Appendix II

SHORT-TERM TESTS*

INTRODUCTION

A number of short-term tests have been developed to aid in evaluating the potential of substances to cause cancer. These tests can be conducted quickly, often requiring only a few weeks. Short-term tests examine the capacity of a substance to cause mutations or other genetic alterations. Most chemical carcinogens are either mutagens and/or can be shown to interact with DNA. Most short-term tests are designed to detect one or the other of these properties. The tests are in varying stages of development, and some have been more widely used than others.

Until this study, saccharin had not been systematically or extensively examined in short-term tests. About 20 studies of saccharin have been reported in the scientific literature, and they fall into three general types of short-term tests:

(1) Tests using Drosophila;

(2) In vitro and *in* vivo tests (in mammals) for induction of chromosome abnormalities; and

(3) Tests for the induction of dominant lethal mutations in mice.

The published results available through 1975 were reviewed by Kramers (85), who stated that an unequivocal conclusion about the mutagenicity of saccharin was not possible. Although some reports suggested that saccharin might have weak mutagenic activity, the reports contained limited or conflicting data, and positive results were of only borderline significance. In one study in which genetic effects were found (positive dominant lethal effects and chromosomal translocations in spermatocytes in mice), Kramers suggested that chemical impurities in the saccharin preparation, and not saccharin itself, could have been the responsible agent.

Reports subsequent to Kramers' review have not clarified the situation. Chinnici (29) did not detect crossing over in the X chromosome after growing Drosophila *melanogaster* (fruit flies) on culture medium containing 5-percent saccharin. It is unlikely that this dose level was achieved, however, because Drosophila do not ingest food containing saccharin at such high levels (172). Van Went-de Vries and Kragten (174) did not detect any chromosome abnormalities in bone marrow 'cells after oral administration of 1.5 g/kg/day of saccharin to Chinese hamsters for 3 days. The study was quite small, however, and only 50 metaphrases were examined for abnormalities. Machemer and Lorke (101) reported no increase in chromosome aberrations in spermatocytes of Chinese hamsters. This report is somewhat in contrast to the earlier

*Joyce C. McCann, a member of the advisory panel for this study, coordinated the OTA short-term test battery and was the principal author of this appendix.

positive results in mice (156); however, comparisons are difficult because species of test animals, doses, and routes of administration differed in the two experiments. Machemer and Lorke (100,101) reported orally administered saccharin to be negative in causing dominant lethal mutations in both male and female mice. The earlier positive study (156) used a different route of administration (intraperitoneal injection), making comparisons uncertain. Two other studies (126,150) reported some positive effect after oral administration of saccharin, but as discussed by Kramers, these results were questionable.

OTA SHORT-TERM TESTS

As part of this study, the Office of Technology Assessment commissioned a battery of 12 short-term tests to be conducted on saccharin, This battery marked the first time that saccharin had been tested by many of these methods. The purpose of conducting these short-term tests was to demonstrate to the Congress the nature of the tests, the speed with which they can be conducted, and their usefulness in making regulatory decisions. The test battery, which took about 3 months to complete, illustrates one way that short-term tests can be applied to a particular regulatory problem.

It also seemed possible that conducting a battery of short-term tests might help to clarify some of the uncertainties regarding the carcinogenesis of saccharin. Since saccharin causes cancer only at high doses in rats, there is controversy about whether saccharin is a carcinogen at the lower doses to which humans are exposed, or whether some secondary effect introduced by high doses causes cancer in rats. Similarly, questions have arisen about whether saccharin itself or impurities in saccharin caused the positive results in the carcinogenesis experiments. Positive results in short-term tests would add weight to the argument that saccharin is a carcinogen. The battery of tests was designed to determine, as definitively as possible within the time limits of this study, whether highly purified saccharin is mutagenic or causes other genetic alterations.

Of the 12 short-term tests commissioned by the OTA, 10 have been completed. The tests were conducted by their developers or recognized experts, who generously donated their time to this study. The tests conducted and the principal investigators are presented in table 38.

Table 38.—OTA Saccharin Short-Term Test Battery

	Collaborating Investigators
Positive Tests	
Sister Chromatid Exchange	Dr. Sheldon Wolff/Dr. Brita Rodin Laboratory of Radiobiology University of California San Francisco, Cal if. 94143
Mouse Lymphoma	Dr. Donald Clive Genetic Toxicology Laboratory Burroughs Wellcome Company Research Triangle Park, N.C. 27709

Table 38.—OTA Saccharin Short-Term Test Battery—Cont.

	Collaborating Investigators
Chromosome Aberration (CHO Cells)	Dr. Abraham Hsie/Dr. Juan San Sebastian Biological Division Oak Ridge National Laboratory P.O. Box Y Oak Ridge, Tenn. 37830
Negative Tests	
Salmonella/Ames	Dr. Bruce Ames/Dr. Joyce McCann Department of Biochemistry University of California Berkeley, Calif. 94720
Mitotic Recombination in yeast (D3)	Dr. Vincent Simmon Applied Microbiology Program Stanford Research Institute Menlo Park, Calif. 94025
Pol test (E. Coli)	Dr. Herbert Rosenkranz Department of Microbiology New York Medical College Valhalla, N.Y. 10595
Drosophila (sex-linked recessive lethal test)	Dr. Seymour Abrahamsen/Dr. Ruby Valencia Department of Zoology University of Wisconsin Madison, Wis. 53706
Unscheduled DNA synthesis (human fibroblasts)	Dr. Hans Stich Cancer Research Centre The University of British Columbia Vancouver, Canada V6T 1W5
In Vitro Transformation (hamster embryo cells)	Dr. Roman Pienta Frederick Cancer Research Center Frederick, Md. 21701
Induction of Plasminogen Activator (HeLa cells)	Dr. 1. B. Weinstein College of Physicians and Surgeons of Columbia University Institute of Cancer Research 99 Fort Washington Avenue New York, N. Y. 10032
Tests in Progress	
In Vitro Transformation (mouse C3H 10T1 /2 cells)	Dr. Charles Heidelberger/ Dr. Suktab Mondal University of Southern California Cancer Research Building 1303 North Mission Road Los Angeles, Calif. 90033
CHO/HGRPT	Dr. Abraham Hsie/Dr. Patrick O'Neill Biological Division Oak Ridge National Laboratory P.O. Box Y Oak Ridge, Tenn. 37830

The battery of tests included many of the most sensitive short-term tests currently available. Criteria for the inclusion of tests in the battery were: (1) sensitivity and validity for detecting carcinogens; (2) complementarily with the other tests in the battery and with test literature on saccharin; and (3) ability to be completed within the time constraints of the OTA study. Saccharin had been tested previously by only 2* of the 12 methods.

