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Gravitational and space biology at the cellular level

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1.1 General Introduction

Space biology can be said to have begun in the late 1940s, with the launch of monkey Albert on a U.S. rocket called 'Blossom 3' in 1948. Several other suborbital flights with monkeys and mice followed before the first manned space flight took place in 1961. Scientists in the then Soviet Union began their programme in space biology with the launches of nine dogs in 1951-52 before the historic flight of the dog Laika which survived one week in orbit in 1957. On the other hand, interest in gravitational biology began well over a century earlier, as reports on the effects of hypergravity (growth in a centrifuge) on plants and oocytes were published in the last century (Knight 1806; Pfluger 1883). Indeed, plant scientists have been using clinostats to study plant gravitropism for most of the past century, and are still using them.

On Earth, there is no way to perform an experiment without gravity, i.e. in the true weightless conditions of free fall, so space flight is essential to provide the opportunity to perform microgravity research. Progress in gravitational biology in general, and space biology in particular, was initially slow because the interest of space agencies in the U.S. and the former Soviet Union was focused on means to monitor and protect the health of astronauts. Experiments with bacteria, fungi or mammalian cells, such as lymphocytes, were seen as probes to detect effects of radiation, acceleration, vibration and/or weightlessness on living cells within the 'space medicine' context, and the experiments were devised accordingly. Nevertheless, these pioneering experiments supplied scientists involved in basic research with sufficient results to awaken their interest. The primary motive inducing the first few scientists to study single cells in space was scientific curiosity; the wish to see how relatively simple living systems react to an environment they had never experienced before throughout evolution. More recently, space flight research has been applied in a more focused analytical manner, the weightless environment being used to study specific biological phenomena, and there have been some notable and important discoveries.

Gravitational biology as a new discipline, then, started with the advent of biosatellites in the early 1960s. In 1960, the U.S. satellite Discoverer XVII and the Soviet satellite Biosputnik II, were loaded with bacteria, plant and animal cells. Failure due to technical difficulties was common in these early experiments, and although some missions (particularly Skylab III (1973)) were outstandingly successful, many of the biological results were of limited value because of engineering constraints on the scope of the experiments and the frequent lack of meaningful controls.

Systematic and extensive investigation became possible in 1983 when the first Shuttle-Spacelab mission was realized. Since then, important steps to increase the credibility and scientific gain from biological experimentation in space have been the integration of the Spacelab and, especially, the Biorack concepts in the Shuttle programme. Biorack provides a wide (and widening) range of laboratory facilities which, though commonplace on Earth, are new to orbital vehicles and provide experimenters with the necessary scope for more meaningful experimentation. Biorack has been flown successfully in three Spacelab missions, D-1 in 1985, IML-1 in 1992 and IML-2 in 1994. Other important biological experiments were carried out in Spacelab SLS-1 in 1991, SLJ in 1992 and D-2 in 1993.

A wide range of cell types have been subjected to space flight, including bacteria and protists as well as fungal, plant and animal cells. In many of these experiments the cells have

shown alterations in behaviour compared to ground based samples (review in Cogoli & Gmünder 1991). However, ground based samples are not the optimal control as it is not always possible to attribute observed changes solely to alterations in the gravitational field strength. Space flight introduces a number additional factors that need to be considered other than microgravity. These include high hypergravity levels at launch and re-entry (brief exposure to 3-25 × g), strong vibration and acoustic levels, and high-energy radiations, all of which may influence the function of the system under investigation (see Hamacher *et al*. 1987 and Chapter 4 of this volume). The full spectrum of effects cannot be simulated adequately on earth, so the only way to eliminate them from the investigation is to provide a 1 × g centrifuge facility for control experiments aboard the spacecraft.

This necessary device which has only recently been incorporated into orbital vehicles (in equipment such as Biorack). Consequently, many of the earlier space experiments which are reported in the literature could not include an on-board 1 × g control. These experiments must be interpreted with caution, but they still make a useful contribution.

The reported experiments also vary enormously in the biological material involved, as well as in the gravitational conditions encountered. Contrasts are obviously to be expected between experiments involving different organisms, different organs or different cells, but there are also disparities in the nature of the gravitational acceleration to which the experimental material is exposed. ***Stavrinidis et al. (1991)*** discuss both the opportunities for use of hypogravity and, importantly, the quality of the microgravity environments the various installations provide. The terms *microgravity*, *µg*, *0 g*, *low gravity*, *hypogravity*, and *weightlessness* do not have a quantitative meaning but are used in this chapter to refer to the gravitational conditions provided by manned space laboratories (10^{-4} to 10^{-2} × g), automatic satellites (10^{-5} to 10^{-3} × g), sounding rockets (10^{-5} to 10^{-4} × g), parabolic flights (10^{-1} to 10^{-2} × g) and drop towers (10^{-6} to 10^{-5} × g), respectively (see Hamacher *et al*. 1987).

Experiments with single cells in microgravity investigate changes in molecular organization, genetics, growth, proliferation and differentiation, and morphological characteristics. Space experiments represent the most spectacular segment in the field of gravitational cell biology in which the effects of gravity on living systems are studied, but experimentation in space must be interpreted in the context of related research. Gravitational biology also encompasses experiments carried out under hypergravity in centrifuges and ultracentrifuges. Low gravity experiments in the range of 0.02 × g can be simulated on Earth in clinostats, which can be accommodated in any biological laboratory. However, gravity conditions below 0.01 × g need elaborate and costly equipment such as drop towers, aircraft flying through parabolic trajectories, sounding rockets, and orbital vehicles.

The clinostat. Although other machines have been suggested (Mesland 1990, 1992), the key device among earthbound 'hypogravity' equipment is the clinostat. This valuable tool has a long history of use though it seems to us that its users do more than their fair share to maintain an unnatural and spurious division between microgravity research on animals and similar research on plants. For example, Brown *et al*. (1976) state that '...the clinostat must be considered an essentially botanical device...' because animals are seen to respond to gravity so much faster than plants. Those who use animal cells for their research are currently most likely to rely on the 'fast-rotating' clinostat although the original reference to which this is usually ascribed (Muller 1958) referred specifically to the human *organism* and a speed of rotation at the '...psycho-physiological optimum for disengaging his vestibular apparatus from an effective pull by gravity...' Preference for such a device over the conventional one for animal ***cells*** would clearly need other justification than the psychological. Fortunately, the basic theory upon which clinostat operation depends is well represented in the literature, very similar detailed mathematical treatments being published at about the same time by botanists (Dedolph & Dipert 1971), a zoologist (Silver 1976), though publication of this was delayed

by the death of the author, and in association with a mycological study (Tobias *et al*. 1973). The main points in these slightly different treatments will be brought together briefly below.

The clinostat provides circular rotation at uniform speed about the horizontal axis. This does not remove the subject from the effects of gravity and care must be exercised to use descriptive terms which do not carry unwarranted implications. A test subject mounted on the clinostat experiences altered vector direction; the normal gravity vector sweeps through 360° in each revolution, so in comparison with a stationary subject which is placed horizontally (and thereby experiences unilateral gravity stimulus) the clinostat subject experiences continuously shifting omnilateral stimulation. The crucial point, though, is that the effect of the treatment is *relative*. It depends on the rate of rotation, on the mass of the object considered, on its size and density and on the viscosity and density (specific gravity) of the surrounding fluid.

In complete contrast to the situation on an orbiting space craft, in which all objects experience the same microgravity environment, it is inevitable that different components of an experimental object on a clinostat experience different conditions. In the literature the clinostat has been variously described as providing gravity nullification, gravity compensation, or a time averaged gravitational force of zero. None of these descriptions can be true for all components of the object on the clinostat. It *is* true for all components that the gravitational stimulus is confused relative to the normal. The clinostat does not provide an exact analogue of the orbital (microgravity) environment but results of experiments using the two approaches are often broadly similar (e.g. Moore, 1991), though care must be exercised in interpreting clinostat experiments as differences of detail have been noted (Brown *et al*. 1976). The use of clinostats in gravitational biological research was discussed in detail at a symposium organized in 1991 by the American Society for Gravitational and Space Biology. The reports presented at the symposium appear in Halstead *et al*. (1992) and the papers (three presenting the physical properties and two reviewing the results) related to the clinostat are briefly discussed here.

Great caution in the use of the clinostat, in particular with single cells, is recommended by Albrecht-Buehler (1992) because '... it (a) generates centrifugal forces, (b) generates particle oscillations with mass-dependent amplitudes of speed and phase shifts relative to the clinorotation, (c) is unable to remove globally the scalar effects of gravity such as hydrostatic pressure, which are independent of the direction of gravity in the first place, and, (d) generates more convective mixing of gaseous or liquid environment of the test object, rather than eliminating it, as would true weightlessness'. Therefore, accurate control of the conditions is required and tests at different rotation speed must be conducted. Also, Kessler (1992a) points out a number of artefacts that may affect the results of clinorotation. Important advice on how to avoid artefacts in the use of the clinostat is given by Briegleb (1992). 'Functional weightlessness' can be achieved (i) with *small* systems, i.e. when the correlation between the quality of the simulated weightlessness and the size of the specimen depend on the threshold of gravitational sensitivity of the process, (ii) when there are only low density differentials between the components involved and (iii) when the viscosity of the medium is sufficiently high.

In a historical overview of the application of the fast rotating clinostat to single cells, M. Cogoli (1992) concludes that there is a good correlation between the results obtained in space and in the clinostat (see following sections). Sievers & Hejnowicz (1992) describe the effect of clinorotation and microgravity on plant cells and organs by distinguishing two types of gravistimulation: static and dynamic. While static as well as dynamic gravistimulation are switched off by microgravity, clinorotation can switch off the first but not the latter if the rotation is not sufficiently fast. In addition, one must take into account that plants are extremely sensitive to the mechanical stresses which may appear during clinorotation and,

therefore, influence the results.

Clinostat dynamics. In a stationary cell an intracellular organelle or particle which is denser than the cytoplasm will sink under the effect of the normal gravity vector; one which is relatively less dense will tend to float. Sedimentation and flotation are physically and mathematically equivalent except for the sign reversal of the vector (see note in Tobias *et al*. 1973). Any organelle which occupies a characteristic position within a cell under normal conditions must do so through the active control of its position by regulation of its density and/or by structural constraint by the membrane or cytoskeletal systems of the cell. The statements above, and those which follow, must, because of ignorance of the forces involved, neglect the possible effects of active cytoplasmic movements (streaming or cyclosis; but see Kessler, 1992b) and any specific restraint imposed by the cytoskeletal or membrane architecture.

Dedolph & Dippert (1971) and Silver (1976) provide detailed mathematical treatments of the theoretical behaviour of sedimenting particles subjected to clinostat rotation. The fundamental purpose of the clinostat is to equalise the effects of gravity on a particle through its circular rotation with uniform speed about a horizontal axis. For any particle there is a theoretical optimum rotation rate at which the rotation sweeps the gravity vector around the particle too swiftly for any sedimentation to occur. Both higher and lower speeds result in the particle executing circular motions; in the former case because of the centripetal accelerations due to centrifugal forces and in the latter case because of sedimentation during the slow sweep of the gravity vector. A major problem in interpreting clinostat experiments is that the cell must contain many particles exhibiting a variety of density differentials with their suspending medium with a consequent potential for a variety of responses to any given clinostat treatment. However, the clinostat can be used in an analytical manner. Theoretically, any quantifiable effect produced by clinostat treatment can be optimised at a specific rotation rate and the physical characteristics of the particle(s) deduced from the experimental circumstances which achieve this. Apart from the early work on plant organs (Larsen, 1962; Audus, 1962) this experimental approach has been applied rarely, most experimenters being content to work at one speed of rotation.

Dissimilar responses to different speeds of rotation have been noted. Increased fresh weight of cell cultures of *Haplopappus gracilis* occurred when they were cultivated on a 50 rpm clinostat, but not when rotated at 2 rpm (Klimchuk, 1984). A decrease in cell division in rapeseed protoplast cultures was recorded on a 2 rpm clinostat, but not on a 50 rpm clinostat (Rasmussen, Baggerud & Iversen, 1989). Lyon (1970) used nine clinostat rotation rates to study root and coleoptile development in wheat seedlings (*Triticum aestivum*) and curvatures of leaves and branches of *Coleus blumei*. The former showed the same effect over the whole range of rotation rates tested, while curvatures in *Coleus* were maximal at 0.3 to 1 rpm.

Evidently, different organisms, and perhaps even separate phenotypes in the same organism, depend upon particles with diverse physical characteristics so experiment at different rates of rotation is crucial. As with many other experimental devices, comparisons of responses to clinostat treatment must be made with care, and this is especially true if the device is to be used as an analogue of orbital conditions. Ideally, some direct evidence should be obtained to show that such a comparison is valid.

Fortunately, there is now a sufficient range of facilities available to the experimenter for him/her to match the vehicle or facility to the specific biological functions and structures investigated. While long-lasting processes (lasting hours or days), such as cell division, differentiation and, sometimes, genetic expression can be studied only in orbiting laboratories, other events, like binding of ligands to the cell membrane, signal transduction, cell movements and morphological aspects can be investigated easily in sounding rockets (which provide 7-12 min of weightlessness), parabolic flights (serial episodes of microgravity

of 15-30 seconds each) and even in drop towers (3-4 seconds of microgravity). On the ground, there is no limit to the exposure time when centrifuges or clinostats are used. Clearly the latter instrument is a useful tool to verify or prepare experiments in space providing it is always remembered that real microgravity is attainable only under free fall conditions. As stressed above, it is essential to appreciate that the clinostat simulates microgravity only through rotational compensation of the gravity vector (Silver 1976; Briegleb *et al.* 1976; Cogoli & Gmünder 1991). As the instrument is still within the Earth's gravitational field, gravity-driven phenomena (like density differentials which are absent in free fall) still occur. In this overview, experiments conducted in hypergravity in centrifuges, in simulated microgravity in clinostats, and in real microgravity in drop towers, parabolic flights, sounding rockets, and in manned as well as automatic orbital flights, will be discussed. Section 1.2 will focus on bacteria, slime moulds, protozoa and mammalian cells; whereas research on protozoal motility and fungi is discussed in Section 1.3 and the gravitational biology of plants and animals will be dealt with in Sections 1.4 and 1.5.

Data have now accrued to provide ample evidence for gravity dependent responses in a wide variety of cell types from simple prokaryotes to complex animal cells in culture (see earlier reviews in Gmünder & Cogoli 1988; Cogoli *et al*. 1989; Cogoli & Gmünder 1991). This is of great interest since such responses imply that gravity may influence biological processes through its action on physical phenomena within individual cells, suggesting that gravisensing at the cellular level could be an ancient and ubiquitous faculty.

1.2 Cell and molecular biology

1.2.1 Introduction

The two main objectives of today's cell biology in microgravity are:

- basic science, i.e. the study of significant changes of cellular behaviour. In certain instances (see Section 1.2.3) experiments in microgravity may help to understand better the complicated systems regulated by cytokines which are expressed by genes under differential control. In fact, at 0 × g, specific genes may be switched off thus permitting identification of the sequence of events leading to cell differentiation or to signal transduction;
- medical diagnostics, i.e. the use of cellular systems to assess the status of certain physiological conditions of humans in space.

Certain preliminary observations made in early missions encouraged hypotheses and speculations on the profitability of bioprocessing in space. So a third goal, namely bioprocessing, i.e. the use of microgravity to produce substances of pharmaceutical interest, might also prompt some experiments. However, it is doubtful that the, still theoretical, gains would balance the high cost of operations in space.

Several experiments described here have been carried out with peripheral blood lymphocytes (PBL), monocytes or with cell lines derived from PBL, monocytes or hybridoma cells. All these experiments appear here under the title 'Cells of the immune system'. In addition, it is important to notice that mitogenic activation of T cells always takes place in the presence of monocytes as accessory cells. More details on the lymphocyte-mitogen system are presented in Section 1.2.4.8.

Fig. 1. Mammalian cell cycle. While certain cells, like cancer cells go trough an indefinite number of cycles, other cells can enter a quiescent G0 status. Resting lymphocytes are in a G0 status until activation is triggered either by the corresponding antigen or by mitogens.

1.2.2 Proliferation

1.2.2.1 Background

Cell proliferation or division is the result of a complex series of processes which constitute the cell cycle. In eukaryotes, the cell cycle is subdivided into four discrete stages as shown in Fig. 1: the G1 period, the S phase, the G2 period and the M period. In the synthetic, S phase, DNA replication and synthesis of histone proteins occur. The S phase (DNA replication phase) is preceded by G1 and followed by G2, two 'gap' periods for what concerns DNA synthesis activity. Cell division occurs in the mitotic, M, period. Under certain circumstances, e.g. when the nutrients are consumed, the cell cycle can come to a stop at a so called G0 stage. Other cells, like lymphocytes, are in quiescent G0 status unless a stimulatory signal (an antigen or a mitogen) triggers the onset of the cell cycle. The regulation and timing of the cell cycle phases varies from cell to cell. While the S, G2 and M periods show little variation for mammalian cells (7-10 h, 2-4 h, and 1-2 h, respectively), G1 may vary from 1 h to many days or even be absent. Whereas yeast cells can double their number within 2 h, mammalian cells may require a minimum of 10-20 h, for instance, HeLa cells, a cell line derived from a human cervical carcinoma, take approximately 16 h to double their number.

Methods commonly used to measure cell proliferation are:

- cells are counted electronically with a Coulter Counter or by eye with a haemacytometer;
- the optical density of a culture suspension is measured spectrophotometrically and related to the number of cells;
- DNA content.

Sometimes, especially when cells form aggregates that cannot be counted, the rate of synthesis of DNA can be related to cell proliferation. For instance, mitogenic response of lymphocytes is measured by incorporation of radiolabelled thymidine into DNA, which, with this material and under these conditions indeed reflects the ^{3}H-thymidine pulse-labelling index (TLI). Immunologists thus often use the term 'proliferation' to indicate the rate of DNA synthesis in lymphocytes exposed to mitogens. This might not necessarily apply to all systems or conditions. In particular, data obtained in microgravity which are based on the TLI procedure are subjected to strong criticism and must be interpreted with great caution because microgravity, or other space flight factors, may change the labelling pattern of the precursor pool and thus indirectly affect the incorporation of label into nucleic acids. Independent assays are required to confirm the data.

1.2.2.2 Hypergravity

In parallel to experimentation in microgravity, cell proliferation and other cell functions have been studied under hypergravity conditions. The rationale is that if one wants to test the hypothesis that the transition from 1 × g on Earth to 0 × g in space causes alteration of cellular behaviour, the transition from 1 × g to hypergravity in a centrifuge should cause changes as well. Therefore, inexpensive and easy experiments in centrifuges could allow us to speculate whether changes may or may not occur also in microgravity. There is, however, an obvious and fundamental qualitative difference between the transition from 1 × g to hypergravity and from 1 × g to 0 × g. Experiments with single cells in centrifuges are reviewed here only as a complement to investigations in space, microgravity remaining that

interesting environment not reproducible on Earth. Nevertheless, several interesting findings have been made in hypergravity at g forces between 2 and 2000 × g, except for some experiments with *E. coli* which were conducted at 50,000 and 110,000 × g, respectively.

Human lymphocytes, HeLa, MC3T3, JTC-12 cells and the ciliate *Paramecium tetraurelia* were among the most extensively studied cells in centrifuges (Cogoli & Gmünder 1991).

Cells of the immune system. Gravitational effects were first discovered in murine spleen lymphocytes exposed to 2 × g and activated with the T cell mitogen concanavalin A (Con A) (Cogoli *et al.* 1979). Studies with human lymphocytes purified from peripheral blood and cultured with Con A at 10 × g showed an increase of the proliferation rate of 20-30% (Tschopp & Cogoli 1983; Cogoli 1993b). The effect was not due to an increase of hydrostatic pressure, as 1 × g controls in which paraffin was over-layered on the culture medium failed to show the effect. When the lymphocytes were kept in whole blood cultures, obtained from fresh blood diluted 1:10 with culture medium, the increase in proliferation rate at 10 × g could be as high as 300% of the 1 × g controls (Lorenzi *et al*. 1986). Interestingly enough, the effect appeared also when the cultures were first centrifuged for 72 h without mitogen and then cultured at 1 × g with Con A. It could be shown that the increase at high g was attributable mainly to the concomitant activation of B cells by Con A molecules attached to the erythrocytes which are present at 1-5% in purified cultures and in overwhelming amounts in whole blood cultures (Lorenzi *et al*. 1988; Cogoli, A *et al*. 1990). It is known that when Con A is presented to B lymphocytes attached to a solid matrix, B cells may also be activated. Maybe this effect is strongly enhanced at 10 × g, however, there is no explanation yet and why the effect occurs at high-g and not at 1 × g remains to be clarified.

HeLa cells. The proliferation rate was increased by 14% and 30% at 10 × g after 24 h and 48 h of culture, respectively. Cell counts and radioactive thymidine incorporation data correlated fairly well (Tschopp & Cogoli 1983). In other studies, conducted by Japanese scientists, it was seen that at 35 × g proliferation was 60-80% higher than at 1 × g after 4 days. At 18 × g and 70 × g the increase was 20% and 30% of the respective controls. It was shown that the change was due to a shorter G1 phase, 10.4 h at 1 × g and 8.0 h at 35 × g. Thus, the cell generation time was 22.4 h at 1 × g and 19.5 h at 35 × g (Kumei *et al.* 1987, 1989). In a further study (Sato *et al.* 1992) proliferation rate increased by 34% after 72 h. In contrast to the response of MC3T3-E1 cells (see below), no effect of indomethacin (IND), a suppressor of prostaglandin (PG) synthesis, on the proliferation of HeLa cells was detected.

Friend cells. An experiment conducted at 10 × g with this murine erythroleukemic cell line in the presence of dimethylsulfoxide (DMSO), an inducer of haemoglobin expression, for 6 days showed an increase of 35% in cell number, while glucose consumption remained unchanged (Tschopp & Cogoli 1983). Friend cells were chosen for their interesting peculiarity of differentiating along erythroid lines in the presence of DMSO. Indeed, Friend Cells are a widely used *in vitro* model of murine erythropoiesis. When incubated in minimum essential medium, the cell cycle lasts approximately 16 h. If 1.3% DMSO is added to the culture cell proliferation stops and haemoglobin is produced and stored intracellularly. Induction of haemoglobin synthesis is measured spectrophotometrically after lysing the cells with the detergent Triton X-100 and treatment of the supernatant with tetramethylbenzidine hydrochloride. Haemoglobin-positive cells are counted after staining with benzidine hydrochloride. The induction of Friend cells with DMSO can be regarded as a typical *in vitro* differentiating system which is analogous to the mitogenic activation of T lymphocytes.

Human lung adenocarcinoma cells A 549. The cells have the property to grow as nodules of 3.5 mm diameter and to express the normal differentiation of pneumocytes. Incubation at 2 and 15 × g for up to 27 d did not reveal any change of cell growth, protein and DNA content. Also the parameters indicating cell differentiation such as phospholipid composition,

particularly phosphatidyl content, were the same as in the controls (Croute *et al*. 1990; Gaubin *et al*. 1991). However, structural changes were detected as discussed in section 1.2.4.2.

Human embryonic lung fibroblasts (HEL). Exposure to 15 × g resulted in a significant decrease (17%, $P < 0.05$) of proliferation rate determined as number of cells on the 5th and 7th day of culture. Exposure to 5 × g gave less evident differences. The data correlate fairly well with the DNA content determined spectrofluorometrically (Gaubin *et al*. 1991). Surprisingly, there was no difference in protein content.

Human dermic fibroblasts (F 106). Exposure to 5 and 15 × g for up to 7 days at various stages of the life span of the cells did not show differences in cell growth rate measured in terms either of number of cells or as DNA content (Gaubin *et al*. 1991).

Other mammalian cells. There are reports (reviewed by Cogoli & Gmünder 1991) on other mammalian cells exposed to hypergravity conditions. Cells like V-79 (Chinese hamster), sarcoma Galliera (rat), CEF (chicken) showed increases in proliferation rate between 20% (sarcoma) and 70% (V-79) compared to the controls. Another erythroleukemic cell line, K-562 cells (human), showed no change or reduction of the proliferation rate at 10 × g.

Hypergravity (2.6 × g) depressed the development of limb elements in cultured mouse limb rudiments. There was a proximo-distal gradient of sensitivity to excess gravity in the limb with proximal structures being less susceptible than distal ones (Duke 1983). In some cases, proximal limb elements present prior to explantation disappeared upon exposure to excess gravity. The effect of hypergravity was assumed to operate via changes in mechanical tension and/or pressure on the cells, accompanied by alterations in cell size and morphology and membrane properties.

Three cell lines (MC3T3-E1 from neonatal mouse calvaria, HeLa (see above), and JTC-12 from monkey renal tubules) were investigated in hypergravity (Sato *et al.* 1992). Cell growth was determined in cultures of all cell lines kept at 5 × g, 10 × g, 20 × g, 40 × g for 72 h, respectively. The effect of conditioned medium on MC3T3-E1 cells was studied as follows: cells were incubated at 40 × g for 24 h, the supernatant was used as conditioned medium for stationary cultures for 48 h. The effect of IND on all cell lines was studied during 48 h at 40 × g. The effect of PGE2 was studied on MC3T3-E1 cells for 8 h.

