Tab-30-(1)-IV

Present d as a part of the Status Report on Vinyl Chloride, Dow Chemical of Canada, Limited, Fort Saskatachewan, Alberta to Officials of Department of Health and Environment of Alberta, Canada on September 11, 1980 by V. K. Row .

Good afternoon, Ladies and Gentlemen.

It has been a few years since it was my privilege to discuss with you the toxicological data on vinyl chloride. Today I am not going to burden you with a whole lot of detail but rather I want to highlight some of the observations in toxicology that I believe are pertinent to the assessment of hazard -- or safety as I prefer to think of it.

I believe the most significant recent observations are those dealing with carcinogenicity recently reported by Prof. Maltoni, teratology reported by John et al. and others, and metabolism and pharmacokinetic data reported by Gehring et al. and Butcher et al. Consequently I will focus my attention on the recent developments in these areas.

As I am sure you all know that in January of this year, OSHA submitted a series of questions to the industry about VC and PVC and asked the industry to respond. A comprehensive response to each question was developed and submitted by the industry under auspices of SPI. The Dow Chemical Company had a major part in the preparation of the review. Sections of this submission relevant to the toxicology of VC are included in the document we are filing with you today. In this presentation, we will present a summary of the information pertinent to this meeting and not burden you with all of the detail.

Acute Toxicity. Vinyl chloride, with boiling point of $-13.8^{o}C$ is a gas at ordinary temperatures. Consequently the major route of exposure is by inhalation. Vinyl chloride was investigated as a possible anesthetic in the 1920's to 1930's. But it was considered to be unsatisfactory as an anesthetic because of the high concentrations required (10-20%) which caused circulatory and cardiac effects as well as a fire and explosion hazard. If liquid vinyl chloride were spilled on the skin, there may be severe cooling and frost bite.

With respect to mutagenicity, Vinyl chloride has been shown to be mutagenic in a number of microbial test systems and in vitro Chinese hamster cell cultures, but not in neurospora. However, vinyl chloride is not mutagenic in in vivo dominant lethal assay in mice but is positive in Drosophila. That vinyl chloride is negative in any of the in vitro tests particularly where closed systems are involved or where metabolic activation is inherent in the test procedure is a mystery to me. It is also not surprising that it is negative in the D.L. test since in the whole system, defense and excretory mechanism can come into play.

Chronic Toxicity. Torkelson, et al. report repeated exposures of animals for 4 hours/day, 5 days/weeks for 6 months. At 500 ppm rats showed increased liver weight and histopathological changes. At 100 ppm and 200 ppm exposures, increase in live weight was observed in rats but no changes could be

observed in dogs or guinea pigs. All animal species showed no adverse effect at 50 ppm for 6 months.

Carcinogenicity. As you are aware, investigations sponsored by the industry have been going on in Prof. Maltoni's lab in Bologna, Italy for several years. He reported his most recent data in detail at the OSHA/NIOSH/NIEHS sponsored symposium, March 20 and 21, 1980 in Bethesda, Maryland. I will summarize it here.

In Maltoni's studies, laboratory animals were exposed to vinyl chloride vapour concentrations ranging from 30,000 to 1 ppm or they were fed olive oil solutions of vinyl chloride by gavage.

Slide 1 Summarize the results of Dr. Maltoni's studies. Description.

Slide 2 Recent inhalation work.

Slide 3 Recent oral work.

Slide 4 The lowest cancer effect dose by inhalation is 10 ppm which calculates to approximately 1.5 mg/kg/day. The experimentally determined lowest cancer effect level by oral gavage is 0.3 mg/kg/day. The differences between the two values could be due to: (I) the inexact nature of the assumptions used in the calculation; and (II) oral gavage exposures would be expected to yield higher transient levels of vinyl chloride and its activated metabolites in contact with sensitive reaction sites.

Metabolism. For some time I have felt that it is easy to criticize someone else's conclusions, but it is not always so easy to justify one's own conclusions for personal biases are always present and they are hard to exclude from rational thinking. Consequently, if one is to criticize, he should have some scientific rationale for doing so. It was with this objective in mind that the rather extensive metabolic and pharmacologic studies were begun in the Dow Toxicology Lab on VC and a number of other controversial materials. Other investigators have been intrigued with this approach and many contributions have been made. With respect to VC, most of our work has been published. Basically, from all the data available, it appears that VC undergoes metabolic activation via the route shown in the next few slides.

Insofar as metabolites are concerned these are shown in the next slide.

Why is this important? Because glutathione and sulfhydryl compounds are biochemical scavengers of free radicals, such as are formed in the metabolism of VC. The next slide shows what happens to the sulfhydyl content of the liver following exposure to VC. This is important for its depletion reduces natural defense capability.