All tests were conducted using the same sample of saccharin that was used in the most recent Canadian carcinogenicity tests in rats. This material is referred to as "impure saccharin;" even though highly purified, it still contains very small amounts (about 20 ppm) of impurities. For this reason, all participating laboratories also received a sample of saccharin that had been specially purified to remove essentially all traces of impurities. This material is referred to as "pure saccharin."**

Results from three tests-sister chromatid exchange, mouse lymphoma, and chromosome aberration—were positive. Highly purified samples of saccharin were weakly active in these tests, and the results are clearly suggestive that saccharin itself has mutagenic properties. The results should be regarded with some caution, however. The responses were very weak in the three tests, even at the high dose levels tested. And the value of the sister chromatid exchange, mouse lymphoma, and chromosome aberration tests in predicting carcinogenicity has not yet been firmly established. However, validation of the tests has begun by testing a number of carcinogens and mutagens and a few noncarcinogens, with promising results.

The results of 7 of the 10 completed tests in the OTA test battery were negative; that is, saccharin did not cause mutagenic or other genetic alterations in the tests. These negative results, even in well-validated tests such as the Salmonella/Ames test, do not invalidate or cast suspicion upon the positive results for several reasons. Saccharin is detected as a carcinogen in rats only at high doses and is therefore called a "weak carcinogen. " Mutagenic effects were detected only at very high dose levels (5-10 mg/ml), and this fact, coupled with the results in the seven negative tests as well as the preponderance of negative results in the published literature, indicates that any mutagenic properties of saccharin are very weak. Thus, this property might not be detected in most short-term tests. Each of the short-term tests has its own set of limitations, both in sensitivity and in the range of chemical classes it can detect. Although it would be surprising if a potent carcinogen were negative in many different kinds of short-term tests, it is not surprising that a carcinogen such as saccharin might be detected in only a few systems.

Saccharin was detected only at very high dose levels in the three positive tests, In all but two of the seven negative tests, these dose levels either were not tested or could not be tested because lower doses were toxic. In the Pol test, the highest dose tested was about 0.02 mg/ml; in the plasminogen activator test, 0.05 mg/ml; and in the *Drosophila* sex-linked recessive lethal test, the highest concentration of saccharin that was well ingested was 2.5 mg/ml. In other tests, the highest nontoxic doses found were about 1 mg/ml in the Salmonella/Ames test; 3 mg/ml in the hamster embryo

*Several sex-linked recessive lethal tests in *Drosophila* have been published, with somewhat conflicting and uncertain results. Results obtained by Stoltz et al. (162) using the Salmonella/Ames test were independently confirmed for the battery.

**Both the "impure" and "pure" saccharin were generously provided by D. Stoltz and B. Stavric.

fibroblast *in vitro* transformation test; and 2 mg/ml in the 10T 1/2 in vitro transformation test. High doses (up to 50 mg/ml) were tested in the mitotic recombinant test in yeast, but the absence of any toxic effect even at such high dose levels suggests that saccharin may not have penetrated the yeast cell wall sufficiently. Only in the unscheduled DNA synthesis test were doses of 5-10 mg/ml tested.

It was somewhat surprising that saccharin was not positive in the unscheduled DNA synthesis test. This method is quite sensitive and measures changes that are likely to occur during the sister chromatid exchange and chromosome aberration processes. Additionally, the unscheduled DNA synthesis and sister chromatid exchange experiments were conducted in human cells (albeit different cell types) over similar dose ranges, *

Interpretations of the validity of the carcinogencity tests on saccharin are complicated by the presence of variable, and usually unspecified, amounts of impurities present in different batches of saccharin. The possibility that impurities might be responsible for the positive carcinogenicity test results on saccharin has long been debated. Even results from the most recent Canadian studies (in which saccharin containing only approximately 10 to 20 ppm impurities was tested) cannot unequivocally be considered as caused by saccharin itself, rather than by unknown carcinogenic impurities.

If carcinogenic impurities are present in preparations of saccharin, their detection and identification can be facilitated by demonstrating their mutagenicity in short-term tests. Uncertainties regarding impurities are characteristic of the published short-term data on saccharin, almost all of which are negative or of borderline validity.

Stoltz et al. (14,162) have demonstrated that the impurities found in the saccharin used in the Canadian study are mutagenic in the Salmonella/Ames test. The impurities are only weakly mutagenic, and it is not clear that they are sufficiently potent to have caused the positive carcinogenicity result. However, samples of saccharin used in the Canadian cancer tests contained far lower levels of impurities than commercial saccharin (to which humans are exposed), and it is possible that carcinogenic impurities in commercial saccharin pose a greater human health hazard than saccharin itself.

POSITIVE TESTS

1. **Sister Chromatid Exchange (SCE)**

The sister chromatid exchange (SCE) test (134,161) is similar to other cytogenetic procedures in that it measures changes in chromosomal structure. However, it employs a special staining technique to detect subtle changes that do not affect gross chromosomal structure. Classical cytogenetic techniques depend upon such gross changes in structure for detection. In many cases, SCEs have been shown to occur more frequently than gross chromosome aberrations after treatment of cells with chemical mutagens. For this reason, the SCE test may be a more sensitive method than standard cytogenetic procedures for detection of chemicals which have weak

*Similarly, the CHO/HGPRT test (still in progress) is closely related to the mouse lymphoma test, and it will be of interest to see if saccharin is detected in this system.

cytogenetic activity. The role of SCEs in the generation of mutagenic events in cells has not been proven. But considerable experimental evidence, consistent with the theoretical understanding of how mutations are likely to occur, indicates that events which cause SCEs also can cause mutations. A number of carcinogens and a few non-carcinogens have been tested using the SCE procedure (1,134,161), and the correlation looks promising. However, the method needs to be thoroughly validated to demonstrate its value as a predictor of carcinogenicity and mutagenicity.

The SCE data on saccharin (tables 39 and 40 and figure 5) are convincing, especially the data obtained on human cells (table 39), which shows a clear dose-response effect (figure 5). The effect is weak in both Chinese hamster and human cells, but the observation is well documented. * The fact that both impure and highly purified saccharin produced essentially the same results suggests that saccharin itself, and not impurities, caused the increase in SCEs. The possibility that either pH or ionic-strength effects is responsible for the increase is being considered in assessing the possible significance of these results. The medium was well buffered, there was no pH change in the saccharin-treated samples, and therefore pH changes were apparently not a factor (190). Control experiments in which the concentration of sodium chloride was varied suggest that ionic-strength effects are not likely to have been a factor (190).

