses up to 8 Gy on day 10 of the 20-day spaceflight of the biosatellite Cosmos-690 in order to study radiosensitivity and radiation injury under the combined action of ionizing radiation and microgravity. Endpoints under investigation were mortality, changes in mobility, weight, behavior, hemopoietic system, metabolism, muscles, and morpho-histology. For the majority of endpoints studied, the effectiveness of γ-radiation in microgravity was similar to that in normal gravity on Earth. However, after irradiation in-flight, the regeneration of the hemopoietic system was remarkably delayed compared to the animals irradiated on the ground. From these experiments, it was inferred that the modifying effects of microgravity on the radiation response of whole animals might be moderate. However, the delayed recovery process observed during the period of re-adaptation to terrestrial conditions might be a point of concern.

4.2.4 Repair Process

It has been conjectured that microgravity might interfere with the operation of some cellular repair processes, thereby resulting in an augmentation of the radiation response (Figure 7-14). Experimental support in favor of this hypothesis has been provided in a space experiment utilizing a temperature-conditional repair mutant of the yeast *Saccharomyces cerevisiae* in which the extent of repair of DNA *Double Strand Breaks* (DSBs) was

reduced by approximately a factor of two compared to the ground control. However, this observation could not be confirmed in a follow-up experiment.

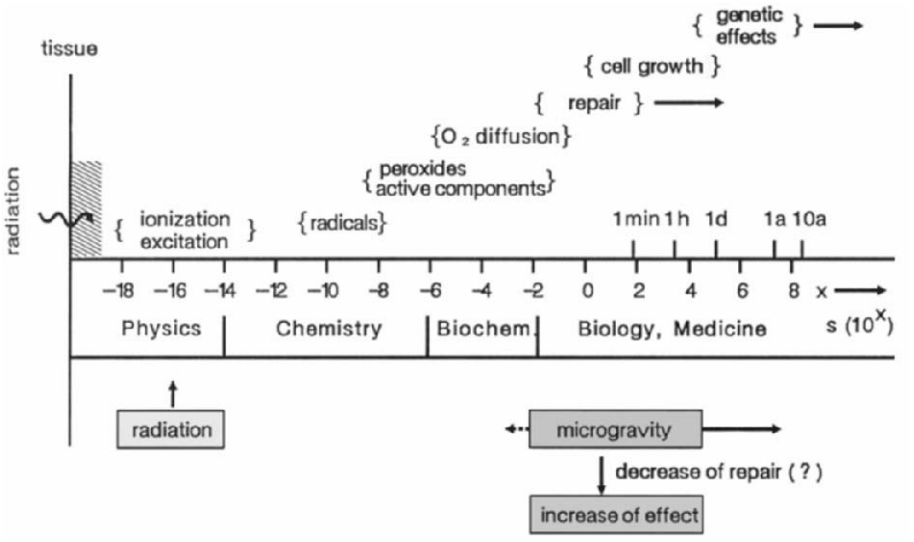

Figure 7-14. Time scale of the radiobiological chain of events and possible impairment of DNA repair by microgravity.

Examining several different repair pathways in different unicellular systems that were irradiated prior to the space mission, evidence was provided that cells in the microgravity environment possess almost normal ability to repair radiation-induced DNA damage (Figure 7-15). In this study, the following repair functions were investigated:

a. The kinetics of rejoining of radiation-induced DNA strand breaks in *Escherichia coli* and human fibroblasts;
b. The induction of the SOS response[7] in *E. coli*;
c. The efficiency of repair in cells of *Bacillus subtilis* of different repair capacity.

The enzymatic repair reactions were identical in cells that were allowed to repair in microgravity and those in normal gravity (both onboard 1-g centrifuge and corresponding ground controls) (Figure 7-15). Although after being irradiated on ground, the samples were kept inactive (e.g., frozen, as spores, or at a repair-prohibiting temperature) until incubation in space, it

[7] The *SOS response* is the synthesis of a whole set of DNA repair, recombination, and replication proteins in bacteria containing severely damaged DNA, e.g., following exposure to radiation.

cannot be excluded that the very first steps of repair initiation, such as gene activation, already occurred on ground. Therefore, studies on gene activation related to DNA repair require irradiating of cells directly in space.

If however, the synergistic effects of microgravity and radiation in biological systems, which has been observed in several instances, cannot be explained by a disturbance of DNA repair in microgravity, other mechanisms must be considered:

a. At the molecular level, as consequences of a convection-free environment;
b. At the cellular level, as impact on signal transduction, on receptors, on the metabolic/physiological state, on the chromatin, or on the membrane structure;
c. At the tissue and organ level, as modification of self assembly, intercellular communication, cell migration, pattern formation or differentiation.

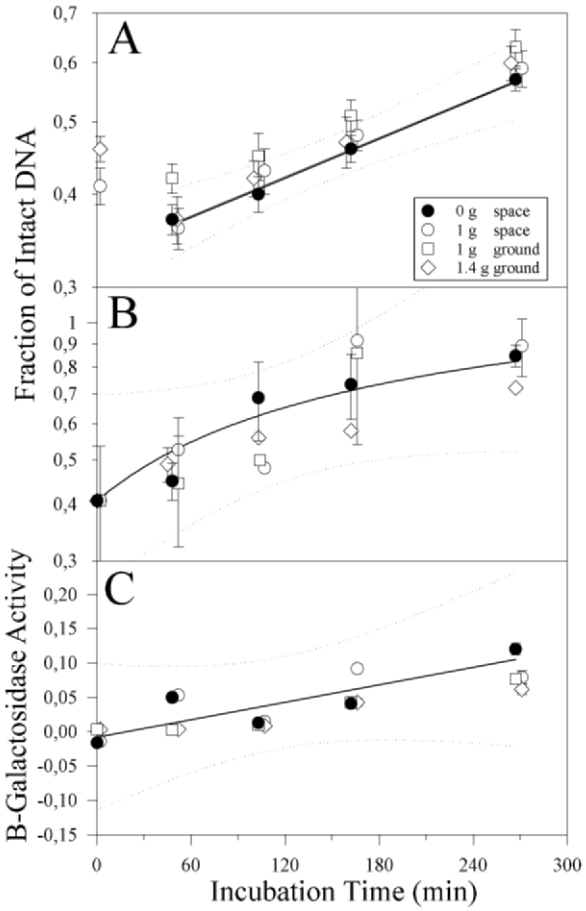

Figure 7-15. Repair of radiation-induced DNA damage under microgravity conditions. A: Rejoining of DNA strand breaks in cells of E. coli B/r. B: Rejoining of DNA strand breaks in human fibroblasts. C: Induction of SOS response in cells of E. coli PQ37.

Further studies are required to interpret the synergistic effects of microgravity and radiation observed preferentially in embryonic systems, using both an onboard radiation source under well-defined conditions and appropriate controls. These studies can be expected to involve both cellular systems as well as whole organisms including mammals.

As far as radiation protection of astronauts is concerned, it must be kept in mind that several defense mechanisms against radiation damage operate above the cellular level, i.e., on the tissue or immune system level. The established physiological changes brought about by microgravity, in particular in the humoral system, may well modify the response to radiation, especially late response after long-duration missions. So far, this aspect has not been addressed experimentally.

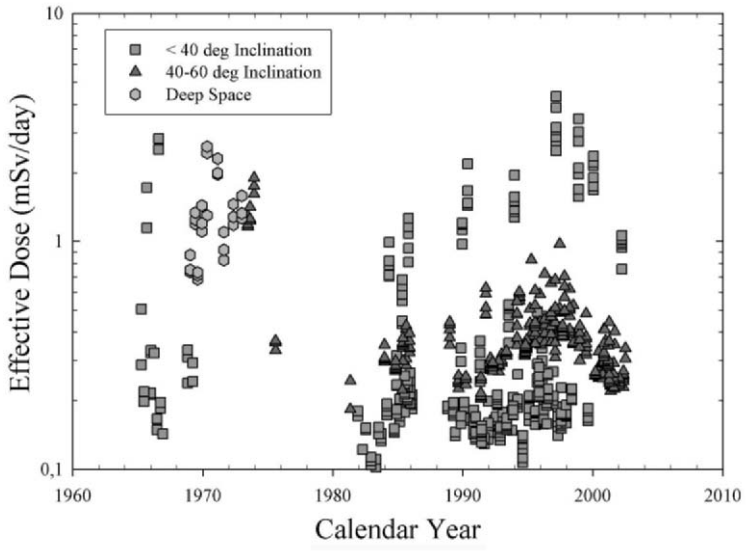

Figure 7-16. Effective radiation doses measured during LEO and Moon missions.

5 RADIATION DOSIMETRY IN SPACE

5.1 Physical Radiation Monitoring

Knowledge of the radiation situation inside of a space vehicle is mandatory for each mission under consideration and shall be based on in-flight dosimetry data. Such measurements of radiation exposures were performed during manned spaceflights at various altitudes, orbital inclinations, durations, periods during the solar cycle, and mass shielding (Figure 7-16) (McCormack et al. 1988, Swenberg et al. 1993, Reitz 1994).

The deposition of energy by radiation strongly depends on the type of radiation under consideration, both macroscopically and microscopically (see Figure 7-06). Because of the complex mixture of radiations occurring in space, comprising sparsely ionizing components (photons, electrons, pions, muons and protons) and densely ionizing components (heavy ions, neutrons and nuclear disintegration stars) (see Section 2), different dosimetry systems have been applied, that specifically respond to the quality of the radiation under consideration. The contribution of the sparsely ionizing component of the radiation in space has been mostly determined by lithium fluoride *Thermoluminescence Dosimeters* (TLD). A TLD is an (usually doped) inorganic crystal. It "absorbs" radiation dose by its valence electrons being excited to a higher energy state. The number of electrons at the higher energy state is directly proportional to the amount of ionizing radiation the crystal is exposed to. When the crystal is heated, these electrons fall back to their resting energy and emit photons, causing the crystal to glow. The emitted light intensity as a function of the temperature is called the *glow curve*. In a heating cycle the amount of emitted light, i.e., the integral of the resulting glow curve, is proportional to the total dose received by the crystal since the last time it was heated ("annealed"). The sensitivity of TLDs is nearly constant in the energy range of interest (Apathy et al. 2002).

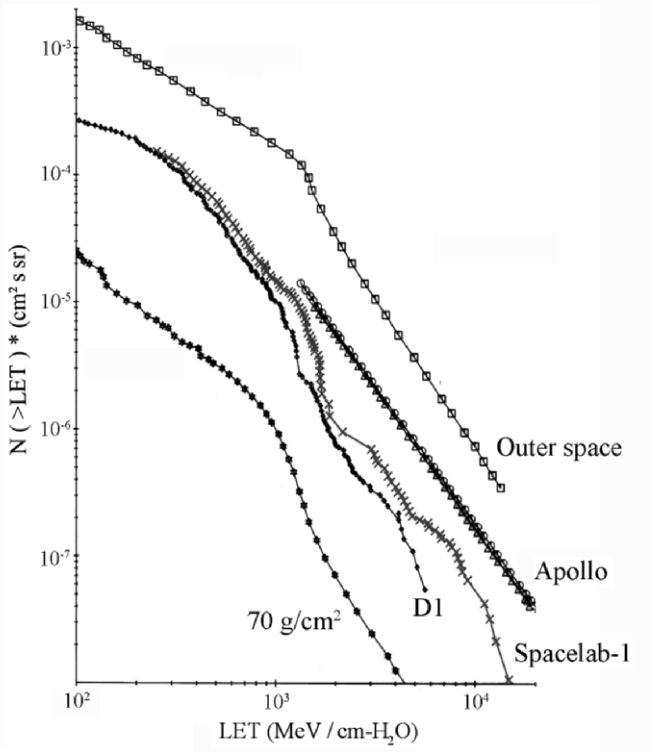

Figure 7-17. Integral LET spectra of heavy charged particles measured inside the spacecraft with plastic track detectors during space missions outside of the geomagnetic (Apollo-16 and -17), in low Earth orbit (Spacelab-1 and D1), and calculated for outer space without shielding and behind a shielding of 70 g/cm². The nomenclature of the spectra refers to the mission and the location of the detectors inside the spacecraft.