The next slide shows the pattern of excretion of C^{14} following various oral doses of C^{14} labeled VC.

The following slide shows the major changes in excretion of VC with dose.

It is well accepted that metabolic activation rates of different species varies greatly and this has much to do with the amount of active metabolites of VC that are available to react in the tissues from given exposures.

Recent studies by Butcher et al. and Gehring et al. have shown that this activation rate in rats is substantially greater than in man. These data would predict that rats are more susceptible to tumor induction by vinyl chloride than humans. This is indeed confirmed by human epidemiological studies.

A series of studies have been conducted in the Dow Toxicology Research Laboratory to assess the hazard associated with exposure to vinyl chloride as well as to investigate the mechanism by which this monomer might exert its toxic effects. The purpose of the studies was to assess the embroyotoxic potential of inhaled vinyl chloride in mice, rats, and rabbits. These studies were supported in part by the Chemical Manufacturer's Association.

Since previous studies in this laboratory suggested that the primary metabolic pathway for vinyl chloride is blocked by ethanol, it was considered possible that administration of ethanol might alter its metabolism in a manner which would enhance its toxic

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TERATOLOGY STUDIES WITH VINYL CHLORIDE*

	Vinyl chloride, ppm	Ethanol, Percent
Mice	500	0
	500	15
	50	0
	50	15
Rats, rabbits	2500	0
	2500	15
	500	0
Mice, rats, rabbits	0	0
Mice, rats, rabbits	0	15**

*John, et al, Tox Appl Pharmacol 30, 497-513 (1977), CMA
**Schwetz, et al, Teratology 18, 385-392 (1978), NIEHS

or teratogenic potential. Thus, some of the vinyl-chloride exposed animals were given 15% ethanol in their drinking water during the days of exposure. The teratogenic potential of inbibed ethanol in these three species was previously studied in our laboratory and reported at the Washington meeting.

In SUMMARY, the results of these studies indicate that exposure of pregnant mice, rats, or rabbits to vinyl chloride by inhalation at concentrations sufficiently high to cause maternal toxicity did not cause a teratogenic effect in any of the three species tested. Fetal effects consisted of delayed skeletal development in mice at 500 ppm, an exposure level which was maternally toxic, and an increase in the incidence of dilated ureter in rats exposed to 2500 ppm.

In mice exposed to 500 ppm of vinyl chloride, the incidence of fetal resorptions was increased over concurrent, negative controls. This incidence was at high end of the range for historical control groups in our laboratory.

Ingestion of 15% ethanol in the drinking water enhanced the toxicity of inhaled vinyl chloride. Fetal body measurements were lower among mice and rats given the combination and the increases in skeletal variants, indicating delayed development, were observed in both species. The incidence of resorptions was increased in rabbits given ethanol, and maternal toxicity was enhanced by ingestion of ethanol in all three species.

In 1979, a report on teratology studies in mice and rats exposed to vinyl chloride appeared in the Hungarian literature. Their work is summarized from an English translation on this last slide. Among mice exposed to 1000 ppm for 4 periods of 2 hours duration each day during organogenesis an increased fetal loss was reported. I'm not sure what was meant by fetal loss. The author reports that no teratogenicity was observed in mice.

Several experiments were conducted with rats. Exposure to 1500 ppm for 24 hrs/day during organogenesis was reported to have no fetal or teratogenic effects apart from an increased liver weight, presumably in the fetuses.

Exposure during the third part of pregnancy produced no deleterious effects, whereas exposure in the first part caused increased fetal mortality and decreased fetal body weights. The exact days of gestation during which exposures were conducted were not indicated. The author reported that vinyl chloride did not potentiate the teratogenicity of trypan blue in rats. Vinyl chloride plus alcohol, reported as given as large doses, during the neurulation period, which I presume to be approximately days 9 through 12 of gestation, caused some skeletal retardation in rats. Thus, these results as summarized in the Hungarian report are quite similar to those observed in our studies with rats and mice.

More recently, a multi-generation reproduction study was sponsored by Consumer Products Safety Commission in which

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1979 UNGVARY

Mice . . .

- 1000 ppm 4 (2 hr) periods/day, organogenesis: Increased fetal loss, no teratogenicity

Rats . . .