Proliferation of MC3T3-E1 cells was depressed (- 8%) after 72 h at 10 × g, whereas at 20 × g and 40 × g it was increased by 11 and 27% respectively. In JTC-12 cells, centrifugation at 40 × g for 72 h caused an 8% increase in proliferation. Conditioned medium favoured slightly but significantly more (+16%) growth of MC3T3-E1 cells at 1 × g suggesting that humoral factors may play a role in gravitational effects. IND significantly suppressed proliferation of MC3T3-E1 cells at 1 × g as well as at 40 × g. At 40 × g the suppression brought proliferation at the level seen at 1 × g without IND (i.e. the hypergravity effect was abolished by IND). IND abolished the 40 × g effect in JTC-12 cells also. IND and PGE effects seem to be related to humoral factors. Other authors reported that low PGE concentrations (10^{-7} M) suppressed DNA synthesis whereas high PGE (10^{-5} M) activated it. However, the concentrations used by Sato *et al.* (1992) were lower than those used by the other authors in MC3T3-E1 cells. In addition, the amounts of PGE measured by Sato *et al.* (1992) at 20 × g and 40 × g are not consistent with those of the other authors. It is concluded that some other unknown factors must be involved. These interesting data need further verification.

Hypergravity (5 × g) stimulates proliferation (possibly through enhanced production of prostaglandin E2) but suppresses differentiation of osteoblast-like cells. The signalling pathway involved was independent of the activation of protein kinase C and the production of cyclic nucleotides, and distinct from the pathway through which IGF-I stimulates proliferation of these cells (Miwa *et al*. 1991). Further evidence for hypergravity

enhancement of the proliferation of the same osteoblast-like cell line being mediated via a prostaglandin E2-mediated mechanism is provided by Nakajima (1991). In this study cells were exposed to 5 and 18 × g for 1 to 3 days, or cultured at 5, 10, 20 and 40 × g. Migration was increased by 18 × g, and proliferation was enhanced at 20 and 40 × g but reduced at 10 × g.

Paramecium tetraurelia. This protozoan lives in ponds and can swim against gravity. There is a negative linear correlation between gravitational field strength and proliferation time measured at 1 × g, 2.5 × g, 5 g, 7.2 × g, 10 × g, and 20 × g. At 20 × g the number of cells after 3 days of incubation was one fifth of the value at 1 × g (Tixador *et al.* 1984b). When the generation time is determined 24 h after the centrifugation was stopped, the effect disappeared.

E. coli. Incubation in an ultracentrifuge at 50,000 × g for 2 h did not affect DNA, RNA or protein synthesis (Pollard 1971). However, at 110,000 × g, Montgomery *et al*. (1963) observed increased lag phases and prolonged generation times (between 1.41 and 1.58 h in the centrifuge and between 0.71 and 0.9 h in the control, respectively) after 24 h of incubation. A control to test whether the effect was due to packing of the bacteria at the bottom of the tube was conducted at 1,000 × g. No change of growth pattern was detected thus ruling out simple mechanical effects.

In conclusion, the data obtained in hypergravity strongly suggest that alterations of the gravitational environment have an important impact on several cell functions and thus justify the investment of resources to conduct similar studies in space.

1.2.2.3 Hypogravity in clinostats

Cells of the immune system. Reduction of proliferation by 50%, measured as the rate of DNA synthesis as TLI, was observed in cultures of purified as well as of whole blood lymphocytes in the fast rotating (90 rpm) clinostat in the presence of Con A (Cogoli A. *et al.* 1980, 1990; Cogoli 1993b). The data were confirmed by two independent qualitative analyses. First, blast cells (e.g. activated lymphocytes) appeared in large amount in the 1 × g controls, whereas none or very few were seen in the clinostated cultures. A quantitative analysis was not possible since cells cannot be counted when clustered in the typical aggregates formed by the polyvalent intercellular binding of Con A to the <a>-glycosides on membrane proteins. Second, activated lymphocytes were identified in large amount also in transmission electron micrographs of the 1 × g controls whereas very few or none were found in the clinostated samples.

Other mammalian cells. Proliferation did not change in the erythroleukemic human cell line K-562 exposed to haemin (Wiese *et al*. 1988). This was expected since haemin is known to trigger the synthesis of haemoglobin with concomitant halt of proliferation. Conversely, proliferation was increased in Friend cells exposed to DMSO (Lorenzi *et al*. 1988).

Paramecium tetraurelia. Growth rate during 3 days at rotation speeds between 50 and 110 rpm in the fast rotating clinostat was increased by approximately 20% at 90 rpm, lower (or no) differences were seen at the other rotation speeds (Ayed *et al.* 1992). In earlier work (Tixador *et al*. 1984b) no effect was seen in a slow rotating (1 rpm) clinostat. These data support calculations indicating that, for particles of the size of *Paramecium*, low-gravity simulation with the clinostat needs 30-100 rpm to generate the desired tumbling motion (Schatz *et al*. 1992; Björkman 1992.)

Salmonella typhimurium. Growth was increased by 62% in the clinostat (Mattoni *et al.* 1971).

1.2.2.4 Hypogravity - in orbit

The proliferation of representatives of nearly all classes of organism has been investigated in

space.

Cells of the immune system. Extensive studies on several space flights were conducted in the last decade with lymphocytes and monocytes from human peripheral blood as well as with derived cell lines (for review see Cogoli 1993b). Activation of T lymphocytes by Con A, purified from human peripheral blood, was carried out for the first time in space in an experiment in Spacelab-1 in 1983 (Cogoli *et al.* 1984; Cogoli & Tschopp 1985). Surprisingly, it was discovered that activation, measured as the rate of DNA synthesis as TLI, was inhibited by 93%, compared to synchronous 1 × g controls on the ground, despite the fact that the cells formed aggregates in microgravity. Again, the results were confirmed by qualitative light and electron microscopic analyses. Unfortunately, due to the limited resources, a 1 × g control in space was not conducted. However, the results were confirmed in two experiments performed in Biorack in Spacelab D-1 (Bechler & Cogoli 1986; Cogoli *et al.*1988) which included 1 × g controls on board.

A step towards understanding the strong inhibition of activation in microgravity was taken in an experiment conducted on Spacelab SLS-1 in 1991 (Bechler *et al.*1992; Cogoli *et al.* 1993). Using a new technology, lymphocytes and monocytes were attached to Cytodex 1 microcarriers prior to exposure to Con A in microgravity. Activation was more than doubled compared to the flight and ground controls at 1 × g. As described in Section 1.2.3.6, analysis of the cytokines secreted established that the lack of activation in free cells is due to the failure of monocytes to deliver IL-1 as the second signal required for T lymphocyte activation. Not yet explained is the fact that attached T cells double their activation in microgravity when sufficient IL-1 is available. A summary of the changes observed on lymphocyte activation under altered gravitational conditions is shown in Fig. 2.

Fig. 2. Gravitational effects on lymphocyte activation. The diagram gives an overview of what we know to date on the effects of space flight (0 × g/space and 1 × g/space respectively), hypergravity (10 × g/centrifuge), simulated low gravity (0.02 × g/clinostat) and cosmic radiation (1 × g/stratospheric balloon) on mitogenic activation by Con A of T lymphocytes. The experiments were conducted either with resuspended (-MC) or microcarrier-attached cells (+MC). The bars give the percent of activation (measured on day 3 after exposure to Con A at 37°C as counts min^{-1} of tritiated thymidine incorporated into DNA) with respect to the relative control (taken as 100%) on the ground in the absence of MC beads (-MC/1 × g/ground). Since the number of independent experiments shown in the diagram varies from one to 20, standard errors are not given here.

Proliferation of hybridoma cells (derived from the fusion of myeloma cells and B lymphocytes) has been determined in two Spacelab flights. In both experiments the results in 0 × g were compared to in flight 1 × g control. AM2 mouse hybridoma cells, cultured in Biorack in D-1, did not show differences in cell counts after 5 days in space (Beaure d'Augères *et al.* 1986).

The cell line 7E3-N, producing monoclonal antibodies against a lipopolysaccharide binding protein, was cultured in Biorack in IML-1 (Bechler *et al.* 1993). Being derived from lymphocytes, it was thought that hybridoma cells may also be gravity sensitive. The number of cells ml^{-1} after 4 days in culture (independent duplicates) was increased from 7.0×10^5 (1 × g in flight) to 9.8×10^5 (0 × g). This difference is significant ($P = 0.05$). No significant difference was detected between the 1 × g controls in flight and on the ground. The increase of the proliferation rate (+ 40%, $P = 0.05$) at 0 × g was confirmed by the DNA synthesis data. Conversely, no important differences were seen after 2 days of incubation. This may indicate that microgravity effects became important only after a longer exposure (48 h) to 0 × g.

Friend cells. Friend leukaemia virus transformed cells were chosen for their interesting peculiarity of differentiating along erythroid lines in the presence of DMSO. The major objective of the experiment was to test the hypothesis that, in analogy with another *in vitro* differentiating system, namely T lymphocytes exposed to mitogens, important cellular functions would change in microgravity (Bechler *et al.*1993). The numbers of cells found in 1 ml volumes of culture after 140 h of incubation in the presence of DMSO were 12×10^5 in both the in-flight 0 × g and the 1 × g centrifuge controls, and 10×10^5 in the 1 × g ground controls; these differences were not significant. In addition, the cells grew normally, the starting concentration being 0.75×10^5 cells ml^{-1} (contrast these results with the increase in cell number of cells cultured at 10 × g discussed in Section 1.2.2.2).

WI38 human embryonic lung cells. These cells were cultured for 28 days in Skylab, in a sophisticated instrument consisting of incubation chambers with automatic medium supply, fixation and time-lapse cine-camera. No effect was observed on the lag phase or growth rate, although glucose consumption from the medium was 20% higher in the flight cultures than in the ground control (Montgomery *et al.* 1978).

HeLa cells. Cultures were exposed successively to five to six orbital flights, though the duration of the flights and the intervals between them are not specified (Zhukov-Verezhnikov *et al.* 1971). No alteration of the mitotic index was detected.

HaK cells. Hamster embryonic kidney cells were grown for 7 days in Spacelab IML-1 in a newly developed cell culture chamber (DCCS - dynamic cell culture system). No change was detected in cell proliferation, measured as cell counts, in comparison with in-flight 1 × g controls as well as ground controls (Gmünder *et al*. 1988; Lorenzi *et al*. 1993).

Paramecium aurelia. On Spacelab D-1, microgravity was found to have a stimulatory effect on proliferation (Richoilley *et al.* 1986, 1988a, 1988b). Maximal growth rate of zero gravity cultures was 0.67 per day compared to 0.41 per day of 1 × g controls both in the Biorack centrifuge and on the ground. The *Paramecium* cells grown at 0 × g had a 15-20% larger cell volume during early log-phase than cells grown at 1 × g (both in the gravity simulating centrifuge and on the ground). In contrast, the cells of 0 × g cultures were 10-20% smaller in the late log-phase than the 1 × g controls. These findings are in line with earlier experiments carried out on Salyut 7 (Richoilley *et al.* 1988a & b; Tixador *et al.* 1981). Radiation was found to have a weak growth-stimulating effect (Tixador *et al.* 1984b). On a stratospheric balloon flight, the number of cells per culture was significantly higher than in earth-based synchronous cultures (19 vs 17 cells per culture, means of 64 cultures) (Tixador *et al.* 1984b).

Pelomyxa carolinensis. Of particular interest is the behaviour in microgravity of this giant amoeba, 5 mm in length when fully extended and containing 100 to 1,000 nuclei. No significant difference in growth rate was detected in two experiments on Biosatellite II (Ekberg *et al.* 1971; Abel *et al.* 1971). Unfortunately, the flight had to be shortened from 3 to 2 days due to weather conditions at the landing site. Thus the exposure to microgravity was too short to provide conclusive data on the growth rate. It is surprising that this unicellular organism which, due to its size, appears to be an ideal object for gravitational studies has not been flown in space again so far.

E. coli. In an experiment conducted on board the US Biosatellite II and designed mainly to investigate effects of radiation, viable bacterial densities were 19% higher in flight samples than in ground controls when measured by plate counts, but not different when measured by Coulter counter, after a flight lasting two days (Mattoni *et al.* 1971). In samples exposed to 2 Roentgen of chronic γ-radiation, the density of the flight samples was 45% and 41% higher as determined by the same methods, respectively. Samples of *E. coli* were cultured for 24 h at 37°C in an automated instrument during the flight of the Biocosmos 2044 satellite which lasted 15 days (Bouloc & D'Ari 1991). Cultures were inoculated by breaking ampoules containing the bacteria. The medium contained glucose or glycerol as limiting carbon source.

Growth was stopped by lowering the temperature to 4°C. Analysis revealed that cell growth, calculated from the OD of triplicate cultures at 600 nm, did not change in space. Using a different procedure (cell counts of cultures held at 5° after incubation for various times in flight), Gasset *et al*. (1994) also came to the conclusion that microgravity (during the STS-42 mission) has no effect on exponential growth of *E. coli*.

Bacillus subtilis. Increases of yield and growth rate were noted in Spacelab D-1 (Mennigmann & Lange 1986, 1988). The authors were not able to give exact numbers, because the optical density reading of the 0 × g culture went out of range. However, they found that *B. subtilis* yielded far fewer spores under microgravity conditions than at 1 × g (0.5×10^5 ml^{-1} against 8×10^5 ml^{-1}). Thus, under microgravity the spore forming bacillus does not develop spores at the end of the stationary growth phase as easily as it normally does. This suggests that under microgravity conditions cell differentiation processes may not proceed normally.

Salmonella typhimurium. In an investigation in Biosatellite II (Mattoni *et al.* 1971) the increase in viable density of the flight samples was 30% (from plate counts) and 15% (by Coulter counting), respectively, thus confirming the results in the clinostat. In addition, in samples irradiated with 265 R, 654 R, and 1630 R the increase in cell density was 91%, 88%, and 84% (from plate counts), and 88%, 93% and 62 % (from Coulter counts), respectively.

Proteus vulgaris. The number of cells grown for 2 days in Soyuz-12 was 7 times higher than the corresponding controls (Kordyum *et al.* 1974).

Space experiments fall into two categories. Ideally, experiments should be systematically performed in space, should include 1 × g controls on board and be accompanied by ground experiments, preferably involving use of the clinostat. The most valid conclusions on 0 × g effects on cell proliferation are drawn from this group, but the criteria have been met only rarely and many experiments fall into the second category - being conducted only once and with little or no supporting ground-based investigation.

- Changes of the proliferation rate of single cells in microgravity are now well documented. However, no general pattern of behaviour can be recognised, sometimes opposite effects are observed (e.g. lymphocytes *vs Paramecium*), and in the majority of the cells investigated there is no effect.
- The fast rotating clinostat delivers results which are in fair agreement with those obtained in space as seen in lymphocytes, *Paramecium* and *Salmonella*.
- Among mammalian cells, the lymphocyte/monocyte system appears to be one of the most dramatically affected. Interestingly enough, Friend cells, induced to differentiate with DMSO, are not sensitive to 0 × g in space. Among other unicellular organisms, *Paramecium* shows the greatest effects.
- The partial failure of the Biosatellite II mission in the early seventies, may have discouraged the continuation of studies on interesting objects like the giant amoeba *Pelomyxa carolinensis*.

1.2.3. Genetic expression and signal transduction

1.2.3.1 Background

After the discovery of gravitational effects on the proliferation of single cells, the next step was to deepen the investigations to genetic and metabolic effects at the molecular level. This section deals (i) with effects on the expression of cell-specific products, like cytokines as well as on the early expression of genes like *c-fos*, *c-jun* and *c-myc*; (ii) with effects on the transfer of genetic material between cells; and (iii) with effects on the consumption of nutrients as well as on the secretion of waste products. All these effects are related through signal transduction within the cell. Normally, biochemical signals are received via recognition and

binding of ligands to specific cell membrane receptors, so experiments on the binding of ligands in microgravity are also described in this section. Since the process takes only a few seconds, interesting experiments can be conducted in parabolic flights with episodes of microgravity of 15-30 sec.

The gene products of the c-*fos* and c-*jun* proto-oncogene family are known for their prominent role in cell proliferation and differentiation. Their expression is usually rapidly induced by growth factors and can be induced also by a variety of agents that by-pass the receptor and mimic the partial activation of signal transduction pathways. Lymphocytes and monocytes, A431 epidermoid cells, and HeLa cells are the most extensively studied systems with respect to signal transduction and genetic expression under changed gravitational conditions. The hypothetical mechanism of the T lymphocyte activation by Con A is shown in Fig. 3 as an example of signal transduction in which the G proteins/phospholipase C/phosphoinositol bisphosphate as well as the protein kinase C pathways are involved. A third important mechanism involves the adenylate cyclase/protein kinase A/cAMP pathway. Phorbol esters (e.g. TPA) are known to activate PKC and thus mimic the second signal (IL-1) in T cell activation. Ionophores (e.g. A23187) may bypass the first signal (Con A or antigen) by releasing calcium ions into the cytoplasm from the endoplasmic reticulum. Forskolin is an activator of adenyl cyclase.

Fig. 3. Mechanism of T lymphocyte activation by Con A. Three phases of T cell activation can be distinguished:

- Phase A: Binding of Con A (1st signal) to membrane glycoproteins (MGP), induction of protein G (G) to activate phospholipase C (PLC). Cleavage of phosphoinositol bisphosphate (PIP2) into diacylglycerol (DG) and inositol triphosphate (IP3). Release of calcium ions from the endoplasmic reticulum (ER). Activation of a cytoplasmic kinase. Triggering of synthesis of IL-2 following expression of the oncogenes *c-fos* and *c-myc*. The release of calcium ions from the ER can be induced by calcium ionophores thus bypassing and mimicking the transduction of the 1st signal.
- Phase B: Secretion of limited amounts IL-2 and cell-cell interaction between T and accessory cells (AC), usually monocytes, triggering of the production and secretion of IL-1 (2nd signal), activation of protein kinase C (PKC) upon interaction with IL-1. Probably there is a synergistic effect of DG in the activation of PKC. Activation of a cytoplasmic protein, production and insertion of IL-2 receptors (IL-2R) in the cell membrane and amplification of synthesis and secretion of IL-2. The action of IL-1 can be by-passed and mimicked by phorbol esters.
- Phase C: IL-2 binds (3rd signal) to the IL-2 receptor. Full activation of T lymphocytes is triggered. Cells start to divide and two populations, the effector and the memory cells, are generated

1.2.3.2 Hypergravity

HeLa cells. The induction of the proto-oncogene *c-myc* in response to hypergravity was determined in cells grown in monolayer and centrifuged at 18 × g, 35 × g and 70 × g at 37°C for between 15 and 360 min. Induction of c-*myc* mRNA was determined by electrophoresis of the extracted RNA, followed by hybridization with ^{32}P-labelled c-*myc* probes and densitometry of Northern blots. Elevated levels of c-*myc* mRNA were observed at all hypergravity values tested. The effect was most evident at 35 × g; levels of c-*myc* mRNA were 3.0- and 3.8- fold higher than the control after 30 and 120 min of centrifugation, respectively. The elevated level of c-*myc* mRNA was maintained (3.3-fold greater than the control) even after 360 min exposure to 35 × g (Kumei *et al.* 1989).

In a later study conducted at 35 × g, Kumei *et al.* (1991**)** observed that hypergravity increased the production of inositol 1,4,5,-triphosphate (IP_3) 1.5-fold after 2 min and 2.1-fold after 5 min and reported that intracellular level of cAMP decreased by 11% after 10 min and by 16% after 20 min at 35 × g. Phosphorylation of proteins immunoprecipitated by antibodies recognizing microtubule-associated proteins (ipMAPs) was determined. Phosphorylation of a 115-kDa detergent-insoluble protein was enhanced by 100% after 5-min exposure to hypergravity but did not differ from the control after 80 min. Phosphorylation of a 200-kDa detergent-soluble protein was observed after 20 min exposure to 35 × g. These results suggest that IP_3 and c-AMP may act as second messengers in hypergravity signal transduction.

Phosphorylation of ipMAPs in both detergent-soluble and -insoluble fractions suggests that cytoskeletal structures may be influenced by gravity (Kumei *et al.* 1991). No similar data are available on microgravity effects (though sounding rocket experiments have implicated involvement of actin microfilaments in gravity detection in plants, see Section 1.4.5.7).

Erythropoietic cell lines. K-562 and Friend cells were exposed to haemin and DMSO, respectively, at 10 × g (Lorenzi *et al.* 1988). K-562 cells showed depression of haemoglobin production, but no effects on glucose consumption and proportion of haemoglobin-producing cells were detected. In Friend cells, glucose consumption was reduced though haemoglobin production remained unchanged.

A431 cells. At 10 × g a slight increase (+ 18%) of epidermal-growth factor (EGF) induced *fos* expression and no change of constitutive expression was found by de Groot *et al.* (1991), see following section for more details.

Human dermic fibroblasts (F 106). Cell differentiation in terms of synthesis of elastase was studied at 15 × g, and a moderate but significant increase of elastase enzymatic activity (+8% and +13% on the 8th and 9th day of culture, respectively) was recorded. Since elastase degrades elastic fibres the data suggest that remodelling of the basement membrane reconstituted by fibroblasts is enhanced in hypergravity (Gaubin *et al.* 1991).

Osteoblastic cells MC3T3-E1. In a recent study conducted with cultures kept at 50, 200, 900 and 2000 × g for 5 min the expression of c-*fos*, c-*jun*, and *egr*-1 genes as well as protein kinase C activity were determined (Nose & Shibanuma 1994). A qualitative analysis by electrophoresis and autoradiography of cellular RNA hybridized with molecular probes showed that exposure to 50 × g was sufficient to stimulate transiently osteoblastic cells to induce the early response genes c-*fos* and *egr*-1, whereas c-*jun* was only marginally affected. Induction of c-*fos* in hypergravity was almost completely inhibited by staurosporin (an inhibitor of PKC) whereas that of *egr*-1 was only partially inhibited. Also, TPA inhibited almost completely the induction of c-*fos*, whereas *egr*-1 was not affected. A quantitative analysis showed that PKC activity is increased at 900 × g but not at 5 × g. Altogether these result confirm that hypergravity stimulates multiple signal transduction cascades connected with early genetic expression.

1.2.3.3 Hypogravity in clinostats

K-562 cells. Human K-562 cells were cultured in the fast rotating clinostat in the presence of haemin. A 10% decrease in glucose consumption and a 50% decrease in haemoglobin production (measured in the supernatants of lysed cells) were observed, while the number of haemoglobin-producing cells (stained with benzidine) remained unchanged (Wiese *et al.* 1988).

A431 cells. The induction of so-called ‘immediate early genes’, like c-*fos*, is the earliest detectable nuclear indication of a normally functioning signal transduction cascade. The EGF induced expression of c-*fos* proto-oncogene in A431 cells was studied in the clinostat (60 rpm) (de Groot *et al.* 1991). Under normal laboratory conditions at 1 × g, a 20-fold increase

of c-*fos* mRNA level is seen after 10 min exposure to EGF, after 30 min it is 50-fold (maximum). The induction is transient, returning to pre-stimulation levels after 2-2.5 h. With a more sensitive method, induction can be detected after only 3-6 min. With cycloheximide, the EGF effect is longer-lasting as a result of blocking the expression of proteins responsible for the repression of *fos* transcription and degradation of *fos* mRNA. c-*fos* induction is temperature dependent: there being no induction at 4°C, the maximum being at 30-37°C.

Various agents like TPA (phorbol esters), calcium ionophores (A23187, ionomycin), and mitogenic neuropeptides (bradykinin, histamine, bombesin) can induce the *fos* gene in A431 cells. The efficiencies are different at excess concentrations. TPA and A23187 mimic aspects of the signalling cascade initiated by EGF or mitogenic neuropeptides.
Constitutive *fos* mRNA levels did not change in the clinostat. EGF-induced *fos* expression was slightly depressed (-20%). Based also on results obtained in the centrifuge, in which the opposite effect was found, it is concluded that the results clearly show the EGF-induced *fos* expression in A431 cells is sensitive to change in gravitational acceleration. 'The induction is a rapid nuclear response following activation of the signal transduction cascade by extracellular factors, and is therefore a good indicator to study the influence of gravity changes on this process' (de Groot *et al.* 1991).

In another study by the same team (de Groot *et al.* 1990), it was possible to show, by coupling c-*fos* serum response element (SRE) with the CAT (chloramphenicol acetyl transferase) gene and transfecting into A431 cells, that the reduction of expression of c-*fos* gene is caused by a specific decrease in SRE activity in microgravity. To identify which subset(s) of signal transduction is/are affected in microgravity, induction of c-*fos* expression was tested in the clinostat in the presence of EGF, TPA, activator of protein kinase C (PKC), A23187 (by-passing phospholipase C) and forskolin, activator of protein kinase A (PKA) respectively. EGF and TPA induced expression were reduced by 25% and 30% respectively, no significant difference (from the 1 × g control) was found with A23187 or forskolin.
In parallel with experiments in sounding rockets (see below, Section 1.2.3.5), the question whether microgravity just delays c-*fos* expression or really depresses it, was addressed in an experiment conducted in the clinostat (de Groot *et al.* 1991). Maximum expression was reached after 30 min, incubations were performed for 10, 20, 30, 60, and 90 min (at this time it decreased by 20%). The 1 × g/0 × g ratio of expression remained greater than 1 throughout the incubation, showing that microgravity really depresses rather than delays the response. However, the effect remained low.

1.2.3.4 Hypogravity in parabolic flights

Inflammatory cells. In a device specially developed for experiments lasting a few seconds, it was possible to show that production of superoxide anion induced by phorbol ester (PMA) in peritoneal murine neutrophils was enhanced by a factor of four in microgravity (Fleming *et al.* 1991).

