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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20. A similar experiment was performed aboard the Soviet Cosmos 936 biological satellite, which was launched 3 August 1977 and recovered 22 August 1977. Preliminary results for bone parameters are virtually identical to those presented in this report.
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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Table 1. Testing of saccharin as a possible carcinogenic or cocarcinogenic agent in C3H/10T1/2 cells.

Treatment schedule	Plating efficiency (%)	Number of dishes	
		With type 3 foci	Total
Acetone (0.5 percent)	16	0	42
TPA (0.1 μg/ml) in each medium change	16	0	32
Pure saccharin (100 μg/ml) in each medium change	15	0	41
Impure saccharin (100 μg/ml) in each medium change	15	0	34
MCA (1 μg/ml) for 24 hours	15	5	15
MCA (0.1 μg/ml) for 24 hours	15	0	48
Ultraviolet light (50 erg/mm²)	8	0	11
Pure saccharin (2 mg/ml) for 24 hours	15	0	49
Impure saccharin (2 mg/ml) for 24 hours	15	0	50
MCA (0.1 μg/ml) + TPA (0.1 μg/ml)*	15	4	33
MCA (0.1 μg/ml) + pure saccharin (100 μg/ml)	15	6	36
MCA (0.1 μg/ml) + impure saccharin (100 μg/ml)	15	7	39
Pure saccharin (2 mg/ml) + TPA (0.1 μg/ml)	15	1	28
Impure saccharin (2 mg/ml) + TPA (0.1 μg/ml)	15	0	37
Ultraviolet light (50 erg/mm²) + TPA (0.1 μg/ml)	8	2	12
Ultraviolet light (50 erg/mm²) + pure saccharin (100 μg/ml)	8	0	12
Ultraviolet light (50 erg/mm²) + impure saccharin (100 μg/ml)	8	0	12

*The time intervals between the two treatments are described in the text.

tion at each medium change until the end of the experiments (6 weeks), when the cultures were fixed and stained to score the type 3 transformed foci.

Cytotoxicity experiments. These were done by determining the plating efficiency with 200 cells per dish plated and treated in the same way as above, fixed and stained 10 days later, and the colonies counted.

The results of our experiments are shown in Table 1. At the concentration of 2 mg/ml, both saccharin samples were nontoxic and nontransforming. When saccharin treatment was followed 4 days later by continuous treatment with TPA there was only one transformed focus out of 28 dishes treated with pure saccharin, which is probably not statistically significant. Thus, these two samples of saccharin had no significant transforming nor initiating effect at this high concentration in C3H/10T1/2 cells.

When the cells were initiated with MCA at a concentration (0.1 μg/ml) that did not transform the cells (experiment 6), and were then treated continuously with TPA (0.1 μg/ml) or with either sample of saccharin (100 μg/ml), transformed foci appeared in the dishes, and their numbers were highly significant. Thus, both pure and impure samples of saccharin had cocarcinogenic activity in this system. The finding with ultraviolet-initiated cells was unexpected. TPA promoted the ultraviolet-initiated cells, but neither saccharin sample did. The reason for this difference between the hitherto equivalent initiators is not clear. In our experiments saccharin at 1000 times the concentration of TPA gave rise to a similar number of transformed type 3 foci in MCA-initiated cells.

Repeated or continuous feeding of animals with a diet containing saccharin is more analogous to the combined schedule used in these experiments than to a one-time application of initiator. From the animal studies reported earlier (*2–5*) in which saccharin was fed continuously, it is impossible to ascertain whether saccharin acted as a complete carcinogen or as a cocarcinogen acting together with some unknown carcinogenic initiator already present in the host. However, there are three animal studies demonstrating the cocarcinogenic action of saccharin (*6, 7*). Our experiments in cell culture confirm and extend the above findings, as well as those of R. Pienta (*11*, p. 105), who found no transformation of hamster embryo cells in culture with these samples of saccharin. I. B. Weinstein (*11*, p. 106) did not find any induction of plasminogen activator in HeLa cells with either of these samples of saccharin (at 50 μg/ml). He has proposed this as a model system to test for promoters, in which TPA at 30 ng/ml induced large amounts of plasminogen activator (*20*). However, we do not know whether the induction of plasminogen activator, which is a very indirect method, can be taken as a reliable test for tumor promotion because Weinstein (*21*) has not observed significant induction by TPA in C3H/10T1/2 cells, in which initiation and promotion have clearly been demonstrated (*16*). Nor do we know whether TPA can promote transformation in human cells.

Our finding that saccharin is a weak but significant cocarcinogen in the oncogenic transformation of C3H/10T1/2 cells that have been initiated with MCA, reinforces the animal experiments in which saccharin has been found to enhance bladder carcinogenesis in rats (*6, 7*). Although we have cautiously characterized saccharin as a cocarcinogen, the analogy in the present experiments with TPA, although not perfect, suggests that saccharin acts as a promoter. These observations, although they do not prove that saccharin is a cocarcinogen in human carcinogenesis, taken together with the epidemiological evidence suggest caution in the indiscriminate or long-term use of saccharin in human nutrition.

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