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18. These results should be viewed with caution, since two of the rats died after receiving about two thirds of the full dose of dithizone, and a third rat (the only moderately stained animal) died shortly after receiving the full dose. None of the control animals died prematurely. This differential effect of dithizone on viability raises the possibility that some confounding factor may have reduced the stainability of the deprived animals.
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22. It seems doubtful that the mossy fiber response decrement could be due to nonspecific effects of zinc deprivation on mossy fiber oxidative metabolism. The major oxidative enzyme in mossy fibers (glycerophosphate dehydrogenase; *23*) probably contains iron, not zinc [Y. Hatafi and D. L. Steggall, in *The Enzymes*, P. D. Boyer, Ed. (Academic Press, New York, 1976), vol. 13, p. 175]. In addition, other hippocampal projections showing high zinc metalloenzyme activity (*23*) do not have a zinc content comparable to that of mossy fibers (*1-3*). Finally, the prominently staining mossy fiber zinc is not, for the most part, associated with mitochondria (*2, 24*). It seems unlikely that these results could be attributed to developmental failure or to degeneration of the mossy fibers in the deprived animals, since (i) the mossy fiber system is morphologically [S. A. Bayer and J. Altman, *J. Comp. Neurol.* **158**, 55 (1974)] and chemoarchitectonically [I. L. Crawford and J. D. Connor, (*2*); S. I. Mellgren, *Z. Zellforsch.* **141**, 347 (1973)] mature before the zinc-deficient diet was instituted and since (ii) most mossy fiber zinc is unlikely to have a structural role (*4*).
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Genetic Effects of Impure and Pure Saccharin in Yeast

Abstract. *Yeast cells were grown in media containing impure or purified saccharin preparations. Dose-dependent increases in frequencies of cells possessing aberrant cell morphologies were revealed by light microscopy. At each test dose, cells grown in impure saccharin exhibited up to sevenfold higher frequencies of mitotic crossing-over or gene conversion in three of four assays for genetic recombination than cells grown in purified saccharin from the same lot. With one exception, the sweetener produced by the Maumee process caused larger increases in recombination and gene reversion than the sweetener produced by the Remsen-Fahlberg process. The several test markers did not respond equally to any test saccharin. Cells grown in liquid media containing no saccharin or two of three test concentrations of saccharin produced cell titers that were approximately equivalent.*

The saccharin (lot S1022, Sherwin-Williams) that induced bladder cancer in two generations of rats (*1*) was produced by the Maumee process and contained impurities that, when highly concentrated, were weakly mutagenic in some *Salmonella* test strains (*2, 3*). Both commercial S1022 saccharin and saccharin purified from the same lot did not cause mutagenic or other genetic alterations in several short-term tests (*4, 5*), including a mitotic recombination test in yeast D3 (*4*) and widely used *Salmonella* assays (*2, 4-6*). In contrast, the same impure [organic solvent-soluble impurities, 10 to 15 parts per million (ppm)] and partially purified saccharin (organic solvent-soluble impurities, 1 to 5 ppm) increased frequencies of sister chromatid exchanges in both Chinese hamster ovary cells and human lymphocytes (*4, 7*), caused urines of saccharin-fed mice to be weakly mutagenic to *Salmonella* TA100 (*6*), caused weak mutagenic responses at the TK+/TH− locus in mouse lymphoma L5178Y cells (*4*), and exhibited cocarcinogenic activity in C3H/10T1/2 mouse embryo cells in culture (*8*). There is also evidence that the same highly purified saccharin from lot S1022 induces a variety of chromosome aberrations in Chinese hamster cells in culture (*4*). The National Academy of Sciences' Panel I for the Study of Saccharin and Its Impurities re-

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Table 1. The saccharins used in our tests with yeast cells. Mutagenicity in *Salmonella* is indicated by: −, nonmutagenic; +, mutagenic; ++, very mutagenic.