Table 39.—induction of Sister Chromatid Exchanges (SCEs) by Saccharin in Human Lymphocytes *in vitro*[1]

	Impure Saccharin		Pure Saccharin	
Dose (Percent)	# SCEs per # Chromosomes	SCEs/cell	# SCEs per # Chromosomes	SCEs/cell
Experiment 1				
0	981/4588	9.81 ± 0.31	.....	.....
0.1 : : : : : :	1169/4592	11.69 ± 0.34[3]	.....	.....
-0.5[2]	1711/4594	17.11 ± 0.413	.....	.....
Experiment 2				
0	950/4592	9.50 ± 0.31	950/4592	9.50 ± 0.31
0.3 : : : : : :	1311/4595	13.11 ± 0.36[3]	1322/4590	13.22 ± 0.36[3]
0.5[2]	1607/4598	16.07 ± 0.40[3]	1745/4596	17.45 ± 0.42[3]

[1]Data from S. Wolff and B. Rodin. Procedures were as described by Perry and Evans (1 34). Cells cultured 72 hours in 20 µM bromodeoxyuridine (BUdR.). Saccharin was present during the entire incubation period.
[2]Higher doses were toxic.
[3]p < 0.001.

2. Mutagenesis Tests in Mammalian Cells in Culture

Several mutagenesis tests using mammalian cells in culture are in various stages of development and validation. The two used to test saccharin are the mouse lymphoma (31) and Chinese hamster ovary (CHO)/HGPRT (130) tests. These

*Abe and Sasaki (1), in independent experiments, reported similar results in Chinese hamster cells.

Table 40.—Induction of Sister Chromatid Exchanges (SCEs) by Saccharin in Chinese Hamster Ovary (CHO) Cells *in vitro*[1]

Dose (Percent)	# SCEs per # Chromosomes	SCEs per cell
Experiment 1 (impure saccharin)		
0	875/2027	*8.75±0.30*
0.1	953/2003	*9.53±0.30*
0.5	995/2027	*9.95±0.32*
1.0[2]	1246/2028	12.46±0.35[3]
Experiment 2 (impure saccharin)		
0	845/1 967	8.45±0.29
1.0[2]	1294/1 982	12.94±0.36[3]
Experiment 3 (pure saccharin)		
0	855/1 947	8.55±0.29
0.5	1021/1969	10.21±0.32[3]
1.0[2]	1121/2006	11.21±0.33[3]
Experiment 4 (pure saccharin)		
o	872/1979	8.72±0.30
0.8	1105/1987	11.05±0.33[3]
1.02	1196/1996	11.96±0.35[3]

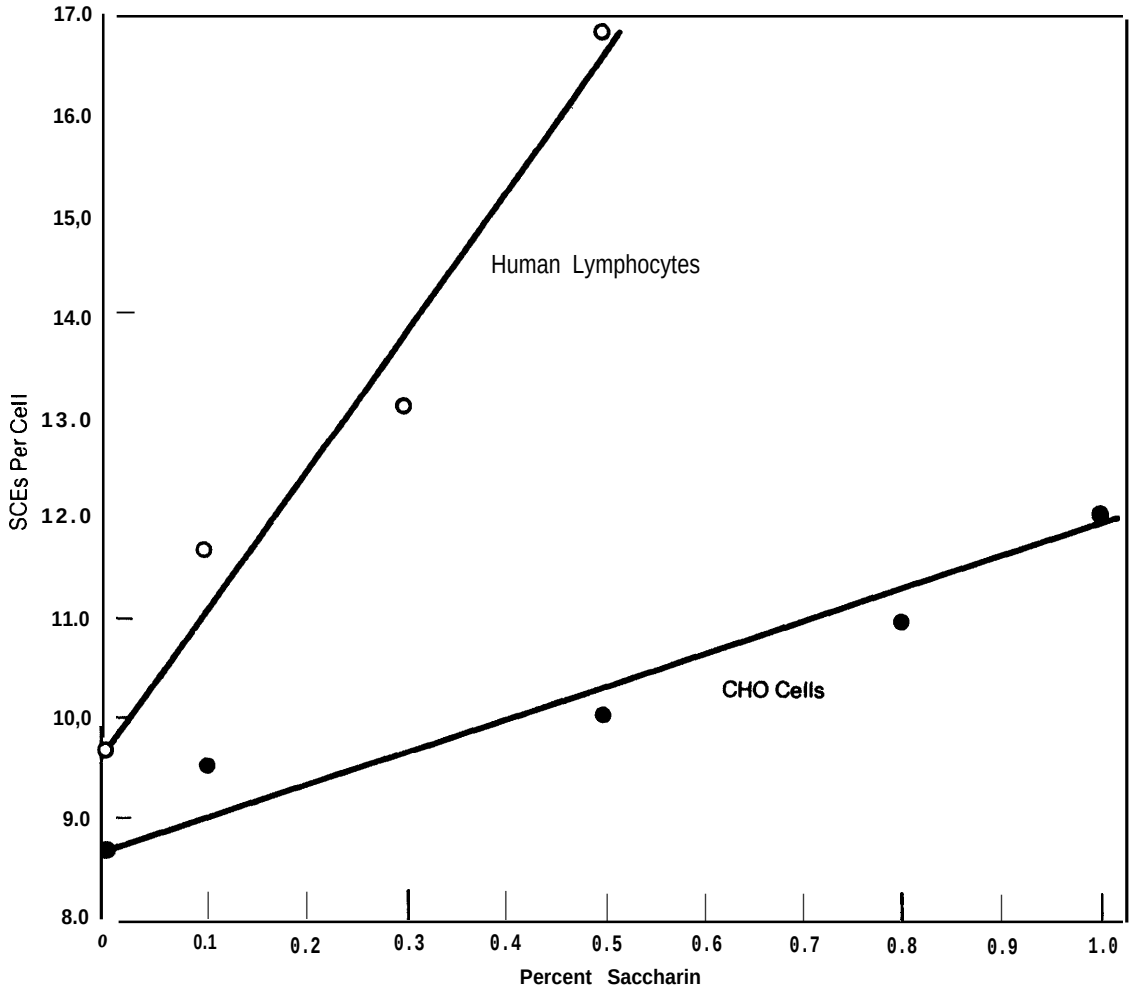
[1]Data from S. Wolff and B. Rodin. Cells cultured 24 hours in I0 µM. BUdr. I00 cells per point were examined.
[2]Higher doses were toxic.
[3]P < 0,001

methods are similar in that both measure mutations at a specific locus in either mouse lymphoma or CHO cells. Both select mutants that are resistant to either purine (HGPRT test) or pyrimidine (mouse lymphoma test) analogues which, if incorporated into DNA, are lethal to the cell. A mutation at the HGPRT genetic locus in CHO cells makes it impossible for a purine analogue to be incorporated into DNA, and a mutation at the TK genetic locus in mouse lymphoma cells prevents incorporation of certain pyrimidine analogues. Currently, the genetics of the CHO cell line is better defined, but the mouse lymphoma system has been more extensively used for mutagenesis testing. A validation study on the CHO/HGPRT system is in progress, and some results have been published (131). Although the mouse lymphoma test is being used by a number of laboratories and many chemicals have been tested, most of the results have not yet been published.