For densely ionizing radiation, the spatial pattern of energy deposition at the microscopic level is important. For example, lesions in the sensitive structures, such as biomolecules or chromosomes, are induced with higher efficiency than by X-rays. The fluence of densely ionizing radiation has been mainly determined by use of plastic track detectors or nuclear emulsions. Plastic detector systems are diallylglycol carbonate (CR39), cellulose nitrate (CN), or polycarbonate (Lexan), which cover different ranges of LET. The tracks of heavy ions are developed by etching in caustic solutions, e.g., in sodium hydroxide 6 N NaOH. The track etching rate grows as a function of the LET. Plastic detectors allow to determine the fluence, charge, and LET spectrum of the heavy ions. Generally different plastic detector systems are arranged in a stack, and the combination of their spectra is used to generate a LET spectrum adequate for dosimetry calculations (Figure 7-17). The density of nuclear disintegration stars has been determined by nuclear emulsions. The absorbed dose deposited by neutrons can be estimated from TLDs differing in their relative contents of the isotopes ^{6}Li and ^{7}Li.

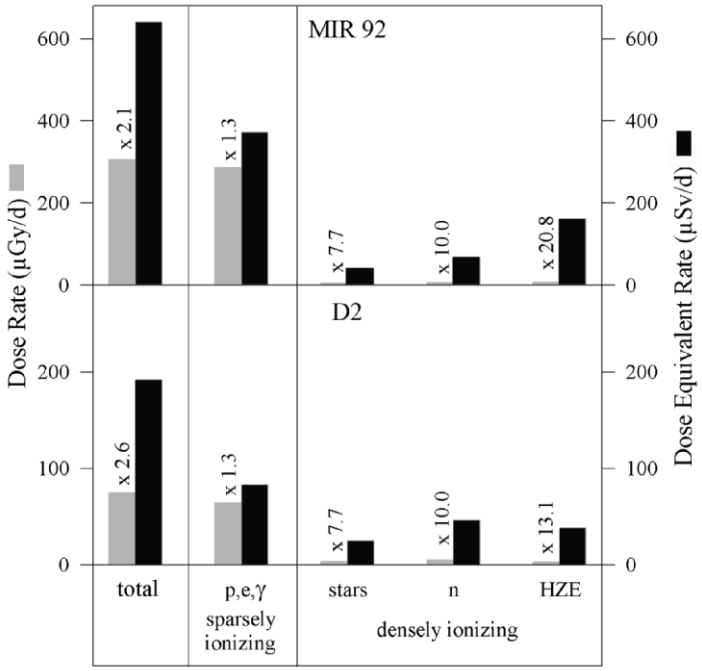

Figure 7-18. Absorbed dose rate and dose equivalent rate (in µGy/d or µSv/d, respectively) of the sparsely ionizing and the three densely ionizing components of the radiation field measured on board the Mir-92 mission (51.5 deg, 400 km) causing a total dose equivalent of 640 µSv/d, and during the Spacelab-D2 mission (28.5 deg, 296 km) with a total dose equivalent of 192 µSv/d. For comparison, the dose equivalent rates are about 60 mSv/3months on board the ISS (51.5 deg, 400 km) and 3 mSv/year at the surface of the Earth.

Figure 7-18 illustrates the contribution of the different types of radiation measured during two different space flights in LEO and estimations for the ISS.

It is important to note that these *passive*[8] dosimetry systems integrate over the time of exposure. Their advantages are their independence of power supply, small dimensions, high sensitivity, good stability, wide measuring range, resistance to environmental stressors, and relatively low cost. However, long duration space missions, such as on board the ISS or future interplanetary missions, require time-resolved measurements, especially for radiation protection purposes. This requirement has been met by the "Pille" device, a small, portable and space-qualified TLD reader suitable for reading out TLD repeatedly on board (Apathy et al. 2002).

In addition to passive dosimeters, *active* dosimeters have been developed to provide real-time dosimetry data. The measurement principle is based either on ionizations (e.g., ionization chamber, proportional counter, Geiger-Müller Counter, semiconductors, charged coupled devices CCD) or on scintillations (e.g., organic or inorganic crystals). A combination of two silicon detectors, the *Dosimetry Telescope* (DOSTEL), has been flown on board the Space Shuttle, Mir, and the ISS. Particle count rates, dose rates, and LET-spectra were measured separately for GCR, the radiation belt particles in the South Atlantic Anomaly, and solar particle events (Beaujean et al. 2002).

During human spaceflight an individual dosimetry is required for each astronaut. Dosimetry varies for *Intra-* and *Extra-Vehicular Activities* (IVA and EVA, respectively), where the astronauts are only shielded by the material of the space suit. A number of active devices such as small silicon detectors or small ionization chambers may be used, but they need power and are difficult to design in sufficiently small dimensions. In most cases, passive integrating detector systems have been used, such as TLDs, also in combination with the "Pille" device (Apathy et al. 2002).

However, these personal dosimetry systems provide only data on the "surface" or skin dose. In order to assess the depth dose distribution within the human body and especially at the most radiation sensitive organs, such as the brain, the blood forming organs and the gonads, human phantoms are required equipped with different dosimetry systems at the sites of sensitive organs. The anthropomorphic phantom "Matroshka" was exposed for one year to the radiation in space outside of the ISS (Figures 7-01 and 7-19) in order to determine the depth dose distribution of radiations within the human body during EVA (Reitz and Berger 2005).

[8] The dosimeters are "passive" in the sense that they do not need power during the mission.

Figure 7-19. ISS Cosmonaut Sergei K. Krikalev holds the anthropomorphic phantom Matroshka, a human-torso-like device, after its retrieval from the exterior of the ISS during a spacewalk, for return to Earth. The experiment is designed to better understand the exposure of astronauts, including those making spacewalks, to radiation. Photo courtesy of NASA.

5.2 Biological Radiation Monitoring

Complementary to physical dosimetry, biological dosimetry systems, i.e., which weight the different components of environmental radiation according to their biological efficacy, have been developed. Biological dosimetry systems are especially important when interactions of radiation with other parameters of spaceflight, especially microgravity, may occur. Basically two types of biological detecting or monitoring systems are available: (a) the intrinsic biological dosimeters that record the individual radiation exposure (humans, plants, animals) in measurable units; and (b) the extrinsic biological dosimeters/indicators that record the accumulated dose in biological model system (summarized by Horneck 1998).

When used in parallel with physical dosimetry, both types of biological dosimetry systems can provide valuable complementary information because of their following properties:

a. Ability to weight the different components of environmental radiation according to their biological efficacy;
b. Ability to give a record of the accumulated radiation exposure of individuals;
c. Capacity to monitor the cumulative biological effects of all environmental stressors present;
d. High specificity;
e. Generally high sensitivity.

5.2.1 Intrinsic Biological Dosimeters

As described above, exposure of G_0 lymphocytes to ionizing radiation leads to chromosome type aberrations such as polycentric and ring chromosomes. The frequencies of these aberrations are correlated with radiation dose and can therefore be used as a biological dosimeter. After long-term spaceflights on board Mir, Obe et al. (1997) observed significantly elevated frequencies of chromosome-type aberrations with indications that the aberrations were radiation-induced. The frequencies of dicentrics found in lymphocytes of Mir cosmonauts before and after their last spaceflight compared well with frequencies expected from doses of low and high LET radiation to which they were exposed during their mission. These data have also been used to predict the radiation risks of astronauts during interplanetary space missions.

Another promising technique is *Premature Chromosome Condensation* (PCC) that allows interphase chromosome painting and the detection of non-rejoining chromatin breaks without going though the first mitosis. This method is especially relevant for biological dosimetry for astronauts that are exposed to high doses of high-LET space radiation which may induce interphase death and cell cycle delay.

In addition to chromosomal aberrations, other intrinsic biomarkers for genetic or metabolic changes may be applicable as biological dosimeters, such as germ line minisatellite mutation rates or radiation induced apoptosis, metabolic changes in serum, plasma or urine (e.g., serum lipids, lipoproteins, ratio of HDL/LDL cholesterol, lipoprotein lipase activity, lipid peroxides, melatonin, or antibody titers), hair follicle changes, and decrease in hair thickness, triacylglycerol-concentration in bone marrow, and glycogen concentration in liver. Whereas the first three systems mentioned are non-invasive or require only blood samples for analysis, the latter systems are invasive and therefore appropriate for radiation monitoring in animals only. Dose response relationships have been described for most of the intrinsic dosimetry systems, yet their modification by microgravity remains to be established.

5.2.2 Extrinsic Biological Dosimeters or Indicators

Cellular bioassays for genotoxic assessment, such as the efficacy of radiation, are based on DNA damage induced in target cells, and are frequently used to infer the mutagenic or carcinogenic potential of environments. Examples are the Ames assay that uses a set of auxotrophic strains of *Salmonella typhimurium* that revert to histidine prototrophy upon exposure to mutagens of specific mechanisms. SOS-dependent bacterial strains make use of the fact that in response to DNA damage a cascade of functions is induced, including the transcription of more than 15 repair

enzymes, known as the *SOS response*. The SOS lux assay utilizes the SOS system as receptor which is sensitive to DNA damage, and the bioluminescence system as rapid optical reporter. The SOS lux system is a suitable environmental monitoring system to be accommodated on board the ISS.

6 RADIATION PROTECTION CONSIDERATIONS

On Earth, the radiation exposure limits are defined by the *International Committee on Radiation Protection* (ICRP). These limits prevent detrimental non-stochastic (acute) effects and reduce the probability of stochastic (late) effects to levels deemed to be acceptable. The annual terrestrial exposure limit for the public, in excess to the natural radiation exposure, lies at 1 mSv. The annual occupational limit is 20 mSv with a lifetime limit of 400 mSv. It is important to note that this level is an upper limit, according to a principle known as the ALARA (for *As Low As Reasonably Achievable*) Principle. The same guidelines are also used to set the limits of allowable radiation exposure during space missions.

6.1 LEO Missions

The current guidelines for radiation protection in LEO missions have been derived from a postulated “acceptable” risk for late cancer mortality, which had been justified by comparison with mortality rates from “normal” terrestrial occupations (NCRP 1989, 2000). For space missions in LEO, a lifetime excess risk for fatal cancer due to radiation exposure of 3% was judged reasonable, taking into account the fact that space crews have to cope with other serious risks besides the radiation risk. This risk of 3% is comparable with the risk in less safe but ordinary industries, such as agriculture and construction.

Furthermore, NCRP recommends age- and gender-dependent limits. For example, if the career of an astronaut extends over 20 years, the total risk decreases because the susceptibility for radiation-induced cancer decreases with age. Correspondingly, the risk is higher per unit exposure for shorter periods of exposure. The career whole-body dose equivalent limit (in Sv) for a lifetime excess risk of fatal cancer of 3% as a function of age and gender has been recommended as follows: for males at age of 25, 35, 45, or 55 years the limit has been set at 1.5, 2.5, 3.25 or 4.0 Sv, respectively; for females the limit are lower at 1.0, 1.75, 2.5 or 3.0 Sv, respectively.

In no circumstances, pregnant females are allowed to fly. The special risks for the embryo-fetus are malformations and particularly mental retardation, and the risk of cancer is expected to be greater than for the adults. The dose measurements obtained from previous space missions inside a space vehicle in LEO within the geomagnetic field have shown that the exposures

are sufficiently low that so far no special actions were necessary to keep the dose equivalent limit within these NCRP limits.