Rat osteosarcoma. A rat osteosarcoma cell line (ROS 17/2.8 osteogenic cells) was exposed to gold labelled epidermal growth factor at 37°C during 20 sec microgravity in a parabolic flight (Schmitt *et al.* 1991). EGF was added 3 sec before 0 × g, glutaraldehyde was added immediately before the end of the microgravity period. Gold particles were counted on electronmicrographs. No difference in binding of EGF was observed in microgravity.

Internalization of EGF via receptor mediated endocytosis (RME) was observed during the 25 min flight (8 parabolas). RME is an active mechanism closely related to the cytoskeleton.

In another study with the same cell line, the effect of gravitational stress on RME was studied with cells exposed continuously from 18 min to 5 parabolas consisting of 5 periods of microgravity and 10 periods of hypergravity (1.8 × g) (Malouvier *et al.* 1992). Three phases

of RME were determined: binding, clathrin-mediated internalization of the ligand-receptor complex, and recycling or degradation via endosomes and lysosomes. Membrane binding (1st phase) of EGF was lower in gravity-stimulated cells (-19%). No difference was seen for the coated structures (2nd phase). More cytoplasmic labelling (21%) was seen in the gravity-stimulated cells (3rd phase).

Blood platelets. The PKC signal transduction pathway has been studied on the PKC-dependent phosphorylation of the 40 kDa protein plekstrin. Phosphorylation occurs within a few seconds upon activation of ^{32}P-labelled platelets with thrombin or phorbol esters like PMA (Schmitt *et al.* 1993). Under the experimental conditions the 40 kDa band was clearly visible 10 sec after activation on SDS-PAGE autoradiographs. Quantitative analysis of the radiolabelled bands from samples exposed to 1 × g, 1.8 × g and microgravity did not show significant differences. The results indicate that neither Ca^{2+}-calmodulin-mediated activation nor the PKC-dependent pathways are inhibited during these short periods of microgravity.

Gap junction channelling. Gap junctions extracted from cardiac tissue (chicken) were inserted in liposomes containing FITC-microperoxidase and incubated at 1 and 0 × g with Azure C/H_2O_2 as substrate (Claassen & Spooner 1989). The oxidation of Azure C was followed spectrophotometrically at 612 nm. No effect was observed during 20 sec microgravity.

Rhizobia. In an experiment on the binding of lectin to the cell membrane, no effect of microgravity was observed on the interaction between lectin and rhizobia (Henry *et al.* 1990).

Antigen-antibody binding. The binding of α-fetoprotein (AFP) to immobilized monoclonal antibodies (mAb) was determined (Spooner *et al.* 1990). Microgravity lasted 20 sec, AFP was mixed with monoclonal anti-AFP bound to latex microparticles. Bound AFP was determined with mAb anti-AFP marked with alkaline phosphatase using nitrophenylphosphate as substrate. No effect of microgravity on binding was observed.

1.2.3.5 Hypogravity in sounding rockets

Cells of the immune system. The hypothesis that changes in membrane function and, consequently, in the availability of membrane glycoproteins to Con A may occur in microgravity, was tested in two experiments conducted on sounding rockets (Cogoli, M. *et al.* 1990). The instruments provided automatic injection of fluorescence-labelled Con A, followed by fixation at given times with paraformaldehyde during seven minutes in microgravity. Both experiments showed that binding of the mitogen to the membrane was not affected, only a slight delay of patching and capping was observed.

A431 epidermoid cells. Important studies were carried out by Dutch investigators with A431 cells exposed to EGF on MASER 3 and MASER 4, respectively. In MASER 3, the parameters measured were expression of c-*fos* and c-*jun*, and, in the clinostat (see above), activity of SRE. Expression of c-*fos* gene was depressed by 50% as well as that of c-*jun* (the product of c-*jun* forms a heterodimeric complex with the product of c-*fos*) (de Groot *et al.* 1990). Expression of the β-2-microglobulin gene, which is not modulated by EGF, did not change indicating that the effect on proto-oncogenes is a specific one. SRE is present in the 5' regulatory region of the *fos* gene.

In MASER 4, the protocol was essentially the same as that in MASER 3, except that incubation with EGF, TPA, A23187 and forskolin were performed in real microgravity on the sounding rocket and not in the clinostat. EGF- and TPA-induced expression of c-*fos* was decreased by 47% and 26% respectively. No effect with A23187 or forskolin, nor effects on the expression of β-microglobulin were detected. Similar results were obtained with c-*jun*: with EGF and TPA, depression was 56% and 51% respectively, no effect was seen with A23187. Forskolin was not tested because it does not induce the expression of c-*jun* (de Groot *et al.* 1991).

1.2.3.6 Hypogravity in orbit

Cells of the immune system. The first to study cultures of lymphocytes in Space were Tàlas *et al.* (1983) who discovered that the production of interferon-α (IFN-α), induced by polynucleotides in human lymphocytes cultured on the Soviet space station Salyut 7 was increased by 500% compared to the ground controls. However, the experimental conditions were far from optimal because the incubator was switched off during the sleep time of the crew for safety reasons. In addition, no 1 × g reference was available on board.
Dramatic effects on ^{3}H-thymidine incorporation were discovered in T lymphocytes, as discussed in Section 1.2.2.4. It was with the experiment conducted in Spacelab SLS-1, that a systematic determination of the production of cytokines by free floating as well as by microcarrier-attached cells contributed to understanding of the nature of the effects (Bechler *et al.* 1992; Cogoli *et al.* 1993). In the resuspended cells, the production of interleukin-1 (IL-1) (believed to be the second signal required for T cell activation) by monocytes was almost nil, thus indicating that the function of the accessory cells in delivering the second signal for activation had failed (see also Fig. 3). In correlation with the increased activation of T lymphocytes on microcarriers, the production of IFN-γ and of IL-2 were also remarkably increased, IFN-γ 2.5-fold and IL-2 two-fold compared to the 1 × g flight and ground controls (Cogoli *et al.* 1993). In addition, the pattern of tumour necrosis factor production by monocytes was different from that of IL-1. The most relevant data on the secretion of cytokines by T lymphocytes and monocytes in space are summarised in Fig. 4.

Fig. 4. Production of cytokines by T lymphocytes and monocytes in space. Cultures of lymphocytes with monocytes as accessory cells, either attached to microcarrier beads (+MC) or free floating (-MC), were incubated with Con A in space either at 0 × g or at 1 × g in a reference centrifuge. The data relative to IL-2 are from samples frozen with dimethylsulfoxide 46 h after incubation with Con A at 37°C, those relative to IFN-γ, IL-1 and TNF-α are from samples cultured for 65 h. The results are expressed as a % of the respective control (1 × g, -MC) which is taken as 100%.

In conclusion, the data from the experiment on mitogenic activation of the T lymphocyte/monocyte system support the following hypotheses:

- IL-2 is produced independently of IL-1 in Con A-induced activation;
- IL-1 production is low in cultures of free cells at 0 × g, an effect that is reversed by attachment to microcarrier beads;
- the expression of IL-2 receptors depends on IL-1;
- provided sufficient IL-1 is available, activation is enhanced in microgravity;
- it appears to be a selective effect of microgravity on the secretion of IL-1 and tumour necrosis factor (TNF) by monocytes and in the production of IL-2, IL-2R and IFN-γ by T lymphocytes.

A number of interesting effects were observed by Chapes *et al.* (1992, 1994) in cultures of three types of immune cells in space. However, the cultures were kept in the mid-deck 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-α in space than on the ground. Murine spleen cells, stimulated with poly I:C released significantly more IFN-α in space than on earth. Also, human PBL as well as murine lymph node T cells activated with Con A produced

significantly more IFN-γ 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 (Woods & Chapes 1994). The authors conclude that space flight ameliorates the action of TNF by affecting PKC in target cells, but none of these experiments were accompanied by on-board 1 × g controls (only ground controls were available) so what aspect of space flight is effective has not been established.

Another important investigation (Limouse *et al.* 1991) was carried out on the Soviet biosatellite Cosmos 2044 with THP-1 monocytes and Jurkat T cells, phenotypically similar to T cells. Cells were incubated at 37°C at a low starting concentration (2×10^5 cells ml^{-1}) in plastic bags for 5 days before launch (due to operational constraints). Activation occurred 6 h after launch. Jurkat T-derived lymphocytes were activated to produce IL-2 either by monocyte-derived THP-1 cells + mAb anti CD3/T cell receptor, or by A23187 + PMA. THP-1 monocytes were activated to produce IL-1 either by Jurkat cells + anti-CD3 mAb or by PMA. Although in the 1 × g controls IL-2 production under normal culture conditions was 10-fold higher than under the experiment conditions, it was still 20 to 40 times above the detection limit of the test. IL-2 production in the presence of THP-1 cells + mAb anti CD3/T was not different from 1 × g ground controls, but it was inhibited by 100% with A23187 + PMA. Similarly, IL-1 production in the presence of Jurkat cells + anti-CD3 mAb was not different from the 1 × g control, but was inhibited by 85% when PMA alone was present. Glucose consumption was nearly identical (85%) in all cultures, thus showing that cell growth and metabolism were not affected. A control experiment conducted on the ground showed that 85% of the cells were still viable after 5 days in culture. The results show that (1) cell-cell contacts (Jurkat/THP-1) work normally in microgravity; (2) binding of mAb to the T-receptor works normally; (3) cosmic irradiation effects are unlikely; (4) PMA, an activator of PKC, binds to cells in microgravity as shown in parabolic flights. However, anti-CD3 mAb are also potent activators of PKC in T-lymphocytes. Nevertheless, data in the literature do not implicate a PKC-dependence of IL-2 production by anti-CD3 mAb. The data are compared to those of de Groot *et al.* (1990, 1991) and direct effects of microgravity are considered to be the likely cause of the effects (Limouse *et al.* 1991).

The metabolic data of the experiment with Hybridoma 7E3-N cells in Spacelab IML-1 and described in Section 1.2.2.4 (Bechler *et al.* 1993) reveal another interesting behaviour 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.

Friend cells. In the experiment in Spacelab IML-1, mentioned in Section 1.2.2.4 (Bechler *et al.* 1993), the amount of haemoglobin produced upon induction with 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 behaviour in microgravity.

HaK cells. Production of tissue plasminogen activator (t-PA) by these cells cultured in the DCCS chamber was determined in Spacelab IML-1 (see Section 1.2.2.4). 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 at 1 × g in the reference centrifuge in flight or in the ground laboratory (Lorenzi *et al*. 1993).

Bone cells and tissues. Astronauts lose significant amounts of skeletal calcium during space flight (see Section 2.7). Orbital experiments with rats has shown that even short term space flight results in dramatic inhibition of bone formation but no detectable change in bone resorption (Wakley & Turner 1993). Normal gene expression (in terms of mRNA levels of bone matrix proteins) is not restored in such animals even 48 hours after return to Earth (Westelind *et al*. 1993). Such bone remodelling could be responsible for space flight induced bone loss, and this type of result has prompted considerable interest in the development of cell and tissue cultures to study, *in vitro*, the behaviour of the specialised cells which are responsible for the formation (osteoblasts) and resorption (osteoclasts) of bone.

Experiments on the Biobox facility on Biocosmos 10 (including on-board 1 × g centrifuge controls) showed retarded growth of embryonic bone cultures and altered morphology of osteoblasts in culture. After six days of incubation, the cell bodies displayed a round and compressed morphology, with abnormal extensions protruding from these contracted cell bodies (Demets 1993; Genty *et al*. 1993). Very similar morphological changes have been reported in cultured osteoblasts subjected to clinostat treatment (Al-Ajmi *et al*. 1994).

Something else which might be of immediate interest to astronauts is the question of wound repair during orbital flight. The Animal Enclosure Module on the Space Shuttle has been used for initial experiments on this topic and preliminary results indicate that microgravity reduced the chemotactic effect of two cytokines and diminished collagen formation in a model of deep wound healing, suggesting that the orbital environment retards growth factor responses and reduces wound healing in the rat (Davidson *et al*. 1994).

Sf9 cells. These cells (of the army worm, *Spodoptera frugiperda*) were flown in the BioServe Fluids Processing Apparatus aboard Shuttle flights STS-50, 54, and 57. Cell proliferation was measured with and without exposure to a cell regulatory sialoglycopeptide inhibitor (CeReS). Cell proliferation in flight samples was the same as ground controls (there was no on-board 1 × g control), the CeReS inhibitor bound to its specific receptor and its signal transduction cascade was not gravity sensitive (Moos *et al.* 1994).

E. coli. Mechanisms of genetic recombination were investigated in Biorack on Spacelab D-1 with a 1 × g control centrifuge in flight (Ciferri *et al.* 1986, 1988). There are three types of interaction between bacteria: the exchange of chromosomal DNA via sex pili (conjugation), the transfer of short stretches of DNA by a bacteriophage (transduction), and the uptake of extracellular DNA fragments (transformation). No gravity effect was found with respect to transduction and transformation (though the authors point out that attempted transformation of *Bacillus subtilis* with chromosomal DNA fragments, rather than plasmids, would have been a more sensitive test system). On the other hand, conjugation in *E. coli* was enhanced in microgravity, there being a 3 to 4-fold increase in conjugations lasting 40 minutes or more over the 1 × g in-flight centrifuge and ground-based controls (Ciferri *et al*. 1986, 1988). The same investigators observed an increased growth rate in microgravity, though this does not

account for the observed increase in the frequency of recombinants.

In another experiment on Biocosmos 2044 (Bouloc & D'Ari 1991, see also Section 1.2.2.4) DNA damage (cosmic radiation) was determined by analysing the expression of SOS response. A gene fusion in which *lac*Z, the structural gene of β-galactosidase is transcribed solely from the promoter of the SOS gene *sfi*A; β-galactosidase has the advantage of being extremely stable, thus permitting long-term storage of the cells (the flight lasted 15 days, the experiment 270 min). No effects of space flight (microgravity and cosmic radiation) were observed on the three parameters measured.

1.2.4 Morphology and motility

1.2.4.1 Background

Two questions have always intrigued researchers in gravitational biology: first, do shape and morphology of a cell change in microgravity? Second, are free floating cells which are not provided with locomotion organelles like cilia, capable of autonomous movements in microgravity? Biophysical calculations of the forces necessary to alter the shape of a cell show that gravity is not strong enough to influence cell shape. This means that the flattening of a cell sedimented to the bottom a cell culture flask at 1 × g is not due to gravitational forces but rather to the spreading of the cell on the surface. Nevertheless, shape and morphology changes could be the result of small alterations in the pressure that single organelles with higher density than the cytosol may exert on the cytoskeleton. In this case, shape and morphological changes may occur when the gravitational environment is altered.

The answer to the second question is: yes, cells may display autonomous movements in microgravity generated either by Brownian motion or by Marangoni convection (Napolitano 1984; Scriven & Sternling 1960). While Brownian motion is difficult to observe, due to the large size of the cells compared to the length of each impulse, Marangoni convection is generated by temperature or concentration gradients in a fluid and causes macroscopic displacements of the cell. It is conceivable that the microenvironment of a cell in a culture medium changes continuously due to the cell metabolism with consequent generation of concentration gradients of nutrients as well as of waste products.

Shape changes and motion may alter important cell functions including cell-cell interactions and processes related to cell structures, like the formation of the mitotic spindle.

Of great importance for morphological and dynamic studies has been the advent of NIZEMI, the low-speed centrifuge microscope which was used for the first time in space in Spacelab IML-2 in 1994.

1.2.4.2 Hypergravity

HeLa cells. Movements on a substratum coated with colloidal gold were tracked after 48 h in dark-field illumination at 1 × g and 10 × g (Tschopp & Cogoli 1983). While at 1 × g the cells showed normal patterns of migration, at 10 × g the cells did not change their position. In addition, whilst after mitosis the daughter cells go in opposite directions following a symmetrical pattern at 1 × g, at 10 × g the cells remained almost motionless forming aggregates by successive division. The focal contacts between cell and substratum did not differ between 1 × g and 10 × g., and no shape differences were observed by light microscopy. Absence of shape changes was also reported by Kumei *et al.*(1987) after 4 days at 35 × g.

V79 and JTC-12 cells. The lack of shape changes in hypergravity was confirmed also in V79 Chinese hamster lung cells (3 days at 18 × g) and in JTC-12 monkey kidney cells (4 days at 35 × g) respectively (Kumei *et al.* 1987).

Human lung adenocarcinoma cells A 549. As reported in section 1.2.2.2, Croute *et al.* (1990) carried out experiments at 2 and 15 × g lasting up to 27 d. While no changes in cell

proliferation were measured, interesting morphological alterations were detected. Electron micrographs of the A549 were composed at the start of the experiment of small translucent areas (alveolar structures) evenly distributed among a dense cellular mass. After 27 d of centrifugation, 1 × g controls exhibited larger alveoli which protruded at the periphery. Conversely, in the hypergravity cultures large alveoli developed only in the middle part of the nodule, surrounded by a dense cellular mass encircled by a thin layer of cells.

Paramecium. The swimming behaviour has been studied recently in the low-speed centrifuge microscope, NIZEMI at 1-5 × g using a computer-controlled image analysis system (Hemmersbach-Krause *et al.* 1992). Cells retained their swimming capability, did not sediment, and even increased the precision of their negative gravitaxis but reduced their mean swimming velocity.

In an earlier experiment, conducted in culture tubes separated by a septum and fixed in a centrifuge so that the direction of the vector of the centrifugal force coincided with the longitudinal axis of the tube, it was possible to show that *Paramecium* was able to swim even against an acceleration of 8 × g (Tixador *et al.* 1984b).

Amoeba proteus. The growth, shape and movements were observed without interruption (except for daily feeding) during 36 days in a microscope centrifuge at 40 × g. Despite a partial stratification of cytoplasmic particulate material, no effects on growth rate and motility were observed (Montgomery *et al.* 1965).

1.2.4.3. Hypogravity in clinostats

Cells of the immune system. Human peripheral blood lymphocytes were exposed to Con A in the fast rotating clinostat (Cogoli *et al.* 1980). Electron transmission micrographs of cells cultured in the clinostat were compared to those of control cells after fixation with glutaraldehyde. Cells activated for 3 days at 1 × g were characterized by the presence in the cytoplasm of widely spread vacuoles, by the formation of several pseudopodia and by a rather low number of mitochondria. The formation of vacuoles was more marked on day 4 of culture and can be interpreted as an indicator of cell ageing. Cells tended to swell up to a diameter of 10-15 µm. Cells grown in the clinostat appeared to be equally distributed in two populations. The first population was very similar to the control cells. The second populations appeared to be remarkably different and, after 3 days, it was characterized by the presence of a high number of tightly packed and well-developed mitochondria. Cinematographic recording of the rotated cells showed amoeboid movements as well as cytoplasmic streaming within the cells.

A431 epidermoid cells. It is known that EGF causes rapid rounding of A431 human epidermoid carcinoma cells. The effect is temperature dependent. At 37°C lower concentrations of EGF are required for 80% rounding than at 20°C. A431 cells were exposed to EGF in the clinostat and in the centrifuge at 10 × g at 20°C and 37°C. At 1 × g 72% of the cells showed rounding, and at 0 × g rounding was significantly increased to 85%. A slight, non-significant depression of rounding was seen at 10 × g (Rijken *et al.* 1991, 1992).

Epithelial cell adhesion seems to be unaffected by simulated microgravity (Jessup *et al.* 1994). Human colorectal carcinoma cells were cultured in the Rotating Wall Vessel - a clinostat which provides randomization of the gravity vector to mammalian cell cultures but with low shear stress. After 6 -7 days, cells were assayed for binding to various substrates and compared to cells grown in standard tissue culture flasks and static suspension cultures. No differences in epithelial cell adhesion were observed.

Neurones. In a first study neurones and myocytes were isolated from *Xenopus laevis* embryos (Gruener & Hoeger 1990). Myocytes were seeded on collagen-coated glass fibre cover slips. Spinal neurones were added 6 h later. The formation of nerve-associated acetylcholine receptor patches (NARPs) was observed by phase contrast microscopy and by

fluorescence microscopy of myocytes stained with rhodamine α-bungarotoxin. Nerve-associated accumulation of acetylcholine receptor (AChR) patches was characterized by fluorescence bands along the neuritic paths. Samples were kept at 1 × g (static), 1 rpm and 10 rpm in a horizontal clinostat. Controls for mechanical perturbations were kept (i) in a vertically rotating clinostat, (ii) at 100 rpm in a centrifuge, (iii) in an inverted configuration, and (iv) on an oscillating platform. Three types of synapses were considered: (a) mature, cultured for 36 h before rotation, (b) immature, cultured for 16 h before rotation, and (c) *de novo*, cultured for 6 h before rotation. Significant reduction of NARP incidence and NARP area was observed in immature (both -80%) and *de novo* (-45%, -80%, respectively) NARPs. In contrast, the incidence and the area of NARPs did not differ significantly in the mature synapses. The data suggest the existence of a window of sensitivity to microgravity during synaptogenesis.

In another study by the same authors (Gruener & Hoeger 1991) co-cultures of myocytes and neurones were observed at rotation rates between 1 and 50 rpm. When spinal neurones were co-cultured with myocytes, nerve cells produced robust neurites which frequently made physical and functional contacts with myocytes. No significant alterations were observed in survival of neurones or in the formation of neuritic extensions, or in establishment of contacts with myocytes. However, neurones in the clinostat frequently showed large, bean-shaped swellings along their neuritic shafts. In control neurones, swelling had a mean major diameter of 8.6 µm, in rotated samples the mean major diameter was 11.9 µm at 1 rpm and 12.3 µm at 10 rpm. Swellings were significantly larger (120%) in rotated cultures.

Myocytes. In the same experiments described above (Gruener & Hoeger 1991) rotated myocytes were larger, the nuclei were often more prominent and their nucleoli were larger. Whilst control cells changed their shape as a function of time from a rounded to an elongated shape, rotated myocytes appeared rounded at all times. Surface area increased significantly at 1 and 5 rpm, cell thickness remained unchanged.

Physarum polycephalum. An interesting model to study gravity effects on cells is the slime mould *Physarum polycephalum*, which forms side branches that can extend over a distance of more than one metre. These branches consist of an ectoplasmic tube in which the endoplasm streams back and forth in regular oscillations. This intracellular transport is driven by actin-myosin interactive filaments. Particles in the stream can move as fast as 10 mm h^{-1}. Briegleb and coworkers (Briegleb *et al.* 1986; Block *et al.* 1986, 1988) devised an experimental system to detect gravity effects on the amplitude and frequency of these oscillations. In the fast rotating clinostat (30 - 100 rpm) which was connected to a microscope, they were able to monitor the intracellular fluctuations of endoplasmic streaming under conditions of simulated low gravitational acceleration. An alternative to the clinostat was to turn the samples by 180° relative to the gravity vector. Distinct disturbances and changes in the kinetics were detected. In the initial phase of 0 × g simulation (20 min) and after the 180° turn, the rhythmic contractions showed an average increase in frequency of 10%. This initial phase was followed by further increase in the frequency of contractions and, later, by oscillations around the mean frequency. In addition, a 40% increase of the velocity of cytoplasmic streaming compared to the 1 × g control was measured. These data show the existence of gravisensitivity in *Physarum*, without suggesting a mechanism.

By applying inhibitors of respiration, it was possible to show in the clinostat that the receptor site of gravisensitivity may be correlated with the mitochondria (Block & Briegleb 1989; Block *et al.* 1986).

Dictyostelium discoideum. The height of the fruit body of this slime mould was measured after clinorotation or centrifugation at 2 × g and 3 × g for 4 days, with the acceleration vector oriented both normally and inversely to the normal direction of the gravity vector (Kawasaki

et al. 1987). The height of the fruiting bodies was least (less than 1 mm) under simulated microgravity conditions and greatest as acceleration was increased, both in normal and inverse directions (heights were up to 1.4 mm in inverted cultures at 3 × g and 1.6 mm in normally-oriented cultures at 2 × g). The differences were significant at $P < 0.01$. The authors suggest that the height of the fruiting bodies is controlled by two factors, one is gravity-dependent the other gravity independent.

1.2.4.4 Hypogravity in parabolic flights

Blood platelets. It is known that stimulation with thrombin and ADP causes shape changes within 15 sec. The platelets are transformed to spherocytes with appearance of pseudopodia via a calmodulin-dependent mechanism. The effect of microgravity on cell shape and aggregation has been studied recently (Schmitt *et al.* 1993). The activators were injected into the cell suspensions after 5 sec in microgravity, fixation followed 15 sec later. Scanning electron micrographs of platelets activated by phorbol ester PMA, ADP or thrombin showed no difference between 1 × g controls and microgravity samples. Also, transmission electron micrographs of platelets exposed to thrombin did not reveal differences.

1.2.4.5 Hypogravity in drop towers

Paramecium. The swimming behaviour was recorded with a CCD-camera in drop tower experiments conducted by ZARM at the University of Bremen during free fall episodes lasting 4.5 sec each (Machemer *et al.* 1992). The data support the following conclusions: (i) at 1 × g, the rates of upward swimming are lower than that of downward swimming. The difference is clear although it is only 20% of the gross speed. (ii) During microgravity, differences in swimming rate did not subside instantaneously. Only after 3 sec did the upward and downward rates of swimming adjust toward a common value indicating that relaxation of the gravikinetic response takes several seconds. The results are in agreement with an electrophysiological hypothesis on graviperception in cells. Similar data were obtained with *Loxodes*, but not with *Didinium*. The results, although preliminary, show that drop towers may be useful in certain studies in gravitational biology (see also Section 1.3.1).