In tests with saccharin, a weak positive result was obtained in the mouse lymphoma test (tables 41 and 42).* The results are difficult to interpret as unequivocably positive because the effect is very weak, and there is no clearly reproducible dose-response. Also, the effect occurred only at doses that were quite toxic to the cells, as is shown by the percent survival. However, nearly all mutagens require such

*Final results of the CHO/HGPRT tests are not yet available. Several experiments have been completed and they did not detect any statistically significant mutagenic effect of saccharin. However, for technical reasons the results were not conclusive, and more experiments are in progress.

Figure 5– Induction of Sister Chromatid Exchanges by Saccharin



All data combined. For human lymphocytes the linear regression is given by Y = 9.64 (± 0.39) + 14.01 (±1.14)D where D is the % saccharin. **For** CHO cells the regression **is** given **by** Y = 8.66 (±0.21) + 3.37 (±0.32)D. **The coefficient of correlation** is **0.98 for human lymphocytes and 0.96 for CHO cells. (Figure 5** is **from Wolff and Rod in.)**

toxic doses for detection in this system *(30).* The results suggest, however, that both impure and highly purified forms of saccharin may be mutagenic. This result occurred at approximately the same dose levels as positive results obtained using the sister chromatid exchange and chromosome aberration test.

3. In *Vitro* Cytogenetic Tests for Chromosome Aberration in Mammalian Cells in Culture

Cytogenetic tests measure changes in the morphological structure of chromosomes. A number of different types of structural changes can be produced. Many of these changes are lethal to cells, but some can lead to stable mutagenic changes. Many cytogenetic methods are available, using a variety of cell types from

both mammals and humans and using both in vitro and *in* vitro procedures. For example, some of the most frequently used *in vitro* methods measure changes in chromosome structure in bone marrow cells, peripheral lymphocytes, or spermatocytes in animals. In vitro human peripheral lymphocytes are often used, as well as a wide variety of cell lines from both humans and animals.

Table 41 .—Induction of Mutations by Saccharin at the TK+/TK Locus in Mouse Lymphoma L5178Y Cells[1]

Concentration of Na Saccharin (mg/ml)	Impure Saccharin		Pure Saccharin	
	Percent survival	Mut. freq.[2]	Percent survival	Mut. freq.
o	100	o	o	o
10".0"	63	26	36	6
11.0	60	24	.....	.....
11.5	.....	.....	23	29
12.0	41	56	.....	.....
12.5	33	39	16	40
13.0	.....	.....	9.3	32
13.5	20	43	6.3	19
14.0	7.9	41	.....	
2-AAF[3](50pg/ml)	25	222	24	" "1 60" " "

[1]Data from D. Clive. Procedures were as described by Clive and Spector (1973). Procedures for use of the rat liver S-9 Mix are in preparation (30). Results shown for impure saccharin were replicated, and those for pure saccharin were from a single experiment. Aroclor-induced rat liver S-9 Mix was present.
[2]Number of mutants/1 0[5] survivors, after correcting for spontaneous background. The spontaneous background was about 40.
[3]2-AAF = 2-acetylaminofluorene is a known mutagen and was included as a positive control.

Table 42.—induction of Mutations by Saccharin at the TK+/TK Locus in Mouse Lymphoma L5178Y Cells[1]

Concentration of Impure Na Saccharin (mg/ml)	With Microsomes		Without Microsomes	
	Percent Survival	Mut. freq.	Percent Survival	Mut. freq.
0	100	0(Spont=58)	100	0(Spont=70)
7.6	55	20	.....	.....
8.5	35	32	.....	.....
10.0	22	45	.....	.....
11.5	6.9	76	.....	.....
13.0	2.1	99	.....	.....
14.5	.....	.....	25	17
16.0	.....	.....	66	30
17.5	.....	.....	9.6	45
19.0		.....	5.3	37
2-AAF(50µg/ml)	" "33 "	189	.....	
EMS[2](620µg/ml)	.....	.....	.....	1 0 9 8

[1]Data from D. Clive. Microsomes were Aroclor-induced rat liver S-9 Mix. For other details, see footnote to table 41.
[2]EMS = ethyl methane sulfonate, a known mutagen, which served as a positive control.

In the saccharin test battery, preliminary evidence for cytogenetic effects of highly purified saccharin has been obtained using CHO cells in the presence of a rat liver activation system (table 43). A variety of chromosome aberrations and an apparent dose response have been found. This cell line is also being used by the same investigators to test saccharin for its ability to induce point mutations (the CHO/HGPRT test, not yet concluded).

Table 43.—induction of Chromosome Aberrations by Saccharin in CHO Cells[1]

Concentration of Pure Na Saccharin (mM)	Chromosome Aberrations					
	Cytotoxicity (Percent of viable cells)	Chromatid Gaps	Chromosome Breaks	Translocations	Ring Formations	Abnormal Metaphrases per total metaphrases examined?
0	100	2	3	1	0	3/100 (3%)
25	98	3	6	4	0	9/120 (7.5%)
50	83	3	5	7	1	10/110 (91%)
100	45	8	14	24	9	25/128 (19.5%)
200[3]	<lo	6	18	15	6	23/110 (20.9%)
DMN (100µg/ml)	not tabulated -					23/140 (16.4%)

[1] Results are preliminary data from experiments still in progress (J San Sebastian J P O'Neill, and A W Hsie) All experiments were conducted in the presence of rat liver S-9 from Aroclor reduced animals (130,131) The sodium ion concentration was kept constant, at all doses of sodium saccharin, by varying the amount of NaCl added to the assay mixture Similar results have been Independently obtained and have been recently reported (Ishidate, M and Odashima, S., Mutation Res. 48, 337-354 (1977))

[2] The usual spontaneous background of abnormal metaphrases, in the absence of S-9 IS about 1 percent

[3] Nonspecific cytotoxic effects cannot be ruled out at this high dose level.

NEGATIVE TESTS

1. The *Salmonella/Ames* Test

This test is currently the most widely used of the short-term tests. A large number of known carcinogens have been tested and shown to be mutagens in this system (96,97,139,155,163). The method is very efficient for detection of organic chemical carcinogens (about 90 percent of those tested can be detected), but it does not detect all classes of carcinogens with equal efficiency. For example, metals, some chlorinated hydrocarbons, and the hydrazines are poorly detected.