Shielding (g/cm²)	***0.3***	***1.0***	***5.0***	***10.0***
GCR (Sv)				
Solar minimum		*0.195*	*0.177*	*0.161*
Solar maximum		*0.074*	*0.070*	*0.066*
"Worst case" SPE (Sv)				
Interplanetary travel	*4.21*	*3.52*	*1.93*	*1.26*
Lunar surface	*2.11*	*1.76*	*0.97*	*0.63*

Table 7-01. Estimated radiation equivalent doses (Sv) for the blood forming organs during a 6-month lunar mission behind different shielding thicknesses and at different time of the solar cycle (Horneck et al. 2003b). Note: NCRP limits for LEO: 0.25 Sv for 30 days; 0.50 Sv annual dose; and 0.4-4.0 Sv for career dose, depending on gender and age (NCRP 1989).

6.2 Exploration Missions

In contrast with the LEO conditions, the doses expected during exploration mission to the Moon and Mars are likely to infringe the NCRP limits in certain instances. For a lunar base, the estimates for the total radiation doses received at the *blood forming organs* (BFO) from SPE irradiation indicate that some exposure levels would exceed the limits for radiation guidelines in LEO.

In general, the exposure levels for GCR while on the Moon would range within the exposure limits recommended for LEO. As an example, let's consider a male lunar "colonist" who starts his space occupation at the age of 45. His career limit amounts to 3.13 Sv (calculated from NCRP 1989). This career limit of 3.13 Sv would be reached only after 16 turns of the nominal 195-day mission shift, assuming an average 1 g/cm^2 thick Al shield which gives an average GCR dose of 0.195 Sv per mission (Table 7-01). This assumption of an average shield of 1 g/cm^2 is highly pessimistic, because the lunar habitat will provide a much better shielding against GCR. The value finally depends on the number of EVAs with much lower shielding. In other words, this astronaut most likely would reach his retirement age before exhausting the "acceptable" limits of radiation exposure from GCR.

However, the doses expected when encountering a large SPE could possibly induce acute radiation injuries even behind 5 g/cm^2 Al and even in a shelter with 10 g/cm^2 Al, as shown for the doses deposited by the worst case reference event in deep space (Table 7-01). If such an SPE event would be encountered with only 1 g/cm^2 Al or even less shielding, e.g., in a spacesuit during EVA, severe incapacitating acute radiation injuries could ensue, with a substantial probability for a fatal outcome unless adequate medical support could be supplied.

Late effects, such as enhanced morbidity or mortality from malignant cancers occurring up to 20 and more years after exposure have to be considered, as well as early effects which may comprise anorexia, fatigue, nausea, diarrhea, and vomiting (the symptoms of the prodromal syndrome), or cataract formation, erythema, and early mortality within days to a few weeks from failures of the hematopoetic, pulmonary, and gastrointestinal systems. Depending on the phase of the mission, when such symptoms occur, they may well be associated with performance losses of the affected crew, which in turn might increase the risk for critical failures.

A human mission to Mars will add a new dimension to human space travel, concerning the distance of travel, the radiation environment, the gravity levels, the duration of the mission, and the level of confinement and isolation to which the crew will be exposed. Table 7-02 lists the equivalent doses for the blood forming organs estimated for the different missions during different phases of the solar cycle. For missions during solar minimum as well as during a "worst case" SPE, the expected radiation dose exceeds the annual equivalent dose limit of 0.5 Sv, which has been established for missions in LEO with the aim of keeping the radiation induced lifetime excess of late cancer mortality below 3% (NCRP 1989).

Mission duration	***Type of radiation***	***Shielding (g/cm²)[1]***				
		0.3	***1.0***	***5.0***	***10.0***	***20.0***
1000 day	*GCR*					
	Solar minimum[2]		***0.993***	***0.918***	***0.852***	***0.769***
	Solar maximum[3]		*0.402*	*0.383*	*0.364*	*0.339*
500 day	*Solar minimum[2]*		***0.828***	***0.754***	***0.687***	***0.605***
	Solar maximum[3]		*0.317*	*0.299*	*0.280*	*0.255*
	"Worst case" SPE [4]					
	Interplanetary travel	***4.21***	***3.52***	***1.93***	***1.26***	
	Mars surface	*0.32*	*0.31*	*0.28*	*0.25*	

Notes:
[1] aluminum
[2] based on 1977 solar minimum data
[3] based on 1970 solar maximum data
[4] based on SPE from September 1989 data multiplied by a factor of 10

Table 7-02. Estimated equivalent doses (in Sv) received during a mission to Mars at the blood forming organs as consequence of exposure to galactic cosmic radiation (GCR) or to one "worst case" solar particle event (SPE). Values ***in bold*** *exceed the annual dose limits for LEO given in NCRP (1989). Adapted from Horneck et al. (2003a, 2005).*

6.3 Research Needed

In order to minimize the risk from space radiation during exploration missions, future research and development are required within the following categories:

a. An adequate quantitative risk assessment for accurate mission design and planning in order to minimize the expectation value of *Healthy Lifetime Lost* (HLL);
b. The surveillance of radiation exposure during the mission for normal and alarm operational planning and for record keeping;
c. Surrounding crew habitats with sufficient absorbing matter;
d. Countermeasures to minimize health detriment from radiation actually received by selecting radiation resistant individuals or by increasing resistance, e.g., by radioprotective chemicals.

The opposite selection process whereby individuals with identifiable genetic disposition for increased susceptibility to spontaneous, and implied, radiogenic cancerogenesis are detected, will in any case be part of the standard crew selection.

In addition to the standard countermeasures such as avoidance of exposure by adequate shielding and mission planning or by chemoprotective and even nutritional measures, it is important to foster radiobiological research activities with the aim to reduce significantly the uncertainty of our risk estimates. These uncertainties are related to the potentially unique radiobiological properties of galactic heavy ions or to the possible modifications of space radiation effects, either synergistically or antagonistically, brought about by the changes in the whole body status during spaceflight. This status is not only shifted to a new set point by microgravity but may also be altered in response to general stress, including psychological stress.

Terrestrial research using heavy ion accelerators, such as the recently developed microbeam techniques, will focus on the effects of single heavy ions on individual cells. However, a definite answer concerning the modification of radiation effects by the exposure conditions in space will only be found in properly designed, most likely animal, experimental studies on the ISS or a lunar base.

Finally, the criteria currently used in deriving space radiation exposure limits need to be redefined in order to allow for an integrated, unified risk management and design approach which, among other advantages, explicitly considers the repercussions of radiation protection measures like shielding design or mission planning on the overall mission success probability. The probabilistic expectation value of the HLL, i.e., the number of healthily lived years lost due to an exploration space mission,

would serve such purposes more neatly than the presently invoked criteria, and its minimization would allow for a combined balanced treatment of early and late radiation effects on an equal footing.

7 SUMMARY AND CONCLUSIONS

Since the first supposition of the existence of ionizing radiation originating from outer space at the beginning of the last century, a lot of work has been done in order to characterize the radiation field surrounding the Earth. A series of experiments has been initiated on the various components of cosmic radiation, their radiobiological importance, and the role of the other spaceflight factors in radiobiological processes.

Besides offering opportunities for radiobiological experiments in space, the current space exploration program requires the collection of radiobiological data in space as baseline information for estimating radiation risks and establishing radiation standards for humans in space.

The various fields of radiation biology in space include:

a. Radiation detection and measurement;
b. Studies on the biological response to radiation in space;
c. Studies on the impact of spaceflight environment on radiation effects;
d. Radiation protection efforts.

Of special concern are the heavy ions of cosmic radiation, the so-called HZE particles. To understand the ways by which HZE particles interact with biological systems, methods have been developed to precisely localize the trajectory of an HZE particle relative to the biological sensitive site and to correlate the physical date with the biological effects observed along the trajectory of the particle. So far these studies have mainly dealt with biological systems in resting state, such as viruses, bacterial spores, plant seeds or shrimp cysts, as well as with a few embryonic systems. Most observations point at damage to the genetic material, such as mutations, tumor induction, chromosomal aberrations, cell inactivation, or development anomalies.

Using higher organisms including mammals, a few attempts were made to identify tissue damage along the passage of single HZE particles, such as microscopically visible injury in brain or eyes, or the light flash sensation. The latter one, correlated with orbital parameters, showed highest frequency during the passage of the South Atlantic Anomaly. To study potential interactions of ionizing radiation with microgravity, either additional irradiation was applied, pre-, in-, or postflight, or an onboard 1-g centrifuge is utilized in combination with methods of particle effect correlation. Synergistic interactions were observed in producing mutations or anomalies with high frequency, especially in embryonic systems.

Although a substantial amount of information has been accumulated on the effects of cosmic HZE particles on biological systems, many issues in space radiation biology are still unanswered and need further experimentation in space. There is especially a paucity of data on the effects of comic radiation on whole tissues of animals and on their potential interactions with the microgravity environment. This information is one of the prerequisites for establishing reliable radiation protection guidelines for human space exploration missions.

8 OPEN QUESTIONS AND OUTLOOK

In spite of more than 30 years of research on the biological effects of radiation in space, there are still experimental data are lacking for several essential issues as follows:

a. Concerning sparsely ionizing radiation:
   - To what extent do the factors of the spaceflight environment interact with the induction and/or expression of late effects, especially during long-duration missions?

b Concerning densely ionizing radiation:
   - To what extent do the factors of the spaceflight environment interact with the early effects of this radiation component?
   - To what extent differ late effects produced by the densely ionizing component from those of sparsely ionizing radiation with respect to severity and/or kinetics of expression?
   - To what extent are these late effects modified by the factors of the spaceflight environment?
   - To what extent are the effects of nuclear reaction stars comparable to those produced by HZE particles, and if not, in which of the above three cases do they differ?

c. For all radiation components in space:
   - If interactions of the space radiation environment and the other factors of the spaceflight environment do exist, what are the relative contributions of these factors?

None of these questions can be adequately resolved by spaceflight experiments alone. Neither in spaceflight experiments nor in terrestrial experiments can these challenges be attacked by experiments with human subjects. Their solution therefore must rely on results from either animal experiments, tissue culture *in vitro* experiments, or experiments with other biological test organisms suitable for investigations on specific fundamental questions. On this empirical basis, theoretical models are to be established in order to render extrapolation to radiation effects in humans. Until these radiation mechanisms are satisfactorily well understood, comprehensive

dosimetry of the radiation in space should be a *sine qua non* for all human spaceflights.

As a consequence, substantial research and development activities are required in order to provide the basic information for appropriate integrated risk managements, including efficient countermeasures. These activities include but are not limited to:

a. Research during long-duration orbital spaceflights, with emphasis on the ISS or other human missions in LEO;
b. Research on robotic precursors missions to the Moon and Mars, including orbiters and landing vehicles;
c. Research during ground-based simulation studies using heavy ion accelerators (Figure 7-20).

Research Area	***Task***	***Approach***
Risk assessment	*Determine depth dose distribution inside habitats and human body* *Determine interactions of space radiation and other space factors, e.g., microgravity*	*Human phantoms inside and outside of ISS, e.g., MATROSHKA* *Cell and animal experiments with artificial radiation source*
Surveillance	*Determine individual biological significant radiation dose*	*Develop:* *(a) personal dosimeters (passive and active)* *(b) biodosimetry concepts, e.g,. chromosome aberration* *(c) biodiagnostic systems, e.g., cellular biosensors*
Countermeasures	*Determine role of diet in radiation responses*	*Interact with nutrition to develop dietary concepts for minimizing oxidative stress*

Table 7-03. Research required in LEO in preparation of future human exploration missions in the field of radiation biology and radiation health (Horneck et al. 2003a).

As outlined in Table 7-03, substantial information can be gained from studies in LEO to obtain a solid base when approaching the next frontier, namely human missions beyond the Earth orbit to the Moon or Mars. This information will be useful to optimize risk assessment, surveillance, and countermeasures for the crew. Robotic precursor missions to the Moon and Mars are required to improve and validate transport codes for prediction of solar particle and cosmic heavy ion radiation doses inside a given shielding distribution at a given position in interplanetary space at a given time within

the solar cycle. Robotic lander missions will provide data on the radiation climate on the surface, and modes and efficiency of natural (e.g., regolith) and artificial (e.g., habitat) shielding.