1.2.4.6 Hypogravity in sounding rockets

Cells of the immune system. Interesting results were obtained recently in two experiments conducted on MAXUS I which showed that free floating cells are capable of autonomous movements and of aggregation in microgravity, implying that cell-cell contacts occur as well, though the number of aggregates is reduced at 0 × g (Cogoli-Greuter *et al.* 1994). In the first experiment, movements of purified human resting lymphocytes kept at 37°C were recorded during 12 min microgravity by use of a microscope telemanipulated from the ground station. Focus regulation and object selection could be operated manually during flight. Images were recorded by an on-board camera. The recorded images clearly show that the free floating cells were able to display autonomous motion in random directions. A more accurate analysis (one image every 13 sec) shows that the movements were much more complex. The cells often changed direction, moved back and forwards and sometimes crossed the same point several times. The average velocity, calculated from the displacement in the 13 sec increment, was 0.14 ± 0.02 µm sec^{-1}, with a range of 0 - 0.49 µm sec^{-1}. Also of interest is the observation that the cells in microgravity were not all rounded. Very often they exhibited longitudinal forms, rotated around their axis and also showed contraction waves similar to those described in the literature for lymphocytes moving under 1 × g conditions. This result is of primary importance to interpretation of the behaviour of T lymphocytes in microgravity.

The origin of the movement can be attributed to two major causes: First, as discussed above, the changes of the cytoskeleton may determine at least in part the random

displacements observed. Second, Marangoni convection due to differences in the concentration of components dissolved in the medium may generate the movements of resuspended cells in microgravity (Section 1.2.4.1). Concentration gradients are generated by the metabolism of the cells which are consuming nutrients (mainly glucose and glutamine) and producing waste (like lactate and ammonia).

In the second experiment, tubulin and vimentin were marked in Jurkat cells exposed to microgravity with FITC-labelled monoclonal antibodies at preselected times followed by fixation with formaldehyde. Cytoskeleton analysis of Jurkat cells revealed the formation of large and compact bundles of intermediate filaments of vimentin. Analogous structures appeared in the 1 × g controls, but to a much lesser extent. The changes occurred after 30 sec exposure to microgravity and remained stable throughout the flight. Similar but less evident changes occurred also in tubulin. These data are in favour of direct effects of gravity on the cell. Thus, gravitational unloading does not cause changes of the cell shape. However, changes of the cytoskeleton, probably due to the change in pressure of dense organelles may occur (Cogoli-Greuter *et al*. 1994).

The fusion of the antibody-producing hybridoma cell line G8 with the hypoxanthine-aminopterine-thymidine sensitive SP2/0-UZ cell line was attempted in microgravity on a TEXUS flight (Schnettler *et al.* 1989). The fusion rate was substantially increased and the yield of viable cell hybrids was 2-fold enhanced compared to the ground control experiment. The higher yield is related to a better alignment of the parent cells resuspended in a weightless electric field compared to a system at 1 × g in which cells rapidly sediment to the bottom of the fusion chamber.

A431 epdermoid cells. The clustering of EGF receptors was used as a marker to investigate whether the effects detected on the EGF-induced c-*fos* and c-*jun* expression in microgravity (see Section 1.2.3.5) are due to the inhibition of processes occurring at the beginning of the EGF-induced signal transduction. The clusters were visualized in the electron microscope by immunogold labelling with mAb of samples in a sounding rocket flight at the beginning of and after 5 min exposure to microgravity (**Rijken *et al.* 1993**). No difference was detected between the flight and ground control samples. This suggests that the influence of microgravity on EGF signal transduction occurs downstream of EGF binding and receptor redistribution, but upstream of early gene expression; inducer binding and clustering of signal receptors are not affected in microgravity.

1.2.4.7 Hypogravity in orbit

Cells of the immune system. Samples of lymphocytes activated for 3 days with Con A and frozen in flight in Spacelab 1 with hydroxyethylstarch showed the formation of cell aggregates in microgravity (Cogoli *et al.* 1984). Transmission electron microscopic analysis of samples activated with Con A and fixed in flight (Spacelab D-1) with glutaraldehyde confirmed the formation of aggregates. As shown in Fig. 5, lymphocytes appeared to be highly damaged under microgravity conditions whereas cells cultured at 1 × g in flight undergo mitosis and blastogenesis. Ultrastructural changes observed in lymphocytes may be related to increased apoptosis in microgravity (Cogoli *et al*. 1988; Cogoli *et al.* 1993). This is in agreement with the data on DNA synthesis (see Section 1.2.2.4). Monocyte ultrastructure appeared to be intact, though with a stronger membrane activity, i.e. display of a large number of pseudopodia, in the 0 × g samples (Cogoli *et al.* 1988; Cogoli *et al.* 1993). Finally, as shown in Fig. 6, transmission and scanning electronmicrographs of cultures flown in Spacelab SLS-1, in which cells were attached to microcarrier beads, revealed that (i) cells had a strong interaction with the substratum; and (ii) that attached cells did not deteriorate in microgravity as free floating cells did (Bechler *et al.* 1992; Cogoli *et al.* 1993).

Fig. 5. Electron micrographs of lymphocytes and monocytes exposed to Con A in Spacelab D-1. Top, cells in microgravity fixed after 36 h of culture (× 5100): dead lymphocytes and two monocytes (larger cells) with a large number of vacuoles. The monocyte on the right is displaying intense membrane activity. Bottom, cells in the 1 × g reference centrifuge fixed after 96 h of culture (× 8200): one lymphocyte is in metaphase (left) and another is transformed into a lymphoblast (right) following mitogenic activation. Micrographs by O. Müller & E. Hunzinger from Cogoli *et al.* 1988.

Fig. 6. Electron micrographs of microcarrier-attached lymphocytes and monocytes exposed to Con A in Spacelab SLS-1. Cells were cultured for 72 h in microgravity before freezing at -20°C in the presence of dimethylsulfoxide. After thawing in the ground laboratory, the cells were fixed with glutaraldehyde. Control samples were kept at 1 × g in-flight (not shown here). Top, scanning electron micrograph showing cells on three microcarrier beads (× 730). Bottom, transmission electron micrograph showing, from left to right, one lymphocyte, two blood platelets and one monocyte (× 3400). Micrographs by O. Müller & E. Hunzinger from Cogoli *et al.* 1993.

Ultrastructural analysis of an experiment with hybridoma cells conducted in Spacelab D-1 did not give conclusive results (Beaure d'Augères *et al.* 1986).

Human embryonic kidney cells. The attachments to a substratum of adhesion-dependent cells was tested in microgravity in an experiment carried out in an incubator installed in the flight deck of the Space Shuttle flight STS-8 (Tschopp *et al.* 1984). Microcarriers were added in flight 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 (see Section 1.2.4.3).

Friend cells. In an experiment conducted in Biorack in Spacelab IML-1, extensive analysis (scanning, transmission, volume measurements) of the ultrastructure of cells cultured for 6 days in the presence of DMSO did not reveal differences between the cell cultures at 0 × g and in the reference centrifuge at 1 × g (Bechler *et al.* 1993).

WI38 human embryonic lung cells. In an experiment carried out in Skylab and described in Section 1.2.2.4, cinematographic recording, phase, electron and scanning microscopy produced no observable differences in ultrastructure and in cell migration between flight and ground controls (Montgomery *et al*. 1978).

Erythrocytes. The peripheral blood from several donors (either healthy or with history of a number of diseases) was diluted with autologous plasma. Storage at ambient temperature on two Space Shuttle flights showed a dramatic decrease of red blood cell aggregation compared to the ground controls. Without specifying the nature of the effect, the authors concluded that membrane function is altered in microgravity (Dintenfass *et al.* 1986; Dintenfass 1990).

L8 rat myoblasts. 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 (Kulesh *et al*. 1994). Unfortunately, the space flight cultures were accommodated in an automated cell culture apparatus in a mid-deck locker on the Space Shuttle, and there was no on-board 1 × g control. Consequently, the cause of the altered phenotype is unknown.

Paramecium. Interesting observations are reported from experiments carried out in Spacelab

D-1 and in Soviet space laboratories (Tixador *et al.* 1984b; Richoilley *et al.* 1986, 1988a & b). The cell membrane of cells exposed to microgravity appeared thicker than that of the controls in transmission electron micrographs. Changes were seen also in exocytosis which under normal conditions is characterized by the extrusion of trichocysts docked beneath the plasma membrane. Trichocysts are spindle-like organelles the function of which is unknown. In a number of cells cultured at 0 × g exocytosis was reversed and decondensed trichocysts were detected inside the cytoplasm.

Pelomyxa carolinensis. Transmission electron micrographs of cell cultures aboard Biosatellite II for two days did not show differences between flight and ground control samples (Ekberg *et al.* 1971). Locomotion on a substratum seems to be independent from gravity. In this context, it is interesting to note that the speed of amoeba *Naegleria gruberi* was the same on the top surface and the bottom surface of a horizontal perfusion chamber. This shows that cell-substratum adhesive forces must be considerably greater than gravitational forces (Davies *et al.* 1981).

Physarum polycephalum. Two experiments were conducted in Biorack in Spacelab D-1 and IML-1 respectively, using a photodiode and a microscope with camera (Briegleb *et al.* 1986, 1987; Block *et al.* 1986, 1992). In space, the cells were first adapted to 1 × g during 6 h in the Biorack centrifuge and then the cytoplasmic streaming was recorded at 0 × g. The experiment in D-1 was only partially successful due to operational problems in flight. Nevertheless, the data obtained in the clinostat, i.e. increase of frequency of the cytoplasmic oscillations and of the streaming velocity (Section 1.2.4.3), were qualitatively confirmed. In the second experiment, carried out in IML-1, the effect of white light stimulation was studied. White light is known to have an opposite effect to microgravity, i.e. a decrease of cytoplasmic frequency and velocity in *Physarum*. The transition from 1 × g to 0 × g induced - as seen before - an increase of frequency that was maximum 18-26 min after onset of 0 × g. The mean period decreased from 88 sec to 60 sec, after backregulation it rose to 98 sec. This high value persisted for nearly 2 h with mean period values of 98-99 sec. Except for a more pronounced reaction to 0 × g, the data from the clinostat were qualitatively and quantitatively confirmed. When the opposing effects of microgravity and white light were measured simultaneously, a mutual suppression of both responses was observed. Another test, in which *Physarum* was first adapted to 0 × g and then exposed to white light, provided further evidence that the two responses have a strong mutual influence on each other.

1.2.4.8 Experimental models

As described in the previous Sections, few single cell systems have showed significant changes in space. In this section, the characteristics of systems which are good candidates for future research in gravitational biology are described.

Cells of the immune system. Several investigators in different laboratories have used either peripheral blood lymphocytes (PBL) or derived cells lines for experiments in gravitational biology, especially the lymphocyte-monocyte system (Beaure d'Augères *et al*. 1986; Chapes *et al*. 1992; Cogoli & Tschopp 1982, 1985; Cogoli, 1993b; Gmünder *et al.* 1990, 1992; Limouse *et al*. 1991; Tàlas *et al.* 1983). Lymphocytes are easily separated as purified resting (i.e. non-activated) cells from peripheral blood of healthy donors and upon exposure in culture to mitogens, can be activated polyclonally to proliferate and, in the case of T cells, to produce a number of lymphokines. The separation from fresh blood is based on centrifugation on Ficoll/metrizoate gradients and leads to a cell population consisting of 90-80% lymphocytes (T and B cells), 10-15% monocytes and 5% granulocytes. Alternatively, activation can be triggered in samples of whole blood obtained by diluting fresh blood with culture medium. In some cases, lymphocytes can be isolated from the spleen of mice or rats. The terms activation, stimulation or proliferation refer to the measurement of the amount of

tritiated thymidine incorporated into DNA (precipitated with cold trichloroacetic acid) after a 2-5 h pulse (see Section 1.2.2.1).

The mitogenic activation of lymphocytes *in vitro* constitutes a system of interest, since the activation *in vitro* mimics the events occurring *in vivo* during antigenic challenge, the mitogenic activation assay is also used as a diagnostic tool to assess the efficiency of the specific immune system. The test was introduced to determine the effects of space flight on the immune system of astronauts more than two decades ago and it is currently used in space. A discussion of the effects observed on T lymphocytes drawn from space crew members prior to, during and after flight go beyond the scope of this Chapter. For reviews see Cogoli (1993a & c), Cogoli & Tschopp (1985), Gmünder & Cogoli (1995, in press). It could be useful to detect alterations of the immune system on future long-duration space flights and to investigate *in vitro* neuroimmunological interactions under the physical and psychological stress of space flight.

Moreover, the transition from the status of resting to that of activated cells can be considered an example of cell differentiation that can be controlled *in vitro* by addition of the mitogen. T lymphocytes are activated by a number of mitogens of different origins. Most of the results presented here were obtained with the lectin Con A from lentil seeds. The mechanism of activation is very complex and not yet completely understood. Studies in microgravity may contribute to the understanding of a basic mechanism underlying lymphocyte differentiation. In fact, certain steps of activation, such as the production of IL-1 (the second signal required for T cell activation as shown in Fig. 3) may be switched off at 0 × g, and thus the process leading to activation might be dissected into separate phases which could be analysed in detail (Cogoli *et al*. 1993).

An alternative to the lymphocyte/monocyte system from PBL are derived cell lines as the Jurkat (T cells) or THP-1 (monocytes) cell lines. Cell lines have the advantage of consisting of homogeneous and well characterized cell populations (Limouse *et al.* 1991; Chapes *et al.* 1992). PBL, in contrast, comprise several T and B cell populations. Nevertheless, while the possibility of regulating *in vitro* the function of derived cell lines is rather limited, the PBL system is more flexible to mitogenic activation and certainly much closer to the *in vivo* function of the cells of the immune system.

Less interesting for experiments in microgravity and possible bioprocessing applications (in terms of proliferation rate and antibody production) appear to be hybridoma cells. This is not surprising, since hybridoma cells are ultimately committed to divide and to secrete antibodies with no, or little, possibility of regulation by external agents (Beaure d'Augères *et al.* 1986; Bechler *et al.* 1993). Conversely, the electrofusion of immune cells to produce antibodies gave promising results in microgravity (Schnettler *et al.* 1989).

A431 epidermoid cells. The use of these carcinoma cells in combination with EGF proved to be very useful to study gravitational effects (de Groot *et al.* 1990, 1991; Rijken *et al.* 1991). In particular, important data were gathered, first, on the intracellular signal transduction cascade involving either protein kinase A or protein kinase C; second, on the binding of EGF to the cell membrane; and, third, on the early expression of oncogenes. Similar studies are certainly needed also on the T lymphocyte/monocyte system.

HeLa cells. Similar conclusions can be drawn for these cells from the work of Kumei *et al.* (1987, 1989) on the expression of oncogenes and on the study of the cell cycle. HeLa cells however, were not subject to regulation by mitogens or growth factors as PBL and A431 cells, respectively.

Neurones. *In vitro* systems using neuronal cell cultures are particularly important for inclusion in experiments involving change in gravitational acceleration because of the anticipated rapid developments in neurobiology. Studies with the clinostat which led to the identification of windows of gravity sensing are very promising (Gruener & Hoeger 1990,

1991; see Sections 1.2.4.3 and 1.5.2).

Physarum. Regulation of rhythms and cytoplasmic streaming are important elements of gravitational biology and *Physarum* may be a good experimental system (Briegleb *et al.* 1986; Block *et al.* 1986, 1988, 1992; Block & Briegleb 1989). The discovery of its gravisensitivity is an important step in this analysis.

1.3 Taxes and tropisms in microbes

Much of the early research on the space biology of microorganisms was concerned with microbial assay of lunar samples (Taylor *et a*l. 1971; Taylor & Wooley 1973); with the microbial biota of spacecraft and astronauts (Taylor 1974a & b; Taylor *et al*. 1974; Carmichael & Taylor 1977); and with the use of microorganisms as test organisms for the mutagenic effects of exposure to the space environment (Hotchin *et al.* 1965; Orlob & Lorenz 1968; De Serres 1969; De Serres *et a*l. 1969; Jerger & Volz 1977; Horneck 1992; and see Chapters 4 and 5 of this book).

Over the last 20 years, more attention has been given specifically to the effect of gravitational disturbance on the behaviour of microbial cells, in part as contribution to study of their taxes and tropisms (Carlile 1975), but with increasing emphasis on study of the overall gravitational biology of relatively simple multicellular systems and, most importantly, single cells. Most of this work is reviewed in Section 1.2 but there are a few other observations which are worth recording here.

Extremes of hypergravity (10^3 to $10^5 \times$ g) increased enzyme activities but reduced endogenous respiration in *Staphylococcus aureus* (Nemec *et al*. 1983); perhaps not surprising at this level of acceleration stress! Sporulation of *Bacillus subtilis* was suppressed in microgravity, but higher growth yields were recorded (Mennigmann & Lange 1988). In contrast, Bouloc & D'Ari (1991) and Gasset *et al*. (1994) report no increase in growth in space-flown cultures of *Escherichia coli* (see also Section 1.2.2.4). Greater growth has also been observed in space flight cultures of *Nocardia meditterranei* (Gu *et a*l. 1989), though antibiotic titres and the stability of recombinant plasmids were unaffected. Minimum inhibitory concentrations of the antibacterial colistin (= polymyxin E, which inserts into the bacterial membrane and collapses the proton gradient) have been claimed to double, implying increased bacterial (*E. coli*) resistance to or tolerance of the antibiotics in microgravity conditions during Salyut and Spacelab D-1 missions (Tixador *et a*l. 1984a, 1985; Lapchine *et a*l. 1986, 1988; Moatti & Lapchine 1986). As alterations in membrane structure are a common response to acceleration stress (see Section 1.4.5.2), some such change might be suspected as a cause of this antibiotic tolerance. However, it is disturbing that the only differences observed were between ground based control cultures and space flown cultures. The authors do not discuss the fact that there were no differences between in-flight static and in-flight centrifuged cultures (Lapchine *et al*. 1986).

Cellulose synthesis by *Acetobacter xylinum* is affected by the 20 sec of microgravity during parabolic flights. Controls produced compact ribbons of microbial cellulose, but during the parabola regime cellulose ribbons became splayed. This observation suggests that the crystalline cellulose is normally produced under strain, and the microgravity exposure relieves the stress, perhaps by reduction of intermicrofibrillar hydrogen bonding, to produce splayed ribbons (Brown *et al*. 1992). This observation may be relevant to the changes in plant cell wall composition and structure discussed in Section 1.4.5.1.

The majority of research on eukaryotic microbes has been done with protozoa, including the cellular slime mould, *Dictyostelium discoideum* (Kawasaki *et al*. 1987) and, especially, the plasmodial slime mould *Physarum polycephalum*. The latter has been chosen because of the readily-visible cytoplasmic movements which occur in the plasmodia (Hejnowicz & Wohlfarth-Botterman 1980; Block *et al*. 1986). The effect of the gravitational

field on these movements has been studied (Block & Briegleb 1989; and see Sections 1.2.4.3 and 1.2.4.7) in rather similar ways to the investigations which have been made of cytoplasmic streaming in plants (Section 1.4.5.7). The *Physarum* plasmodium is essentially an abnormally large amoeboid cell. An unfortunate consequence of its size and its abnormality is the perpetual question of whether phenomena observed in this organism relate to (widely applicable) general cell biology, or result from the peculiar specialisations of the organism. Ironically, *Physarum* does not appear to have been employed in gravitational biology for the sort of research for which it is most suited as an experimental object - the study of nuclear division.

Among unicellular protozoa, *Paramecium tetraurelia* was flown aboard Salyut 6 (Planel *et al.* 1981, 1982), the experiment involving measurement of changes in the ionic environment of the cells during flight (Tixador *et al*. 1981). A significant observation was increased proliferation in the *Paramecium* cultures (Tixador *et al*. 1984b). Controls indicated that microgravity was the major factor responsible of this response (no stimulation of proliferation in cultures placed on a 1 × g in-flight centrifuge aboard the Spacelab D-1 mission), and, interestingly, exposure to hypergravity on ground centrifuges resulted in a reduced cell growth rate (Planel *et al*. 1986, 1990; Richoilley *et al.* 1988a & b). Stimulation of *Paramecium* proliferation has also been observed in cultures on a fast rotating clinostat (Hemmersbach-Krause *et al*. 1991; Ayed *et al.* 1992) (see Section 1.2.2).

An isolated observation, which is potentially interesting for the experimental possibilities it raises, is that hypergravity (1.9 × g) influences the crystal structure of the calcite synthesised by foraminifera (Le Campion & Very 1980). The authors suggest a mechanism operating at the crystal/liquid interface at the cell membrane.

1.3.1 Motile cells

Motile cells can move towards or away from the source of stimulus, thereby exhibiting a *taxis*. In +ve taxis the organism remains in the region of stimulus, homing in on the source; the opposite is a -ve taxis. Responses such as photo- and chemotaxis depend upon the cell sampling the environment through its own movement, and so characterising the stimulus gradient. In the absence of gravitational field gradient over biological distances, gravitaxis results from displacement of the centre of buoyancy and/or centre of propulsion of the cell relative to its centre of gravity. Such displacement results in a torque leading to orientation of the cell within the fluid stream (Kessler 1986, 1989, 1992; Pedley & Kessler 1987). Kessler demonstrates (theoretically and experimentally) that orientation of cells in a flowing fluid can be entirely physical, requiring no sensory processing but depending on cell shape. Under normal conditions on Earth, even cyclical changes in cell behaviour can be accommodated in a physical model because as cells aggregate the local fluid density changes, creating a convection current which can mix the medium and redisperse the cells (Kessler 1989). This analysis does not exclude active signalling processes, but if gravity-dependent aggregation patterns can be achieved entirely through physical interactions - i.e. without cellular sensory perception and/or intercellular signalling, it does add an additional level of complexity to studies of the dynamics of cells in suspension. It also imposes the additional discipline that if such experiments are aimed at studying cell signalling, the purely physical explanation must be rigorously excluded.

This has not been done adequately in instances such as experiments with *Euglena* aboard the TEXUS sounding rocket (Häder *et al*. 1990) where absence of aggregation was ascribed to diminished ‘cooperativity between the individuals’.

Many motile protozoa exhibit -ve gravitaxes which are believed to act as mechanisms enabling the cells to find the air/water interface, the reaction to the gravity vector supplementing phototaxis and chemotaxis in oxygen gradients (Häder & Vogel 1990;

Hemmersbach-Krause *et al*. 1990; Vogel & Häder 1990).

The motion of ciliates lends itself to analysis even in the short periods of microgravity available in drop-tower experiments (Machemer *et al*. 1992, 1993). Unfortunately, although undoubtedly technically elegant, these experiments have not yielded any particularly incisive insights. The bias normally shown by *Paramecium* and *Didinium* for swimming vertically (negative gravitaxis) was absent in microgravity; so were gravity-induced changes in velocity of these cells (gravikinesis), which tend to compensate passive sedimentation at 1 × g. The swimming rates of *Paramecium* and *Loxodes* in microgravity were the same as their corresponding rates in the horizontal direction at 1 × g, but in *Didinium* the speed of swimming in microgravity exceeded the horizontal speed at 1 × g. The authors interpreted the data in terms of an electrophysiologically regulated gravisensor, but the evidence is weak and circumstantial.

Longer-term swimming behaviour of a number of ciliates and flagellates has been studied in microgravity, clinostats and hypergravity conditions but with no greater yield of understanding (ciliates: Hemmersbach-Krause *et al.* 1993; Hemmersbach-Krause & Briegleb 1994; and flagellates: Häder *et al*. 1991a & b; Horneck *et al*. 1991; Vogel *et al*. 1993).

Gravitaxis of *Euglena gracilis* increased at accelerations up to about 3 × g, but decreased, and some of the cells were sedimented, by accelerations of 4.5 to 5 × g. UV irradiation drastically impaired gravitactic orientation, suggesting to the authors that the taxis is an active physiological perception rather than a physical effect such as an asymmetry of the centre of gravity within the cell (Häder *et al*. 1991b). However, UV damage to metabolism (e.g. altered distribution of reserve materials) could also shift the centre of buoyancy so this is not a compelling reason to believe in intercellular signalling systems. Gravitaxis was lost within a few minutes of first exposure to real microgravity or onset of rotation on a fast clinostat. After the onset of microgravity, cells persisted in their original swimming direction until they collided with other cells or made random course corrections. The mean velocity was higher under microgravity than at 1 × g (Vogel *et al*. 1993).

Despite the varied and considerable investments in these protozoal motility experiments, it seems that the systems are intrinsically too complex to serve as helpful experimental models until sufficient is known for them to be used to test very specific predictions. Being highly adapted and specialised organisms, the protozoa themselves are difficult to understand in terms of any generalised cell biology. The cells are beyond the limit for easy observation of individuals so that most of the experiments have to rely on observing the behaviour of potentially heterogeneous populations, and the experiments have to be designed to distinguish purely physical interactions of small particles in fluid from a variety of (potentially conflicting) tropic responses.

1.3.2 Sessile microorganisms

In sessile organisms response to external stimuli involves tropisms - directed growth towards (+ve) or away (-ve) from the stimulus (see also Section 1.4.1). Amongst microorganisms, this phenomenon is particularly evident in fungi. Gravimorphogenetic responses are known, too. For example, in the apple-scab pathogen, *Venturia inaequalis*, which over-winters in fallen leaves, over 90% of the fruiting structures (ascomes) in the leaves were directed towards the surface that had faced upward during winter (Gadoury & Machardy 1985). In a similar phenomenon, Petri dish cultures of the resupinate polypore *Phellinus contiguus*, which normally forms its fruiting bodies (hymenophores) on the lower surfaces of tree branches, produce disorganised masses of tissue when incubated mycelium-side up, but normal hymenophores when inverted and incubated mycelium-side down (Butler & Wood 1988).

