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# Carcinogenesis Testing of Saccharin. No Transformation or Increased Sister Chromatid Exchange Observed in Two Mammalian Cell Systems*

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**Abstract**—*Saccharin was assayed in two mammalian cell systems for possible transforming or mutagenic effects. In the mouse embryo fibroblast C3H/10T½ cells, saccharin was without transforming effects over a large concentration range. On transformed, but non-oncogenic C3H/10T½ type* I *cells, a slight effect on transition to the growth pattern morphology of types* II *and* III *cells was found, but this was not accompanied by characteristic changes in plasma membrane structure when studied by scanning electron microscopy. In human lymphocytes, saccharin did not induce any sister chromatid exchange. Addition of a metabolic activation system did not change the sister chromatid exchange pattern as usually seen after positive control with* 3,4-*benzopyrene. Based on these studies, saccharin cannot be classified as a carcinogen, not even a weak one. Neither does it appear to be a mutagen.*

## INTRODUCTION

DUE TO the broad use of saccharin as an artificial sweetener, and the current debate over risks and benefits of its use [1, 2], we have studied whether saccharin (*o*-sulfobenzimide) may exert any measurable effect in two *in vitro* cell systems often used in carcinogenesis tests. We scored for transformation of the mouse embryo fibroblast C3H/10T½ cells in culture [3], and for increased sister chromatid exchange (SCE) in human lymphocytes [4]. Saccharin had no significant effect in the two systems.

## MATERIALS AND METHODS

### *Cell source and culture conditions*

The origin and method of culture of the C3H/10T½ clone 8 mouse embryo cells were as previously described [5]. The origin of the type I cells has been described earlier [6, 7]. The medium in the cell cultures was changed every 3½ days. Human peripheral lymphocytes were all drawn from the same healthy donor.

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### *Transformation and scoring of morphological transformation*

The transformation system and the scoring of the three types of transformed foci were as reported elsewhere [3]. The saccharin was added to final concentrations of from 0.1 to 100 μg/ml. The saccharin was purchased from British Drug House and was further purified by recrystallization. Only the solvent ethanol was used in the control series. The C3H/10T½ cells were seeded at 500 cells/ml. The already transformed, but non-oncogenic type I cells were seeded at 100 cells/ml. All the cultures contained 5 ml. The foci were counted after 6 weeks.

Preparation of cells for scanning electron microscopy and evaluation of micrographs were as previously reported from this laboratory [6]. Non-synchronized cells in the subconfluent, late logarithmic phase of growth were used.

### *Chromosome preparations*

The whole blood cultures, the preparation and treatment with the rat liver homogenate reaction mixture (S-9 mix), and the staining of the slides with the FPG-technique have been described elsewhere [8]. The activity of

the S-9 mix had been tested with 3,4-benzopyrene, which proved to induce SCE in the presence of the S-9 mix, but not alone.

## RESULTS

*Transformation assays*

Saccharin was assayed for transforming effect in a series of 12 cultures at 0.1–1.0–10.0 and 100 μg/ml. Three series without saccharin served as blanks. The cultures were fixed and stained with Giemsa after 6 weeks. In case of the C3H/10T½ cells there was a complete lack of transformed foci (Table 1). In case of the type I cells, a certain transition to types II and III growth pattern morphologies was found during 6 weeks in postconfluent cultures. A very slight increase in this transition occurred in the presence of saccharin (Table 1). In a positive control experiment type I cells were exposed to 7,12-dimethylbenz(a)anthracene (DMBA) at 0.1–1.0 and 10.0 μg/ml. A certain transition to types II and III growth pattern morphologies occurred and was dependent on the concentration of the carcinogen.

*Scanning electron microscopical evaluation of the type I cells*

After 6 weeks in the postconfluent cultures, one dish was picked at random from each series, trypsinized and passaged once to cultures with cover slips, prepared for SEM and quantitatively evaluated as previously reported [6]. The distribution of the concentration of microvilli and their appearance was essentially the same in all the series (Table 1). In no case were long microvilli found.

*Sister chromatid exchange in human lymphocytes*

The results of the SCE analyses are given in Table 2. It is apparent that neither 24 nor 48 hr treatment with 1, 10 or 100 μg/ml of saccharin increased the SCE frequency. Also negative was the treatment 24 hr before harvest with saccharin and S-9 mix for 2 hr, after 1 hr previous incubation of the reaction mixture.

