haps the most similar reported effect is the promotion of citrus bud callus formation by ABA (*17*).

The reported increases in endogenous levels of ABA in water-stressed terrestrial plants (*18*) and in the aerial rosettes of the aquatic angiosperm *C. stagnalis* (*19*) suggest interesting theories of the normal development of heterophylly in *P. nodosus*. In the natural habitat, the vegetative tubers usually germinate when completely underwater and the plant does not produce floating leaves until the submersed-type leaves have reached the surface. It is possible that desiccation of the uppermost surfaces of those submersed leaves when they reach the surface initiates production (or accumulation) of ABA, which in turn promotes the development of floating leaves at the apical meristems. This would also account for the observation that floating leaves are first produced in germlings at the aerially exposed, damp margins of ponds.

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Inhibition of Bone Formation During Space Flight

Abstract. *Parameters of bone formation and resorption were measured in rats orbited for 19.5 days aboard the Soviet Cosmos 782 biological satellite. The most striking effects were on bone formation. During flight, rats formed significantly less periosteal bone than did control rats on the ground. An arrest line at both the periosteum and the endosteum of flight animals suggests that a complete cessation of bone growth occurred. During a 26-day postflight period, the defect in bone formation was corrected. No significant changes in bone resorption were observed.*

Changes in calcium homeostasis present a potential problem during prolonged space flights. Significant decreases in bone density have been documented after space flight in humans by photon absorptiometric techniques (*1*) and in monkeys by x-ray densitometry (*2*). Microscopic examination of long bones of young Wistar rats after a 22-day space flight aboard the Soviet biological satellite Cosmos 605 suggested that bone growth was inhibited during flight but returned to normal by 27 days after flight (*3*). Metabolic studies of Skylab astronauts indicated that during flight there was a significant increase in urinary calcium (*4*), similar in degree to that observed during bed rest (*1*, *5*), but no change in hydroxyproline (*6*). Since mechanical forces imposed by muscle utilization and gravity influence bone turnover (*7*), prolonged recumbency or prolonged weightlessness with continuous hypercalciuria and decreased bone mass could ultimately result in osteoporosis. To further define bone changes during space flight, parameters including bone formation and resorption were measured in tetracycline-labeled rats orbited for 19.5 days aboard the Soviet Cosmos 782 biological satellite.

Specific pathogen-free, male Wistar

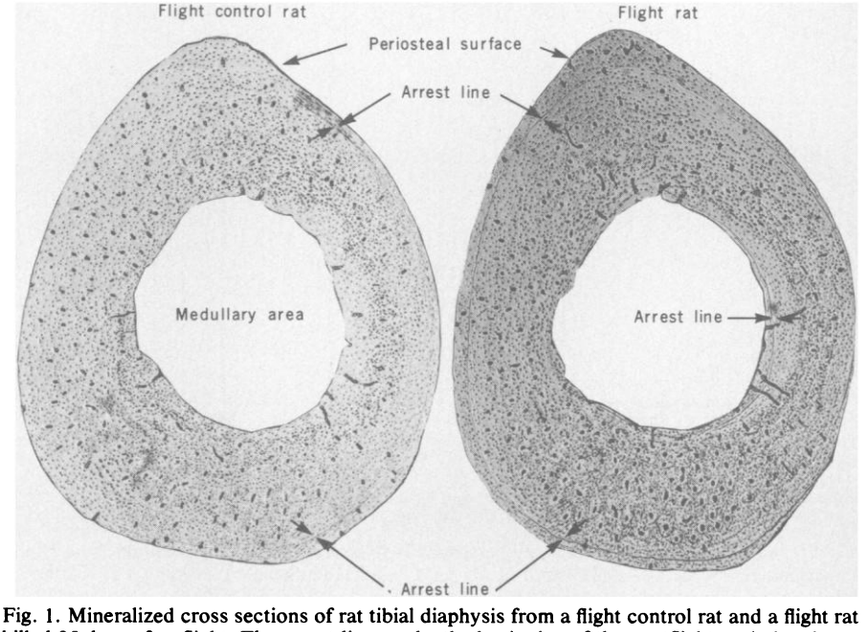

Fig. 1. Mineralized cross sections of rat tibial diaphysis from a flight control rat and a flight rat killed 25 days after flight. The arrest line marks the beginning of the postflight period and was not discernible in animals killed immediately after flight.