Saccharin	Lot No.	Production method	Source	Mutagenicity in *Salmonella**	
				Saccharin	Impurities
A	65C-0129	Maumee	Sigma	−	++
B	191010	Remsen-Fahlberg	HPB†	−	+
C	Purified 191010	Remsen-Fahlberg	HPB	−	−

*Mutagenicity of saccharin or concentrated organic solvent-soluble impurities for *Salmonella typhimurium* (*2, 3*). †Health Protection Branch, Ottawa.

cently concluded that saccharin itself—and not one or more of its associated impurities—possesses carcinogenic potential, although the panel acknowledged that absolutely pure saccharin has never been tested (*5*).

We have tested the effects of impure and purified saccharin on mitotic intergenic and intragenic recombination and reverse mutation in the yeast *Saccharomyces cerevisiae*, and have compared the extents to which these genetic events are affected by impure saccharins produced by both the Remsen-Fahlberg (R-F) and Maumee processes (Table 1). These two major processes for producing commercial saccharin can result in final products with distinct types and amounts of impurities.

For this study, we used an impure preparation produced by the R-F process [Diawa (Japan) lot 191010, saccharin B] and saccharin purified from the same lot (saccharin C). Saccharin C is the purest sample ever prepared by the Toxicology Research Division, Health Protective Branch, Health and Welfare, Ottawa (*3*). Both preparations were nonmutagenic in the *Salmonella*-mammalian microsome test; the highly concentrated water-soluble impurities extracted from saccharin B were also nonmutagenic in this assay, but the highly concentrated extracts from this lot that were soluble in organic solvents were weakly mutagenic (*2, 3*).

Saccharin produced by the Maumee process [Sigma, lot 65C-0129 (saccharin A)] was also nonmutagenic in the *Salmonella* test (*3*). The organic solvent-soluble impurities (10 to 15 ppm) extracted from this lot are mutagenic for *S. typhimurium* TA1538, but only when they are very highly concentrated (*3*). Since a large part of the water-soluble impurities cannot be extracted from this preparation (*3*), we did not test purified saccharin A.

It is important to note here that, in order to avoid bias in favor of recombinant or revertant cells, we made allowances for the fact that yeast cells grown in liquid complete media with and without sweetener grow at equal rates and undergo equal numbers of divisions. For each experiment, equal portions of a fresh suspension of yeast [diploid strain CM-1293 (*9, 10*)] were inoculated into 1 percent yeast extract, 2 percent peptone, and adenine sulfate (0.16 mg/ml) (YPA); this medium was made within 18 hours of being used and contained the test concentrations of saccharins. Yeast cells grown in this medium to which we added 0.005 or 0.1 percent ethyl methanesulfonate anesulfonate (Sigma; EMS) were used as positive controls. Genetic activities in repeated experiments with fresh media were comparable, whereas genetic activities assayed with stored media containing saccharin were always lower and frequently not reproducible. The mutagen, therefore, is labile.

Yeast grew to nearly the same titers in YPA containing, per milliliter, 0, 2, or 20 mg of saccharin A, B, or C (Tables 2 and 3). Figure 1 shows that the growth rates and final cell titers obtained from inocula of five cells per milliliter were almost equivalent by the early stationary phase (60 to 65 hours). With high concentrations (100 mg/ml) of saccharin A, B, or C, we used starting inocula of approximately 500 cells per milliliter, because such concentrations inhibit growth (Fig. 1). Colony-forming abilities of cells grown in the presence of the two lower test concentrations were similar for all three saccharins, though consistently less for cells grown in the presence of saccharin A than cells grown with saccharins B and C (Tables 2 and 3). Since viabilities were high, it is unlikely that saccharin acted as a selective agent for severely damaged cells.