- 1500 ppm 24 hours/day, organogenesis: No fetal or teratogenic effect apart from increased liver weight
- Third part of pregnancy: No effects
- First part of pregnancy: increased fetal mortality and decreased fetal body weight
- VCl + trypan blue: No potentiation
- VCl + alcohol, neurulation period: Increased skeletal retardation

Sprague-Dawley/Wistar rats were exposed to vinyl chloride. It did not cause adverse effects in reproductive performance. Dr. Robert Hehir of the CPSC reported on these studies at the Washington meeting.

We have also seen an abstract of a Bulgarian paper which was presented at the 19th International Congress on Occupational Health in which the effects of maternally inhaled VCI on embryonal, fetal, and postnatal development of rats were reported. The authors report that inhalation of 6.15 mg/m^3/day or about 2.5 ppm throughout gestation produced increased fetal mortality, decreased fetal weight and increases in a number of fetal anomalies including hemorrhages, encephalocele, hydrocephalus, and delayed skeletal ossification.

Postnatal effects consisted of biochemical effects (decrease enzymatic activity of dehydrogenases, decreases in enzymatic activity of bile) and injury to the nervous system.

Since this exposure level is equal to only 2.5 ppm, or 1/1000 of the levels tested in our laboratory and since details of the test methods, especially as it pertains to vapor generation and analysis are lacking in the abstracted form, we cannot fully evaluate the findings that are reported, except to say that they are not what we would have expected on the basis of any data available.

Finally, it should be noted that the maximum dose levels tested in the study of John et al., 2500 ppm in rats and rabbits and 500 ppm in mice without teratogenic effects, provide reasonable assurance that the likelihood of VC causing teratogenic effects under present conditions of human exposure is extremely remote.

To our knowledge, there are no reports which clearly relate birth defects in humans to maternal, or for that matter, paternal exposure to VCM. Two relevant studies which have been published [15, 16] indicate no relationship between population exposure to VCM in the area surrounding PVC formulation plants and birth defects. Infante et al., [17] have suggested a causal relationship between paternal exposure to VCM in the industrial setting and pregnancy outcome. The adequacy of the date supplied by Infante et al. was almost immediately questioned[18]. More recently, Haas and Schottenfeld[19] have leveled severe criticisms at the Infante study, the following is extracted from their paper: ".. the inferences made cannot be sustained and little light is shed on the possible association of abnormal pregnancy outcome with paternal occupational exposure to VCM."

Likewise, others have raised serious doubts regarding the validity of the conclusions drawn by the Infante et al. study. For example, Downs, et al.[20] completely discredited the study; their ultimate conclusion was as follows: and I quote --

"...it does not seem possible to salvage anything from study..."

"The authors' analytical methods are invalid since the... test requires that the two rates being compared be independent, and this is not the case..."

"...The methods used by the authors to test significance are inappropriate since pregnancies are clustered..."

"...The misleading conclusions drawn by the authors were brought about through the selection and use of their control group."

"...The purpose of adjusting rates is to make them comparable. Adjusting a rate r to a population A and another rate(s) to a population B, and then comparing the adjusted rates r and s is contrary to the purpose of adjusting rates, and the resulting comparison does not make any sense.

"...the comparisons of rates made by the authors are irrelevant to the hypotheses tested by them and to their corresponding conclusions.

Similarly, McMahon[21] opens his review of the Infante paper with the following comment:

...It is disappointing to see an article of such poor quality as this published in the Lancet. The data are subject to serious criticism on several counts.

and, after detailed point-by-point analysis of the study, presents the following conclusory statement:

> In short, this paper deserves...no consideration whatsoever in weighing the question of whether there is or is not a genetic risk associated with exposure to VCM.

The foregoing reviews, two of which were available in 1977, completely rebut the scientific validity of the paper by Infante, et al. in this matter. In the absence of a scientifically sound defense of this paper by the authors or their scientific peers, continued citation of this paper is inappropriate and misleading to the public, especially.

On a positive note, Edmonds et al. of the U.S. CDC attempted to establish a correlation between parental occupation and congenital effects in and surrounding a PVC plant in West Virginia. They were unable to confirm Infante's conclusions and concluded themselves that "...no relationship between infants with malformations and parent's exposure to vinyl chloride could be established."

Thank you, ladies and gentlemen, for your attention.