Whereas radiobiological research on board the ISS is an inevitable condition for all questions concerning the possible impact of microgravity, hypogravity, radiation, other space specific factors, and potential interactions between them, several basic questions can be more appropriately addressed by ground-based studies using heavy ion accelerators. These studies include improving and validating transport codes, determining the effects of single heavy ions by applying microbeams, and determining the interaction of radiation and microgravity by installing facilities providing simulated functional microgravity at heavy ion accelerators.

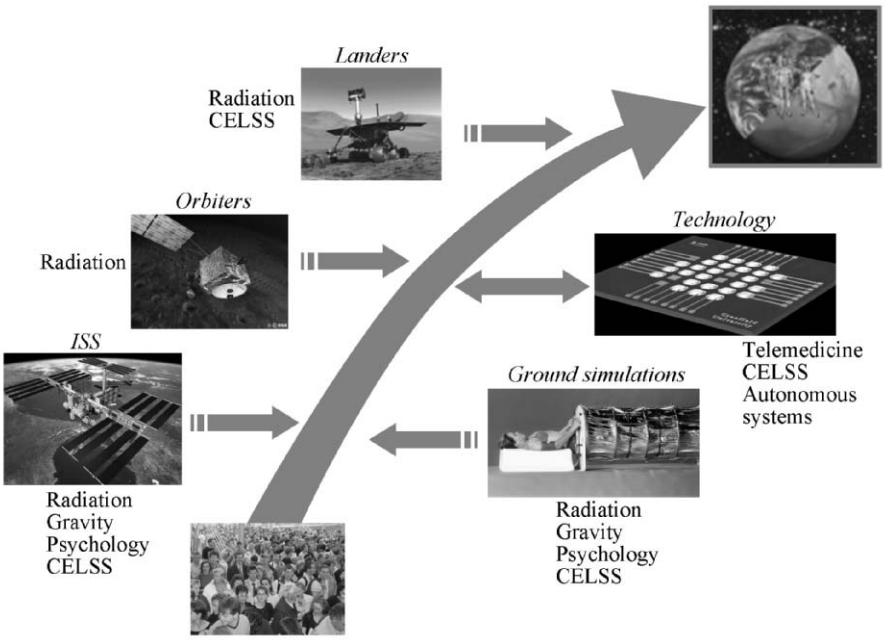

Figure 7-20. Roadmap in human health issues for ESA's exploration program, as recommended in the HUMEX study (Horneck et al. 2003a).

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FURTHER INFORMATION:

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NASA Space Life Sciences Research Highlights. Space Radiation:
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The Space Environment:
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Chapter 8

BIOTECHNOLOGY IN SPACE

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Often praised as the most promising field for the commercial exploitation of space, this domain today has two primary fundamental aspects: the use of microgravity as a tool for separation processes and techniques (including protein crystals growth), and the production of cells for medically valuable proteins like immuno-reactive molecules, hormones, enzymes, and vaccines. Results of space experiments and their terrestrial applications are presented, and their advantages and disadvantages compared to Earth-based techniques, such as genetic engineering, are discussed.

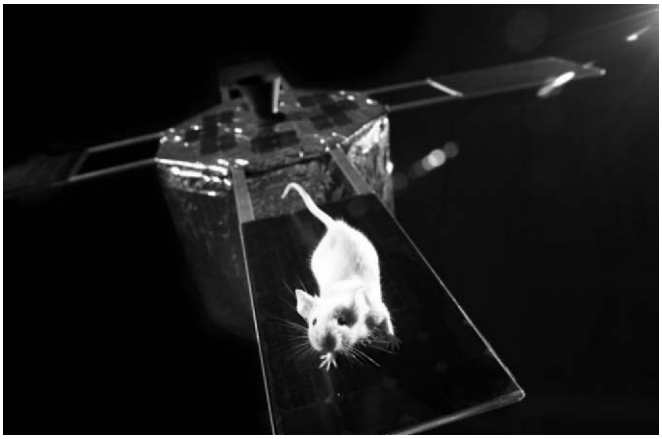

Figure 8-01. A mouse-astronaut candidate poses atop a model solar panel. Source: http://science.nasa.gov/headlines/y2004/20jan_marsmice.htm. Credit: Marsgravity.org

1 INTRODUCTION

Biotechnology is an applied multidisciplinary research that includes biological science, engineering, and biochemistry to provide biomolecules, cells, tissues, and other bioproducts to enhance human health and welfare. As demonstrated in the previous chapters, microgravity offers a unique environment for research on the growth of cells, tissues, and biological materials.

Space biotechnology focuses in two areas: cell culture and protein crystal growth. The cell culture work is a basic research that contributes to understanding how microgravity affects the fundamental behavior of cells, particularly in relation to tissue formation on living organisms. The protein crystal growth work uses microgravity to produce higher quality biological macromolecular crystals for structure determination and for improving that process in terrestrial laboratories.

2 CELL CULTURE

2.1 Objectives

The culture of cells in microgravity opens up opportunities to develop new understanding of the way macromolecules function, cells respond to their environment, and tissues form via tissue engineering studies. Such understanding can open new strategies for probing disease processes, developing medical countermeasure for space travel, and advance our knowledge of cellular processes. More specifically, the research focuses on: (a) the engineering of tissue for research, transplantation, and biopharmaceutical production; (b) the production or tissues for disease modeling such as cancer; (c) the vaccine production through propagation of microorganisms; (d) tissue engineering and bioreactor design; and (e) studies on changes in gene expression.

The research also has implications for ground-based systems, as perturbations of biological systems by microgravity can provide insight into physiological control in the absence of mechanical forces and in the absence of convection. This work could give scientists insight into how cellular processes respond to mechanical and chemical manipulation, eventually allowing them to design more efficient bioprocesses and to develop a new generation of high-resolution biosensors. Finally, the program also allows investigators to compare various tissue culturing techniques to determine which of them produce systems that most effectively mimic the characteristics of genuine tissue.

2.2 Results of Ground and Space Experiments

The cell culture biotechnology program encompasses a wide range of research topics, from cancer cells to parasites, from chondrocytes to lymphocytes. Originally, research focused on the generation of three-dimensional cultured tissue and on the rough characterization and comparison of these engineered tissues to natural tissues, envisaging the commercial exploitation of space for generating large amounts of tissue. Although some scientific success in this area has been achieved both in space and in ground-

based experiments, the commercialization of space products has not been successful yet.

Many ground-based studies have been performed using bioreactors for exploring cellular and tissue responses to low-stress growth environments and simulated microgravity. These systems have been used primarily to produce three-dimensional self-assembling aggregates that retain some of the cell-cell interactions present in tissues. A wide variety of culture systems have been tested, leading to several advances, such as the propagation of parasites and studies on impaired activation of lymphocytes in space (see Chapter 4). Investigators have also compiled a large list of tissues that have been cultured in bioreactors, including cancer cells, cartilage, liver, kidney, lymphoid tissue, thyroid, skin, pancreatic islet cells, neuroendocrine cells, hematopoietic cells, and intestinal epithelium, as well as microorganisms (Figure 8-02).

Figure 8-02. The Generic Bioprocessing Apparatus on board the Spacelab Science Module in the Space Shuttle Columbia (STS-94), shortly after arriving on orbit. The bioprocessing reactions involved sequential mixing of fluids for phase processing, incubation, optical monitoring, and temperature storage. This facility could host up to 132 individual experiments. Experiments included studies of how collagen fibers could be used more effectively as artificial skin or blood vessels, how the assembly of liposomes and virus capsids could be used to target a drug to specific tumors, or how mineralization occurs and influences the embryonic bone tissue of rodents. Photo courtesy of NASA.

To date, studies on cell cultures in space in have demonstrated that microgravity and the space environment affect cell shape, signal transduction, replication and proliferation, gene expression, apoptosis, and synthesis and orientation of intracellular and extracellular macromolecules (see Chapter 4, and the following review articles: Dickson 1991, Moore and Cogoli 1996, Cogoli and Cogoli-Greuter 1997, Lewis et al. 1997, Freed et al. 1997, Hammond et al. 1999). Cell culture technology has made substantial contributions to the artificial engineering and growth of human cartilage, cardiac muscle, and kidney tissue. Macromolecular studies of insulin crystals grown in space have enabled researchers to obtain a previously unavailable molecular model that can be used to develop more effective drugs for diabetic patients (see this Chapter, Section 3.3).

2.3 Limitations

At the same time, tremendous progress has also been made in three-dimensional tissue development in normal gravity on Earth, using, for example, scaffolds and extracellular matrix gels. In experiments done in space, cell cultures experience a different gravitational environment, which reduces convection, buoyancy-driven flows, and sedimentation (see Chapter 1, Section 2.2), and it is difficult to separate the specific factor(s) causing differences between space- and Earth-grown samples. Researchers are also limited by the difficulties inherent in distinguishing the effects of launch, flight, and reentry on samples. As for the other areas of space biology, studies on cell cultures in space require experimental controls. These controls include the use of bioreactors on Earth, culture bags in microgravity, bioreactors in space, three-dimensional structures grown on Earth from scaffolds, and the same experimental setup operated in an onboard 1-g centrifuge.

Bioreactors were originally developed to simulate the microgravity environment, and provide appropriate predictions of the behavior of cells and tissue in such an environment. However, the validity of this model will not be known until comparisons are made with experiments that have been subjected to microgravity and not modified by launch and reentry. Such research is now made possible by the long-duration microgravity capability of the ISS.

Also, while bioreactors have been an important tool for generating aggregates in cell culture for three-dimensional engineered tissue, they are limited in many respects. First, the tissue synthesized is lacking many of the minor cells and elements formed within the intact organism. Second, in cell cultures, the cells that die are not generally removed, creating some artifacts. Third, tissues grown in bioreactors are not subject to the environmental signals that they might sense *in situ* (e.g., growth factors, vascular changes, neuromuscular changes), yet these signals are apt to change in microgravity.

In addition to these systemic and environmental drawbacks, the bioreactor has technical limitations. The limited oxygen transfer capabilities

make bioreactors inappropriate for systems with high oxygen demand. Also, it has not yet been determined if rotating-wall vessel bioreactors can provide an appropriate environment for tissues such as osteoblasts that only grow properly when the distances between the cells are maintained.

2.4 Research Facilities

The hardware for cell and tissue culturing falls into three main categories: basic incubators, perfused stationary culture systems, and rotating wall vessels. There is also a variety of supporting equipment, including refrigeration, monitoring, and analytical instruments. A description of various pieces of hardware relevant to cell and tissue culturing on board the ISS is provided in Chapter 3. A summary of these facilities is shown in Table 8-01.

Cell Culture Systems
• *Biopack*
• *Biolab*
• *Bioreactor*
• *CBEF (Cell Biology Experiment Facility)*
• *CCM (Cell Culture Module)*
• *CCU (Cell Culture Unit)*
• *CGBA (Commercial Generic Bioprocessing Apparatus)*
• *Incubator*
• *MCS (Modular Cultivation System)*
• *Rotating Cell Culture Vessel*
Imaging
• *NIZEMI (Slow Rotating Centrifuge with Microscope)*
• *Life Sciences Dissecting Microscope*
• *ISS Compound Microscope*
• *ISS Dissecting Microscope*
• *Cameras/Video*
In-Flight Sample Preparation & Preservation
• *GN2 Passive Freezer*
• *Quick/Snap Cryogenic Freezer*
• *Cryogenic Storage Freezer*
• *-80°C Freezer (MELFI)*
• *-20°C Freezer*
• *4°C Refrigerator*
• *SIGB - Standard Interface Glovebox*
• *Life Sciences Glovebox*
• *Flight Approved Preservatives for Cell Cultures*

Table 8-01. Hardware available on board the ISS for research in biotechnology. Source: http://astrobiology.arc.nasa.gov/genomics/technologies/available_hardware.html

The incubators are designed to provide refrigeration as well as to allow preserving and incubating of multiple cell cultures simultaneously. The

cell culture bags are transparent to allow visualization of the samples by light microscopy. The bioreactors provide temperature and pH control and allows for continual feeding and waste medium harvest from perfused stationary cultures. They also provide automated sample collection and injection, and high-quality video microscopy. The bioreactors can generally accommodate multiple cell culture chambers. Individual perfused culture chambers can be replaced on orbit. Specimens are loaded in chambers on the ground, and inoculation and subculture can occur in space. Unlike ground-based, rotating-wall bioreactors, in which laminar flow is set up to randomize the force vectors and to minimize the shear stress, space-based vessels have rotating walls in order to augment mass transport. Observation and video recording are possible through windows in the front of the unit, and cell and media samples can be removed on orbit through sample port (Figure 8-03).