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rats from the Institute of Experimental Endocrinology of the Slovakian Academy of Sciences were 63 days of age and weighed an average of 215 g at the beginning of the experimental period. Two weeks before flight, all rats were placed on a paste diet required for the flight hardware; 10 g of food was dispensed every 6 hours. Water was freely available. The lighting schedule was 12 hours light and 12 hours darkness throughout the experimental period. Flight rats were loaded into individual cylindrical cages (about 20 cm long and 10 cm in diameter) 20 hours before the launch because of constraints imposed by prelaunch activities. Five cages shared common water and food sources. Activity was monitored by changes in coupling between electromagnetic coils wrapped around the circumference of each cage.

In addition to the flight group, two ground-based control groups were included. The ground control group designed to simulate flight conditions, called the flight controls, were placed in an identical spacecraft, which was kept on the ground. The flight control experiment was started 5 days after the launch so that this group could be exposed, as closely as possible, to the spacecraft environment and sequentially to the acceleration, noise, shock, and vibration of launch and reentry; a 5-day period was necessary to obtain data telemetered from the spacecraft and to make corresponding changes in the spacecraft containing the flight control group. The second control group, the vivarium controls, were housed three to four rats to a cage in the animal quarters.

The modified Vostok spacecraft was launched in the Soviet Union at Plesetsk on 25 November 1975 and achieved an altitude of 210 to 230 nautical miles at apogee by 119 to 122 nautical miles at perigee with an orbital inclination of 62.8°. The craft was recovered on 15 December 1975 in the Soviet Union. During a 26-day postflight period, remaining flight and flight control rats were kept in the vivarium in individual cages.

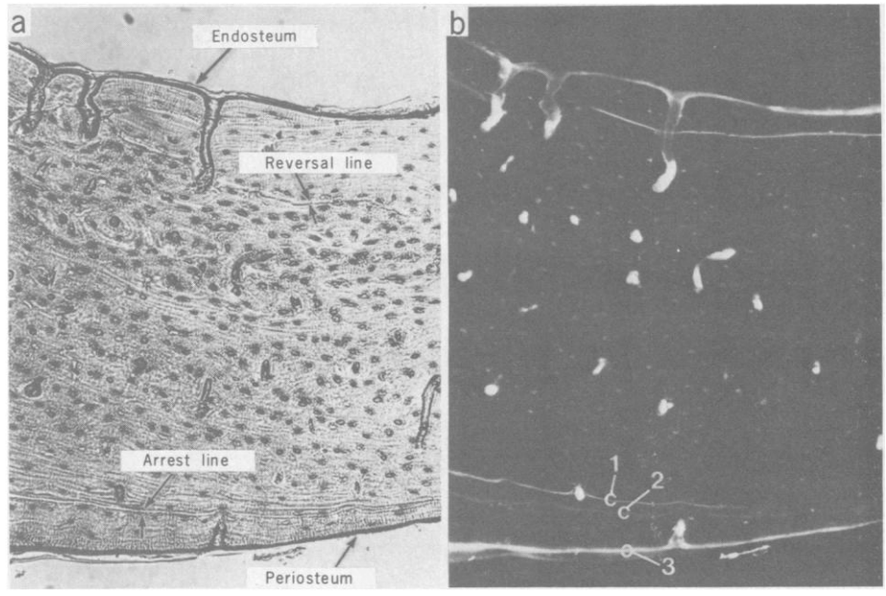

Fig. 2. Higher-power photomicrographs of a field from the tibial cross section of the flight rat in Fig. 1. (a) Section photographed under bright-field illumination; (b) same section, using fluorescence microscopy. The difference in appearance between the arrest line and the reversal line is apparent in (a). In (b), *1* corresponds to the first tetracycline label, which was given 3 days before the launch; *2* is the second label, which rats received 3 days after flight; and *3* is a label given in vitro that marks the end of the postflight period. The distance between the first and second labels, which were given 26 days apart, represents the width of bone formed during the flight period; it is much shorter than the distance between second and third labels, which represents the width of bone formed during 23 days of the postflight period.