These fascinating phenomena have never been subjected to microgravity research, not even with clinostats. Indeed, there is an even more remarkable ‘lost opportunity’ in

mycological gravitational biology in that no space experiments seem to have been done with *Phycomyces* sporangiophores. The sporangiophores of *Phycomyces* are essentially individual (though unusually large) hyphal tips. They show a number of interacting tropisms and avoidance growth-responses which have been analysed in great detail (Varjú *et al*. 1961; Johnson & Gamow 1971; Lafay *et al*. 1975; Gamow & Böttger 1982; Gyure *et al*. 1984), and as early as 1961, Dennison (Dennison 1961) used a centrifuge (with accelerations up to 4.35 × g), conducting some experiments with sporangiophores submerged in a buoyant fluid to distinguish two separate sensory systems; a transient reaction extending over about 5 minutes and the long term, 'normal', gravitropic response. As the direction of the transient response was reversed in the buoyant fluid it was concluded to be a by-product of mechanical forces, probably exerted on the cell wall, arising from the action of gravity on the sporangium (such forces are reversed in direction between experiments in air and in a dense buoyant fluid). This was subsequently shown to be a response to a stretch receptor (Dennison & Roth 1967). Later experiments suggested that the gravitropic sensory mechanism may involve displacement of the vacuole changing the distribution of wall synthetic potential (Dennison & Shropshire 1984). Although it is doubtful that such a mechanism would have the sensitivity to correct small changes in orientation, to date this remains the only published suggestion for a gravireceptor mechanism in fungi.

Despite the fact that the above-described experiments were contemporaneous with developing space flight opportunities, *Phycomyces* has never been flown. However, there have been a variety of space flight and clinostat experiments with much larger fungal fruit bodies (for reviews see Molitoris 1990; Moore 1991; Kern & Hock 1993; Moore 1994).

Ground based experimentation was recently resumed after a gap of almost 25 years with studies of gravitropic bending in *Coprinus cinereus* stipes (= mushroom stems) (Kher *et al.* 1992; Greening *et al*. 1993; Moore *et al*. 1994a). Bending is a two-stage process, the first stage being an elastic deformation, the second a rigidification process, and it seems to be different from growth processes concerned with normal extension of the stipe. Control of gravitropic bending is exercised by the apical half of the *Coprinus* stipe and the same tissues are responsible for gravity perception and gravitropic response. The reaction time is about 21 minutes (Moore *et al*. 1994b). Perception of the gravity vector does not involve modulation of Ca^{2+} metabolism, proton channels nor stretch-activated ion channels (Novak Frazer & Moore 1993). The perception time for gravitropic bending is 7 minutes. Gravistimulation is not additive and the angle of bending attained depends on gravistimulation dose (implying that sustained stimulation is required for sustained bending). There is no relationship between extent of gravistimulation and rate of response or reaction time (Hatton & Moore 1992, 1994). Broadly similar results have been obtained with *Flammulina velutipes* (Monzer *et al*. 1994), although the much slower growth and different tissue distributions in this organism result in the kinetics being very different from those observed in *Coprinus*. Experiments with *F. velutipes* were flown on the D-2 Spacelab mission (Hock *et al*. 1993). Although the cultures fruited extremely well and many samples were returned for analysis, only preliminary results have so far been published (Kern & Hock 1994). However, mushroom cultures of a different sort, of the polypore *Polyporus brumalis*, have been flown on a number of Salyut missions (Dudchenko *et al*. 1978), and this species, together with *C. cinereus* and some others, has been the subject of experimentation with clinostats (Gorovoi *et al*. 1989a & b; Moore 1991; Hatton & Moore 1992, 1994). The indications from both clinostat and space-flown experiments are that the basic form of the mushroom (overall tissue arrangement of stem, cap, gills, hymenium, and veil) in agaric and polypore alike is established independently of the gravity vector. Abnormalities of stem growth have been observed in clinostat cultures of *Panus (= Lentinus) tigrinus* and *P. brumalis* (Gorovoi *et al*. 1987; Gorovoi *et al*. 1989a & b), but the morphogenetic event which seems most dependent on

gravity is sporulation (in the broadest sense). Cultures of *P. brumalis* on orbiting space craft fail to produce the poroid hymenophore and in clinostat experiments on the ground even karyogamy was rare in similar cultures. *C. cinereus* grown on the clinostat was able to produce apparently normal fruit body primordia which failed to produce spores and then aborted, forming a new flush of primordia on the old (Gorovoi *et al*. 1989a). Taken together with the clear association between observation of gravitropism and the onset of sporulation the implication is that commitment to the pathway incorporating the meiotic division and subsequent spore formation both requires the gravity vector and couples it in some way to fruit body maturation.

An unexpected feature of clinostat experiments with *C. cinereus* fruit bodies is that stipes placed on the clinostat after various gravity exposure times 'relaxed' by 5° immediately after reaching maximum curvature (Hatton & Moore 1994). This relaxation process has parallels in the 'tropic reversal' of *Phycomyces* sporangiophores (Galland & Russo 1985), 'springback' of plant roots (Leopold & Wettlaufer 1989), 'spatial memory' of maize coleoptiles (Nick *et al*. 1990b) and 'autotropisms' of seedlings (Heathcote 1987; Chapman *et al*. 1994). Such similarities suggest that in all these otherwise very different cases, gravitropic bending has an initial, reversible, phase of plastic bending which is followed by a 'fixation' phase if the tropic stimulus has been maintained but which relaxes if the tropic stimulus has been removed.

Other fungal experiments with a bearing on morphogenesis have concerned the persistence of diurnal cycles of conidium formation in *Neurospora crassa*. The conidiation pattern generally persisted in microgravity but there were strain differences. One strain had a relatively robust rhythm though its period increased by several hours and alterations in morphology were occurred. Another strain gradually lost its rhythm (Sulzman *et al*. 1984; Reiss-Bubenheim 1990). This suggests that sensitivity to the gravitational field may have a genetic component which could account for cases where different responses are observed in experiments with the same organism. The unicellular alga *Chlamydomonas reinhardtii* also exhibits a diurnal cycle, but of photo-accumulation (tendency to accumulate in the light alternating with no accumulation). The rhythm is enhanced in microgravity (Mergenhagen 1986; Mergenhagen & Mergenhagen 1986), and this is taken to imply that the rhythm is driven by endogenous factors rather than by environmental cues. In *Chlorella*, clinostat treatment causes alterations in the chemistry of membrane lipids (Baraboi *et al.* 1991).

1.4 Plant growth and development

1.4.1 Introduction

In so far as the clinostat was first developed to study plant gravitropism it can be said that microgravity experimental work originated with plant gravitational biology. Inevitably, there is an enormous literature on the topic, of which only a fraction can be reviewed here. This section will attempt to provide a context within which the more recent work on gravitropism especially that involving space flights can be emphasised, but the section will begin with a consideration of broader aspects of plant developmental biology.

Being sessile organisms, plant response to external stimuli involves orientation and growth towards (+ve) or away (-ve) from the stimulus, known as a tropism. This is generally considered to result from the differential growth of parts of the plant (Barlow 1992a & b) in response to differential distribution of growth hormones - the classic Cholodny-Went theory of the early 1930s (Went & Thimann 1937). Higher plants, mosses, filamentous algae (and fungi too, though they are in a different Kingdom to the plants) all exhibit several types of tropism including response to light (phototropism), chemicals (chemotropism), physical surfaces (thigmotropism), airflow (anemotropism) and gravity (gravitropism) and even strong magnetic fields (magnetotropism) (Schwarzacher & Audus 1973; Jenkins *et al*. 1986;

Heathcote & Bircher 1987; Moore 1991; Perbal & Driss-Ecole 1993). Plant primary roots are positively gravitropic, whilst shoots are negatively gravitropic although the characteristics of the response are similar in both. If a vertically growing root is re-oriented to the horizontal, i.e. gravistimulated, then there is a minimum period of stimulus required to elicit a response, known as the perception time (Audus 1962). Mechanistically, this is the time period required for the sensor system to react to the stimulus. There follows a lag period between initial stimulation and the first signs of response, termed the reaction time. This is the collective time it takes for gravity perception to generate a physiological signal, the signal to travel (if appropriate) from the site of perception to the site of response and the response then to be implemented. In plant roots the tropic response occurs a few millimetres back from the root tip in an actively elongating region, but gravity is believed to be sensed in the root cap since removing the cap virtually abolishes the response (Audus 1962; Moore & Evans 1986). This implies that a signal must travel from the tip to the response zone.

Very little research has been done on modulation of the gravitropic reaction. This occurs to ensure that secondary branches of the main axis (whether shoot or root) grow outwards, i.e. inclined away from the vertical (= plagiotropism) and is arguably an important component of mathematical models of plant gravitropism (Stockus, 1992, 1994, 1995). The 1 × g centrifuge on board Spacelab was used during the IML-1 mission to expose roots of lentil seedlings grown in space to varied gravitational stimuli (Perbal & Driss-Ecole 1994).

The kinetics of root gravitropism in orbital and ground experiments were different. Root tips exposed to 1 × g in orbit could overshoot the direction of the acceleration vector, ground controls did not, suggesting that a gravity dependent compensation (or inhibition) of root curvature is a component of the normal root response.

1.4.2 Plant growth in microgravity

Levine & Krikorian (1992) grew plantlets of day lily (*Hemerocallis*) (clones regenerated from cell suspensions before flight), and clonal populations of *Haplopappus gracilis* during a 5-day mission aboard the Shuttle Discovery. Statistically significant differences in shoot growth were found (the controls growing more than plantlets subjected to space flight conditions), and between the different plant populations (day lily gaining more shoot material than any of the *Haplopappus* populations and *Haplopappus* seedling clones outperforming the tissue culture-derived *Haplopappus* lines). There were also differences between different growth chambers, implying environmental heterogeneities within the culture apparatus. On the other hand, the fresh weights and hormone contents (abscisic acid and indole-3-acetic acid) of seedlings of *Zea mays* grown for five days during a Shuttle flight were, with minor exceptions, not statistically different from seedlings grown in normal gravity. The tissues of the Shuttle-grown plants appeared normal and the seedlings differed only in the lack of orientation of roots and shoots (Schulze *et al*. 1992).

The diversity of these recent results echoes earlier studies. Cowles *et al*. (1984) found stem and root growth and fresh and dry weights to be reduced in comparison with ground controls in mung bean, oat and pine seedlings grown on Shuttle and Spacelab missions, and coleoptile growth was reduced, compared with ground controls, in lettuce, cress and *Arabidopsis thaliana* seedlings grown on Salyut missions (Merkys *et al*. 1981, 1984). In these Salyut experiments, more coleoptile growth occurred in microgravity than in an on-board centrifuge, though all flight-grown material was considerably shorter than ground-based controls. Paradoxically, cucumber seedlings grown on an in-flight centrifuge on Cosmos 1667 had longer roots and coleoptiles than those grown in microgravity, and lettuce seedlings grown in space on the same flight were larger than those grown on the ground (Sitnik *et al*. 1984).

Diversity is to be expected, given that the organisms have evolved independently

within the Earth's gravitational field and their evolutionary origins have led them to respond to gravitational stresses in different ways. Also, some of the physical effects of microgravity especially lack of gravity-driven convection and the consequent relative increase in importance of concentration-driven effects and surface-surface interactions, will inevitably influence fluid distributions and gas exchange on which plant growth depends. Further, these influences will almost certainly differ between plants depending on their sizes and the geometrical structures of their different tissues.

However, the diversity of experimental results coupled with the relatively small number of (mostly) short duration experiments which have been completed combine to make generalised interpretations extremely difficult. Longer duration plant growth experiments have been few in number and often suffered from technical difficulties (Halstead & Dutcher 1987). *Arabidopsis thaliana* has been successfully cultivated through a complete life cycle (Merkys & Laurinavichius 1983; Merkys *et al*. 1984) aboard Salyut-7. Seeds produced in orbit were subsequently germinated on Earth. They showed reduced germination, increased frequency of embryonic lethality, generally poorer seedling growth and greatly reduced ability to develop into healthy mature plants. Similar results were obtained with seeds produced on plants flown aboard Cosmos 1129 (Parfenov & Abramova 1981). Second generation seeds produced on Earth by the progeny of the space-flown plants were apparently normal. Seemingly, the adverse effects of microgravity on plant development and fecundity are transient, but would they be cumulative in continuously space flown crops? Significantly perhaps, endosperm and embryo nutrition in wheat normally depends upon sedimentation of vesicles within the embryo sac (Huber & Grabe 1987).

1.4.3 Plant cell proliferation and differentiation

In lentil roots, the normal gravireaction leads to an increase of the mitotic activity in the whole proximal part of the meristematic zone and also in the lower half of its distal part. Moreover, gravistimulation brings about an increase of nuclear DNA synthesis of cortical cells in the upper half of the distal part of the meristematic zone. The cell cycle of cortical cells is thus changed during the gravitropic curvature. In the roots of *Zea mays* grown aboard the biosatellite Cosmos 1667, the mitotic activity of cortical cells and their differentiation occurred faster than in the ground controls (Darbelley 1986, 1988) although other workers reported a decrease in the number of dividing cells in meristems of maize root tips on the same flight (Barmicheva *et al*. 1989). Roots of lentil seedlings grown for 25 h on board the Spacelab D1 Mission showed an increased mitotic index in microgravity in comparison with the flight controls, due either to a shortening of the interphase in roots grown in microgravity or to a lengthening of mitosis. Cell length and mitotic activity were reduced when roots were first grown in microgravity and then subjected to centrifugal acceleration (Darbelley *et al*. 1989). Clinostat treatment of similar seedlings of lentil caused cell elongation to occur closer to the tip than in the vertical roots, but the mitotic index was not modified, nor were the frequencies of the G1, S and G2 phases of the cell cycle (Legue *et al*. 1992). Similarly, spore germination, direction of growth, and cell division in protonemata of the fern *Adiantum* were not affected by growth on a 3-dimensional clinostat (Kasahara *et al*. 1994) although clinostated protonemata were shorter than the controls. Such differences between true microgravity and its clinostat simulation raise the question as to whether adverse effects on mitosis might depend on density differentials being abolished in true microgravity, rather than on absence of a directional gravity vector.

Earlier work suggested that microgravity might cause a block during the later stages of mitosis (Halstead & Dutcher 1987), but the data are clearly not unanimous in indicating this specifically. Nevertheless, there are frequent reports of disturbances to the progress of mitosis and to increases in the frequency of chromosomal abnormalities (often with sufficient

controls to eliminate radiation as direct cause of the latter). Thus, it may well be that plant cell proliferation requires positional cues which are absent in microgravity; the response to this absence varying between species. In this context, it should be remembered that plant developmental biology depends on the exact positional placement of the new cell wall which separates the daughters produced from each dividing cell. That, in turn, depends on the positioning and orientation of the mitotic division spindle. Perhaps it is these intracellular positioning processes which are gravity-dependent for their correct execution.

There was no increase in protein content of cells of sunflower (Bara 1977) or corn (Tairbekov & Rozov 1978) grown on clinostats, though the former showed enhanced growth and water content. Although growth of cells of *Haplopappus gracilis* cultivated *in vitro* for 35 days on a 2 rpm clinostat had no effect, on a 50 rpm clinostat an increase in wet weight was observed as compared to the control with relatively the same amount of dry weight gain (Klimchuk 1984). Root cap cells of *Arabidopsis* grown on board the Salyut 6 orbital space station had increased vacuolization compared with the ground *g*rown control (Tarasenko *et al*. 1982).

Microgravity may also influence the progress of plant cell differentiation (Kordyum 1994; Laurinavichius, Kentstaviciene *et al*. 1994). Aniseed cell cultures differentiated more rapidly during the D-1 Shuttle flight (Theimer *et al*. 1986) and there are other reports in the literature of accelerated development which has been suggested to parallel reports of accelerated aging in animals reared in space (Halstead & Dutcher 1987). On the other hand, experience with protoplast regeneration in a number of more recent missions suggests that this aspect of plant cell differentiation is severely retarded in microgravity. Alterations in wall synthesis in microgravity contributed to reduced protoplast regeneration in material flown on the 14 day flight of Biocosmos 9 (Cosmos 2044) (Iversen *et al*. 1992; Klimchuk *et al*. 1992; Rasmussen *et al*. 1992). Regeneration of flight rapeseed (*Brassica napus*) was only 56% of the ground control, and with carrot (*Daucus carota*) regeneration in orbit was 82% of the ground control. Peroxidase activity and the amount of protein was lower in flight samples than in the ground controls; the number of different isoenzymes was also decreased in the flight samples. A 54% decrease in the production of cellulose was found in rapeseed, and a 71% decrease in carrot. Hemicellulose production was also decreased in the flight samples compared to the ground controls. Fluorescence analysis showed that cell viability was unaffected by space flight.

One general effect of the stay on board the space vehicle was a retardation of the regeneration process. Callus cultures obtained from the flight samples grew very slowly compared to callus regenerated from the ground controls, and two years after the Biocosmos flight there was no further growth in the samples exposed to microgravity, although callus cultures from the ground controls continued to grow well and in related experiments gave rise to regenerated plants. There was a decrease in the calcium content of cell cultures subjected to space flight compared to the ground controls and a variety of (minor) ultrastructural changes, including folding of the plasmalemma, more cell inclusions and microbodies and greater heterogeneity in mitochondria in the flight samples. The most consistent ultrastructural observation, though, was a 2 to 3-fold decrease in wall thickness in the flight samples, which seems to correlate with the changes in wall constituents referred to above. Potentially the most interesting microgravity-induced developmental alteration in plants appears to be the least well examined. This is that regeneration of the root cap seems not to occur in orbit (Moore *et al*. 1987c). Podlutsky (1992) reported normal root cap development in roots regenerating from callus cultures of *Arabidopsis thaliana* grown on a clinostat, whereas, in similar cultures exposed to 8 days of microgravity in space (flight not specified, but possibly Salyut-6), the root cap was abnormal, lacking the gravireceptor cells normally located in this tissue. This clear implication that a specific and well-defined developmental

process needs the normal unilateral gravity vector deserves more extensive investigation.

1.4.4 Plant gravitropism

It is over 100 years since Noll (1892) first suggested that plants might sense gravity by means of a mechanism which depends on sedimentation of microscopic intracellular particles. It is now widely assumed (Wilkins 1984), but by no means fully proved or completely accepted (Poff & Martin 1989), that the mechanism of graviperception in plants is the sedimentation of starch-containing amyloplasts (statoliths) in specific cells (statocytes) in the columella (central core) of the root cap and in various locations in shoots (e.g. near vascular bundles or the inner cortical or endodermis layers of the stem). So, while there is discussion about the mechanism (or mechanisms - see Firn 1992) by which plants eventually sense statolith movement (Björkman 1988; Poff & Martin 1989; Trewavas 1992), there is no debate over the basic premise that gravity perception is the responsibility of particular specialised plant cells and that it depends upon redistribution of intracellular organelles (Perbal & Driss-Ecole 1994).

The large size and relative density of the amyloplasts allow them to sediment rapidly. The rate of sedimentation (determined directly and theoretically) is comparable with the perception time (Sack *et al*. 1984, 1985; Moore & Evans 1986). Statolith sedimentation polarises the cell, through interaction either with the cytoskeleton, organelle membranes or the plasma membrane (the potential mechanism is discussed below). The physiologically important outcome is differential enhancement of cell elongation in only one side of the growing zone leading to a tropic curvature (downwards in roots, upwards in stems). The Cholodny-Went theory ascribed this specifically to lateral transport of auxin and tropic curvatures can be mimicked experimentally by lateral application of auxin. Migliaccio & Rayle (1989) demonstrated that applied auxin gradients as small as 1:1.3 produced >60° curvatures in hypocotyls of sunflower (*Helianthus annuus*) seedlings. Asymmetric auxin distributions (Harrison & Pickard 1989), redistribution of auxin-regulated RNA (McClure & Guilfoyle 1989) and movement of calcium towards the lower side of the organ (Lee & Evans 1985a & b; Moore 1985) have all been recorded as responses to reorientation in plant organs which comply with the Cholodny-Went theory. However, asymmetric (i.e. polarised) distribution of many metabolites have been demonstrated in tropically-responding plant organs, including concentration gradients of potassium and phosphorus (Goswami & Audus 1976), Ca^{2+} and H^{+} (Wright & Rayle 1983; Gehring *et al*. 1990), Na, Cl, and Mg (Moore *et al*. 1987a), gibberellins (Hestnes 1979; Rood *et al*. 1987) as well as auxin (Audus 1962; Jackson & Barlow 1981; Moore, Evans 1986; Briggs 1992; Evans 1992). Even ethylene has been implicated as a modifier of tropic curvature in *Mimosa* (Roblin & Perault 1985) though not in tomato (Harrison & Pickard 1986). Extrusion of ionic species induces polarised electrical current flows across reoriented plant organs to which particular importance has been attached because they occur so early in the gravitropic response sequence (Behrens *et al*. 1985; Lühring *et al*. 1986; Iwabuchi *et al*. 1989; Collings *et al*. 1992).

The great variety of rapid metabolic reactions to reorientation makes it difficult to understand the exact sequence of events in the gravitropic response, despite over a century's research. The debate about how gravitropic bending is effected continues to rage. Even its claimed dependence on a concentration gradient of a hormone is not universally accepted. Differential growth of soybean hypocotyls occurred without free auxin redistribution (Rorabaugh & Salisbury 1989), which is interpreted as evidence for differential change in *sensitivity* to auxin (Evans 1991). Bruinsma (1992) discusses altered distribution of *inhibitors* of auxin activity as a cause of plant phototropism. This controversy about the way in which the gravitropic response is regulated is beyond the scope of the present review. We will attempt now to concentrate attention on aspects of plant cell physiology which seem to react

directly to change in the gravity vector.

1.4.5 Graviperception in plants

It is important to start, perhaps, with the recognition that the perception system may be complex and not necessarily identical even between different phases of the same organism. Jenkins *et al*. (1986) isolated several mutant strains of the moss *Physcomitrella patens* which had partial or complete impairment in gravitropism of the caulonema (main stem of the moss sporophyte). Complementation analysis resolved the mutations into at least three genes, but none of the mutants was altered in gametophore gravitropism, suggesting that the gravitropic response of caulonemal filaments may require some gene products that are not required for the response of the multicellular gametophores. These observations issue a warning against seeking a single graviperception mechanism, yet in the absence of detailed genetic analysis in any other system there is no way of knowing whether different observations actually relate to the same mechanism.

The gravisensing cells of plants are very specialised, but they do not contain any unique cell structures. Thus the gravisensing cells are still essentially using general cell structures to sense gravity. Amyloplasts are the most obvious candidates as statoliths (Björkman 1992), but calculations by Audus (1962) showed that structures as small as mitochondria could, in theory at least, act as a very weak gravisensing system. Since structures such as the nucleus, endoplasmic reticulum, Golgi complex and other inclusion bodies are sufficiently large and dense these, too, could form the basis of a gravisensing system in an otherwise non specialised cell. In both space-flown and clinostat treated roots of lentil (*Lens culinaris*), as well as the expected change in distribution of amyloplasts when gravistimulated, the shape and position of the nucleus was also altered systematically (Perbal *et al*. 1987; Perbal & Driss-Ecole 1989; Lorenzi & Perbal 1990a). In the account which follows, systematic physiological changes in response to change in the gravitational field will be documented.

1.4.5.1 Cell walls

Among the changes which occur most rapidly in plant cells under the simulated hypogravity conditions of the clinostat are alterations in structure and chemistry of the cell wall matrix. Clinostat grown sunflower (*Helianthus annuus*) hypocotyls had increased elongation (28%) and weight (18%) compared to controls. Cellulase activity was also increased but β-1,3 glucanase was not (Bara & Gordon 1972). When [U-^{14}C]-proline was fed to shoots of intact marigold (*Tagetes patula*) grown on 15 rpm clinostats they showed a 2-to 9-fold increased incorporation of ^{14}C into salt-extractable material from the cell walls in comparison to controls (Oputa & Mazelis 1977).

Growth of marigold (*Tagetes patula*) under experimental hypogravity is characterised by increased peroxidase activity and decreased lignin content (Siegel *et al*. 1978). The specific activity of peroxidase was also elevated significantly in marigold seedlings grown on clinostats or with buoyancy compensators to reduce the mechanical stresses caused by gravity (Waber *et al*. 1975) so it is possible that this response arises intracellularly and is independent of the mechanical effects of gravity. Elevation of peroxidase activity of the cell wall, but not of the protoplast, is a short-term response to clinostat treatment (Siegel & Siegel 1983); long-term effects include changes in cell wall composition of Ca, lignin, and protein-bound hemicellulose (possibly cell wall glycoprotein). Lignin contents were also reduced in mung bean seedlings exposed to 194 h of microgravity on the Shuttle STS-3 flight, though this was coupled with reduced phenylalanine ammonia-lyase and peroxidase activities (Cowles *et al*. 1984).

An early change in ultrastructure of protonema cells of the moss *Funaria*

hygrometrica grown on a 2 rpm clinostat is that the fibrils of the cell walls became thinner and loosened. Populations of peroxisomes in protonema cells increased (Nedukha 1986) and there was an increase in the deposition of callose (amorphous β-1,3 glucan) in the cell walls (Nedukha *et al*. 1988) and a considerable increase in polygalacturonase and pectin esterase activities (Nedukha & Trutneva 1988). Cell wall lysis has also been observed in cells of the root cap of *Arabidopsis thaliana* grown in orbit (Tarasenko *et al*. 1982).