Other ground-based methods for generating three-dimensional tissue constructs, such as the use of scaffolding constructed from biomaterials or micro-patterned substrates, may prove to be more effective sources of samples for multiple-chamber hardware such as the existing incubators and culture

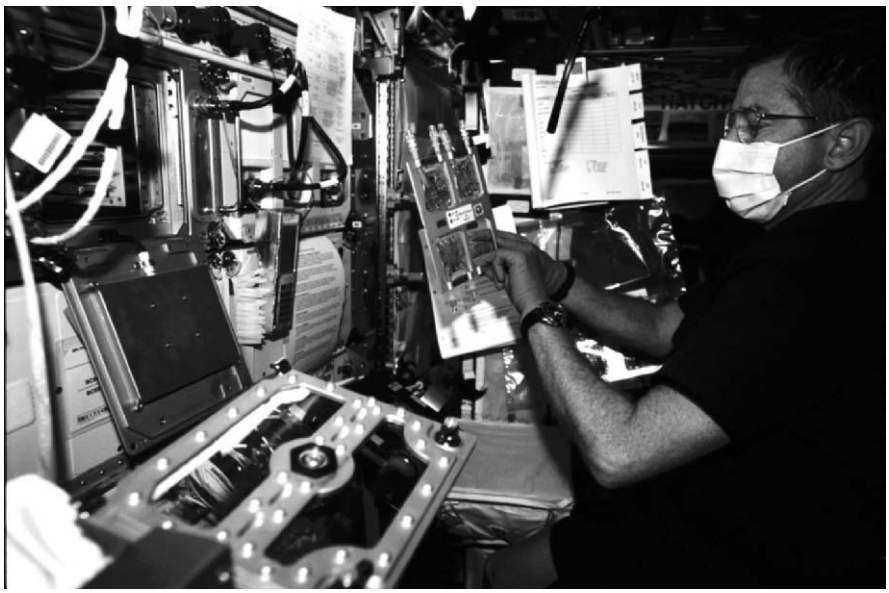

Figure 8-03. Astronaut Frank L. Culbertson works at the NASA bioreactor rotating wall vessel in the Destiny laboratory on board the ISS. The bioreactor comprises four incubation/refrigeration chambers. Every 7 to 21 days (depending on growth rates), an astronaut uses a shrouded syringe and the bags' needleless injection ports to transfer a few cells to a fresh media bag, and to introduce a fixative so that the cells may be studied after flight. The design also lets the crew sample the media to measure glucose, gas, and pH levels, and to inspect cells with a microscope. Photo courtesy of NASA.

systems. In addition, the amount of data produced by these new systems in a given period of time and amount of volume on the ISS will be significantly greater than would be produced by a bioreactor system.

2.5 Perspectives

With the increased availability of research opportunities on the ISS and the new hardware developed, further investigation of these processes will clarify how cells behave in microgravity. A better understanding of how the cells in the physiological systems, such as muscle, bone, balance, and cardio-vascular, sense and respond to microgravity would have immediate relevance for the manned space program.

Potential research topics would not be limited to areas that have already been explored, but could come in other areas, including the adaptive responses of cells in microgravity to factors such as: (a) radiation; (b) induced phenotypic and genotypic changes; (c) effect of the space environment on replicating cells; (d) and the effect of microgravity on plant cells and tissues, on microorganisms that cause disease or that will be used for waste treatment on long-duration flights, and on cells (e.g., osteoblasts) that may not proliferate in bioreactors as they are currently designed (Unworth and Lelkes 1998).

As mentioned above, the key areas in which perturbations of cell structure and function in microgravity are observed are components of nuclear architecture, cytoarchitecture, and the extracellular matrix. It is becoming increasingly evident that the organization of genes and regulatory proteins within the nucleus, the organization of nucleic acids and signaling proteins in the cytoplasm and cytoskeleton, and the organization of regulatory macromolecules within the extracellular matrix contribute to the physiologically responsive fidelity of gene expression. Consequently, the functional interrelationships between cell structure and gene expression within the three-dimensional context of cell and tissue organization can be rigorously and systematically studied under microgravity and regular Earth-gravity conditions. The corollary is that microgravity can provide valuable insight into structure-function interrelationships that connect control of gene expression to cell and tissue architecture (National Research Council 1998).

The microgravity environment has shown a unique utility to facilitate cultures of virus and pathogens. Examples of flown viruses include the Norwalk virus, a gastroeneric pathogen, the influenza flu virus, and the respiratory syncytial virus that causes pneumonia and severe upper respiratory infection. Specimens derived from the space-grown virus can be injected in selected cell lines obtained from tissue culture differentiation studies, and the infected cell cultures are characterized for evidence of virus replication. The ultimate phase is to generate these adapted strains of the virus with enhanced replication properties in conventional tissue culture lines and systems.

Another technique is to better determine the atomic structure of the antibody by growing crystals of the virus's antibody (see section on crystal growth below). Knowing the structure of the antibody will accelerate the development of an effective vaccine against the virus. Thus, microgravity, as an experimental tool, may provide insight into fundamental aspects of biological regulation that will be important in the space as well as terrestrial environment (Volkman et al. 1995, Kaysen et al. 1999).

Figure 8-04. On board the ISS, ESA astronaut Pedro Duque of Spain watches a water bubble floating between him and the camera, showing his reflection (reversed). Photo courtesy of NASA.

3 PROTEIN CRYSTAL GROWTH

3.1 Objectives

This area of space biotechnology has both a basic science component and an applied science component. The basic science component seeks to understand the fundamental physics and chemistry of macromolecular crystal growth by utilizing microgravity to study aspects of the crystal growth process that are masked by gravity on Earth. The applied science component of the program uses microgravity to produce higher quality crystals that are subsequently used in ground research to produce more detailed and more

accurate atomic structures of macromolecules. Examples of this research include structural biology research, biological nanotechnology, and biomolecular self-assembling materials.

Biological macromolecules such as proteins, enzymes and viruses play a key role in the complex machinery of life. They possess active sites which make them bind or interact with other molecules in a very specific manner that determines their biological function. They intervene in the regulation, reproduction and maintenance mechanisms of living organisms, and they can be the cause of diseases and disorders. Pharmaceutical drugs are molecules that inhibit the active sites of macromolecules and, in principle, are intended to affect only the targeted macromolecule.

The vast majority of current drugs are the result of systematic testing, first at molecular level, then at a clinical level. This extensive process significantly increases the cost of the product. With a detailed knowledge of the 3D structure of a macromolecule, biochemists can restrict the range of drugs to be tested. Furthermore, with a rational drug design approach, one may attempt to synthesize a drug targeted exclusively on a specific macromolecule. That means a drug will perfectly bind to the macromolecule and inhibit its biological function while remaining inert vis-à-vis other macromolecules.

The 3D structure of the macromolecule can be discovered through the analysis of crystals by X-ray diffraction: X-rays are passed through a single crystal at various angles. The resulting diffraction patterns are analyzed using computers to estimate the size, shape, and structure of the molecule. A flawed crystal will yield a blurry and/or weak diffraction pattern, whereas a well-ordered crystal will yield a sharp and/or strong diffraction pattern and thus useful information about the structure. So, the better the quality of the crystal, the faster and the more accurate the determination of the structure and the faster the identification of a drug (Binot 1998).

During the 1990's, there was explosive growth in the number and complexity of macromolecular structures being determined by X-ray crystallography, as evidenced by the exponential increase in the number of structures published and submitted to the Protein Data Bank[1]. This growth has been made possible by the convergence of a large number of new technologies, including the following:

a. Improved systems for cloning and expressing wild-type and mutant proteins;
b. Improved protein and nucleic acid purification techniques;

[1] The *Protein Data Bank* is a repository for 3D structural data of proteins and nucleic acids. This data, typically obtained by X-ray crystallography or NMR spectroscopy, is submitted by biologists and biochemists from around the world, is released into the public domain, and can be accessed for free at http://www.wwpdb.org/

c. Immortalization of crystals by cryogenic freezing;
d. Very high brilliance X-ray synchrotron sources;
e. Fast, accurate area detectors with high dynamic range;
f. Super fast, inexpensive computers;
g. Readily available software packages for data acquisition and reduction, phasing, and refinement.

For the most part, however, protein crystallization is done in much the same trial-and-error manner it was a decade ago, although easier and faster since the introduction of reagent kits and the growing use of automated systems. It is still more art than science. One of the main goals of crystallization in microgravity has been the growth of crystals in space that are of better quality than those available on the ground (National Research Council 2000).

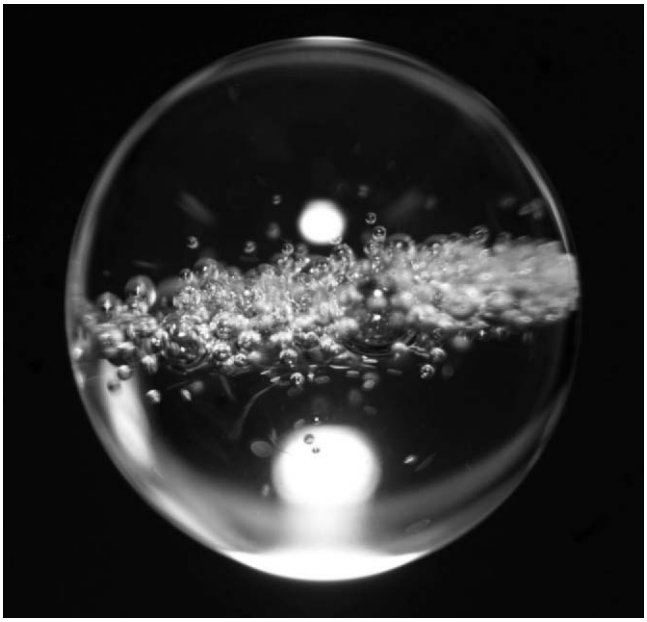

Figure 8-05. View of a bubble formed as a result of a Zeolite Crystal Growth experiment in the Destiny laboratory on the International Space Station. This experiment has shown that the bubbles could cause larger number of smaller deformed crystals to grow. Photo courtesy of NASA.

3.2 Minimal Resolution

The determination of macromolecular structures by X-ray crystallography at a level of detail sufficient for the construction of reliable atomic models requires crystals that diffract X-rays by 3.5 Å[2] or better. A resolution of at least 3.5 Å is required to see structural elements in proteins,

[2] 1 Å (Ångström) = 0.1 nm = 10^{-10} m

such as alpha-helices or beta-sheets, which can be directly visualized in electron density maps calculated using the X-ray data.

Where the general fold of the protein chain is desired, an analysis at 3.5 Å may suffice to determine the protein structure. However, at this resolution the orientation of hydrogen bonding groups is not well determined, and detailed questions regarding the structural architecture of the protein cannot be answered reliably until a resolution of ~2.5 Å or better is achieved. The precise calculation of the energetics of ligand binding or intermolecular interfaces requires structure determination carried out to an even higher resolution, making possible the mapping of ordered water molecules and an accurate description of hydrogen bonding geometries (Geierstanger et al. 1996). The most accurate protein structure determinations are carried out at a resolution of 1.5 Å or better. In the relatively rare cases where data to better than 1 Å are obtained, individual hydrogen atoms can often be distinguished and the disorder within the protein structure can be described in detail.