The experiment consisted of two test periods, the flight period and the postflight period. Bone was labeled with tetracycline (*8*) as close as possible to the beginning and end of each period, as described below. All rats were injected intraperitoneally with tetracycline (*8*), 1 mg per kilogram of body weight, 3 days before the beginning of flight because of constraints of the flight schedule. Half the rats in each group were killed immediately after the end of the flight period. A second tetracycline injection was to be given to remaining rats at the beginning of the postflight period, but weather problems in the recovery area delayed this injection for 3 days. At the end of the test period, rats were guillotined and their tibias were removed and frozen. Bones were transported frozen to the United States and kept frozen until analyzed. Mineralized sections were prepared from a sampling site at the tibiofibular junction and were labeled by immersion in tetracycline solution. Hence, animals killed immediately after flight had two labels, an in vivo and an in vitro label encompassing a 23-day flight period while those killed 26 days after flight had three labels, two in vivo (encompassing

Table 1. Periosteal formation, periosteal apposition, medullary area, and length of arrest line in flight and control rats. Data are expressed as mean ± standard deviation; *N* is the number of animals.

Rats	Periosteal formation rate (10^{-3} mm³/day)		Periosteal apposition rate (10^{-3} μm/day)		*N* for rates*	Medullary area† (mm²)	Arrest line length† (mm)
	Bone	Matrix	Bone	Matrix			
			Flight period				
Flight	9.4 ± 2.8‡	7.2 ± 2.8‡	1.3 ± 0.4‡	1.0 ± 0.4‡	11	0.96 ± 0.22	
Flight control	15.8 ± 1.5	13.8 ± 1.4	2.2 ± 0.2	1.9 ± 0.2	7	0.86 ± 0.10	
Vivarium control	16.0 ± 1.4	14.0 ± 1.4	2.1 ± 0.2	1.8 ± 0.2	4	0.86 ± 0.11	
			Postflight period§				
Flight	17.1 ± 2.2‖	17.8 ± 2.1‡	2.2 ± 0.2‡	2.3 ± 0.2‡	5	0.82 ± 0.07	5.3 ± 0.6‡
Flight control						0.82 ± 0.08	2.1 ± 0.6
Vivarium control	11.3 ± 1.4	11.2 ± 1.4	1.4 ± 0.4	1.4 ± 0.2	4	0.90 ± 0.12	1.5 ± 0.7

*The number of rats per group was 6. The *N* for the rate measurements should have been 12 for the flight period and 6 for the postflight period, but not all rats were labeled. †*N* = 6. ‡Significantly different from controls at $P < .001$. §The majority of the flight control group did not receive a second tetracycline label, and hence bone formation parameters could not be measured. ‖Significantly different from controls at $P < .005$.

a 26-day flight period) and one in vitro. Area, width, and surface length measurements were made with a digitizer, which was interfaced with a PDP-8 computer. The area of bone between the labels was divided by the length of the labeling period in days to give the rate of periosteal bone formation. Quantitative histology techniques used in this study have been described in detail (*9*).

All bone parameters associated with formation were decreased significantly during flight (Table 1). The bone formation rate decreased about 40 percent relative to either control group; however, if adjusted for the 3-day preflight period, the rate decreased about 47 percent. The dramatic decrease in bone formation during flight was accompanied by an arrest line (Fig. 1). Indications of arrest lines were also evident in the control groups, but these lines were neither as distinct (Fig. 1) nor as extensive (Table 1) as those in the flight rats. They seem to occur normally in growing rats and are exaggerated by space flight. Arrest lines occur when bone formation ceases and is later reinitiated (*10*). An arrest line differs from a reversal line in several aspects (Fig. 2a). A reversal line is created when resorption reverses to formation; it is characterized by an irregular appearance and by acid phosphatase activity. An arrest line is a smooth, distinct demarcation that does not stain for enzyme activity. It is not an artifact caused by section preparation, since the second tetracycline label is consistently superimposed on this line (Fig. 2, a and b). Although the cause of cessation is not known, starvation was not a factor because not all the food was consumed during flight and the average weight of the flight group was not significantly different from the average weight of either control group. Also, immobilization was not a factor since flight rats were, if anything, more active than flight controls. Cessation of bone formation was probably due to inhibition of either matrix formation or mineralization or both; however, since periosteal matrix apposition rate in flight animals was not greater than periosteal bone apposition rate (Table 1), a defect in mineralization alone is unlikely. It is not known when the arrest occurred, but flight animals formed an average of 181×10^{-3} mm^3 of bone during the 19.5-day space flight, in 1-mm-thick mineralized sections. If formation proceeded normally until arrest—that is, at 15.8×10^{-3} mm^3 per day (Table 1)—then the earliest time when cessation could have occurred would be day 11 of flight. However, bone formation in flight rats may not have been constant before cessation; it was more likely gradually decreasing.