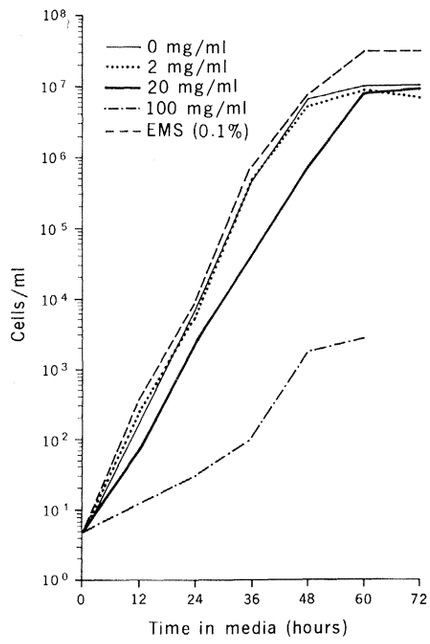

Fig. 1. Growth of yeast in 0, 2, 20, and 100 mg of (pure) saccharin C and 0.1 percent EMS per milliliter of medium. Cell titers at 0, 12, and 24 hours are based on cells plated and forming colonies on complete medium. Titers at 36, 48, 60, and 72 hours are based on hemacytometer counts of cells.

Microscopic examinations of cells grown in the three saccharin concentrations revealed dose-dependent increases in the frequencies of cells with abnormal morphologies at the logarithmic and stationary phases. Cultures grown in media containing saccharin A showed higher frequencies of aberrant types than those grown in media containing saccharin B, and cultures from saccharin B possessed higher frequencies of abnormal cell types than those grown in saccharin C. Such irregularly shaped and multiply budded cells were rarely observed in cultures grown in YPA without saccharin or with EMS. Spore formation was not observed in test cultures, but enhanced mutation frequencies in experiments with haploid cells grown in the presence of saccharin would corroborate our genetic data from diploids.

Intergenic exchanges between homologous chromosomes revealed by the formation of twin-sectored, pink-red colonies in the CM-1293 diploid strain (*10–12*), were 11 to 12 times more frequent in cells grown in the presence of the lowest (2 mg/ml) dose of impure saccharins, than in cells grown without saccharin (Tables 2 and 3). Half as many exchanges occurred among cells grown in the same dose of saccharin C. Cells grown on media containing canavanine sulfate (100 μg/ml) showed a second class of intergenic exchanges (*13*), and such cells formed colonies up to twice as frequently when grown with saccharin (2 mg/ml) than without saccharin. In cultures grown with high saccharin concentrations, we observed up to 100-fold increases in red-pink sectors and up to 40-fold increases in canavanine-resistant colonies.

By monitoring the appearance of prototrophic colonies on selective media,

we found that *TRP5* (*14, 15*) gene convertants increased twofold in the presence of low (2 mg/ml) concentrations of each test saccharin and up to 25-fold with higher concentrations of saccharin A. With saccharin A and B, *CYC1* (*16, 17*) gene convertants increased two- to fivefold with low (2 mg/ml) concentrations and up to 18-fold at high concentrations; in purified saccharin, three- to sevenfold fewer convertants appeared. Reverse mutations at the *ilv1* locus (*15, 18*) increased 2- to 30-fold. Other classes of aberrant colonies that could be scored visually as red, pink, or sectored, and indicating a variety of other genetic events such as deletions, aneuploidy, point mutations, and mitotic gene conversion (*9, 13*), also increased to this extent usually, but not always, as a function of dose. Our finding that all test markers did not respond equally to any of the three saccharin preparations emphasizes the need for testing a variety of genetic effects, because of the possibility of encountering site specificity.

At all three concentrations, cells grown in the presence of saccharin B showed higher frequencies of mitotic crossing over or gene conversion in three of the four assays for the recombination than cells grown in saccharin C. With the exception of *CYC1* gene convertants, saccharin A usually caused larger increases in both recombination and mutation than saccharins B and C, indicating the greater potential mutagenicity of saccharin A.

Differences between these studies with growing yeast cells and the two previous (negative) tests of recombination in nongrowing yeast cells (*4, 19*) exposed to saccharin have been described elsewhere (*20*).

From our data we conclude that the impurities (*2, 3, 6, 21, 22*) in saccharin caused some or all of the observed genetic effects. Even saccharin C is not entirely free of water-soluble impurities, and in all likelihood, entirely pure saccharin probably cannot be made. Indeed,

Table 2. Frequencies of genetic events in *Saccharomyces cerevisiae* strain CM-1293 (*10*) after growth in liquid saccharin containing saccharin B (impure) or saccharin C (purified from the same lot). Saccharins B and C were tested together. After incubation with aeration at 30°C for 60 and 65 hours, the cells were harvested, washed twice, sonicated, pooled, and counted in a hemacytometer; several different dilutions of cells were then plated on synthetic complete media and media selective for recombinant and revertant cells (*9*). Colonies were counted and recombinant and revertant classes scored after incubation of plates at 30°C for periods of time appropriate for each type of medium (2 to 6 days). Data are representative of results obtained in four to eight experiments. Estimates of standard errors were omitted from this table to save space.