The intrinsic resolution of a protein crystal can be thought of as arising from two factors. One is the *mosaicity*, a parameter that is a measure of the misalignment between small coherent blocks of individual molecules within the larger crystal. While crystals that are highly mosaic may diffract to high resolution, the high mosaicity leads to a broadening of the diffraction spots, which can complicate or even foil their measurement. The other crystal characteristic that affects resolution is the *Debye-Waller Factor*, also known as the overall temperature factor, which reflects *disorder* and *mobility* within the individual molecules that make up the crystal.

Many protein molecules that are of interest today, particularly those that are studied in the form of relatively unstable complexes, are expected to have intrinsically high Debye-Waller factors, limiting the resolution of the resulting diffraction pattern. In such cases, if the size of the perfectly aligned mosaic blocks can be increased, the resulting increase in the sharpness of the diffraction pattern can effectively improve the resolution of the diffraction pattern. In such situations, if growth in microgravity produces crystals with larger mosaic blocks (lower mosaicity), then there may be a significant improvement in the quality of the diffraction measurements. These added levels of detail would enable researchers to see the functional groups and water molecules and thereby more fully understand the interactive mechanisms of macromolecular assemblies (Chayen and Helliwell 1999).

3.3 Results of Space Experiments

It is estimated that in about 20% of the different proteins and other biological macromolecular assemblies that have been studied in space, the resolution of the crystallographic analysis was better than that of the best ground-based results available at the time. The proteins whose resolution improved in space range from well-understood test cases, such as *lysozyme*, to

proteins that present significant challenges for contemporary structural biology, such as the EcoRI-DNA complex, the nucleosome core particle, and the epidermal growth factor receptor. Enhanced resolution has also been obtained for proteins of importance for drug design, including the HIV protease complexes with lead compounds, and the influenza neuraminidase. Improvements in resolution of crystals of canavalin, *Satellite Tobacco Mosaic Virus* (STMV), and insulin in microgravity were also substantial.

For example, the analysis of tetragonal lysozyme crystals grown on two Space Shuttle missions showed that the mosaicity of the crystals was improved by factors of 3 or 4 over that observed for lysozyme crystals grown on Earth (Snell et al. 1995) (Figure 8-06). This is a very significant improvement because it can allow the measurement of very weak reflections that would otherwise be too broad to be observed over background. Although crystals of lysozyme with very low mosaicity can occasionally be obtained on Earth, only about 1 in 40 of them have properties comparable to those of the crystals grown in space.

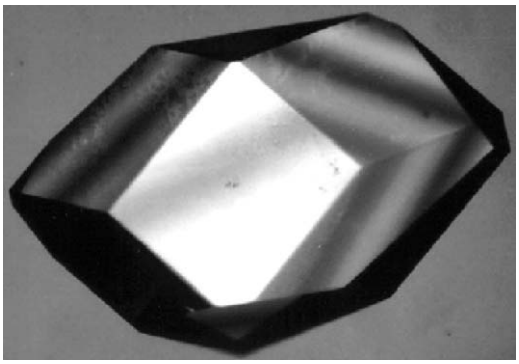

Figure 8-06. A lysozyme crystal grown in orbit. Photo courtesy of NASA.

Another example is STMV, a small icosahedral plant virus consisting of a protein shell made up of 60 identical protein subunits of molecular weight 14,000, which has been studied extensively on Earth (Figure 8-07). Its crystallization in microgravity was investigated during two Space Shuttle missions in 1992 and 1994. Using a liquid-liquid diffusion technique with careful temperature control, crystals of STMV obtained in space were about 10 times larger in volume than the largest crystals of STMV previously grown in ground-based laboratories (Day and McPherson 1992). In contrast to the crystals grown on Earth, the crystals grown in microgravity were visually perfect, with no striations or clumping of crystals. Furthermore, the X-ray diffraction data obtained from the space-grown crystals was of a much higher quality than the best data available at that time from ground-based crystals. STMV also crystallizes on Earth in a cubic crystal form that diffracts poorly; at the time of the 1994 shuttle flight, the best available ground results gave

only about 6 Å resolution (Koszelak et al. 1995). Cubic crystals of STMV obtained on board the Space Shuttle were 30 times larger than those obtained on Earth. These crystals diffracted X-rays to 4 Å resolution, a significant improvement over the ground-based crystals.

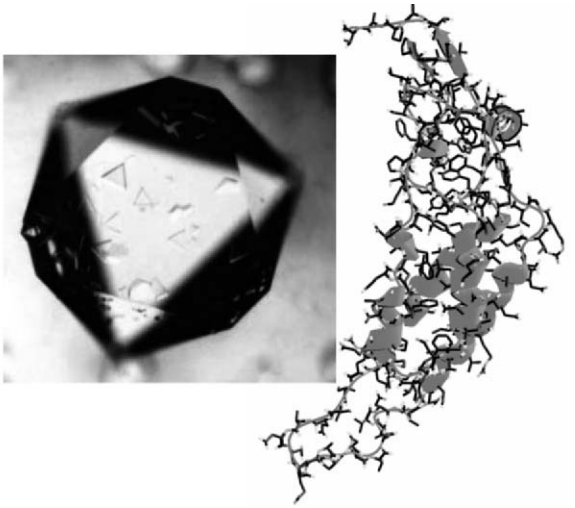

Figure 8-07. Crystal of the Satellite Tobacco Mosaic Virus (STMV) and computer-generated model of its protein structure.

As with STMV, large crystals of *canavalin*, a plant storage protein, were obtained in space (Koszelak et al. 1995). Visually perfect crystals of canavalin were obtained in large numbers (Figure 8-08), with significantly better diffraction properties than those of crystals grown on Earth. The diffraction limit was extended from 2.7 Å (Earth) to nearly 2.2 Å (space), and the total number of useful X-ray measurements essentially doubled.

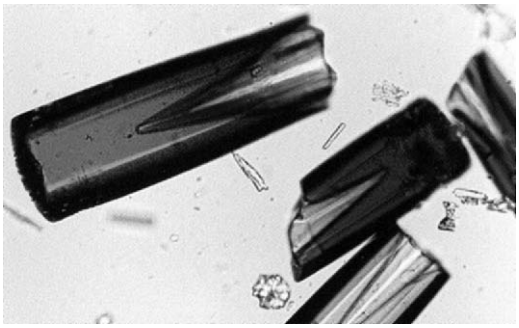

Figure 8-08. Hexagonal crystals of canavalin grown in the Protein Crystal Growth (PCG) facility on board the Mir station. Photo courtesy of NASA.

Another example is with *insulin*, a hormone released by the pancreas in response to increased levels of sugar in the blood. Insulin aggregates to form hexamers, which undergo a change from one three-dimensional arrangement of atoms and bonds to another. The switching between the two states is altered by the presence of particular ions and organic molecules, and there is interest in identifying additives that would stabilize one state, which

would lead to insulin preparations with greater stability. To this end, high-resolution crystallographic analyses of insulin have being carried out. Crystals of human insulin grown in space were larger and free of imperfections compared with crystals grown on Earth (Figure 8-09). A resolution of 0.9 Å has been obtained using synchrotron X-ray radiation on T6 insulin crystallized on board the Space Shuttle, whereas data to 1.9 Å resolution were obtained using the crystals grown on Earth and a laboratory X-ray source (Smith et al. 1996). This ultrahigh resolution data is allowing very detailed analysis of the molecular structure, including the study of electronic distributions within the protein molecule. This data will add information for the development of new therapeutic insulin treatments for the control of diabetes. Such treatments would greatly improve the quality of life of people on insulin therapy by reducing the number of injections they require and by reducing the cost of treatment.

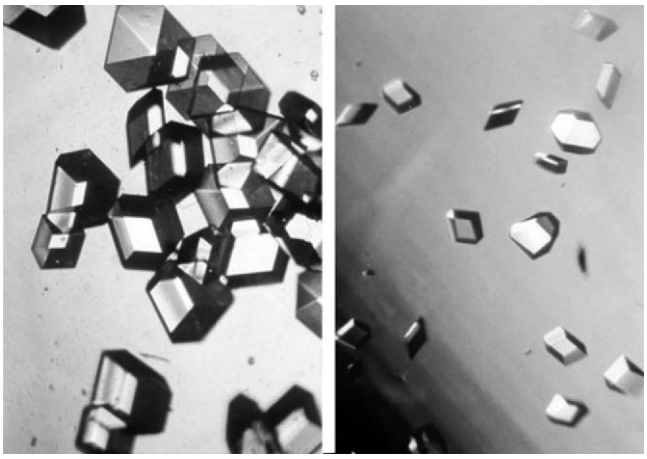

Figure 8-09. Space-grown (left) and Earth-grown (right) insulin crystals. Photo courtesy of NASA.

The four cases described above provide the most convincing data currently available on the benefits of growing protein crystals in microgravity. Several dozens of other experiments produced space-grown crystals with improved resolution, e.g:

- Factor D protein crystals – led to development of a drug that may aid patients recovering from open heart surgery.
- Antithrombin – a protein which controls blood coagulation in human plasma, which has important implications for medicine.
- HIV protease/inhibitor complex – may have applications for designing new drugs for AIDS therapies.
- Influenza protein crystals and neuraminidase – a target for the treatment and prevention of the flu.

- Proteins associated with Chagas' disease – a debilitating and deadly disease that affects more than 20 million people in Latin America and parts of the United States.

3.4 Limitations

However, in some of the cases described above, the investigators were not able to make the comparisons needed to demonstrate that growth in microgravity was indeed the factor responsible for producing higher quality crystals. The incorporation of additional features in the analysis of the space-grown crystals, such as the use of cryogenic techniques and synchrotron radiation, makes it difficult to be certain that the improvements are due to microgravity and not to some of these additional factors (DeLucas et al 1989). Also, it is not enough to compare space-grown crystals to crystals grown on Earth in the same equipment and solution over the same time period. The microgravity-grown crystals must also be compared to the best result from all Earth-based attempts at growing the crystal regardless of crystallization conditions, equipment, or time of growth. This latter comparison is the baseline standard for defining success.

Today's high-energy synchrotron[3] sources have, in general, eliminated crystal *size* as the key factor in increasing the diffraction resolution limit. This was not the case when the space crystallization program began in the mid-1980's. Scientists today are interested in crystallization methods that provide higher quality crystals, where quality is measured by disorder and mosaicity. Therefore, a well-ordered crystal of average dimensions (around 30 to 50 μm) is all that is needed for effective diffraction studies. Synchrotron technology continues to improve, and the target crystal size may decrease even further before the ISS is completed. Crystal quality, rather than crystal growth, is thus the primary focus of the biological macromolecular research community.

Another limitation is that all research on protein crystallization in space so far has been done under less than optimal conditions. Most of the work has been done on fairly short Space Shuttle flights, with a few experiments occurring on the Russian Mir space station. The crystallization work has been generally restricted to a matter of days, which is not enough time in most cases to complete the crystallization process, especially in space, where crystals appear to nucleate and grow more slowly. Except for Spacelab missions devoted exclusively to microgravity research, the environment has generally not been free of noise and vibration. No mechanism has been

[3] A *synchrotron* is a ring-shaped accelerator in which charged particles are accelerated by a magnetic field and an electric field. The high-density X-rays produced by this particle accelerator are used for gathering crystallographic data for structural determination.

provided to stabilize the crystals that do grow and to protect them from the stresses of reentry. In general, the ability to visualize crystal growth in space has been extremely limited, preventing investigators from determining if flawed crystals examined after landing had failed to grow well in space or if crystals with good morphology had indeed been grown but later had been damaged during reentry. Like for the other disciplines in space biology, the irregular schedules of space missions and the long lead times have made it difficult for scientists engaged in an extremely competitive research field to seriously consider participation in space experiments. The slow and uncertain progression of space experiments has disconnected them from the even more rapid tempo of contemporary protein crystallography research (National Research Council 2000).