The procedure uses several specially constructed strains of the bacterium *Salmonella typhimurium*. These strains contain different mutations that inactivate the genes necessary for the synthesis of the amino acid histidine, and as a result the bacteria cannot grow unless this amino acid is added to the growth medium. The test is carried out by exposing the bacteria to the chemical to be tested and measuring the number of bacterial colonies that are able to grow in the absence of histidine. Each such bacterial colony is the product of a mutational event. A correlation between increasing dosage of a chemical and increasing numbers of colonies shows the chemical to be mutagenic. The method also incorporates rodent (or human) liver extracts into the assay mixture to provide "activating enzymes, " which are necessary to metabolize some carcinogens to their active forms.

Saccharin was tested in *Salmonella* over a wide dose range by Stoltz, et al. (162) and found to be negative. These results are independently confirmed by the data in table 44, Impure saccharin was tested on the five standard tester strains (TA100, TA1535, TA1537, TA1538, TA98) over a wide dose range (.001 to 100 mg per petri plate) and in the presence of activating enzymes from either aroclor or phenobarbital induced rat liver homogenate.

Table 44.—Negative Assay of Saccharin in the Salmonella/Ames Test[1]

Dose (mg/plate)	TA100			TA1535			TA1537			TA1538			TA98		
	S-9	PB[2]	PCB[3]	S-9	PB	PCB	S-9	PB	PCB	S-9	PB	PCB	S-9	PB	PCB
0	155	149	141	29	21	19	6	9	11	8	20	25	33	35	39
.001	174	173	148	24	19	18	6	9	14	17	21	31	41	57	56
.01	128	160	124	28	15	14	7	12	12	11	25	34	30	49	56
.1	163	148	141	31	14	18	15	18	8	17	14	25	34	36	49
.5	149	132	140	18	14	23	13	11	15	9	16	33	28	43	48
1.0.	141	138	137	27	15	14	6	19	10	8	21	24	39	38	48
10.0	116	153	124	20	18	7	10	8	9	14	22	44	51	32	43
20.0.	163	135	126	19	15	11	10	11	8	9	15	25	55	41	48
50.04	108	120	108	19	14	13	6	10	13	14	15	23	33	39	55
100	89	82	91	6	5	10	3	4	4	10	6	8	29	29	27
MMS (2µl)	(+)														
MNNG (lµg)				(+)											
9-AA(50µg)							(+)	•	•						
2-AA(10µg)											5009				
B(a) P(5µg)															827

[1]Data from Yamasaki, J. McCann and B N Ames The standard Plate assay was used, as described in Ames, McCann, and Yamasaki, 1975 MMS = methyl methane sulfonate, MNNG = N-methyl -N-nitronitrosoguanidine, 9-AA = 9-aminoacridine, 2-aminoanthracene, B(a)P = benzo(a)pyrene.
[2]PB = phenobarbital reduced S-9 Mix (100 µl/plate)
[3]PCB = aroclor reduced S-9 Mix (20 µl/plate)
[4]The lower number of revertants at the 50 and 100 mg/ml dose levels indicate toxicity
+ = positive in a spot test

The Salmonella/Ames test was also used to test impurities in saccharin, and these results are discussed in a separate section.

2. **Mitotic Recombination in Yeast**

A number of different types of genetic events can be assayed in yeast. The most widely used assays for testing carcinogens and mutagens are those that measure mitotic recombination (194). This process involves breaking and rejoining parts of homologous chromosomes and can lead to changes in the genetic characteristics of the organisms. Mitotic recombination can result in chromosomal mutations that affect large numbers of genes, as compared to point mutations, which affect single genes. The basic molecular processes involved in these two mutagenic events are most likely related because chemicals that induce mitotic recombination, almost without exception, also cause point mutations. The yeast that has been most commonly used to detect mitotic recombination events is *Saccharomyces cerevisiae* D3. Over 100 carcinogens and noncarcinogens have been tested in this strain as part of an NCI-sponsored contract (154) to evaluate short-term tests. The method does detect a number of carcinogens and is useful, but it is not as sensitive as many of the other short-term tests.

Mitotic recombination tests using the D3 system with saccharin up to 5-percent dose levels, both with and without aroclor-induced rat liver activation, were negative. Both the impure and pure samples were tested, and each test was conducted three times, The results are given in table 45.

3. **Tests for DNA Repair Activity (Unscheduled DNA Synthesis and the Pol Test)**

These tests measure the ability of chemicals to interact with DNA in a way that causes DNA repair to occur. The unscheduled DNA synthesis test (149) measures DNA repair directly in cultured human fibroblasts (cells derived from human skin) after treatment with chemicals. This method is quite sensitive and has been shown to

Table 45.—Negative Assay of Saccharin for Mitotic Recombination in *Saccharomyces cerevisiae* D3[1]

Concentration (Percent)	Mitotic Recombinants per 10^5 Survivors[2]					
	Experiments Without S-9[3]			Experiments With S-9		
	1	2	3		2	3
Saccharin (impure)						
0	<2.3	9.6	5.7	6.1	12.2	14.6
0.1	11.6	3.1	21.6	6.3	2.9	16.7
0.5	13.5	1.8	9.7	2.4	7.3	15.8
1.0	11.1	3.1	6.4	22.9	1.7	37.0
2.5		3.6			1.8	
5.0	10.0		5.3	8.0		9.8
Control, Diepoxybutane	.....	1429	2000	.....	763	2440
Saccharin (pure)						
0	<2.3	9.6	5.7	6.1	12.1	14.6
0.1	9.8	2.1	8.1	7.3	12.0	15.4
0.5	9.1	2.4	33.3	4.2	6.4	27.3
1.0	3.0	3.3	13.3	6.5	2.0	22.7
2.5		1.9			1.7	
5.0	2.6		17.2	2.3		6.3
Control Diepoxybutane	.....	1429	2000	.....	763	2440

[1]Data from V. Simmon. Procedures were as described bv Zimmerman (1941).
[2]Saccharin had no significant toxic effect.
[3]S-9 Mix rat liver homogenate, activating enzymes, from Aroclor induced rats.

detect a wide range of chemical carcinogens and mutagens. The Pol test is used to detect chemicals that cause particular types of damage in DNA. This type of damage can be repaired by an enzyme present in the bacteria. The Pol test determines the toxicity of the test chemical in mutants that lack the DNA repair enzyme. A positive result is inferred if the chemical under test is more toxic to the bacteria lacking the repair enzyme than it is to the parent strain that contains the enzyme. The Pol test, though useful, is not applicable to detection of a wide range of carcinogens and mutagens. The method has recently been improved (145), and the modified as well as the standard procedures were used in testing saccharin.