Cellulase activity (both endo-1,4-β-glucanase and exo-1,4-β-glucanase) increased in the periplasmic space and protonema cell walls of *Funaria hygrometrica* grown in a horizontal clinostat (2 rpm) for 30 days (Nedukha 1992). A decrease of total cellulose content, its crystalline form, and pectic substances as well as an increase of hemicellulose content was observed in the clinostated material compared to controls.

No significant changes were detected in the cell wall polysaccharides of the peripheral cell layers on the upper and lower side of sunflower hypocotyls which had undergone gravicurvature. Neither the relative rates of synthesis of the main polysaccharides nor the turnover of recently incorporated major polysaccharides were influenced significantly by short term changes in growth rate of the peripheral cell layers (Carrington & Firn 1985). Although β-D-glucan content increased in proportion in lower halves of gravistimulated oat stems, in concert with an increase in glucan synthase activity in lower halves, the asymmetry arose too slowly to account for initiation of the graviresponse (Gibeaut *et al*. 1990). These authors postulated that asymmetrical changes in osmotic solutes act as the driving factor for growth promotion in the graviresponse, while wall extensibility acts as a limiting factor during growth. A similar conclusion, that gravitropic curvature is not driven by new cell wall synthesis but, rather, by changes in cell wall plasticity and osmotic potential, was reached by Lu *et al*. (1992).

Overall effects of such structural changes may be small, or negligible, however. Masuda *et al*. (1994) studied seedlings of several species of plant growing on a three-dimensional clinostat (with two rotational axes) and in a centrifuge (30-135 × g). Growth rate and mechanical properties of the cell wall were little affected by either treatment, although the normal gravitropic response of the organs was markedly altered. Far from being a mere enclosure, the plant cell wall is an important organelle which essentially brings the immediate region of the environment under cellular control. There is a constant exchange of information between the cell wall and the cell (see discussion in Trewavas & Gilroy 1991), so it is inevitable that changes in the cell wall induced by change in gravitational acceleration will be complex and may well be compensatory.

1.4.5.2 Membrane chemistry

There are also indications of changes in membrane structure. Palladina *et al*. (1984) recorded a considerable decrease in the protein content of plasma membrane preparations isolated from the roots of 5-day old pea seedlings grown on a 2 rpm clinostat. Zhad'Ko *et al*. (1988) report a 20% decrease in the chemiluminescence of pea seedlings in the first 15 to 30 minutes of clinostating, suggesting that lipid peroxidation is one of the first responses of seedlings to clinostating. Similar changes in lipid peroxidation were recorded in *Chlorella* cells, tissue cultures of *Haplopappus* and pea seedlings subjected to slow clinostating, and in tissue cultures of *Haplopappus* and wheat seedlings after space flight (Baraboi *et al*. 1991; Zhad'Ko *et al*. 1994).

Polulyakh (1988) exposed 5 to 6 day-old pea seedlings to a 2 rpm clinostat for 24-48 h and found that phosphatidic acids and phosphatidylinositol decreased in the root cell membranes. Phosphatidylcholine and phosphatidylethanolamine increased during the first, and decreased during the second day of clinostating. The reverse applied to phosphatidylethanolamine and diphosphatidylglycerol. C14:1, C18:0, C18:2, C18:3, and the

unsaturation index of cell membrane lipids consistently increased during both days, whereas C14:0, C16:0, and C18:1 decreased. On the other hand, the range of fatty acid changes was wider than that of phospholipids. Clinostat treatment thus prompts major changes in the chemical composition of membranes which are related to an increase in microviscosity (of lipid bilayer of vesicles prepared from root plasmalemma) from 0.05-0.07 to 0.51-0.56 Pa s^{-1} during the first 24 h, though this decreased to 0.02-0.05 Pa s^{-1} after a further 24 h treatment (Polulyakh & Volovik 1989). The decrease in microviscosity during the second day of clinostating was associated with an increase of short- and branched-chain fatty acids, of double bonds in a *cis*-configuration, and of polarity of the hydrophilic portion of the molecule. These changes increased intermolecular distances in the lipid bilayer, increasing its fluidity and permeability. We see here evidence for adaptation of the membrane to maintain normal fluidity under the hypogravity conditions provided by the clinostat.

Membrane alterations and/or changes in the physical properties of the fluids in microgravity presumably account for the significant increases in fusion products in electrofusion experiments with mesophyll protoplasts of *Nicotiana tabacum* performed aboard the TEXUS 17 sounding rocket (Mehrle *et al*. 1989; Hampp *et al*. 1990). Microgravity during the TEXUS 21 and TEXUS 25 sounding rocket flights similarly increased the yield of fusion products between *Digitalis lanata* and *Digitalis purpurea* about 8-fold in comparison to terrestrial conditions (Baumann *et al*. 1990). Interestingly, very similar results have been reported for electrofusion of mammalian (mouse hybridoma) cells. During the flight of TEXUS 18, both fusion frequency and hybrid viability were increased by about 2-fold in the microgravity-exposed samples (Schnettler *et al*. 1989).

The most frequently reported membrane phenomenon is a change in ion channels and ion distributions. Tairbekov *et al*. (1980) reported only slight differences between ATPase (mitochondrial and cytoplasmic) activities of maize cells formed on the clinostat and control cells whereas these activities were about 50% higher in cells formed on a 2 × g centrifuge. Increased gravity intensified ATP decomposition and at 2 × g there was a relatively low rate of oxidative phosphorylation. This discrepancy between synthesis and degradation of ATP may lead to structural changes in cell organelles; it was not observed in control or clinostat grown cells. In later work, however, Tairbekov *et al*. (1984) reported the ATP content of clinostat grown cultures of developing maize sprouts to be only 63% of the control. This relatively low ATP content in cells grown on a 2.4 rpm horizontal clinostat was associated with an elevated rate of respiration as measured by CO_2 evolution. These authors pointed out that aerodynamic effects on even slowly-rotating clinostats may increase respiratory activity by eliminating dead zones close to the epidermis and consequently improving gas exchange. Changes in activity of plasma membrane-bound ATPases are commonly reported. In plasma membrane preparations isolated from the roots of 5-day old pea seedlings grown on a 2 rpm clinostat the activity of Ca^{2+}-ATPase decreased, that of Mg-ATPase increased, and K^+ stimulated the Mg^{2+} effect on the ATPase activity (Palladina *et al*. 1984). Electron cytochemical analysis (transmission electron microscopy using the pyroantimonate precipitation method) showed that in control roots the reaction product from Mg^{2+}-activated ATPase was mostly localized on the plasmalemma, in plasmodesmata, on nuclear chromatin, endoplasmic reticulum, Golgi vesicles; reaction product of the Ca^{2+}-ATPase was localized on the plasmalemma of statocytes. After clinostating, (2 rpm) the Mg^{2+}-ATPase reaction product was found as large granules on the plasmalemma, and as a fine precipitate on vesicles and cisternae of the dictyosomes, internal membrane system of plastids, nuclear membranes, chromatin, and nuclei (Palladina *et al*. 1984; Kordyum *et al*. 1984). The intensive reaction for Ca^{2+}-ATPase activity characteristically limited to the plasmalemma of statocytes of pea roots was greatly reduced in roots grown on a 2 rpm clinostat (Podlutsky 1986). Indeed, Belyavskaya *et al.* (1988) state that no Ca^{2+}-ATPase activity was detected along the

plasmalemma in clinostat grown material. Instead it was found in the nuclear envelope, plastid membrane reticulum, granular endoplasmic reticulum, and Golgi membranes, and dictyosomal vesicles. Similar differences in localization of Ca^{2+}-ATPase activity, detected by TEM cytochemistry, were noted between normally grown protonema cells of the moss *Funaria hygrometrica* and those grown for 20 days on a 2 rpm clinostat.

1.4.5.3 The calcium connection

Many of the above observations suggest some disturbance in plasmalemma structure of cells grown on the clinostat which might affect the distribution of Ca^{2+} ions within and around the cell. Sitnik *et al*. (1989) used the pyroantimonate precipitation method for detecting free and weakly-bound calcium in cells of 7-day pea seedling roots grown stationary (control) or on a 2 rpm clinostat. Deposits of calcium pyroantimonate were localized inside organelles and in the periplasmic space in the control. In clinostat grown material, deposits of calcium pyroantimonate were found in the cytoplasm as well. Indeed, chains of deposits were found between amyloplasts in some cases, an interesting observation in view of the localization of calcium in amyloplasts of root cap cells (Chandra *et al*. 1982) and the observation that the region of cytoplasm surrounding sedimenting amyloplasts in maize roots stains intensely with toluidine blue, indicating a transient increase in polyanions in the vicinity of the amyloplast as sedimentation commences (Moore 1986b). Changes in calcium ion distribution have also been noted in protonema cells of the moss *Funaria hygrometrica* grown for 20 days on a 2 rpm clinostat (Nedukha 1987, 1989a & b). Cultivation of protonema on a clinostat led to a considerable intensification of precipitation of the reaction product in membranes, vacuoles, periplasmic space, and cell walls. At the same time, analysis of pectinase localization using TEM cytochemistry showed that a high reaction intensity in cell walls in comparison to that in the control was distinctive of cells cultivated on a clinostat. Increase of Ca^{2+} content in the periplasmic space of cells grown on the clinostat was suggested to result from hydrolysis of pectins (implied by a decrease in cell wall thickness and loosening of cellulose fibrils) with consequent transfer of calcium from the bound state into the free one (Nedukha 1989a, b). Using a fluorescent calcium probe, Sitnik *et al*. (1989) found that the calcium distribution gradient in cells of *F. hygrometrica* changed considerably during clinostating, and it is suggested that gravity may participate in redistribution or activation of calcium ion channels in the plasmalemma.

1.4.5.4 Coleoptiles, stems and leaves

Slocum & Roux (1983) showed that an accumulation of Ca^{2+} in the upper halves of horizontal, gravistimulated oat (*Avena sativa*) coleoptiles occurs within 10 min of their being placed horizontal, about 20-30 min before large-scale visible bending. Gravitropic curvature was well developed by 3 h and was accompanied by further redistribution of Ca^{2+} to tissues along the upper coleoptile half, centred on the bend. Ultrastructural studies indicated that large amounts of Ca^{2+} were accumulated in cell walls at the upper side of the organ in the region of maximal bending, active transport of Ca^{2+} from cells into walls being performed by a Ca^{2+}-dependent ATPase localized in the plasma membrane (Roux *et al.* 1983). Antagonists of calmodulin (Biro *et al*. 1982) and calcium chelators (Daye *et al*. 1984) blocks gravitropism in oat and maize (Medvedev & Shtonda 1989) coleoptiles without inhibiting growth. These observations suggest that differential growth resulting in the establishment of gravitropic curvature may be the consequence of Ca^{2+} antagonism of auxin-mediated cell wall loosening and elongation growth at the upper side of aerial plant organs.

Migliaccio & Galston (1987) showed that inhibitors of IAA movement blocked development of both IAA and Ca^{2+} asymmetries, but substances known to interfere with normal Ca^{2+} transport did not significantly alter either IAA or Ca^{2+} asymmetries in

gravitropically stimulated pea epicotyls (the part of the seedling stem between the cotyledon and the first leaf). These authors concluded that Ca^{2+} was bound to the cell walls, and that auxin movement is primary and Ca^{2+} movement secondary in stem gravitropism. The hypothesis being that apoplastic Ca^{2+} changed during graviresponse because it was displaced by H^{+} secreted through auxin-induced proton release, Ca^{2+} being released from the wall adjacent to the region of high IAA concentration, proton secretion, and growth. In this interpretation, therefore, the asymmetric distribution of Ca^{2+} is a *consequence* of growth stimulation, not a critical step in the early phase of the graviresponse.

Clinostat treatment of plants induces specific changes in the pattern of soluble leaf proteins of *Vicia faba* (broad bean), eight polypeptides increased in amount, whereas four proteins decreased or disappeared. Using immunoblotting, it was shown that the amount of β-tubulin was not affected, but the pattern of polypeptides recognized by calmodulin antibodies was changed by clinostating (Schulz *et al.* 1992). The authors interpret these changes as resulting from altered protein turnover as a stress reaction to the rotating gravity vector.

1.4.5.5 Root responses

The pattern of localization of Ca^{2+} in gravistimulated corn roots was quite different from that seen in gravistimulated coleoptiles. In horizontally positioned roots, Ca^{2+}, initially localized within the stele, became localised within the vacuoles of cortical cells along the upper root surface. There was little staining in the walls and, in the root cap, where graviperception is thought to occur, there was little staining of any cellular organelles (Dauwalder *et al*. 1985). Nevertheless, calcium ions are firmly implicated in gravity signal perception and transduction (Poovaiah *et al*. 1987). Belyavskaya (1992) claims that clinostating and true microgravity exert their disorienting effects by causing unregulated increases of Ca^{2+} in the statocytes of pea roots.

Gravistimulation under normal circumstances induces polar movement of Ca^{2+} across the root tip from the upper side to the lower side in primary roots of maize (*Zea mays*) and pea (*Pisum sativum*). Treatment of maize or pea roots with inhibitors of auxin transport prevents both gravitropism and gravity-induced movement of Ca^{2+} across the root tip (Lee *et al*. 1984). Moore (1986a) used the pyroantimonate precipitation procedure to localize Ca^{2+} in maize roots. Within 5 min of horizontal reorientation, staining became associated with the portion of the cell wall adjacent to the distal end of the cell. This asymmetrical staining persisted throughout the onset of gravicurvature. No staining of lateral cell walls of columella cells was observed at any stage of gravicurvature, suggesting that a lateral flow of Ca^{2+} through the columella tissue of horizontally-oriented roots does not occur. Nevertheless, root cap mucilage of *Zea mays* contains effector(s) that induce gravitropic-like curvature (Fondren & Moore 1987; Moore & Fondren 1986, 1988; Marcum & Moore 1990). Seemingly, the transverse movement of gravitropic effectors, including Ca2+, to the lower sides of tips of horizontally-oriented roots occurs through the apoplast in the outermost layers of the root. It most strongly occurs in the cytoplasm, cell walls and mucilage of epidermal cells (Moore *et al*. 1989).

Björkman & Cleland (1991) used calcium-specific microelectrodes to measure the Ca^{2+} gradient in maize roots. The calcium activity on the upper side of the root tip remained constant for 10 min after gravistimulation (initial pCa about 2.5 mM), then decreased by about 50%. On the lower side, after a similar lag, the calcium activity increased 1.6-fold. The calcium activity gradient (and gravitropism) was eliminated by applying a mobile calcium-binding site (dinitro-BAPTA) to the root cap. Conversely, preventing the formation of apoplastic pH gradients, using MES buffer or 10 mM fusicoccin, did not inhibit curvature, indicating that the calcium gradient is not a secondary effect of proton-induced calcium

desorption by a proton gradient [compare with the Migliaccio & Galston (1987) pea epicotyl experiment described above].

Gravistimulation is followed by alterations in the external current symmetry (Lühring *et al*. 1986; Iwabuchi *et al*. 1989) in *Lepidium* roots. Upon gravistimulation, the membrane potential of statocytes is temporarily depolarized (lag time = 2 s) from a resting potential of *ca* -118 mV to a potential of *ca* -93 mV. This only occurs in statocytes located on the physically lower flank of the root, those on the corresponding upper flank become weakly hyperpolarized (*ca* -13 mV)(Sievers *et al*. 1984). Inhibitors of auxin transport blocked gravitropic curvature but not the change in current density, whilst inhibitors of calmodulin activity blocked both curvature and gravity-induced current. The results suggest that auxin transport is not a component of gravity sensing in the root cap, but that calmodulin plays an intrinsic role in gravity sensing (Björkman & Leopold 1987) [compare with the Migliaccio & Galston (1987) pea epicotyl experiment described above]. Treatment of maize root tips with Ca^{2+} increased the basipetal movement of auxin (Evans *et al*. 1992). Since gravistimulation causes Ca^{2+} movement toward the lower side of the root cap, this observation supports the idea that gravity-induced Ca^{2+} asymmetry is a key step linking gravistimulation to the establishment of auxin asymmetry during root gravitropism.

An important role for calmodulin in root gravitropism was demonstrated by Stinemetz *et al*. (1987), who directly assayed calmodulin in primary roots of maize (whole tissue segments, crude extracts and purified extracts) and found that the time course of the development of gravitropic sensitivity followed the time course of the increase in calmodulin activity in the apical 1 mm of the root. Application of chelators and calcium-ionophores to pea roots (Belyavskaya 1988) and roots of *Zea mays* (Reddy *et al*. 1987) resulted in the loss of gravisensitivity. Furthermore, 5-hydroxytryptamine, which is known to promote the hydrolysis of phosphoinositides, sensitized dark-grown maize roots to gravity and increased inositol trisphosphate levels, suggesting roles for both Ca^{2+} and inositol phospholipid turnover in gravity signal transduction. Phosphorylation of two specific polypeptides also seems to be involved in transduction of the gravity signal in maize roots (Friedmann & Poovaiah 1991).

Prolonged treatment (2 h) of roots of *Lepidium* with the calcium ionophore A23187 caused a complete loss of graviresponsiveness which was accompanied by loss of statocyte polarity and displacement of the usually distal endoplasmic reticulum (Wendt & Sievers 1989). In chelator-treated (50 mM EGTA) roots of *Zea mays*, development of the Golgi apparatus was severely reduced and there was no evidence of a granular secretory product which was encountered in the dictyosomes of control roots (Bennet *et al*. 1990). Reduction in amyloplast numbers brought about by EGTA occurred alongside reduced Golgi secretory activity.

1.4.5.6 Organelle distributions

One of the first ultrastructural features observed when protonema cells of the moss *Funaria hygrometrica* are placed on a 2 rpm clinostat is that grana disappear from plastids and their content of starch decreases (Nedukha 1986). Similar decreases in reserve polysaccharides in *Chlorella* grown on the clinostat result from intensified hydrolysis due to an increase of the specific activity of amylase (Popova & Shnyukova 1988, 1989). Soybean seedlings grown on a 1 rpm clinostat or 5 × g centrifuge also exhibited changes in starch metabolism (Brown *et al*. 1994; Brown & Piastuch 1994). Starch concentration in the cotyledons was 28% lower in clinorotated plants and 24% higher in centrifuged plants than in control (vertically rotated) plants. The opposite relationship was noted for total lipid concentration. Of six starch metabolic enzyme activities measured, only ADP-glucose pyrophosphorylase was affected by the treatments; being 37% lower in clinorotated plants and 22% higher in those which were

centrifuged.

Accelerated chloroplast destruction was noted in tissue cultures of *Haplopappus gracilis* on a 50 rpm clinostat (Lozova *et al*. 1989). On the other hand, mitochondrial volume, assessed by morphometric analysis, increased from 3% to 13% of the cell volume in cultures of *Chlorella vulgaris* grown on the clinostat. The electrophoretic spectrum of succinate dehydrogenase also changed with an increase in the composition of structurally bound forms being observed on the clinostat. The fast and slow electrophoretic variants of the enzyme were most affected by clinostating; those with intermediate rates of migration were present in all samples (Popova *et al*. 1987). Changes in the distribution of succinate and malate dehydrogenases (detected cytochemically with nitrotetrazolium blue) was studied in cells of 5-and 10-day protonema of *Funaria hygrometrica* grown on a 2 rpm clinostat (Nedukha *et al*. 1985).

Morphometric analysis of organelle redistribution after maize roots have been placed horizontally emphasises the correlation of amyloplast sedimentation with graviperception, but also indicates that movement of the nucleus towards the proximal end of the cell (that is, the end of the cell furthest from the root cap) is also correlated with the gravitropic response (Moore 1986b). However, the integrity of the statocyte, as well as this polarity in its organelle distribution, seems to be essential for the gravitropic response of the roots of cress (*Lepidium sativum*). Prolonged growth on a 2 rpm clinostat caused damage to the statocytes for as well as redistributing organelles, such treatment caused loss of amyloplast starch, aggregation of lipid droplets and lysis of cell walls (Hensel & Sievers 1980). Similar results have been described for pea roots grown on both fast and slow clinostats (Belyavskaya 1983) and cress roots exposed to microgravity on the Bion-10 mission (Laurinavichius *et al*. 1994a & b).

Sievers and co-workers (Sievers & Volkmann 1972; Volkmann, Sievers 1979; Sievers, Heyder-Caspers 1983) maintained for a number of years that amyloplasts are effective in cress (*Lepidium*) roots because they exert pressure on a complex of rough endoplasmic reticulum consisting of parallel cisternae near the distal (closest to root tip) cell wall. A major reason for this suggestion was the short presentation time (only 12 seconds) in this material, which must mean that the graviperception mechanism is extremely sensitive and cannot depend on sedimentation over large distances.

Endoplasmic reticulum membranes isolated from *Lepidium* roots accumulated Ca^{2+} when incubated with micromolar concentrations of calcium. The accumulation was ATP-dependent and ATP-specific and was not inhibited by protonophore treatment (Sievers *et al*. 1984; Sievers 1986). Furthermore, *Lepidium* root gravitropic response was drastically inhibited by treatment with 20 µM cyclopiazonic acid, a known inhibitor of Ca^{2+}-transporting ATPases of the endoplasmic reticulum (Sievers & Busch 1992). This mechanism could, therefore, be related to the calcium polarisation which is discussed above. Indeed, Lawton *et al*. (1986) claim that their thick section high voltage electron microscopy shows that statoliths in statocytes in the leaf sheath bases from several different grasses sediment onto cushions of tubular endoplasmic reticulum, so the mechanism might may not be limited to the root. However attractive this idea might be, it is not the end of the story. Audus (1962) had already calculated that the pressure exerted by amyloplasts is extremely small and pressure sensing is, theoretically, a poor basis for a graviperceptive guidance mechanism. Hensel & Sievers (1981) showed that even very short periods of contact between these organelles leads to disintegration of the distal ER complex, loss of amyloplast starch, and confluence of lipid droplets, suggesting that contact rather than pressure may be important. Centrifuge experiments (Wendt *et al*. 1987) have shown that gravitropic bending of cress roots occurs without contact between amyloplasts and endoplasmic reticulum. Sedimenting amyloplasts showed no consistent contact with any other cellular structure in maize (Moore 1986b), and,

indeed, the onset of root gravicurvature in maize does not require a major redistribution of plastids in columella cells (Moore 1987).

Furthermore, gravitropic responses still occur in plants free of amyloplast starch because of nutritional or genetical alterations (Roberts 1984; Moore *et al*. 1987b; Caspar & Pickard 1989; Kiss *et al*. 1989), although the fact that such mutants *do show* a gravitropic response has been interpreted as evidence that starch is unnecessary for gravity perception (Caspar & Pickard 1989). A starchless mutant of *Arabidopsis thaliana* in which amyloplasts did not significantly sediment in either upright or inverted seedlings produced gravitropic curvatures of at least 70% of the wild type (Caspar & Pickard 1989). This parallels a much earlier study which showed that physiologically destarched plants can still make gravitropic responses (Pickard & Thimann 1966). However, Kiss *et al*. (1989) showed that although starch is not required for gravity perception in *Arabidopsis thaliana* roots, it is necessary for full sensitivity; the starchless amyloplasts still function as statoliths. Starch deficiency in a mutant of tobacco (*Nicotiana sylvestris*) greatly reduced gravitropic sensitivity, the mutant bending to an angle of 10^{o} in the time the wild type reached 70° (Kiss & Sack 1990).

In lentil (*Lens culinaris*) roots grown in microgravity in orbit during a Spacelab mission, amyloplasts were not randomly distributed as they become on the clinostat, but were more localised in the proximal part of the cells than in 1 × g controls (Perbal *et al*. 1987; Perbal & Driss-Ecole 1989). These authors maintain that contact between amyloplasts and the distal complex of endoplasmic reticulum was not required for graviperception, but direct attention to a network of microfibrils, which seems to bind the nucleus to the cell periphery, which may be able to respond to acceleration. Hensel (1984) found little evidence for involvement of microtubules in graviperception in statocytes from roots of cress (*Lepidium sativum*). Development of the roots on a horizontal clinostat preserved a youthful state of the microtubular system. Compression of the distal network of microtubules after centrifugation of the roots indicated that microtubules might function in stabilizing the distal complex of endoplasmic reticulum.

An observation which appears to have been given very little attention is that sedimenting amyloplasts in maize roots are associated with a surrounding region of cytoplasm which stains intensely with toluidine blue in sections of resin-embedded material (Moore 1986b); the author stresses that this differential staining was unique to the early stages of amyloplast sedimentation, being observable only in material fixed immediately after reorientation. Toluidine blue is a basic dye which reliably stains anions in the presence of resin embedding media. This observation indicates a *transient* increase in polyanions in the vicinity of the amyloplast as sedimentation commences. Perhaps some conformational change in microtubules or microfilaments around the amyloplasts is generated immediately they begin to sediment.

1.4.5.7 Cytoskeleton

Until very recently, the most widely held interpretation of the sorts of results described above has been that gravity perception in plants involved direct interaction between the statoliths and endoplasmic reticulum membranes normally located beneath them (review - Sievers & Hensel 1990). The position of these membranes seems to be determined genetically. When displaced by centrifugation, they relocate to their original position within a few minutes, independently of the direction of gravity in relation to the root axis, or the direction of the applied centrifugation vector (Wendt & Sievers 1986). Cytochalasin treatment inhibited relocation of the endoplasmic reticulum, so the cytoskeletal system was seen as being involved simply in locating the endoplasmic reticulum (Wendt & Sievers 1986). More recent experiments have certainly changed this view.