3.5 Protein Growth Facilities on Board the ISS

Although there have been some intriguing successes from the experiments carried out to date, at least as many crystals were lost before they could be returned to Earth-based laboratories for study. This is because in the past, most microgravity experiments were consigned to the middeck lockers of the Space Shuttle. Generally, fewer than a hundred crystallizations per flight were attempted, and most were allowed to run for only a week or less.

It should be different when the ISS is completed and in full operation swing. Regular Space Shuttle flights to and from the ISS will allow for considered planning of crystallization experiments. Improvement and standardization of the crystallization hardware will allow laboratory scientists to optimize crystallization procedures for the specialized hardware, maximizing the chances for success.

On board the ISS, experiments will be carried out in dedicated racks in the sciences modules. Many more crystallizations will be set up and allowed to proceed for weeks or months, with periodic visual monitoring both on the ISS and from the ground. In addition, it will be possible to automate the process of crystal growth, monitoring, mounting, and freezing, and of obtaining diffraction data in microgravity.

For example, the *X-ray Crystallography Facility* (XCF) is a multipurpose facility designed to provide and coordinate all elements of protein crystal growth experiments on board the ISS, including sample growth, monitoring, mounting, freezing, and X-ray diffraction. A module for the growth phase is designed to house vapor diffusion experiments. The visualization unit uses magnified still photographs of samples that have completed growth to determine whether the resulting crystals are worth preserving. The *Crystal Preparation Prime Item* (CPPI) is a robotic system that mounts the crystals on hair loops for cryopreservation or on hair loops inside a capillary, unfrozen. Finally, the *X-ray Diffraction Module* employs a low-power (24 W) X-ray source and has a maximum resolution of 1.1 Å. The

various modules are controlled remotely from the ground; crew time is only required to move samples from unit to unit.

The incorporation of microscopic examination, a crucial element of successful crystallization in space, means that the crystallization process can be monitored and successful crystallization recognized when it occurs. The coupling of microscopic examination with automated procedures for crystal recovery and freezing will dramatically improve the ability of scientists to bring back high-quality crystals from space.

Figure 8-10. Astronaut Donald R. Pettit, during his spare time on board the ISS, built a small laboratory to experiment and observe the behavior of fluids and crystals in microgravity. This activity used simple materials that would not impact the programmatic supplies and was dubbed "Saturday Morning Science". In this photograph, he is shown looking closely at a water bubble within a 50-millimeter metal loop. Photo courtesy of NASA. Source: http://spaceflight.nasa.gov/station/crew/exp6/spacechronicles18.html

3.6 Perspectives

The main potential advantage of microgravity is the possibility of obtaining crystals that diffract to higher resolution or crystals that have more favorable morphology. This could be especially important in structure-based drug design. There is now a certain amount of evidence that crystal growth in

microgravity can have beneficial effects on the size and intrinsic order of macromolecular crystals. In many cases, crystals obtained in space are larger, have lower mosaicity, and diffract to higher resolution than comparable crystals grown on Earth. However, space-based crystallization programs have been very limited in scope in terms of the total number of samples compared with the enormous reach of modern protein crystallography on Earth. In addition, space-based crystallization efforts have been carried out under extremely adverse conditions. However, despite the greatly increased sophistication of ground-based protein crystallization projects, the crystals of many important targets have suboptimal diffraction characteristics. Improvements in diffraction that move a system from the margins of structure determination (3.0 to 2.5 Å) to well beyond that boundary will have a significant impact on the ability of the resulting structure to provide important insights into biological mechanisms.

If the protein or proteins being crystallized are soluble and relatively stable, there is little doubt that extensive experimental manipulation in the laboratory will eventually lead to better-diffracting crystals. However, it is very difficult to obtain membrane protein crystals that diffract to high resolution, so membrane proteins, such as the potassium channel, are attractive targets for investigation in microgravity. Potassium channels are integral membrane proteins that are important elements in the functioning of neuronal cells, and they also play diverse roles in the physiology of many different cell types. The potassium channels of greatest interest are those found in mammalian, particularly human, cells. However, it has not yet been possible to obtain crystals of mammalian potassium channels that are suitable for X-ray crystallographic analysis. There would be enormous value in improving the structural accuracy of the model for potassium channels (MacKinnon 2004).

Other proteins yielding crystals that diffract very poorly are those that form transient complexes during dynamic events, such as during cellular signaling. There is great interest in obtaining high-resolution structural analyses of such protein complexes, and these may benefit from the particular conditions of microgravity.

Drug design projects are another case where microgravity may be important. In the design of inhibitors it is usually important to see the stereochemistry by which binding occurs, and it is also necessary that the crystal structure be obtained for the precise target in question rather than for a closely related protein. This is a restriction that is usually avoided in practice, since the protein crystallographer will often search a set of closely related proteins for a protein with optimal crystallization characteristics. It is not at all uncommon to find that the particular protein that is most interesting, for example, the human variant of a family of proteins, does not yield suitable crystals.

The relatively poor diffraction obtained for such systems can arise for one or more reasons. These include the intrinsic flexibility of the macromolecular system being crystallized, as well as impurities or other factors that impede optimal crystal growth. At present there is no direct information on whether crystallization in microgravity will have a positive impact in cases where the sole inhibitor of crystallization is the intrinsic flexibility of the molecules involved. Further experimentation will help resolve this question, but the controlled manner in which crystals grow in microgravity may be beneficial in these cases.

Also, because protein crystals are up to 80% liquid, they can be used as a "sponge" to soak up drugs. After these crystals are injected into a patient, the drugs they embrace are released at a fairly constant rate as the crystal dissolves. This both extends the life of a single injection and eliminates or reduces the peaks and valleys of drug introduction, so harmful to those now undergoing drug treatment for diabetes and hepatitis.

Growing protein crystals in space helps to better understand their structure, as well as investigate their utility for a number of medical applications, such as a time-release vehicle for drugs (e.g., insulin and interferon). Although none of these protein crystals grown in space are ready for the market, several are undergoing clinical testing. However, there are around 100,000 protein crystals in the human body. Thus far, 2,000 structures have been defined. Importantly, new knowledge and techniques are increasing the effectiveness of protein crystallography through DNA studies and a number of related research efforts. Although it is certainly true that not all

Figure 8-11. Astronaut Sergei K. Krikalev, holds a sample tube within the Commercial Protein Crystallization Facility-2 in the Zvezda Service Module of ISS. Photo courtesy of NASA.

protein crystals are of interest, and perhaps there are some which will resist crystallization, it seems there will be a continuous need for space-based research.

4 SPACE COMMERCIALIZATION

4.1 Potential

In general, efforts to find commercial application from manned space activity have had limited success so far. Space activity is almost entirely dedicated to government missions, thus dominated by government procedures and funding. The ISS is usually justified by appealing to objectives other than its furtherance of commercial activity. These objectives include scientific research, national prestige, and establishing a platform for further exploration of the solar system. But surely the expenditure of more than $15 billion for the construction of the ISS should also yield commercial payoffs. As of today, the most promising areas of opportunity for space commercialization include crystal growth and biomedical research efforts.

Biotechnology is the application of biological research techniques to the development and manufacture of products which improve human health, animal health, and agriculture. One important question is what role commercial users and industry might play in using the biotechnology research performed in microgravity?

It is believed that commercial users of a macromolecular crystal growth facility on the ISS would come almost exclusively from pharmaceutical and biotechnology companies, with perhaps an occasional user from a contract research organization or an instrument manufacturer. Worldwide there are currently more than 100 companies with research programs in macromolecular crystallography. These industrial organizations employ approximately 500 scientists and technicians with all levels of expertise in crystallography. Most are located in countries that already participate in development of the ISS.

All of these companies employ crystallographers to aid in the design of biologically active molecules for use in human and animal health care or agriculture for the production of food and fiber. Industrial research programs in macromolecular crystallography fall within two broad categories. In structure-based drug design, the three-dimensional structure of a target macromolecule is determined to help in the design of a compound, most often a small molecule, which will bind tightly and selectively to the target, modifying its activity. In macromolecular engineering, the structure of a macromolecule is determined in order to guide research aimed at changing its structure. The goal is to alter its properties in some desirable way, with the final commercial product being the mutant macromolecule itself.

In 2000, a study by the National Research Council revealed that, although many pharmaceutical and biotechnology companies had participated in the microgravity crystallization research, not one had yet committed substantial financial resources to the program. The study concluded that "this is likely to remain the case until the benefits of microgravity can be convincingly documented by basic researchers and until facilities in space can handle greatly increased numbers of samples in a much more user friendly manner".

In other words, "We need a home run to get more interest in space commercialization".

Figure 8-12. Dr. Albert Sacco flew as a Payload Specialist operating his own zeolite crystal growth experiment onboard STS-73 in 1995. On this photograph, he is inspecting a crystal in a cylindrical autoclave on the middeck of Space Shuttle Columbia. Photo courtesy of NASA.

4.2 Problems and Solutions

In 1995, Dr. Albert Stacco, a professional crystallographer, flew on board the U.S. Microgravity (USML-2) Spacelab mission to complete an experiment on growing zeolite crystals (Figure 8-12). As such, he gained unique insights through serving as a scientist, astronaut, and commercial entrepreneur at the same time. The Zeolite Experiment was a success, yielding zeolite crystals that were superior to those grown on Earth (Figure 8-13).

However, the eventual commercial outcome of the Zeolite Experiment was not as dependent on the quality as it was on the relative cost and availability of zeolites produced on Earth, and the business relationship of the sponsors of the program. At this point, the zeolites produced on Earth are of adequate quality for commercial uses, and their price and availability are better than those produced in microgravity. Furthermore, current oil industry cracking equipment is designed for the existing zeolites, and the industry would be faced with large costs to convert to the use of the ones produced in microgravity. However, several potential customers expressed that should the need arise for microgravity production, they would not hesitate to invest the money required to facilitate production in space.

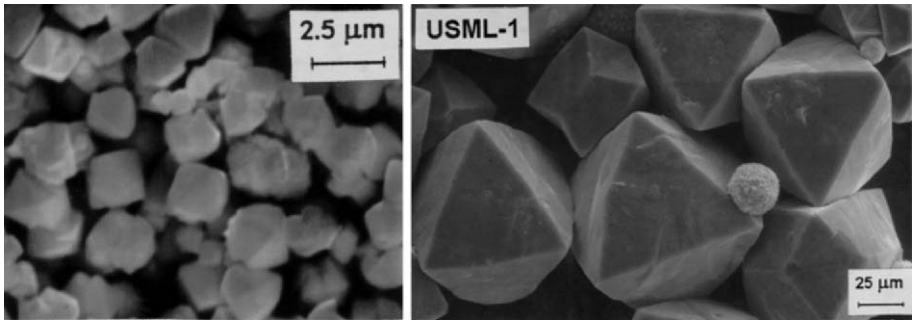

Figure 8-13. Zeolites are used as catalysts in the chemical processing industry, and gasoline is produced or upgraded using zeolites as catalysts in the refining process. Zeolites break up large, heavy oil molecules, making them smaller, and add hydrogen to the structure of the oil molecules so they burn more effectively. Zeolite crystals grown in microgravity (right) were at least 10 times larger than those produced in similar ground-based processing (left). Photo courtesy of NASA.

Dr. Sacco stated that commercialization in space should not be measured against the criteria used on Earth. On Earth, a company may have as many as 10,000 potential products in R&D, with experiments performed 24 hours a day. Of these 10,000, the vast majority will generally be canceled for one reason or another before reaching production and market. However, a vast number of experiments and samples will have been processed to bring the successful products to term. By comparison, space offers few opportunities to experiment (38 samples in the case of zeolites) and offers sporadic access to the "lab" as opposed to round-the-clock availability. There is also the problem of weighting commercial value against scientific value. This is not a necessary or even valid comparison. In fact, the majority of science ends up having some commercial value (National Research Council 2000).

Another problem might come from the selection process of the candidate experiments for space bioprocessing. Space experiments are often selected based on judgments on which science will benefit from the

microgravity environment. Because of a lack of good models in the area of bioprocessing, the peer review has often restricted the scope of science accepted for flight. This has probably resulted in missing the "wave" of new possibilities in discovery and potential market advantages for industry.