Saccharin was negative in both the unscheduled DNA synthesis and the Pol tests. Unscheduled DNA synthesis was measured in bacteria exposed to a dose range from 0.002- to 2-percent saccharin, Neither the pure nor the impure sweetener was positive at any dose. An additional experiment to determine whether saccharin (over the same dose range) would interfere with ultraviolet radiation-induced repair of DNA was also negative. Results of the Pol tests are given in table 46. A positive result would have produced a larger zone of inhibition in the Pol A^- test in the upper half and a reduction in Pol A^- viable bacteria in the lower half when compared to the Pol A^+ data. In these experiments, saccharin was tested at only one dose level (0.5 mg/ml) which, considering the weak activity saccharin has shown in other systems, is not sufficiently high to constitute a definitive test. Urine from rats treated with impure saccharin was also tested in the Pol test, with negative results (table 47).

Table 46.—Negative Assay of Saccharin in the Pol Test[1]

	S-9[2]	Zone of Inhibition (mm)	
		Pol A+ (wild type bacteria)	Pol A- (mutant bacteria)
Disc Diffusion Assay			
Saccharin (impure)			
500 µg.	-	0	0
	+	0	0
Positive control			
(MMS,[4] 0.13 µmol)	-	41	68
Negative control			
(CAP,[5] 30 µg).	-	29	29
Modified Suspension Assay[3]		Viable Bacteria per ml	
Dose (µg/ml	s-9	Pol A+	Pol A-
0	-	7×10^8	7.4×10^8
	+	8×10^8	7.6×10^8
500.	-	6.3×10^8	6.9×10^8
	+	7.1×10^8	8.3×10^8

[1]**Data from H.** Rosenkranz. Procedures were as described by Rosenkranz, et al. (1 45)
[2]S-9 Mix rat liver homogenate from Aroclor induced rats.
[3]Cells were exposed for 24 hours, 37°C.
[4]**MMS = methyl methanesulfonate**
[5]**CAP = chloramphenicol**

Table 47.—Negative Assay of Urine from Rats Treated with Saccharin in the Pol Test[1]

Sample	Viable Cells/ml	
	Pol A+	Pol A-
Solvent Control.	3.6×10^8	1.6×10^8
Solvent Control + ß-glucuronidase.	4.3×10^8	1.8×10^8
Solvent Control + S-9 + ß-glucuronidase	4.3×10^8	2.2×10^8
Pre-treatment Urine + ß-glucuronidase	4.2×10^8	1.7×10^8
Pre-treatment Urine + ß-glucuronidase + S-9. . . .	3.6×10^8	1.7×10^8
6 hour Urine + ß-glucuronidase	3.8×10^8	1.8×10^8
6 hour Urine + ß-glucuronidase + S-9.	3.5×10^8	1.7×10^8
24 Hour Urine + ß-glucuronidase.	4.5×10^8	1.8×10^8
24 Hour Urine + ß-glucuronidase + S-9	3.8×10^8	1.8×10^8

[1]Data **from H. Rosenkranz. Procedures were as described by Rosenkranz, et al. (1 45) and by Durston and Ames (1975). Rats (ea. 150 grams each) each received 1 to 2.5** grams saccharin by gavage. Pre-treatment as well as **pooled 6 to 24 hour urines were assayed** in both the disc diffusion assay (results not shown) and modified suspension assay.

4. Drosophila Sex-Linked Recessive Lethal Test

The fruit fly, Drosophila, can be used to detect a variety of mutagenic and chromosomal breakage events (2). The test used here employs the whole animal and is an *in vitro* test in contrast to most of the other, *in vitro,* short-term tests. *Drosophila* has enzymes that are capable of carrying out many of the same reactions that activate chemical carcinogens in mammalian systems. The most sensitive assay using *Drosophila,* which detects the broadest range of mutagens at the lowest concentrations, is generally considered to be the sex-linked recessive lethal test (2,176), which detects lethal mutagenic changes in the X chromosome. Usually, in this method, male flies are treated with the chemical to be tested (in this case saccharin was added to the nutrient medium), and they are then mated to female flies that have not been treated. Female progeny receive one X chromosome from the female parent and one X chromosome from the male parent; male progeny receive one X from the female parent and one Y from the male parent. Thus, all males carry one X and one Y chromosome, and all females carry two X chromosomes. Even if the one X chromosome carried by the parental male flies has suffered lethal damage from the chemical treatment (due probably to chromosome breakage caused by the chemical), this damage will not affect the viability of the first generation progeny. A "lethal X" will, in the progeny females, have another healthy X to carry out the needed functions of the X chromosome. A "lethal X" will not be present in any of the male progeny because the one X chromosome in each male progeny is always donated by the female parent, which was not treated with the chemical. The next step in the assay is to mate the first generation daughters to normal untreated males. Now, if one of the X chromosomes carried by the females is the "lethal X", this chromosome will be passed on to some of the male offspring of this second mating. Because males have only one X chromosome, the males receiving the "lethal X" chromosome will die, and in fact such offspring are never hatched. Since so much is known about the distribution of chromosomes among progeny, the experiment can be designed so that a normal mating will result in two phentoypic classes of male offspring. If the class of male offspring that would have received the "lethal X" is missing, then this fact is evidence that the initial chemical treatment, two generations previous, caused lethal chromosome damage in the X chromosome of the treated males.

Results of the sex-linked recessive lethal tests in *Drosophila* are shown in table 48 and were substantially negative. Although there was a statistically significant increase in recessive lethals in brood 1, if multiples are not included in the calculation, the result is not significant. The normal procedure in *Drosophila* assays is to remove multiples from the calculation because they often result from spontaneous mutations, and for this reason these results must be considered negative. However, multiples occurred at doses of saccharin when only some of the flies ingested saccharin (see footnote in table 48 for details), and an effect due to saccharin, although it appears unlikely, cannot be ruled out.

Any positive effect of saccharin might be expected to be very weak, In order to detect a doubling of the spontaneous rate, about 8,000 flies must be examined in a sex-linked recessive lethal test. Since flies did not efficiently ingest saccharin at dose levels greater than 0.25 percent (table 48), the number of flies receiving a significant dose of saccharin (the group treated with 0.25-percent saccharin) was large enough to detect only about a quadrupling of the spontaneous rate. Thus, if saccharin caused less than a quadrupling of the spontaneous rate, it would not have been detected in this test.