Tropic curvature in maize coleoptiles and sunflower hypocotyls is correlated with a

change of microtubule orientation from transverse to longitudinal at the slower growing organ flank, whereas the transverse microtubule orientation is maintained (or even augmented) at the faster growing organ flank. These directional changes are confined to the microtubules subjacent to the outer epidermal wall (Nick *et al*. 1990a). Application of the antimicrotubular agent, ethyl-*N*-phenylcarbamate, inhibited gravitropism without affecting phototropism, implying a role for the microtubular cytoskeleton in perceiving and transducing statolith movement (Nick *et al.* 1991). Lorenzi & Perbal (1990b) showed that actin filaments are probably responsible for positioning the nucleus in lentil statocytes in experiments involving space-flown material and clinostat-treated samples. However, it is Sievers and his co-workers who have provided the most definitive results (Sievers *et al*. 1991).

Thin bundles of microfilaments were shown encircling the statoliths as a dense envelope in rhizoids of *Chara* (by labelling actin with rhodamine-conjugated phalloidin), and in root statocytes of *Lepidium sativum*, cytochalasin B treatment caused a 3-fold increase in the rate of statolith sedimentation (Hensel 1989; Sievers *et al*. 1989; White & Sack 1990). Statoliths hold a particular position in both these cell types. The distance between the statoliths and the physically lowest point in the cell is up to 25 µm in the *Chara* rhizoid, and up to 4 µm in cress statocytes. Application of cytochalasin to vertical rhizoids or roots causes statoliths to sediment to the lowest point of the cell by displacement of the ER membranes (Hejnowicz & Sievers 1981; Hensel 1985; Bartnick & Sievers 1988). Presumably, under normal circumstances statoliths interact with microfilaments to maintain their characteristic position. Experiments during 5 rocket flights (TEXUS 18, 19, 21, 23, 25) showed that within approximately 6 minutes of microgravity exposure the shape of the statolith complex changed and, more importantly, the complex moved approximately 14 µm (*Chara* rhizoids) and 3.6 µm (cress root statocytes) ***in the opposite direction*** to the originally-acting gravity vector (Volkmann *et al*. 1991). Videomicroscopy of *Chara* rhizoids during rocket flights confirmed that in the weightless state the statoliths moved **away from the tip** (see Fig. 7), possibly due to relaxation of microfilaments which are normally kept under tension by the weight of the statolith. This idea was further supported by the finding that disorganizing microfilaments with cytochalasin D resulted in sedimentation of statoliths onto the apical cell wall under normal gravity and an absence of basipetal displacement during the microgravity phase of a rocket flight (Buchen *et al*. 1993).

Fig. 7. Summary diagrams of statolith movements in the tips of rhizoids of the alga *Chara*. The rhizoids are growing vertically downwards at the beginning of each experiment depicted here, and this gravitropic response is governed by the statoliths which are normally maintained 17 µm above the apex. When treated with cytochalasin the statoliths fall into the apex, indicating that actin microfilaments are responsible for suspending the statolith complex above the rhizoidal apex. In microgravity (TEXUS sounding rocket) the statolith is pulled back from the apex, suggesting that its normal gravitropic function depends upon its weight exerting a tension stress on the microfilaments of the cytoskeleton. The drawings were made from photographs of video stills in Volkmann *et al*. (1991) and Buchen *et al*. (1993).

The gravity-dependent polarity of cytoplasmic streaming in algal cells (Wayne *et al*. 1990a; Buchen *et al*. 1991) has provided a sensitive tool for study of graviperception, even in cells which lack statoliths. Results support the hypothesis that the plasma membrane is the gravireceptor and that Ca^{2+} ions participate in the signal transduction chain that leads to a gravity-induced polarity of cytoplasmic streaming (Wayne *et al*. 1990b). The link with work

described in the previous paragraph is that Wayne *et al*. (1992) present evidence that the cell-extracellular-matrix-junction, which includes the cell wall and the outer surface of the plasma membrane, is involved in gravity perception by the statolith-free internodal cells of *Chara*. Gravisensing was sensitive to protease treatment, indicating that proteins in the cell-extracellular-matrix-junction may be required for gravisensing, and inhibition by the tetrapeptide Arg-Gly-Asp-Ser suggests that the algal gravireceptor may be an integrin-like protein.

In higher fungi, too, there is growing evidence that actin microfilaments are involved directly in the gravity perception component of the gravitropic response of mushroom fruit bodies (Monzer & Haindl 1994; Monzer, 1995; Novak Frazer & Moore, 1994). Sedimentation of nuclei within an actin 'cage' has been postulated to account for gravity perception in *Flammulina* (Monzer, 1995), and the kinetics of response to a spectrum of inhibitors has been used to deduce that actin microfilaments are specifically involved in perception in *Coprinus* (Novak Frazer & Moore 1993, 1994). However, these are all preliminary findings using living material which is extremely difficult to observe. Cells responsible for gravity perception in fungi are not differentiated, as they are in plants. Consequently, the experimental tissue is comprised of a mass of almost indistinguishable cells, and it is difficult to devise experiments which enable observation to focus precisely on the perception event rather than on some later stage in the gravitropic process.

1.5 Animal growth and development

Embryonic development is perhaps the most complex cell biological process, requiring the co-ordination of activities including gene expression, cell division, cell differentiation and cell interactions. Gravity has been cited as being a possible directional cue that could be used for determining pattern formation (Malacinski *et al.* 1989). This proposition has been examined experimentally in a number of animal systems both in ground-based and space-flown experiments.

Hughes-Fulford (1991) has provided an excellent review of altered cell function in microgravity in which she makes the case for drawing comparisons between physiological alterations seen in single cell prokaryotes and eukaryotes and those observed in animal tissues and even whole animals. It is worth reiterating that many of the physiological changes seen in humans, vertebrate and other multicellular organisms in space flight may originate from dysfunction of basic biological mechanisms caused by microgravity (or other aspects of the orbital environment). Since microgravity seems to affect prokaryotic and eukaryotic cell function at a subcellular and molecular level, research in space offers an opportunity to learn more about basic biological mechanisms which are essential to life. This general justification for microgravity research is a parallel to the observation that as aging in humans shares many of the symptoms seen in astronauts during space flight (reduced cardiac function, loss of bone, reduced immune response, orthostatic hypotension), research in these two areas can only be synergistic; each contributing to the other and both together making more rapid advance than either in isolation (Booth, 1994).

1.5.1 Cell physiology in the whole animal

To a considerable degree, this medical relevance has been a major motive in the design of experiments on animal gravitational biology and has formed the proper context of the research. Human physiology is dealt with in detail in Chapters 2 to 4 and reactions of individual cells are detailed in Section 1.2, so in this introductory section we have chosen to highlight a selection of topics, intending simply to illustrate the wide range of animal physiology which is influenced by change in the gravity vector.

Rats exposed to 3-fold normal gravitational acceleration for 8 months adapted with

little chronic stress but at a higher basal metabolic rate. The rats aged faster than controls, the longest survivors living for an average of 520 days on the centrifuge compared to 574 days for controls. The experiment is interpreted as a demonstration of the rate of living theory of aging in mammals (Economos *et al*. 1982). Hypergravity acceleration (1 to 5 × g) did not decrease total fecundity in *Drosophila melanogaster* (Lints & Le Bourg 1989), but despite initial indications of a decrease in longevity (Le Bourg & Lints 1989a) and in the viability of eggs (Le Bourg & Lints 1989b), the effects are not clear and even at 7 × g, flies lived for roughly 40 days (Le Bourg *et al*. 1993). Thus, the flies remain able to lay viable eggs and to live for a fairly long life, even under sustained hypergravity conditions. Examination of three behavioural traits affected by aging (climbing activity, pattern of movement, spontaneous locomotor activity) suggests that flies age faster in hypergravity (Le Bourg & Lints 1992a-c). All of these results also contribute to theories of aging, especially the rate of living theory. Maternal and foetal plasma prolactin levels were reduced on day 22 of gestation in rats exposed to 3 × g (Megory & Oyama 1985). Such an effect on foetuses conceived after adaptation to hypergravity highlights the possibility that gravitational effects on developmental biology might be due to general effects on systemic physiological mechanisms which then impact the developmental processes.

Exposure of chicken embryos to daily 20 min 7 × g acceleration stress had a substantial effect on myocardial differentiation, decreasing Ca^{2+}-stimulated contraction of isolated myocytes, affecting their rhythmic activity and reducing levels of actin, myosin light chain 2, and troponin C in the myocardium (Oganesyan *et al*. 1980).

Articular and growth-plate chondrocytes respond differently to hypergravity. Centrifugation of rabbit growth-plate chondrocytes at 3 × g resulted in a 2-fold increase in incorporation of [^{35}S]sulphate into proteoglycans, but had little effect on [^{3}H]thymidine incorporation into DNA. However, similar treatment of articular chondrocytes caused 1.5-fold increases in both [^{35}S]sulphate incorporation into proteoglycans and [^{3}H]thymidine incorporation into DNA (Inoue *et al*. 1990). Space flight cultures (aboard Space Shuttle mission STS-54) of embryonic mouse pre-metatarsals which had initiated chondrogenesis and morphogenetic patterning before flight, resulted in an increase in cartilage rod size and maintenance of rod shape, compared to controls. Older pre-metatarsal tissue, already terminally differentiated to hypertrophied cartilage, maintained rod structure and cartilage phenotype during culture in orbit (Klement & Spooner 1994). Cultured mouse embryo lung rudiments continued to grow and branch on the same flight and cultured embryonic mouse pancreas underwent characteristic exocrine acinar tissue and endocrine islet tissue differentiation during space flight (Spooner *et al*. 1994).

Osteoporosis was produced in rats by a 19-day space flight on board Cosmos 1129, bones showing no mineralization in the microstructures and a decrease in Ca content (but no change in P content was observed). The adverse effects of the flight continued to develop until the 6th day after return but the bone parameters measured had returned to normal 23 days later (Rogacheva *et al*. 1984).

Chemical measurements and X-ray microtomography indicated a longitudinal gradient of decreasing mineralization toward the distal diaphysis in femurs of rats flown on Cosmos 1887. The techniques identified areas of femoral diaphysis most vulnerable to mineralization deficiencies associated with space flight (Mechanic *et al*. 1990). In the same flight, the proliferative zones of rat tibial epiphyseal plates were larger than controls, while calcification zones were reduced (Duke *et al*. 1990). These experiments raise another difficulty in literature interpretation as the experimental (flight) animals were exposed to 1 × g for 53.5 h following recovery from a 12.5 day flight. Consequently, as cells cycle through the growth plate in 2-3 days at 1 × g, the results represent the outcome both of the microgravity exposure and a partial recovery from any effects the exposure may have had.

The delay was particularly damaging to experiments involving examination of intestinal tissues with rapid cell turnover (Sawyer *et al*. 1990). Although there were no consistent differences between animals in the flight group and those in the control groups, delay in recovering flight animals meant that the only safe conclusion was that any effects of microgravity on jejunal mucosal cells were short-lived. In a subsequent flight (Cosmos 2044), it was established that mucosal cell proliferation in rats was not affected by microgravity conditions associated with space flight. Although the length of villi and depth of crypts were reduced in flight animals this was attributed to changes in the connective tissue core and not to any impairment of proliferation or migration of jejunal mucosal cells (Sawyer *et al*. 1992).

Reductions in human T cell reactivity to mitogens after space flight are well documented and similar results have been obtained using simulations of microgravity (see Section 1.2.2). With rats, the tail suspension model of weightlessness has been used to examine lymphocyte proliferation in response to mitogens including examination of cell surface proteins and assessment of interleukin receptor expression. Significant depression in [^{3}H]-thymidine incorporation by mitogen-stimulated peripheral blood lymphocytes was evident after only 1 week of treatment, but little decrease was observed in the proliferation of lymph node lymphocytes and splenocytes. There were no changes in interleukin-2 production or interleukin-2 receptor expression. The tissue specificity of the decrease in mitogen activation was taken to indicate a compartmentalized response to the simulated microgravity treatment (Nash *et al*. 1991). Comparison of proliferation of lymphocytes from both spleen and lymph nodes of rats following a 4-day flight aboard the Space Shuttle showed reduced proliferation of lymph node lymphocytes from flight animals, whereas splenocyte proliferation was not depressed, again suggesting tissue-specific response to microgravity (Nash & Mastro 1992). Similarly, proliferation of inguinal lymph node lymphocytes from rats flown on the 14-day Cosmos 2044 mission was not significantly different from controls and they produced similar amounts of interleukin-2 (Nash *et al*. 1992). These results all support the view that microgravity acts on lymphocytes in a tissue-specific manner. Differential effects of space flight on the function of natural killer cells has also been observed. The ability of natural killer cells of rats flown on Cosmos 2044 to lyse two different target cell lines differed. Spleen and bone marrow cells obtained from flight rats showed significantly inhibited cytotoxicity for YAC-1 target cells compared with killer cells from control rats, whereas there was no significant difference in cytotoxicity for K-562 target cells (Rykova *et al*. 1992). Thus, experience of microgravity has selective effects on numerous different aspects of the immune response.

Vacek *et al*. (1991) report that the number of erythrocyte and of granulocyte and macrophage progenitors in bone marrow decreased in rats exposed to microgravity during the 14-day flight of Cosmos 2044. On the other hand, on the basis of bone marrow cell differential counts, clonal studies of RBC colony formation, and plasma erythropoietin determinations, Lange *et al*. (1994) report a slight increase in granulocytic cells in the bone marrow of Cosmos 2044 flight animals. These authors also report a slight decrease in the percentage of erythroid cells in animals subjected to centrifugation at 2 × g.
Reduced secretion of growth hormone and prolactin have been recorded in pituitary cells prepared from rats flown on the 12.5-day mission of Cosmos 1887 and the slightly longer mission of Cosmos 2044. Tail suspension of rats for 14 days resulted in changes similar to those from animals flown in space (Hymer *et al*. 1992).

These few, selected, examples illustrate the variability of response to microgravity exposure, not only between organisms, strains or varieties, but also within an organism there is variability in response between tissues and even between cells within those tissues. The extent of this variability implies the existence of a considerable genetic component in the

'control' of the gravitational response. If this is the case, then the morphogenetic and ontogenetic reaction to changed gravitational acceleration is likely to be even more varied because of the number and complexity of the cell-to-cell and tissue-to-tissue interactions involved in normal animal development.

1.5.2 Morphogenesis and ontogeny

Malacinski & Neff (1984) have described the development of animal systems in terms of a series of overlapping phases: pattern specification; differentiation; growth; and aging; and have reviewed briefly the extent to which microgravity affects these phases. The overall impression gained from this review and from subsequently published work (and including clinostat protocols and orbital experiments alike) is that microgravity alters early pattern specification (dorsal/ventral polarity) but subsequent morphogenesis is much more robust and is not adversely influenced.

Much the same seems to be true for the effects of hypergravity (2-30 × g) exposures. Sensitivity of *Rana temporaria* embryos was increased at the beginning of cleavage into 8 blastomeres and at the gastrula-neurula stage (Parfenov & Oigenblik 1988).

Amphibian and other vertebrate embryology has also been studied using clinostats and orbital experiments (Young & Tremor 1968; Young *et al*. 1970; Tremor & Souza 1971; Black & Gerhart 1985; McLaren 1990). *Xenopus laevis* embryos develop normally on a clinostat, demonstrating that the unilateral gravity vector is not necessary for normal axis formation, presumably because organisation of the egg cytoplasm does not depend solely on gravity-driven processes like differences in density between the components (Ubbels & Brom 1984; Neff *et al*. 1985; Smith & Neff 1986). The correlation between the point of sperm entry and the orientation of the first division can be disrupted by clinostating, and a similar phenomenon has been observed in microgravity aboard sounding rockets (Ubbels 1992; Ubbels *et al*. 1989, 1990, 1992). Nonetheless, after fertilization in microgravity *Xenopus* embryos do develop bilateral symmetry, presumably cued by the sperm alone (Ubbels *et al*. 1994).

The position of the first cleavage furrow during amphibian egg development can be shifted by placing embryos in novel gravitational fields. Position of the furrow is characterised by the animal/vegetal cleavage ratio (AVCR), which is the ratio of the height of the animal blastomere to the height of the *Xenopus* embryo at the 8 cell stage. Microgravity (and clinostating) increases AVCR, and centrifugation (hypergravity) reduces AVCR. Embryo inversion and D_2O immersion were found to increase AVCR, and cold shock was found to reduce it. Based on the additive or antagonistic effects of combined treatments, it is thought that the primary cause of AVCR changes is an alteration in the distribution of yolk platelets and the rearrangement of microtubule arrays. Embryos with a decreased AVCR exhibited reduced survival in early developmental stages, indicating serious difficulties in cleavage, blastulation and/or gastrulation (Yokota *et al.* 1992, 1994).

Some cytoplasmic localisations which occur during normal development on Earth, including clustering of mitochondria in the presumptive dorsal side of the embryo during the first cleavage cycle, are sensitive to disruption by change in orientation (Phillips 1994). Changes in cell morphology associated with clinostat treatment at the 8-cell stage are accompanied by altered distribution of mRNA molecules (detected by *in situ* hybridization), suggesting that gravity dependent differential morphogenesis may result from gravity dependent gene regulation (Neff *et al*. 1994).

Frog eggs were fertilized in microgravity and incubated either on the 1 × g centrifuge or in microgravity during a Space Shuttle flight in 1992. Gastrulae developed in microgravity had a thicker blastocoele roof and formed the blastopore lip nearer the vegetal pole than the 1 × g specimens, but neurulation and subsequent morphogenesis were essentially normal.

Living tadpoles returned from the flight differed in some swimming behaviours, but these differences disappeared after one week. Tadpoles from both groups completed metamorphosis (Black *et al.* 1994). In some experiments, despite dramatic differences in early embryogenesis, tadpoles at the feeding stage were largely indistinguishable from controls (Neff *et al.* 1993). In other reports (e.g. involving fertilized *Xenopus* eggs flown on Cosmos 2229), later stage flight animals also exhibited a catalogue of deformities (affecting notochords, lungs and thymus glands) when compared to ground controls (Snetkova & Wasserung 1994).

The catalogue of changes caused by real and simulated weightlessness in *Xenopus* and *Rana* includes:

(i) the location of the first horizontal cleavage furrow was shifted toward the vegetal pole at the eight-cell stage;
(ii) the position of the blastocoele was more centred, and the number of cell layers in the blastocoele roof was increased at the blastula stage;
(iii) the dorsal lip appeared closer to the vegetal pole at the gastrula stage; and
(iv) head and eye dimensions were enlarged at the hatching tadpole stage, notochords were abnormal and tails elongated, lungs smaller (uninflated?) and thymus significantly reduced.

Effects of hypergravity were opposite to those of simulated weightlessness, except that hypergravity reduced the number of primordial germ cells in feeding tadpoles. Abnormalities of early embryos or larvae returned to normal conditions were repaired as the larva developed further and mature animals were essentially normal.

Embryonic development and hatching of Japanese quail proceeded in weightlessness. The main effect seemed to be that the animals were unable to adapt their motor activity to microgravity. Movements, particularly of chicks, were uncoordinated and chaotic. In adults there was an arrest of egg production, dystrophy of ovaries and testes and a decreased testosterone level after 7 days exposure to microgravity (Boda *et al*. 1992; Meleshko *et al.* 1991). Suda *et al*. (1994) reported that only one out of ten chick eggs flown in the Space Shuttle immediately after being fertilized was recovered alive after landing. Viability was better for eggs preincubated for 7 and 10 days on Earth before flight, with all 7-day-old and 9/10 ten-day-old eggs being recovered alive. The high mortality of the 0-day-old eggs is probably related to a need for inner components to separate on the basis of difference in specific gravity to maintain adequate gas exchange during early development (regular turning of bird eggs on Earth is required for the same reason).

Fertilization of eggs of the sea urchin (*Paracentrotus lividus*) occurred normally on a sounding rocket (MASER 4) flight. Although some abnormalities were observed in pluteus larvae developed from the MASER 4 flight samples, these were considered to be artifacts and in a later experiment on MASER 5, larval development was normal to late pluteus stages (Marthy 1990, Marthy *et al*. 1994).

Behavioural changes have been recorded in fish and amphibian larvae which have developed from eggs exposed to microgravity (Neubert *et al*. 1991; Rahmann *et al*. 1992; Slenzka *et al*. 1993) and have been correlated with ontogenetic effects on the inner ear but also with physiological and biochemical changes affecting membrane function in those parts of the CNS which serve the vestibular system. A related observation is that formation of nerve-associated acetylcholine receptor patches between embryonic spinal neurones and myotomal myocytes in clinostat cell cocultures was significantly inhibited (Gruener & Hoeger 1990, 1991). Control cultures indicated that this did not result simply from loss of contact between neurites and myocytes, accelerated diffusion of a putative aggregating factor secreted by neurites, nor from turbulence in the medium. As the authors concluded, these data suggest that embryonic development of the nervous system in space may be markedly

different from that normally occurring on earth (see also Section 1.2.4.3).

Eggs of the stick insect, *Carausius morosus* were exposed to space flight conditions on the 7 day Spacelab Mission D1 and flights of the biosatellites Cosmos 1887 and 2044. Nuclear track detectors allowed effects of heavy ions to be separated from microgravity. Microgravity leads to a reduced hatching rate of eggs exposed in the early stages of development, but embryonic development before hatching showed no major difference between flight and ground control specimens either in speed of development or in morphological anomalies (Reitz *et al*. 1989). On the other hand, microgravity unexpectedly enhanced the production of morphogenetic abnormalities caused by hits by heavy ions (Reitz *et al*. 1990, 1992).

Results from the last successful flight of the Challenger Shuttle, in early November 1985, indicate that oogenesis and embryonic development of *Drosophila melanogaster* are altered in microgravity (Gonzalez-Jurado *et al.* 1986; Vernos *et al*. 1989). Some early stage embryos recovered from the space-flown containers in microgravity showed alterations in the deposition of yolk, and at least 25% of the living embryos recovered from space failed to develop into adults. Studies of late embryos indicated the existence of alterations in the anterior, head and thoracic regions of the animals. The results suggest an interference with the distribution and/or deposition of the maternal components involved in the specification of the anterio-posterior axis of the embryo. Results of Li & Wang (1992), who experimented with *D. melanogaster* during an 8-day flight of a Chinese biosatellite, are in broad agreement with these observations, showing, essentially, freedom from gene mutation in the germ-line but great influence on the embryonic development of the generation bred in space.

An unusual, and isolated, observation is that six days in orbit as a Get-Away-Special in the open bay of the Shuttle Columbia caused a 10-fold increase in hatching of both field-collected and laboratory-reared gypsy moth eggs (Hayes *et al*. 1991). This means that, in some way, conditions in orbit greatly shorten diapause in these eggs. The authors suggest the technique may be useful for rearing gypsy moths for pest control programmes. This seems to be a rather unlikely candidate for biotechnology in space. On the other hand, the observation suggests that research on the control of dormancy might be progressed by experiments in orbit and the pest control aspect would be an obvious justification for using microgravity facilities for fundamental research of this sort.

1.6 General Conclusions

Tables 1 and 2 attempt to summarise the most reliable and best-documented experiments which have been conducted in true microgravity (i.e. in parabolic flights, sounding rockets and orbital vehicles).

Table 1. Single cells flown in sounding rockets, satellites and orbital laboratories

Cell type	Mission (duration of experiment in brackets)	gravitational effect?*
Bacteria		
Escherichia coli	Vostok 4 & 6 (27-30 h), Zond 5 & 7, Biosatellite (2 d), Biocosmos 2044, Salyut 7 (1 d), D-1 (1 d), D-1 (1 h), STS-42	**NO**
Acetobacter xylinum	parabolic flights	YES
Bacillus subtilis	D-1 (3 d)	YES
Proteus vulgaris	Soyuz-12 (2 d)	YES
Salmonella typhimurium	Biosatellite II (2 d)	YES

Staphylococcus aureus	Salyut 7 (1 d)	YES
Slime mould		
Physarum polycephalum	D-1, IML-1 (20 min - 2 h)	**YES**
Protists		
Chlamydomonas reinhardii	D-1 (6 d)	YES
Euglena	TEXUS sounding rocket	YES(?)
Paramecium aurelia	Salyut 7 (4 d), D-1 (5 d)	**YES**
Pelomyxa carolinensis	Biosatellite II (2 d)	NO
Mammalian cells		
Human immune cells (T & B lymphocytes and monocytes	SL-1, D-1, SLS-1, IML-2 (3-4 d)	**YES**
	MASER 3, MASER 4, Maxus 1 (7-12 min)	YES
	STS 37 & STS 43 (24 h)	YES
	Salyut 6 (7 d) (two missions)	YES
Spleen cells, murine	STS 37 & STS 43 (24 h)	YES
Lymph node cells, murine	STS 37 & STS 43 (24 h)	YES
LML929 TNF-sensitive cell line	STS 50 & STS 57 (24-29 h)	YES
AM2 strain hybridoma, murine	D-1 (6 d)	YES
7E3-N hybridoma, murine	IML-1 (4 d)	
G8 & SP2/0-UZ hybridoma, murine	TEXUS (7 min)	YES
B6MP102 bone marrow-derived macrophages	STS 37, STS 43 & STS 50 (24 h)	YES
Jurkat T cells, human	Cosmos 2044 (24 h)	YES
THP-1 myelomonocytic cells, human	Cosmos 2044 (24 h)	YES
Friend leukemia virus-transformed, murine	IML-1 (4 d)	NO
WI 38 Human embryonic lung cells	Skylab (28 d)	NO
HeLa cells, human	Zond 5 & 7	NO
Hamster embryonic kidney cells	IML-1 (7 d)	NO
Chinese hamster ovary cells	Salyut-6 (6 d)	NO
Human embryonic kidney cells	STS-8 (1, 5 d)	NO
Human erythrocytes	STS-51C & STS-26 (3 d)	YES
A431 epidermoid cells, human	MASER 3, MASER 4 (7 min)	**YES**
L8 rat myoblasts	STS-45 (9 d)	YES
Other animal cells in culture		
Sf9 cells (= army worm)	STS-50, STS-54, STS-57	NO

*entries in bold characters indicate significant **and** well documented results

Table 2. Major findings from experiments with single cells, tissues and embryos in parabolic flights, sounding rockets, satellites and orbital laboratories

Cell type	Finding	Remarks
Cell proliferation		
T lymphocytes, human	Depression of activation by 80-95% in free-floating cells.	5 experiments in Spacelab, 4 of them with in-flight 1 × g, in agreement with data from the fast-rotating clinostat .
	Increase of activation by 100% in microcarrier-adherent cells.	1 experiment in Spacelab with in-flight 1 × g.
7E3-N murine hybridoma cells	Increase by 40% of cell counts and DNA synthesis-rate after 4 days in microgravity, no effect after 2 days.	1 experiment in Spacelab with in-flight 1 × g, microgravity effects become important only after prolonged exposure to space flight conditions.
Paramecium aurelia	60% increase of growth rate.	3 experiments, 2 in Salyut 7 without 1 × g control and 1 in Spacelab with 1 × g in-flight control, in agreement with data from the fast-rotating clinostat.
AM2 murine hybridoma cells Friend cells, murine HaK cells, hamster WI38 human embryonic lung cells HeLa cells *E. coli*	No effect detected.	These data show that gravitational effects occur only in certain cells.
Genetic expression and signal transduction		
A431 human epidermoid cells	Depression by 47% and 26% of the expression of *c-fos* gene and by 56% and 51% of the *c-jun* gene induced, respectively, by EGF or TPA. No effect on the induction by calcium ionophore or forskolin.	2 experiments in sounding rockets, in agreement with data from the fast-rotating clinostat.
T lymphocytes, human	Secretion of Il-2 and IFN-<g> correlates with the effect observed on the proliferation rate, i.e. it was strongly depressed in resuspended cells and strongly increased in microcarrier-attached cells.	1 experiment in Spacelab with in-flight 1 × g. The data have contributed to clarification of aspects of the mechanism of T cell activation.
T lymphocytes, human	Binding of Con A to the cell membrane was not altered.	2 experiments in sounding rockets.
Monocytes, human	Strong depression of the secretion of IL-1 in resuspended cells in flight.	1 experiment in Spacelab with in-flight 1 × g.