Another important point is that the success of virtually any venture, business or science, depends on the staff. It is generally accepted that scientists are better at running experiments than non-scientists. No amount of instruction manuals, expert systems and communications can replace the "gut feel" and experience of a scientist. At present there are approximately 40 scientists with the training and qualifications to go into space. Dr. Sacco believes that the performance of ISS and Shuttle experiments would be greatly enhanced if there were always a scientist on the flight crew, or at the very least available as a visiting consultant during crew change-over on ISS (Richardson 1997).

The burden of excessive paper work is unanimously criticized by the investigators involved in space research. Not only are the application processes complicated, they often need to be fully re-filed for each flight. To the uninitiated (or even the experienced) the flight application and certification process can be a minefield of inconsistencies, inter-Code battles, luck and unforeseen delays. None of these characteristics are attractive to business.

Finally, price and schedule remain two of the most important factors in determining the commercial viability of ISS and Shuttle. "Fly early, fly often" is the basic request of any organization wishing to work in space. There is also a willingness to pay a "fair price" for the service, which can best be defined as either *marginal cost*, or *direct cost*. Marginal cost is the additional cost of flying an experiment on a particular mission. This is variable but, in the case of a flight which is due to be launched with room on the manifest, the marginal cost is approximately zero. The cost of launching additional weight is probably inconsequential in terms of overall fuel cost. The crew costs will be identical, and there is no opportunity cost. Direct cost is applicable to the cost of a dedicated flight. In this case the commercial interest would be that NASA does not apply fixed overhead to the flight, but only charges for expenses directly attributable to the flight. This would include fuel, boosters, turn-around, etc. This figure has not been accurately calculated, but it is estimated to be less than $100 million (National Research Council 2000).

Scheduling is affected by the current hiatus due to ISS construction. When fully operational, the ISS will offer ideal mission length, since a 28-90 day period is adequate for performing a meaningful series of experiments. The greater the time spent in space, the more certain costs are spread, and the lower the per day rate, hence the lower the cost of individual experiments. So,

the ISS should greatly improve prospects for successful and affordable biotechnology experiments.

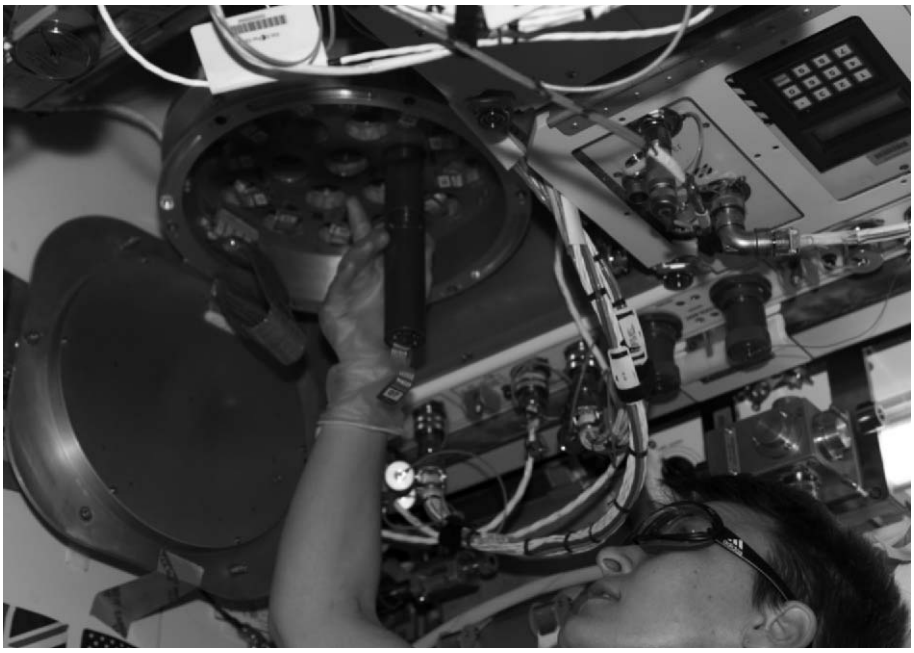

Figure 8-14. Astronaut Peggy A. Whitson inserts an experiment cartridge in the autoclave for the Zeolite Crystal Growth experiment in the Destiny laboratory on the ISS. Photo courtesy of NASA.

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FURTHER INFORMATION:

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http://www.estec.esa.nl/spaceflight/utilint.htm#br136

http://www.estec.esa.nl/spaceflight/access.htm#guide
http://www.estec.esa.nl/spaceflight/utilint.htm#br141
http://www.estec.esa.nl/spaceflight/utilint.htm#brapdx141

ESA Biotechnology Topical Teams:
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http://spacebio.net/Sptopics/index.html

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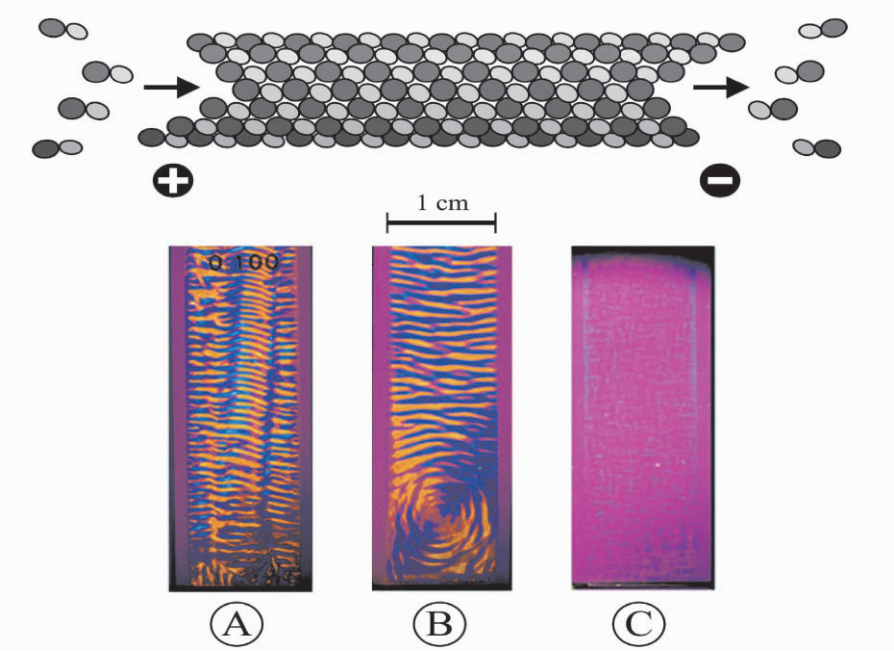

Figure 4-09. Top. A microtubule in a solution of tubulin and GTP is undergoing a dynamic process called "treadmilling". Tubulin is added to one end of the microtubule (+) and is removed from the other (-), at the expenses of GTP that is hydrolyzed to GDP. In such a way, the microtubule grows on one side and shrinks on the other. Bottom. Results of an experiment conducted on a sounding rocket in microgravity. The samples with tubulin and GTP contained in spectrophotometer cells were photographed in polarized light. The samples were kept inside an onboard 1-g centrifuge with the centrifugal force directed along (in A) or perpendicular to (in B) the long axis of the cell. The patterns show the self-organization of the microtubules. In the samples kept in 0 g (in C) almost no self-organization is occurring. Adapted from Tabony et al. (2002).

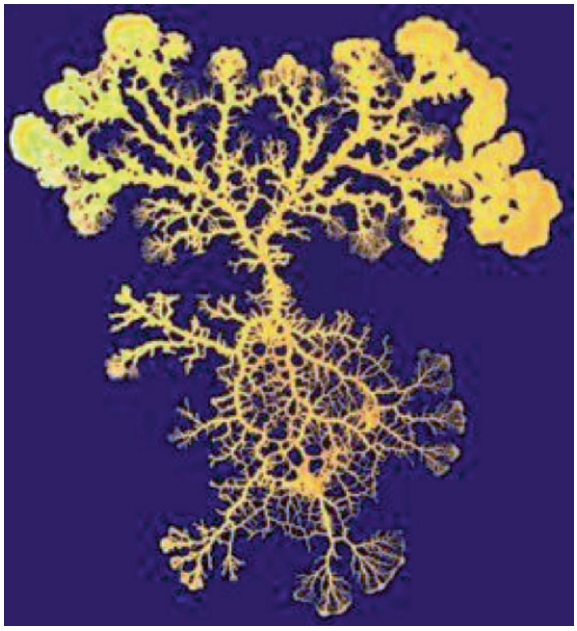

Figure 4-16. Physarum polycephalum. Courtesy of I. Block, Institute of Aerospace Medicine, DLR, Köln, Germany.

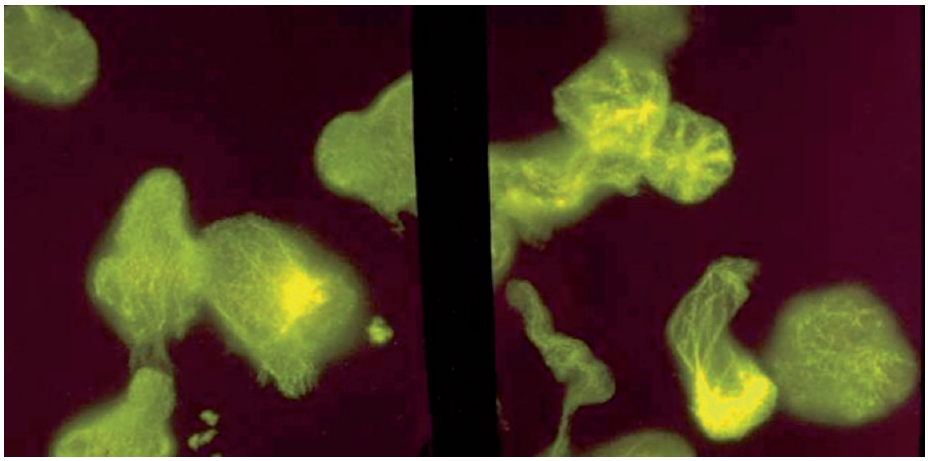

Figure 4-23. Vimentin filaments in Jurkat cells (a derived T-cell line) flown on the sounding rocket Maxus detected with fluorescent antibodies. Courtesy of G. Sciola.

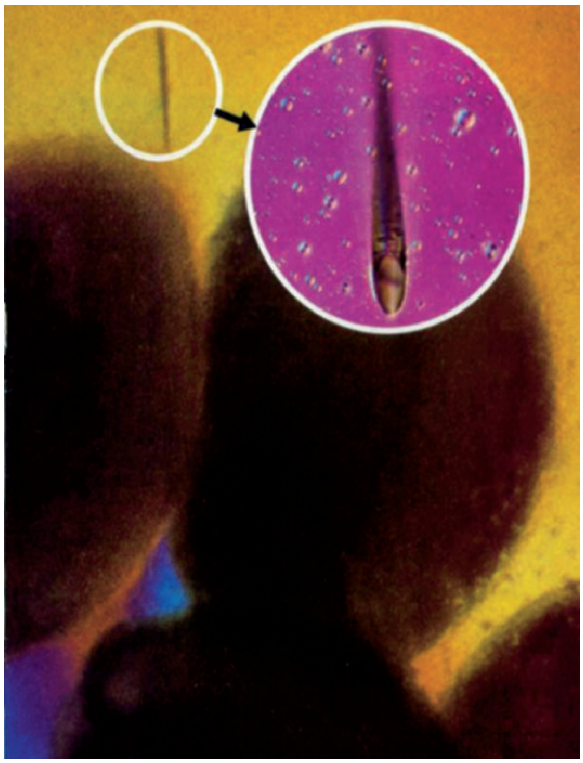

Figure 7-12. Biostack method used to determine the impact parameter for the most sensitive target in plant seeds after exposure to HZE particles of cosmic radiation.