Table 48.—Negative Assay of Saccharin for the Induction of Sex-1 inked Recessive Lethals in Drosophila [1]

Experiment	Cone. (Percent)	Survivors (Percent)	Number of Tests (lethals/non-lethals) Brood 1[3]	Brood 2[3]	Total Tests	No of Lethals	Lethals (Percent)
Treated							
1	0	54[4]	32260	0/1923	4186	3 singles	0.072
	1	96	3/1 841	0/1629	3473	3 singles	0.086
2	1	100	14 / 2228	9/2175	4427	5 singles 1 double 1 multiple of 16	0.512(0.159)[5]
3	0 5	100	13 2439	0 /1205	3657	4 singles 1 double 1 multiple of 7	0.355(0.160)[5]
	025	100	2$1730	1/1449	3182	3 singles	0.094
Totals from exp 1,2,3			35/10498	113/8381	18924	45	0.238
Percent lethals per brood			0 332'	0119			
Controls							
1		100	O 1604	1 1518	3123	1 single	0.032
2		100	52013	8 1896	3922	5 singles 4 doubles	0.331
3		100	2 1589	2860	2453	2 singles 1 double	0.163
Total Controls			715206	11 4274	9498	18	0.190
Percent lethals per brood			0134	0257			

[1]Data from R Valencia and S Abrahamsen. Procedures were as described by Abrahamsen and Lewis (2) Canton-S wild type males were treated for 72 hours with saccharin (Impure) and mated with FM6 females Files were treated im petri dishes m experiments 1 and 2, and to improve ingestion of saccharin in closed vials in experiment 3

[2]Ingestion of saccharin at doses greater than 0.25 percent was incomplete About half the files ingested some saccharin at the O 5-percent level there was only occasional Ingest [on at the 1 -percent level and there was no ingestion at the 10-percent level

[3]Brood 1 mainly assays for effects on mature sperm and Brood 2 also assays for effects on spermatids and younger sperm

[4]Mortality IS most likely not due to saccharin toxicity, but to starvation

[5]In parentheses percent lethals have been calculated after removal of doubles and multiples, which are often due to spontaneous mutations

[6]Statistically significant (Chi. square 5 25) Includes doubles and multiples

5. *In Vitro* Transformation

It is by no means clear that the molecular changes that lead to the altered growth potential associated with transformation of cells *in vitro* are the same as the changes that occur during carcinogenesis *in vitro.* However, several lines of evidence strongly suggest that there is a relationship between the processes and that mutagenesis may be the crucial molecular event. *In vitro* transformation tests measure the ability of a chemical to change a cell from a form that cannot cause a tumor in an animal to a form that will result in a tumor if the treated cells are re-injected into the animal.

Several *in vitro* transformation systems are under development. The hamster embryo (135) and C3H mouse 10T 1/2 (143) systems used to test saccharin have both been used to detect a number of carcinogens and mutagens. The National Cancer Institute has recently completed a validation study on the hamster embryo system (135) in which 54 carcinogens and 21 noncarcinogens were tested. The method was quite efficient and was able to detect transforming activity for all but eight of the carcinogens. It did not detect activity for any of the noncarcinogens. The cells used in *in vitro* transformation assays can activate some carcinogens to their active forms. But in order to detect a broad range of carcinogens, these tests have to be coupled to a metabolic activation system. Work is in progress on this alteration in a number of laboratories.

Saccharin, both the impure and purified samples, was negative when tested in the hamster embryo system at doses up to 10 mg/ml (table 49). Preliminary results in the 10T 1/2 system at 1 and 2 mg/ml were also negative (table 50), and a more extensive experiment is in progress.

Table 49.—Negative Assay of Saccharin for *in vitro* Transformation in Hamster Embryo Fibroblasts[1]

Dose (µg/ml)	Transformed colonies per survivor	
	Impure Saccharin	Pure Saccharin
0	0/781	0/781
1	0/888	0/865
3.2	0/912	0/893
10	0/863	0/912
32	–	0/920
100	0/876	0/881
316	0/938	0/1013
1,000	0/860	0/1018
3,160	0/839	0/971
10,000	0/71	0/228
Positive control: 3-methylcholanthrene (1 µg/ml)	3/629	3/629

[1]Data from R. Pienta. Procedures were as described by Pienta, et al. (135). Rat liver homogenate (activating enzymes) was not present. Plates were scored for transformants after 8 days incubation with saccharin.

Table 50.—Preliminary Negative Assay of Saccharin for in vitro Transformation in C3H Mouse 10T½ Cells[1]

Dose (mg/ml)	Transformed Colonies per Number of Dishes Scored	
	Impure Saccaharin	Pure Saccharin
0	0/5[2]	0/5
1	0/7	0/5
2	0/5	0/9

[1]Data from S. Mondal and C. Heidelberger, Procedures were as described by Reznikoff, et al. (1 43). A more extensive experiment is in progress. Cells were incubated 24 hours with saccharin, about 2,000 cells per dish were plated, and plates were scored for transformants after 6 weeks growth. The doses of saccharin used were non-toxic.

[2]A type 3 (malignant) focus of growth has not been observed in previous control experiments involving a total of over 5000 dishes.

6. Induction of Plasminogen Activator

Plasminogen is an enzyme which, when activated, degrades fibrin, a substance involved in the blood clotting process. Many mammalian cell lines, after transformation with viruses or chemical carcinogens, produce a substance that will activate plasminogen. Recently it has been shown that a number of tumor promoters are very potent inducers of plasminogen activator (187). As discussed earlier in this report, some evidence suggests that saccharin has tumor-promoting activity. For this reason, the plasminogen activator assay was included in the saccharin test battery. The plasminogen activator system has thus far been used only for detecting the plant diterpene tumor promoters, and whether it is capable of detecting other types of promoters is not known.

Both the impure and pure samples of saccharin were negative in this assay when tested from 1-50 µg/ml (table 51). Rather low doses of saccharin were tested in this

system compared to the other in vitro methods. However, the levels were more than 1,500 times higher than active doses of the potent promoter 12-O-tetradecanoylphorbol-13-acetate.