## DISCUSSION

The use of saccharin as an artificial sweetener is currently debated [1, 2], and legislators have concern over the safety of its wide use [9]. Positive results of carcinogenesis bioassays call for long-term experiments with large numbers of animals [10]. The validity of short-term tests for mutagenesis and cell transformation has been discussed elsewhere [10, 11]. One must exercise great care when making conclusions concerning human carcinogenesis, when the positive results are based on few bioassays models only. Final judgements must be based on a series of tests and animal experiments, and when possible, epidemiological evidence.

Since the normal C3H/10T½ cells were not transformed by saccharin, our studies suggest that saccharin cannot be classified as an *in vitro* transforming agent. Consequently saccharin cannot be classified as a carcinogen in our *in vitro* test system, not even as a weak one. Neither did saccharin appear to be a mutagen, since no increased SCE was induced in the human lymphocyte cultures.

The transition of the transformed, but non-oncogenic type I cells to type II and III cells was slightly greater than the spontaneous transition in the non-exposed series (Table 1). There was, however, no increase in the short microvilli by SEM, nor were any long microvilli formed.

Preliminary studies have shown that after exposure to carcinogens, there is a substantial transition of type I cells to type II and III cells, as well as induction of long microvilli. With increasing number of generations in culture, there was an increase in number of both short and long microvilli, but the cultures retained their growth morphology [6]. Morphologically, the pattern of microvilli formation on the one hand and the growth pattern (i.e. the criteria used for typing) on the other, appear to be independent. We have previously shown a positive correlation between the oncogenic potential in DMBA transformed cells and the occurrence of *long* microvilli [6].

In the case of saccharin a slight transition, which gave no changes in microvilli pattern, was induced. Thus no oncogenic transformants had been formed by saccharin from the C3H/10T½ type I cells or from the normal cells. Although saccharin has no effect on normal cells, it does, however, have an influence on the slightly transformed type I cells, which are injured, or pathologic cells, causing the increased transition frequency observed. The significance of this transition remains unclear.

In a paper on cyclamate, Stone *et al.* [12] briefly mentioned that human leukocyte cultures treated with saccharin (500 μg/ml) showed no evidence of increased chromosome breaks. On the other hand, Kristoffersson [13] found that saccharin sodium (100–1000 μg/ml) induced breaks and gaps in Chinese hamster cells.

Abe and Sasaki [14] also found that sac-

*Table 1. Lack of effect of saccharin on transformation of C3H/10T½ cells and its effect on type I cells*

| Series | Saccharin (μg/ml) | Transformed foci from C3H/10T½ cells | Average number of transformed foci per plate from type I cells | | Microvilli concentration | | | | Microvilli short/long | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Type II | Type III | +++ | ++ | + | − | +++ | ++ | + |
| 1 | 0 | 0 | 2.4±1.1 | 1.4±0.9 | 0 | 3 | 21 | 26 | 0/0 | 3/0 | 21/0 |
| 2 | 0 | 0 | 1.8±0.8 | 1.5±0.8 | 1 | 5 | 16 | 28 | 1/0 | 5/0 | 14/2 |
| 3 | 0 | 0 | 2.4±0.8 | 2.1±1.0 | 2 | 6 | 26 | 16 | 0/2 | 5/1 | 26/0 |
| 4 | 0.1 | 0 | 3.0±1.1 | 2.8±1.8 | 0 | 4 | 20 | 26 | 0/0 | 4/0 | 20/0 |
| 5 | 1.0 | 0 | 2.7±1.6 | 3.4±2.3 | 0 | 3 | 23 | 24 | 0/0 | 3/0 | 23/0 |
| 6 | 10.0 | 0 | 2.2±1.0 | 2.7±1.4 | 1 | 6 | 19 | 24 | 1/0 | 6/0 | 19/0 |
| 7 | 100.0 | 0 | 1.1±1.0 | 4.8±2.4 | 0 | 1 | 15 | 34 | 0/0 | 1/0 | 15/0 |