V. K. Rowe

Stanford Research Inst.
SRI Project No. LSC-4378-1

Test Strain:
Bacterial-Salmonella typhimurium — TA98, TA100, TA1535, TA1537, TA1538

Yeast-Saccharomyces cerevisiae — D3

Submitted by: J. M. Norris

S-1	Incomplete (-)	Acrylonitrile From tox. study
S-2	+ D3	Vinylbenzyl chloride Lot #06174
S-3	-	Benzene Bay City Pipeline, 5/28/75
S-4	(+) D3	Styrene tars LUWA, Styrene finishing still tar column, 580 Bldg.
S-5	(+) D3	Chloroacetic Acid Batch 145 (1501), 5/28/75
S-6	-	Styrene From inhal. tox. study (5 ppm TBC), 6/75
S-7	-	Chloroacetyl chloride Lot 05285-5; Batch 3105 tank, 5/28/75
S-8	-	50/50 Polyethylbenzene/DOWFROTH* Ref. #408-3-5-1
S-9	-	Decabromodiphenyl Oxide Lot #09014-317
S-10	(+) D3	Perchloroethylene (DOWPER* formulation, W. Dengler, H. Farber, 6/75)
S-11	-	Divinyl Benzene Lot #05135
S-12	-	Pentachlorophenol (tars) 6/12/75
S-13	-	Ethyl benzene "A", 5/28/75
S-14	-	Pentachlorophenol new (DOWICIDE* EC-7) Lot #08024
S-15	-	#2 Fuel Oil Ref. #408-3-5-2, 5/19/75
S-16	Incomplete	Pentachlorophenol old (DOWICIDE* 7) Lot #705611
S-17	Incomplete	Hydroxyethylacrylate From inhal. tox. study, 6/75

\+ = positive
\- = negative
(+) = marginally positive
(-) = marginally negative

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Saccharomyces Cerevisiae D3

The yeast S. cerevisiae D3 is a diploid and is heterozygous for a mutation in an adenine-metabolizing enzyme.[7] Cells homozygous for this mutation produce a red dye when grown on medium containing adenine. Adenine-requiring homozygotes can be generated from the heterozygotes by mitotic recombination. The frequency of mitotic recombination is increased by many mutagens. Mitotic recombination is indicated by the development of colonies with red pigmentation, and the degree of conversion to this pigmented colony indicates the mutagenicity of a compound or its metabolite.[8]

The Saccharomyces test strain from the liquid nitrogen is grown overnight at 30°C with aeration in 1.0% tryptone and 0.5% yeast extract. The cells are washed twice in 0.067 M PO_4 buffer (pH 7.4) and resuspended in the same buffer at a concentration of 10^8 cells/ml.

The in vitro yeast mitotic recombination assay in suspension consists of 5 x 10^7 washed, stationary-phase yeast cells in 1 ml of 0.067 M PO_4 buffer (pH 7.4) and 50 mg/ml of the test chemical (or a fraction of the concentration required to give 50% killing). The suspension is incubated at 30°C for 4 hours. After incubation, the sample is diluted serially in sterile saline and plated on tryptone-yeast agar plates. Plates of a 10^{-3} dilution are incubated for 2 days at 30°C, followed by 2 days at 4°C to enhance the development of red pigment that is indicative of adenine-negative homozygosity. Plates are screened for red colonies or red sectors by scanning the plates with a dissecting microscope at 10 x magnification. Plates of a 10^{-5} dilution are incubated for 2 days at 30°C for determination of the total number of colony-forming units.

The in vitro yeast mitotic recombination assay in suspension with metabolic activation is carried out as above with the addition of the metabolic activation system to the incubation mixture.

Metabolic Activation--Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic mutagens (e.g., dimethylnitrosamine) are inactive unless they are converted to their active form by being metabolized. The metabolic activation systems we use have been described by Ames. Adult male mice are given a single intraperitoneal injection of a polychlorinated biphenyl (Aroclor 1254) at a dosage of 500 mg/kg.[9] Four days after the injection, the food is removed. On the fifth day, the mice are sacrificed.

The livers are removed aseptically and placed in preweighed, sterile glass beakers. The organ weight is determined, and all subsequent operations to the metabolic activation step are conducted in an ice bath. The organ is washed in an equal volume of cold, sterile 0.15M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernate is removed and stored in liquid nitrogen. To the post-mitochondrial supernate are added $MgCl_2$, KCl, glucose-6-phosphate, TPN, and sodium phosphate (pH 7.4).

RESULTS AND DISCUSSION

Table 1 presents the results of the microbiological assays with S. typhimurium. None of the compounds were mutagenic in these assays. We retested S-1 (acrylonitrile) several times because we had information that this compound had given a positive, mutagenic response in some assays. However, as shown in Table 2, S-1 was not mutagenic with S. typhimurium.