Dose of saccharin (mg/ml YPA) or ethyl methanesulfonate	Cell titer* (cell/ml YPA)	Viability† (%)	Mitotic intergenic recombination (crossovers) and other aberrant colonies on nonselective medium (per 10^4 surviving cells)						Intragenic recombination (per 10^5 surviving cells)‡		Reverse mutation (per 10^7 surviving cells)§
			Sectored, red-pink	Red	Pink	Sectored, other than red-pink	Total	*can*^{r}	*TRP5*	*CYC1*	*ILV+*
Impure											
0	1.6×10^7	100	1.4	13	6.4	0	20.8	4.1	1.5	9.5	1.9
2	1.3×10^7	90	16	35	15	0	66.0	5.1	3.5	49	3.7
20	1.6×10^7	90	31	32	13	0	76.0	8.3	17	118	8
100	2.1×10^6	76	143	163	48	54	408.0	32	4.4	81	31
Pure											
2	1.2×10^7	100	8.7	5.6	4.1	0	18.4	7.1	3.0	7.4	5.9
20	1.3×10^7	100	9.6	16	8.0	0	33.6	11	3.3	30	6.1
100	1.5×10^6	90	112	72	7.6	64	255.6	74	1.2	25	4.1
Ethyl methanesulfonate											
0.005 percent	1.1×10^7	100	1.3	4	0	5.1	10.4	29	1.7	19	3.8
0.1 percent	2.7×10^7	100	2.8	26	23	0.5	52.3	43	14	155	62

*Determined by cell counts in hemacytometers. †Fractions of cells plated that formed colonies. ‡Mitotic gene convertants. §Mitotic gene revertants.

Table 3. Frequencies of genetic events in *Saccharomyces cerevisiae* strain CM-1293 after growth in saccharin-containing liquid media. Experimental procedures were as described in Table 2. Comparable results were obtained in ten experiments with strain CM-1293 and the diploid strain CM-1194 (*8*).

Cell titer* (cell/ml YPA)	Viability† (%)	Mitotic intergenic recombination (crossovers) and other aberrant colonies (per 10^3 surviving cells)					Intragenic recombination (per 10^5 surviving cells)‡		Reverse mutation (per 10^7 surviving cells)§
		Sectored, red-pink	Red	Pink	Sectored, other than red-pink	Total	*can*^{r}	*TRP5*	*CYC1*	*ILV+*
No saccharin										
2.1×10^7	100 ± 9	2.6 ± 0.23	0.9 ± 0.43	0.9 ± 0.39	1.5 ± 1.0	5.9 ± 2.13	2.1 ± 0.4	6.7 ± 0.9	4.9 ± 1.5	1.2 ± 0.5
Saccharin (2 mg/ml YPA)										
1.7×10^7	74 ± 8	30.0 ± 6	14.5 ± 3.2	2.0 ± 0.2	2.1 ± 0.35	48.6 ± 7.9	4.0 ± 0.9	15.2 ± 2.5	8.8 ± 3.0	8.5 ± 1.0
Saccharin (20 mg/ml YPA)										
1.6×10^7	61 ± 8	42.1 ± 4.4	37.7 ± 6.2	10.9 ± 3.0	1.8 ± 0.4	92.5 ± 9	11.0 ± 1.8	166.0 ± 18	30.1 ± 6	9.2 ± 3.0
Saccharin (100 mg/ml YPA)										
3.0×10^6	24 ± 10	53 ± 10	31 ± 5.1	9.8 ± 2.8	5.9 ± 2.4	99.7 ± 12	82.0 ± 10.0	155.0 ± 21	88.0 ± 10	37.0 ± 7.8

*Determined by cell counts in hemacytometers. †Fractions of cells plated that formed colonies. ‡Mitotic gene convertants. §Mitotic gene revertants.

impurities would have to be potent to account for all the effects of saccharins A, B, and C, and thus, saccharin per se or one or more of its metabolites might cause the effects.