Escherichia coli	Exchange of chromosomal DNA via conjugation was enhanced, whereas the exchange via transduction or transformation was not altered.	1 experiment in Spacelab with in-flight 1 × g.
Friend cells, murine	No change in the expression of haemoglobin.	
HaK cells, hamster	No change in the secretion of tissue plasminogen activator.	
Morphology and motility		
Cells of the immune system, human	Cells display autonomous movements and cell-cell interactions.	1 experiment in sounding rocket, with in-flight video camera.
	Rearrangement of microtubule structure immediately after exposure to microgravity.	1 experiment in sounding rocket.
	Formation of cell aggregates, cytoplasmic alterations to the ultrastructure of lymphocytes, intact monocytes.	1 experiment in Spacelab with in-flight 1 × g.
	Strong interaction of cell membrane with the surface of microcarrier beads.	1 experiment in Spacelab with in-flight 1 × g.
WI38 human embryonic lung cells	Normal migration of cells adherent to a substratum.	Cinematographic recording in automatic instrument in Skylab.
Physarum polycephalum	Temporary increase of the frequency of cytoplasmic streaming oscillations and velocity.	2 experiments in Spacelab with video recording.
Friend cells Human embryonic kidney cells *Pelomyxa carolinensis*	No ultrastructural alterations detected.	
Metabolism and cell behaviour		
Acetobacter xylinum	Altered microcrystalline structure of cellulose synthesised in flight.	Parabolic flights.
Euglena gracilis	Gravitaxis lost, swimming velocity higher in microgravity, absence of aggregation.	TEXUS sounding rocket.
Mouse hybridoma cells	Fusion frequency and hybrid viability increased about 2-fold in microgravity-exposed samples.	Electrofusion experiment on TEXUS sounding rocket.
Whole plants and plant tissues		
Mung bean Oat Pine seedlings	Stem and root growth, and fresh and dry weights reduced in orbital plants.	Shuttle and Spacelab missions.

Lettuce Cress *Arabidopsis thaliana*	Coleoptile growth reduced, compared with ground controls.	Salyut missions.
Cucumber seedlings	Longer roots and coleoptiles formed on the in-flight centrifuge than in microgravity.	Cosmos 1667 with in-flight centrifuge.
Lettuce seedlings	Those grown in space were larger than those grown on the ground.	Cosmos 1667 with in-flight centrifuge.
Hemerocallis (day lily) *Haplopappus gracilis*	Controls grew more than plants subjected to space flight.	Shuttle flight.
Zea mays	Fresh weights and hormone contents of orbital seedlings not significantly different from controls	Shuttle flight.
	Higher mitotic activity and faster differentiation of root cortical cells	Cosmos 1667.
Lentil seedlings	Roots showed increased mitotic index in microgravity in comparison with in-flight controls.	Spacelab D1 Mission.
Aniseed cell cultures	More rapid differentiation.	Spacelab D1 Mission.
Rapeseed (*Brassica napus*) Carrot (*Daucus carota*)	Protoplast regeneration severely retarded in microgravity.	Biocosmos 9 (Cosmos 2044).
Nicotiana tabacum mesophyll protoplasts *Digitalis lanata* and *Digitalis purpurea* protoplast mixtures	Significant increases in production of fusion products.	Electrofusion experiments on TEXUS sounding rocket.
Arabidopsis thaliana *Zea mays*	No regeneration of the root cap in orbit.	Salyut-6 and Shuttle mission
Chara rhizoid Cress statocytes	Statoliths interact with actin microfilaments to maintain position.	TEXUS sounding rocket.
Arabidopsis thaliana	Cultivated through a complete life cycle. Seeds produced in orbit showed reduced germination, increased frequency of embryonic lethality, generally poorer seedling growth and greatly reduced ability to develop into healthy mature plants. Second generation seeds produced on Earth by the progeny of the space-flown plants were apparently normal.	Salyut-7 & Cosmos 1129.

Animal tissues, eggs and embryos

Drosophila melanogaster	Generation bred in space showed numerous developmental abnormalities but freedom from gene mutation in the germ-line.	Shuttle and Chinese biosatellite experiments.
Carausius morosus (stick insect)	Space flight results in reduced hatching rate of eggs, but embryonic development before hatching showed no major morphological anomalies, though microgravity enhanced the production of morphogenetic abnormalities caused by heavy ions.	Spacelab Mission D1 and flights of the biosatellites Cosmos 1887 and 2044.
Gypsy moth eggs	Diapause shortened in orbit.	Shuttle flight, no on-board control
Sea urchin (*Paracentrotus lividus*) eggs	Fertilization and larval development normal; some abnormalities in one flight (artifacts?).	MASER 4 & 5 sounding rocket flights.
Frog eggs *Xenopus* eggs	Fertilized in microgravity; in many experiments dramatic differences in early embryogenesis observed, but tadpoles at the feeding stage essentially indistinguishable from controls.	Shuttle experiments and Cosmos 2229.
Japanese quail	Embryonic development and hatching proceeded. Hatchling movements uncoordinated and chaotic. Adults reared from space-hatchlings showed arrest of egg production, dystrophy of gonads and a decreased testosterone level.	Experiments on Salyut and Mir.
Domestic hen eggs	Viability very low in eggs launched immediately after being fertilized.	Space Shuttle experiment.
Fish and amphibian larvae	Developed from eggs exposed to microgravity show numerous behavioural changes, correlated with ontogenetic effects on the inner ear but also with physiological and biochemical changes affecting CNS membrane function.	Space Shuttle experiments.

1.6.1 Mammalian cells

Proliferation. Human T lymphocytes, associated with monocytes as accessory cells showed dramatic changes in the centrifuge, in the clinostat and in space. As free floating cells, mitogenic response is depressed by 90% in microgravity, whereas when attached to a substratum, activation is

enhanced by 100% compared to 1 × g controls on the ground and in flight. The duration of phase G1 of the cycle of HeLa cells is reduced in hypergravity, thus resulting in an increase of the proliferation rate. Other systems like Friend cells and WI38 human embryonic lung cells did not show significant changes.

Genetic expression and signal transduction. Human T lymphocytes and monocytes show important changes in the expression of cytokines like interleukins (IL) IL-1, IL-2, interferon-γ and tumour necrosis factor. The data from space experiments (Spacelab, Space Shuttle mid-deck, Biocosmos) have contributed to clarify certain aspects of the mechanism of T cell activation. Epidermoid A431 cells showed changes of genetic expression of proto-oncogenes c-*fos* and c-*jun* in the clinostat and in sounding rockets. Membrane function, in particular the binding of ligands as first messengers of a signal, is not changed in most of the cell systems in microgravity.

Morphology/motility. Free cells (lymphocytes in particular) are able to move and form aggregates in microgravity. Thus cell-cell contacts and communications do take place in microgravity. Dramatic morphological and ultrastructural changes have not been detected in cells cultured in microgravity.

1.6.2 Animals, plants and fungi: multicellular systems

The conventional approach in reviews of the influence of gravity on living organisms is to consider animals and plants quite separately (the other great Kingdom of eukaryotes, the fungi, are rarely brought into the discussion). There is a conceptual convenience in dealing with similar organisms in the same section, but with a topic like 'response to gravity' distinctions drawn between animals, plants and fungi must, at least in part, be artificial.

This belief has an evolutionary foundation. The mechanisms which are evident in animals, plants and fungi of today have presumably been adapted through evolution to amplify graviperception and optimise it to the particular demands and requirements imposed by the structure, physiology and behaviour of the organisms concerned. Yet it must also be that these different mechanisms have common origins in the primeval response of the general cellular apparatus to the gravitational field. Any common features which emerge from comparison of work on different organisms *may* reflect that basic cellular response.

It must be highly significant, therefore, that from the wide range of research in the literature some consistently-encountered responses in eukaryotes do emerge.

- In plants, frequent reports of disturbances to mitosis and to increases in the frequency of chromosomal abnormalities (often with sufficient controls to eliminate radiation as direct cause of the latter) show that plant cell proliferation requires positional cues which are absent in microgravity.
- In animals, hypergravity stimulates proliferation of various mammalian cells in culture, lymphocytes in microgravity show reduced activation, and microgravity strongly decreases EGF-induced expression of proto-oncogenes implicated in regulation of proliferation and differentiation.
- In fungi, sporulation seems most dependent on the normal gravity vector; cultures on orbiting space craft produced mushrooms lacking spore-forming tissue, implying that commitment to meiotic division and spore formation both require the gravity vector.

There are also common themes in the effects of microgravity exposure on eukaryote development and morphogenesis.

Arabidopsis thaliana was cultivated through a complete life cycle aboard Salyut-7; seeds produced in orbit (germinated on Earth) showed reduced germination, increased frequency of embryonic lethality, and greatly reduced ability to develop into healthy mature plants. Oogenesis and embryonic development of *Drosophila melanogaster* were altered in orbited cultures; observations show no gene mutation in the germ-line but great influence on the embryonic development of the generation bred in space, and behavioural changes in fish and amphibian larvae developed from eggs exposed to microgravity suggest altered development of the nervous system. Clinostat and space experiments show that the basic form of the mushroom (overall tissue arrangement of stem, cap, gills, hymenium, veil) in agaric and polypore alike is established independently of the gravity vector, but particular aspects of development (specifically sporulating

tissues) are defective when grown in microgravity.

The demonstration that the major embryonic axes in animals, plants and fungi form independently of gravity incidentally emphasises that experiments in microgravity are the only way to demonstrate that particular features are *not* affected by gravity. E.g. study of phototropism in space is the only way of separating the light response from the otherwise ever-present gravitropic reaction. The small lateral movements made by shoot and root tips as they grow (circumnutations - Barlow *et al*. 1994) have been shown to occur under weightless conditions (Brown & Chapman 1984; Brown *et al*. 1990) implying that gravity is not essential for their occurrence.
Despite the inevitable difficulties in conducting this sort of work (Table 3), research using microgravity facilities has revealed quite categorically that the normal unilateral gravity vector is not required for:

- establishing embryonic axes in animals, plants or fungi;
- circumnutation or plumule hook formation in plants;

but that altered gravitational acceleration ***does*** cause changes in the following basic cellular processes:

- energy metabolism and intermediary metabolism;
- cell proliferation rates;
- nuclear division;
- membrane composition and function (especially of the plasmalemma);
- cell and tissue differentiation;
- early events in ontogeny;
- rate of aging.

These different effects occur in ***ANIMALS AND PLANTS ALIKE*** and can also be traced in fungi and microorganisms including prokaryotes. The overall conclusion must be that the fundamental cellular machinery is dependent on the normal unilateral gravity vector for some of its functioning and is consequently disturbed when exposed to microgravity. Some of the potential value of research on this topic is discussed by Booth (1994).

Table 3. Key words which emerge from the gravitational biology literature

UNPREDICTABILITY	For the most part, change in the gravity vector produces effects which are unexpected on the basis of prior knowledge.
VARIABILITY	Between organisms.
	Between strains or varieties.
	Between tissues.
	Between cells.
	All implying genetic ‘control’ of the gravitational response.
MULTIPLICITY	There is some (but very little) direct evidence for more than one gravitational target.

1.6.2. Theories and models

Based either on experimental results or on theoretical considerations, several authors have discussed gravity effects at the cellular level. The effects reported may be due to important changes in metabolism and/or molecular organization occurring within the cell which allow it to adapt to a new gravitational environment. Other environmental factors such as radiation may play a role as well. This idea of adaptation is not new as many other changes in the environment such as temperature, solute concentration, pH or pressure are also followed by alterations in cell behaviour which reflect the process of adaptation. Therefore, it is not surprising that single cells adapt to altered gravitational conditions.

Concerning the primary gravity receptor within the cell, two alternatives have been discussed in detail in the literature (Block *et al.* 1992): (a) a system consisting of dense organelles like nuclei, nucleoli, centrioles, dense vacuoles or mitochondria exerting pressure on the cytoskeleton; and (b) a system based on a gravity dependent ion distribution along membranes, e.g.

mitochondrial membranes (Schatz *et al.* 1992). Further detailed analyses of gravity sensing in single cells appear in Albrecht-Buehler (1991) and Todd (1989, 1991). In essence, these discussions depend on consideration of three categories of effect (conclusions being dependent on the relative importance attached to each):

- *indirect effect*, alterations of the gravitational environment cause significant effects on the microenvironment of the cell with consequences for its metabolism;
- *non-equilibrium thermodynamic*, the interaction of gravity with a few organelles may not be sufficient to trigger one event, but a series of small changes could be amplified to generate an important effect;
- *direct effect*, the direct interaction of gravity with one or more cellular organelles of different density generating a mechanical response in neighbouring structures (e.g. the cytoskeleton) and, consequently, a signal that is transduced in a biological event.

Indirect effect (physicochemical effects). With respect to changes in the physicochemical environment, lack of sedimentation and thermal convection in microgravity may result in gradients of nutrient, oxygen and waste products. Schatz & Linke-Hommes (1989) pointed out that electric potential and solute variations may occur at the cell-solution interface. Microgravity conditions favour the formation of stationary films (boundary layers) around the cells. Consumption of oxygen and nutrients at rates exceeding those which can be supplied by diffusion decreases the amounts available and increases the gradients of these substances; thereby the cell metabolism may become markedly affected. Schatz & Linke-Hommes (1989) calculated that in 1 × g density convection may be sufficient to partially counterbalance this effect. For a model they took a phospholipid membrane with a surface charge density $q_s = - 4.824 \times 10^{-6}$ A.s cm^{-2}, in contact with an electrolyte. In this case the positive ions such as sodium, magnesium, and calcium, accumulate near the membrane surface whereas the negative ions are excluded in response to the electrostatic repulsion of the negatively charged phospholipid groups. In other words a concentration gradient is generated by the membrane potential leading to maximal density variations of $\sim 4 \times 10^{-2}$ g cm^{-3} over a range of 1 to 6 nm. In gravity, two configurations are considered, (i) the membrane surface is horizontally oriented, and (ii) the membrane surface is perpendicular. In the first case the convection in the boundary layer can take place along the membrane surface. In this case the movement of ions is not affected by the electric field because the charges move perpendicular to the field lines. In the second case, however, the action of gravity may result in a displacement of the surface layer away from the membrane surface resulting in a change of the surface potential. In gravity, convection continuously supports the supply of fresh electrolytes but in microgravity the supply of these ions is left to diffusion alone. Likewise, a concentration gradient may develop in the close vicinity of the cell surface when the cells are rapidly consuming substrates such as glucose and oxygen. A boundary layer impoverished in glucose and oxygen may develop.

On the other hand, solutal convection due to concentration gradients (Marangoni convection, which is obscured at 1 × g by convection driven by density differences) may develop in cell cultures and thus favour the movement of particles, like cells, in a fluid (Napolitano 1984; Scriven & Sternling 1960; Langbein 1986). Movements of free floating cells have been clearly observed in microgravity, so the lack of sedimentation and thermal convection in microgravity may be compensated, at least in part, by Marangoni convection.

Non-equilibrium thermodynamics (bifurcation theory). An interesting view of a direct action of gravity on single cells has been proposed by Mesland (1990, 1992). This endeavour is a completely new way to explain gravity effects in living organisms. Based on the work of Prigogine & Stengers (1984) and Kondepudi & Prigogine (1983), Mesland applies non-linear non-equilibrium thermodynamics to living cells under changing gravity conditions. Prigogine and coworkers predicted gravitational effects in chemical reactions far from equilibrium. Under the conditions of chemical non-equilibrium the three laws of thermodynamics do not apply, and chemical reactions do not proceed linearly. In thermodynamic terms, this is precisely the principal characteristic of biological systems. Biochemical reaction chains catalysed by enzymes and controlled by complex feedback mechanisms are nonlinear and far from equilibrium. Under these conditions a cell may display quite unexpected behaviour within a given latitude which is often referred to as chaotic. The constituents of a reaction may remain constant, which is, however, very unlikely, they may oscillate

with a known phase, frequency and amplitude, or they may fluctuate chaotically. At the point of such a crossroad, the decision as to which way the reaction develops depends on minuscule differences in the reaction condition such as concentration of substrates, products, catalyst, temperature, and pressure. At the crossroad or point of bifurcation the system is extremely sensitive to changes in the environmental conditions. In fact, the presence of gravity alone can force the reaction to take one direction or the other. Mesland suggested that the lack of gravity alone could cause a cell to behave differently compared with normal gravity conditions. This hypothesis implies that for each cell there must be a threshold gravity force at which the system switches from gravity to microgravity behaviour or vice versa.

With respect to bacteria, yeast, and animal cells, no data are available. In the case of lymphocytes, the space experiments are not in conflict with the bifurcation theory. In microgravity, lymphocyte responsiveness of free-floating cells is virtually nil compared with normal gravity. In a lymphocyte proliferation experiment the individual cell has two possibilities: Upon the addition of the mitogen it can remain dormant in the resting phase of the cell cycle or it can enter it and start to proliferate. With respect to an individual cell, there are no intermediate reactions. Because the lymphocyte population used in the space studies was not uniform (it comprised a great number of subpopulations and clones), the sum of all individual cell reactions may have blurred the transition from microgravity to gravity behaviour. The use of transformed lymphocyte cell lines, which are derived from one single clone, could help to elucidate this interesting question.

Direct effect, gravity receptors. Pollard (1965, 1971) assumed that diffusion and sedimentation inside a single cell might be affected by gravity. Calculations show that the process of diffusion, which is often referred to as Brownian movement, is significant enough to counterbalance gravity effects in cells of roughly spherical shape and below 10 μm in diameter. However, in cells exceeding 10 μm in diameter, sedimentation processes involving organelles may occur. This approach to explain gravity effects by sedimentation and diffusion has become obsolete with the finding that the cytoplasm is not a solution of proteins in a water-like liquid nor a suspension of organelles in the cytosol. Rather, almost no free water is available in the cytoplasm; most of the water is adsorbed to proteins with little bulk water left, which makes the cytoplasm very compact. In eukaryotic cells, the complex latticework of the cytoskeleton further reduces the mobility of organelles. Based on Pollard's considerations, direct gravitational effects in bacteria were ruled out by Kondo (1968).

Nace (1983) proposed that cells may sense gravity by means of the cytoskeleton. Calculations show that gravity exerts a considerable torque on the cytoskeleton. The torque imparted on a cell with a diameter of ~6 μm by starch granules and oil vacuoles is 2.5×10^{-13} dyne cm. The force developed by a bundle of 6 microtubules was found to be $\sim 10^{-6}$ to 10^{-5} dyne. If this force is applied to the lever arm of 6 μm this yields a torque of $\sim 5 \times 10^{-9}$ dyn cm. Neither the actual length of the lever arm of the microtubules (presumably smaller than 6 μm) nor the number of microtubules acting on the organelles are known. Although the torque imparted by the starch granule and the oil vacuole appear to be small compared to the torque microtubules can produce, energy is needed to maintain positional homeostasis under gravity conditions. In microgravity, this requirement for energy expenditure is zero. Thus, cells in space may be expected to use less energy than do cells on Earth and this might be accompanied by structural or biochemical changes.

Until very recently, the most widely held interpretation of gravity perception in plants involved direct interaction between the statoliths and endoplasmic reticulum membranes normally located beneath them. Recent experiments in sounding rockets and in orbit have seriously challenged this view. Theoretically, the pressure of a mass on a surface is a poor candidate as a generator of graviperceptive signals. The reason is that any pressure exerted by the mass is at a maximum when the supporting surface (which is presumed to be detecting the pressure) is horizontal. As the detector tilts the magnitude of the pressure decreases, reaching zero when the detector is vertical. In contrast, for a mobile weight coupled to a shear detector, the shear is zero when the mass is stationary, but increases as tilt increases and gravity causes the mass to move relative to its detector. Further, the *direction* of the shear force relative to the detector depends on the direction of tilt. Consequently, pressure causes effects in the detector which decline as the tilt increases; this is independent of the direction of tilt, and pressure changes sign when the detector surface tilts to the

other side of the vertical. On the other hand shear force increases with tilting angle, is directional and changes direction when tilting to either side of the horizontal. Experiments tend to greatly exaggerate phenomena under study, but for most plants in Nature the gravity perception system will only ever have to detect small deflections. Orientation of the seed after dispersal is the most significant variable. After germination, if an organ is normally held vertically then the perception system will need to be adapted to recognise and correct only small deviations from the vertical. In other words, amplification of small deviations is likely to be an advantage. This leads to the conclusion that a shear detector would provide the most effective gravity perception as it would produce signals that increase in magnitude on increased tilt away from normal, and would offer directional sensing that could be incorporated into a negative feedback system. Shear detection is used in the graviperceptive (balance) organs employed by most metazoans to control posture and movement. It is a mechanism which is likely to be equally widely-used in plants. Direct involvement of the cytoskeletal system in shear-dependent gravity sensing in plants seems most probable from recent research (see Section 1.4.5.7) and the elegant experiments in orbit and in sounding rockets which demonstrated this are the clearest examples so far of the way in which space biology can contribute to better understanding of a basic aspect of cell biology which is of fundamental importance on Earth.

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1.7 Bibliography

This report is dedicated to experiments conducted in hypergravity in centrifuges, in simulated hypogravity in clinostats, and in real microgravity in drop towers, parabolic flights, sounding rockets, and orbital laboratories. The cellular systems considered are single cells like bacteria, protozoa, and animal cells, as well as plant, fungal and animal multicellular systems. The cellular functions analyzed are proliferation and response to mitogens, signal transduction and genetic expression, morphology, motility and morphogenesis.

The data presented in this Chapter are from a total of 471 papers (listed in Section 1.7.1), 279 primary papers published in international journals which are known to be peer-reviewed and 192 appearing in less-accessible journals or as monographs, in books or in reports or proceedings. It is important to note that, at the time of writing, most of the data from experiments carried out in the recent Spacelab missions IML-1, SLJ, D-2 and IML-2 have not yet been published in international journals.

Research on plants, cells and developing embryos in orbital space craft has recently been reviewed by Halstead & Dutcher (1987), Dutcher *et al*. (1994), Gmünder & Cogoli (1988), Malacinski *et al*., (1989) and Cogoli & Gmünder (1991). There have been a number of collected works published (e.g. July 1986 issue of *Naturwissenschaften* dealing with the D1 mission; *FASEB Journal* issue of January 1990 devoted to the Cosmos 1887 flight; NASA *Technical Memorandum 4258* of February 1991 describing NASA's space biology accomplishments in 1989-90; the Proceedings of the 4th (1990) European Symposium on *Life Sciences Research in Space*, (the proceedings of the 5th, 1993, Symposium being on the horizon) and the special issue of *Experimental Gerontology Vol*. 26, numbers 2/3 (1991) containing the proceedings of a 1989 conference on *Correlations of Aging and Space Effects on Biosystems*. Experiments on plants grown in space have featured in a Supplement to *Annals of Botany Vol.*54 (1984) and plant

gravitropism has more recently been featured in a special issue of *Plant, Cell and Environment* (1992, vol. 15, pp. 761-794) dealing with the Cholodny-Went theory that tropic curvatures in plants result from unequal distributions of auxin. A detailed account of biological experiments carried out aboard Soviet orbital stations between 1970 and 1990 has recently become available in English (Nechitailo & Mashinsky 1993).

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