Table 51 .—Negative Assay of Saccharin for Induction of Plasminogen Activator

Dose (μg/ml)	Percent Fibrinolysis			
	Impure Saccharin		Pure Saccharin	
	3 hr	*6* hr	*3* hr	**6** hr
A. In the absence of TPA[2]				
o	**0.6**	**5.8**	**0.6**	**5.8**
1	**0.6**	**6.9**	**1.4**	**5.9**
5	0.5	**6.5**	**0.7**	**5.2**
10	1.8	**7.6**	**1.4**	**6.3**
50	0.1	**6.0**	1.7	10.3
B. In the presence of TPA (30 ng/ml)				
o	**56**	**67**	**56**	67
1	**56.2**	**62**	**57.6**	64.6
5	**54.5**	**61.5**	**57.9**	62.6
10	**58.0**	**60.2**	**64.5**	68.7
50	**46.2**	**55.0**	**56.4**	64.9

[1]Data from I. B. Weinstein. HeLa Cells (human cell line) were exposed to saccharin and TPA for 24 hours. Experiments exposing cells for 48 hours yielded similar results (data not shown). Lysates of exposed cells were prepared, and incubated with ^{125}I-fibrin plates in the presence of purified human plasminogen for either 3 or 6 hours, and the percent digestion of ^{125}I was determined (187).
[2]TPA = 12-0-tetradecanoyl-phorbol-13-acetate, a potent promoter of carcinogenesis, and inducer of plasminogen activator.

RESULTS OF TESTS ON MUTAGENIC IMPURITIES IN SACCHARIN

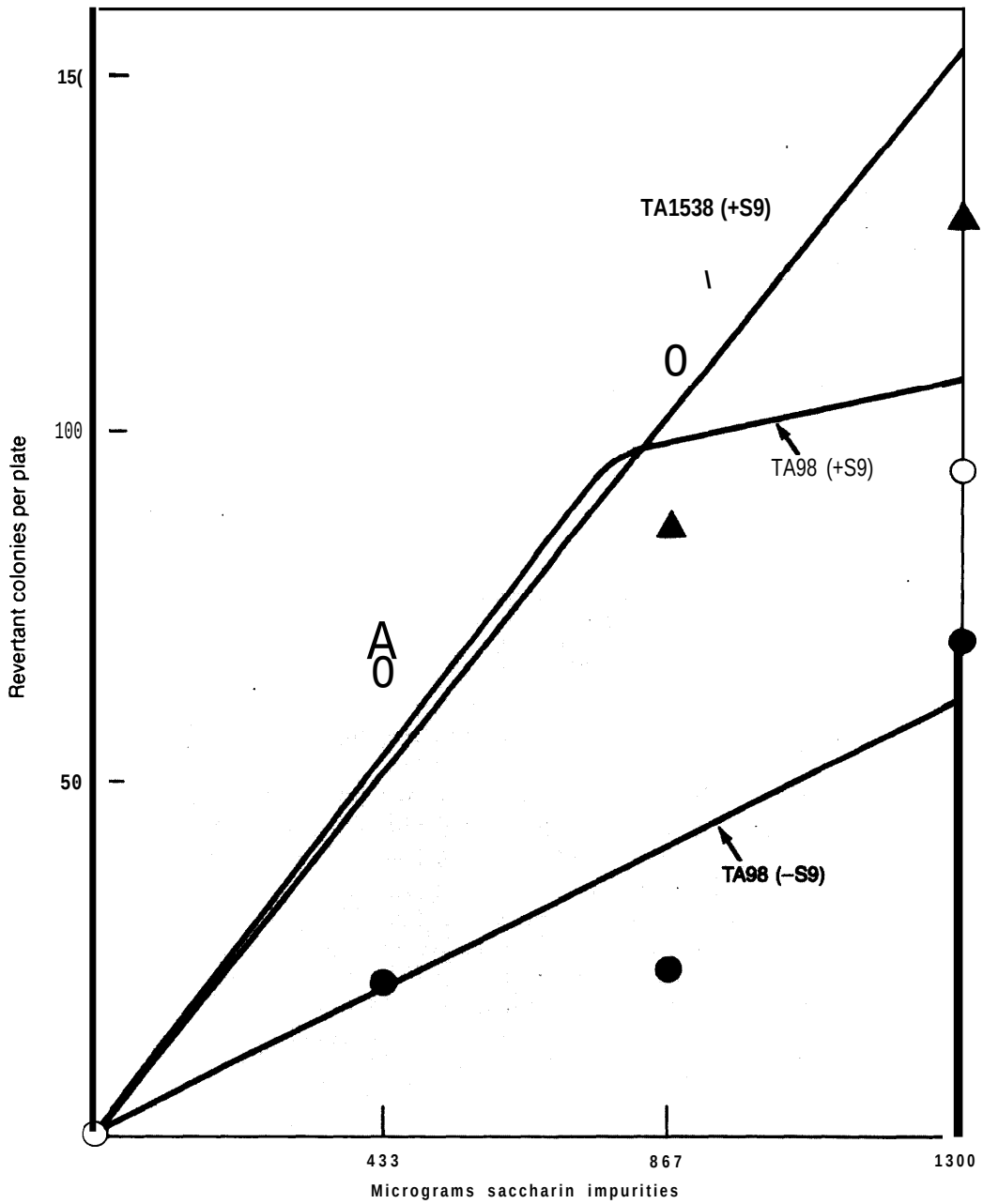
Saccharin used in the recent Canadian carcinogenicity tests, even though highly purified (and much purer than commercial saccharin), still contained a very low level (10 to 20 ppm) of impurities. A number of impurities are present, but as yet none has been chemically identified. Stoltz et al. (162) extracted 1 kg of this saccharin with organic solvents (e.g., chloroform) and recovered about 13 mg of impurities. When tested in the Salmonella/Ames test, this material was weakly mutagenic. These results have been independently confirmed by Yamasaki and Ames (figure 6) using a sample of the chloroform extract (kindly supplied by D. Stoltz).

It is not unreasonable that the weak activity of these impurities was not detected when saccharin itself was tested in the Salmonella/Ames test. About 400/μg of impurities were required in order to detect any mutagenic activity (figure 6). The impurities are present in such small amounts in unpurified saccharin that over 30 g of saccharin would have had to be added to a petri plate in order to expose the bacteria to a mutagenic dose of impurities. In testing saccharin, the highest possible dose was 0.1 g, which produced some toxicity in the bacteria.

More recent, as yet unpublished, work appears to support the conclusion that impure saccharin (and commercial samples of saccharin) contains impurities that are mutagenic in the Salmonella/Ames test. Mutagenic activity was detected in urine of mice after oral administration of several different samples of saccharin. The degree of

mutagenic activity detected varied for the different samples and was least mutagenic for highly purified saccharin (26).

Figure 6-Assay of Saccharin Impurities in the Salmonella/Ames Test



Impurities were from a chloroform extract of saccharin used in the Canadian carcinogenicity test prepared and kindly provided by D. Stoltz. The extract (about 13 mg) was dissolved in 3 ml DMSO. Mutagenesis testing was in the standard plate assay (10) in the presence (20 µl **per plate) or absence of aroclor induced S-9 Mix. For the bacterial tester strains TA98 and TA1538 spontaneous revertants were about 40.**