The saccharins that humans consume in pharmaceutical products, beverages, and foods contain higher concentrations of impurities than the purified saccharin C used in our experiments, and thus could constitute a larger risk than pure sweetener. Moreover, since genetic and toxic effects of nutritional and other environmental agents are cumulative, the contributions of repeated exposures to single compounds are difficult to assess, particularly when a carcinogen is labile or when additive or synergistic consequences of multiple agents result.

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High Concentrations of Glutathione in Glandular Stomach: Possible Implications for Carcinogenesis

Abstract. *In laboratory rodents, concentrations of reduced glutathione (GSH) are exceedingly high (up to 7 to 8 millimolar) in the glandular gastric tissue compared to concentrations in other portions of the gastrointestinal tract or to those of most other organs. Gastric GSH varies diurnally, with the highest levels occurring in the late afternoon or early evening. Starvation, treatment with diethyl maleate, or cold-restraint stress all caused marked decreases in stomach GSH, whereas treatment with cobaltous chloride caused an increase in the GSH concentrations. The physiological significance of the high gastric GSH is unknown, but because this endogenous compound may strongly modulate (decrease or increase) the macromolecular binding of certain chemicals capable of inducing stomach tumors, the possible role of glutathione in the pathogenesis of chemically induced gastric cancer should be considered.*

Adenocarcinoma of the stomach is one of the most important and frequent of human cancers, and the demographic patterns of incidence suggest that its occurrence is carcinogen-related. Many early attempts to develop experimental models of carcinogen-induced gastric cancer in animals failed, or cancers developed only in the squamous portions (forestomachs) of rodent stomachs (*1*). For example, in rodents the forestomach is a major target tissue for carcinogenesis by polycyclic aromatic hydrocarbons (PAH), whereas the glandular portions of the stomachs (the part most analogous anatomically to the human stomach) are highly resistant to these compounds (*1*). In contrast, glandular stomach tissue is highly susceptible to carcinogenesis by certain other kinds of compounds, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (*2*). It is probable that the covalent interaction of the ultimate carcinogenic forms of these types of compounds with cellular macromolecules is a critical initiating event leading to the production of tumors. Reduced glutathione (GSH), an endogenous, nucleophilic tripeptide, may markedly alter the macromolecular binding of these two types of carcinogens in opposite ways: GSH can inhibit macromolecular binding of carcinogenic PAH metabolites (*3*, *4*), but on the other hand it may actually stimulate macromolecular alkylation by agents such as MNNG (*5*). Because of these differences, the relative concentrations of GSH in various portions of the gastrointestinal tract were of interest. We report here that the glandular gastric tissue of laboratory animals contains exceedingly high concentrations of GSH,

Table 1. Treatments causing changes in GSH concentrations in glandular stomachs and livers of rats. Care of animals before the experiment was the same as that described in Fig. 1 legend. All measurements were made at 4 to 6 p.m.; appropriate control groups were run separately for each treatment. All animals for the cobaltous chloride experiment (including controls) were fasted 24 hours prior to and during the experiment. The values shown were calculated from determinations [using the modified Ellman procedure (*7*)] on groups of four to seven rats each. All GSH concentrations represented in the table were significantly different ($P < .01$) from the corresponding control concentrations (analyses by Student's *t*-test on the respective mean absorbance values); S.E., standard error of the mean.

Treatment	Mean percentage of control GSH ± S.E.	
	Liver	Glandular stomach
Food deprivation (48 hours)	80 ± 4	70 ± 3
Diethyl maleate (0.9 ml/kg, subcutaneous, 1 hour prior)	23 ± 10	27 ± 4
Cold-restraint stress (4 hours)	76 ± 1	60 ± 1
Cobaltous chloride (45 mg/kg, subcutaneous, 15 hours prior)	190 ± 10	140 ± 9

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