Two findings suggest that bone formation was reinitiated on about day 3 after flight. First, both the group killed immediately after flight and that receiving a second tetracycline label 3 days after flight formed the same volume of bone (232×10^{-3} and 224×10^{-3} mm^3, respectively). Second, it required active bone formation to entrap the tetracycline label given in vivo after flight (*11*), but the label was less intense and was superimposed on the arrest line (Fig. 2, a and b), suggesting that formation had only recently been reinitiated.

Changes in bone formation were consistent among the individual flight rats (Fig. 3). Without exception, each rat formed more bone after than during flight.

During the postflight period, the flight rats showed a significant increase in all parameters associated with formation when compared to the vivarium group (Table 1). Since flight animals were spatially restricted during the flight period, the rebound in bone formation may have been due to increased activity after transfer to larger cages. The decreased rate of bone formation in the vivarium group between the flight and postflight periods was not unexpected since these animals were about at the peak of their growth curve. In this experiment, the progressive decline in the rate of periosteal bone formation as a function of increasing body weight was linear over the body weight range ($r = -.97$, $P < .001$). The observed decline in tha rate of periosteal bone formation in the vivarium controls (29 percent) was not significantly different from the value (34 ± 11 percent) predicted for Holtzman rats (used in other experiments) over the same weight range. A decreased rate of bone formation with time has also been shown in weanling rats (*12*).

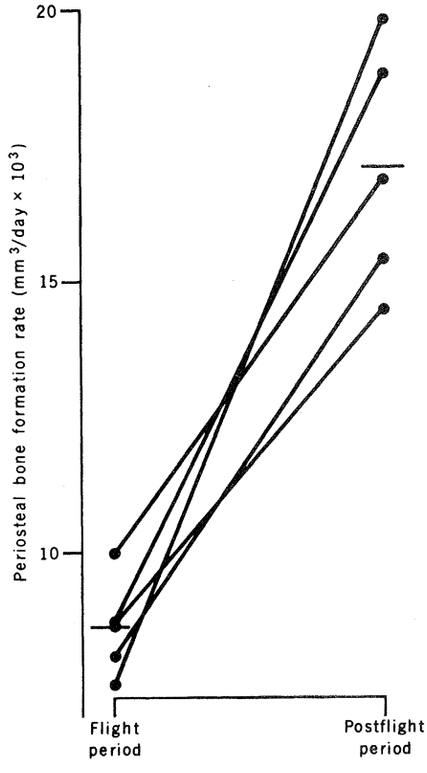

Fig. 3. Rate of periosteal bone formation for each flight rat killed 26 days after flight. Horizontal lines represent the mean periosteal bone formation rate at each period. The difference between the bone formation rates in the two time periods is highly significant ($P < .001$; Student's t-test).

Endosteal bone resorption, which accounts for about 90 percent of bone resorption in this sampling site, could not be quantified since our method also requires measurement of endosteal bone formation. The latter requires either repeated tetracycline injections, which were impossible in this unmanned satellite, or incorporation of tetracycline into the diet, which was not feasible because many other experiments were being performed on these rats. However, past work (*13*) has shown that medullary cavity area will enlarge to reflect any large changes in resorption. No significant intergroup differences occurred in the size of the medullary cavity during either experimental period (Table 1). Thus, no gross change in bone resorption was detected.

A decrease in bone formation or increase in bone resorption during space flight is expected since changes in bone mass have been reported in immobilized animals (*14*) and men (*15*), and in rats (*3*), monkeys (*2*), and men (*1*) after space flight. Because the greatest changes in bone mass during space flight occur in weight-bearing bones, changes in mechanical loading are undoubtedly important. Lack of such forces may also indirectly affect bone by causing changes in blood flow, neural transmission, or hormonal levels. Soviet scientists found no changes in parathyroid or thyroid gland histology or serum calcium in these flight animals, but they did notice that flight rats had significantly larger adrenal glands, reflecting an increase in corticosterone content (*16*). Glucocorticoid treatment has been shown to decrease bone formation and the number of osteoblasts (*17*). Detection of glucocorticoid receptors in bone cells indicates that bone may be a target for direct glucocorticoid effects (*18*), such as inhibition of bone cell proliferation (*19*). Thus, glucocorticoids may also contribute

to changes noted during space flight. Whatever the mechanisms involved, this study demonstrates that diaphyseal bone formation in rats decreases and may even cease during space flight (*20*).

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21. We thank V. Shvets, who removed the bones both at the recovery site and in Moscow, and the many other Soviet scientists who assisted with the experiment by injecting the rats, preparing the samples, and expediting the shipment of biological specimens to this country. We also thank the NASA personnel who made this experiment possible. We are indebed to R. Haller for technical assistance. Supported in part by NASA contract RA18698B and NIH grant DE 02600.