The formation of transformed foci was determined in mouse embryo fibroblast C3H/10T½ cells which were exposed to saccharin. Likewise, morphologically transformed, but non-oncogenic, type I cells were exposed to saccharin and transitions to type II and type III growth pattern morphologies were measured. Series of 12 Petri dishes were seeded with 500 cells/ml of the C3H/10T½ cells, respectively 100 cells/ml of the type I cells. All cultures contained 5 ml. Twenty-four hours after seeding the medium was removed, and saccharin was added in fresh medium. The exposure was allowed to proceed for 24 hr. The medium was then changed and subsequently every 3½ days for 6 weeks. The cultures were then fixed and stained with Giemsa and transformed foci were determined. One or two plates were lost in each series by infections. The number of foci is the mean of 10 or 11 remaining cultures. The type I cells were studied quantitatively by scanning electron microscopy with respect to microvilli concentration in these series. One randomly chosen plate from each series was trypsinized at the end of the 6 weeks and passaged once to dishes with cover slips, and prepared for SEM in the late logarithmic phase of growth. Fifty strictly randomly chosen cells were analyzed with respect to the appearance and concentration of microvilli in each series. The concentration of microvilli on the cell surface was described in four classes: those with none −, with few +, many ++ (still possible to count), and innumerable +++ (counting no longer possible) microvilli.

charin sodium induced aberrations in Chinese hamster cells and gave a slightly increased yield of SCE regardless of dose (0.2–100 mg/ml). Furthermore, Wolff and Rodin [15] found a significant increase in SCE in Chinese hamster ovary cells and in human lymphocytes with doses of saccharin sodium of 1–10 mg/ml.

In a review on the possible mutagenicity of saccharin, Kramers [16] concludes that the evidence for clastogenic properties is stronger than for the induction of point mutation, but the data are "too conflicting and equivocal to classify saccharin as a proven mutagen". In our experiments saccharin did not induce SCE, which indicates that the substance is not mutagenic in human cells in the concentrations used. Our experiments may be at least as meaningful physiologically as the previous investigations, since we used low doses that are closer to the *in vivo* exposure, and saccharin instead of its sodium salt.

No effect was obtained after treatment in the presence of rat liver homogenate reaction mixture. This was not unexpected, since saccharin appears not to be metabolized in man [17], or possibly by less than 1% in animals [18]. Neither does saccharin bind to DNA of liver or bladder in the rat [19]. This is in line with the lack of transforming effect on the C3H/10T½ cells. If saccharin is not metabolized, there would be no generation of electron deficient regions necessary for an ultimate carcinogenic form [20]. However, the slight transition of type II to II and III is difficult to explain if saccharin is not metabolized, at least to a very slight degree, by the pathologic type I cells. Another explanation may be a pure physical effect of saccharin on the type I cells.

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*Note added in proof*

The effect of saccharin on the C3H/10T½ cells was published by Mondal *et al.* [21] while this manuscript was in press. In their assay system transformation was obtained only if the cells had been pretreated with a low concentration (one tenth the transforming dose) of methylcholanthrene before exposure to saccharin (100 μg/ml).

In our studies saccharin exerted a slight transforming effect on the type I cells. The origin of these were normal C3H/10T½ culture cells that had been transformed by DMBA. We have chosen to consider any cell that has been pretreated with a carcinogen as a pathologic cell.

In principle the study by Mondal *et al.* is consistent with our results. However, the latter authors have not tested for the appearance of *long* microvilli, nor assayed for tumorigenicity of these transformed foci. We found that the development of *long* microvilli showed positive correlation to the development of the oncogenic potential [6]. Due to the lack of *long* microvilli (see Discussion), we could only conclude that the effect on the pathologic cell was a morphological transition and not related to any enhancement of oncogenicity by saccharin.

*Table 2. Sister chromatid exchange in human lymphocytes after treatment with various doses of saccharin, either alone or with* S-9 *mix in dialysis bags*

| | SCE/cell | | |
|---|---|---|---|
| Concentration μg/ml | Saccharin 24 hr | 48 hr | Saccharin + S-9 mix |
| 0 | 4.76 ± 1.87 | | 6.23 ± 2.54 |
| 1 | 4.63 ± 2.31 | 4.36 ± 1.94 | 4.34 ± 2.24 |
| 10 | 3.03 ± 1.76 | 4.10 ± 2.11 | 4.83 ± 2.27 |
| 100 | 4.56 ± 2.17 | 4.40 ± 2.24 | 7.03 ± 2.27 |
| 1000 | no mitoses | | — |

BrdU 5 μg/ml. SCE counted in 30 cells.

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