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Enhancement of Oncogenesis in C3H/10T1/2 Mouse Embryo Cell Cultures by Saccharin

Abstract. *Impure and pure samples of saccharin (2 milligrams per milliliter) did not produce oncogenic transformation of C3H/10T1/2, clone 8, mouse embryo fibroblasts. However, after treatment of the cells with a nontransforming initiating dose (0.1 microgram per milliliter) of 3-methylcholanthrene, continuous treatment with either sample of saccharin (100 micrograms per milliliter) led to significant transformation. It is concluded that in this system saccharin is a cocarcinogen, probably functioning as a promoting agent that is 1000-fold less active than the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate.*

In 1970 it was reported that mice developed bladder tumors after cholesterol pellets containing saccharin were implanted in their bladders (*1*). Further evidence for the weak carcinogenic activity of saccharin was provided by long-term feeding experiments in rats (*2, 3*), and particularly by the findings of Canadian investigators (*4, 5*) who demonstrated that rats maintained on a diet containing 5 percent saccharin developed bladder tumors at a significant frequency. Saccharin was also found to have a cocarcinogenic activity in producing bladder tumors in rats that had received a simple instillation of *N*-methyl-*N*-nitrosourea (MNU) into the bladder (*6*) or had been fed with *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (*7*). A recent Canadian epidemiological study (*8*) showed a positive correlation between the consumption of artificial sweeteners, particularly saccharin, and an increased incidence of bladder cancer in human males. Although saccharin is not mutagenic in the Ames-McCann *Salmonella typhimurium* test system, it has been reported that the urine of mice fed purified saccharin is mutagenic to one of the *Salmonella* test strains (*9*).

For the study reported herein [see (*10, 11*)], two samples of saccharin were sent to us by D. R. Stoltz, Toxicology Research Division of the Health Protection Branch, Health and Welfare, Ottawa, Ontario, Canada. One sample (impure) was the same as that used in the Health Protection Branch cancer bioassay in Canada, and the other was this same sample of saccharin after purification (pure).

The C3H/10T1/2 cell line, clone 8, was developed in our laboratory from C3H mouse embryos (*12*) and was found to be transformable by chemical carcinogens (*12*), ultraviolet light (*13*), and x-irradiation (*14*). Two-stage carcinogenesis (*15*) has also been demonstrated in this cell line with polycyclic aromatic hydrocarbons (*16*), ultraviolet light (*17*), and x-irradiation (*18*) as initiators, and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (*19*) and other phorbol esters as promoters. Thus, the cell line now provides the potential of serving as a rapid and relatively economical system for screening both carcinogens and promoters.

The tests for the transforming and promoting activities of saccharin were done in the same way as described elsewhere (*12, 16–18*). Briefly, the procedure was as follows:

Transformation experiments with known initiators or with saccharin. Cells (2000 per dish) were plated in 60-mm plastic petri dishes (Corning) with Eagle's basal medium (BME) supplemented with 10 percent heat-inactivated fetal calf serum (FCS) (Gibco). Twenty-four hours later the culture medium was replaced with medium containing different concentrations of saccharin. To the positive control dishes we added medium containing 3-methylcholanthrene (MCA) (1 μg/ml) and to the negative control dishes, medium containing 0.5 percent acetone. A concentrated solution of the two saccharin samples was always freshly prepared in distilled and deionized water and sterilized through a Millipore filter (0.2 μm). This sterile solution was then mixed with medium to the desired concentration of saccharin. The treatment with these compounds lasted for 24 hours after which the medium was replaced with normal medium (BME plus 10 percent FCS), and the cells were then cultured in the same dishes until the end of the experiments (6 weeks) with weekly medium changes. At this time the cultures were fixed with methanol, stained with 10 percent Giemsa solution, and scored for type 3 transformed foci, which give rise to fibrosarcomas on inoculation into syngeneic mice (*12*).

Transformation experiments with initiators followed by TPA or saccharin. Cells were plated in the same way as above, and 24 hours later they were treated for 24 hours with initiating agents: saccharin (2 mg/ml), MCA (0.1 μg/ml), or ultraviolet light (50 erg/mm²). Four days later the cells were treated with medium containing TPA (0.1 μg/ml) or saccharin (100 μg/ml). Saccharin and TPA were added in the same concentra-

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