Table 3 presents the results of the assays for mitotic recombination in S. cerevisiae D3. One compound--S-2--increased mitotic recombination in these assays. Compound S-2 increased mitotic recombination in three experiments at a concentration of 0.01%, although in one experiment (no. 3) the increase was not significant. Concentrations above 0.02% were toxic. A clear positive response is indicated by an increase of more than threefold in the absolute number of recombinants (mitotic recombinants per ml) as well as the relative number of mitotic recombinants (per 10^5 survivors). Compounds S-4, S-5, and S-10 gave increases in the number of mitotic recombinants per 10^5 survivors. However, none of these compounds increased the number of mitotic recombinants per ml by more than threefold. Therefore, we consider the results to be only marginally positive. None of the other compounds were mutagenic in these assays.

Based on our experience with over 200 chemicals in assays with S. cerevisiae D3, we suggest that Dow Chemical Company consider further tests in other biological assay systems--particularly in vivo or in vitro cytogenetic assays--to measure chromosome breakage. However, because the increase in mitotic recombination induced by S-2 is small compared with the increased obtained with the positive control (1,2,3,4 diepoxybutane), it may be that this compound is of little or no hazard. This conclusion is reinforced by the absence of mutagenicity in all of the Salmonella assays with S-2.

There is no indication of a dose-response in the number of mitotic recombinants per ml with S-4, S-5, or S-10. In previous studies, we have shown that the number of mitotic recombinants per 10^5 survivors increases as survival decreases, even when the decrease in survival is not due to a mutagen. Therefore, the apparent increase induced by these compounds may or may not be an artifact. Since none of these compounds were mutagenic in the Salmonella assays, we believe that if they are mutagens, they are only weakly so.

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Table 1

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
Negative Control	-		17	29	18	37	121
	+		13	19	19	39	128
Positive Controls	-	25			18	19	
4-o-tolylazo-o-toluidine	+				347	306	
N-methyl-N'-nitro-N-nitrosoguanidine		2	3000				4000
S-1	-	0.1	19	21	16	16	82
	-	0.5	15	18	18	15	89
	-	1.0	47	18	27	23	95
	-	5	45	20	17	13	64
	-	10	10	33	18	19	89
	-	50	18	29	23	22	97
	-	100	13	21	30	22	99
	-	500	49	29	24	19	86
	-	1000	43	26	23	21	137
	-	5000	14	26	24	17	74
	+	0.1	13	18	25	24	93
	+	0.5	9	17	21	20	96
	+	1.0	15	25	23	22	110
	+	5	6	16	15	17	95
	+	10	14	34	29	27	146
	+	50	17	27	19	24	111
	+	100	12	22	30	20	122
	+	500	12	23	23	20	133
	+	1000	23	33	16	19	131
	+	5000	19	22	22	24	121

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-2	-	0.1	16	18	49	17	98
	-	0.5	14	19	25	34	145
	-	1.0	12	19	28	30	162
	-	5	10	22	17	39	146
	-	10	9	21	19	24	155
	-	50	12	19	19	27	185
	-	100	5	22	29	33	158
	-	500	4	Toxic	Toxic	Toxic	Toxic
	+	0.1	13	20	11	15	133
	+	0.5	10	17	19	30	173
	+	1.0	12	14	13	30	164
	+	5	13	20	11	30	154
	+	10	8	18	17	26	159
	+	50	10	16	15	35	203
	+	100	7	23	13	14	161
	+	500	6	Toxic	Toxic	14	Toxic

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-3	-	0.1	8	23	27	21	95
	-	0.5	30	21	27	22	140
	-	1.0	20	29	18	16	100
	-	5.0	21	21	12	22	188
	-	10	21	18	22	24	121
	-	50	18	22	12	26	107
	-	100	24	21	19	26	122
	-	500	13	12	11	25	122
	+	0.1	10	18	20	15	111
	+	0.5	18	24	25	31	155
	+	1.0	14	23	20	29	146
	+	5.0	14	18	16	21	133
	+	10	14	19	15	17	143
	+	50	16	22	20	27	152
	+	100	15	22	19	23	122
	+	500	15	19	30	28	152

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-4	-	0.1				20	115
	-	0.5	22	12	19	23	120
	-	1.0	18	10	17	20	115
	-	5	19	16	11	26	127
	-	10	19	16	16	38	130
	-	50	25	12	15	28	115
	-	100	29	10	11	23	125
	-	500	17	12	6	20	99
	+	0.1				15	144
	+	0.5	18	20	16	20	138
	+	1.0	18	10	17	22	103
	+	5	23	15	14	27	130
	+	10	18	16	20	32	96
	+	50	26	12	16	28	113
	+	100	14	13	14	23	155
	+	500	11	11	5	6	117

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-5	-	0.5	10	16	31	28	119
	-	1.0	39	15	29	32	113
	-	5	32	60	31	25	111
	-	10	42	38	21	26	106
	-	50	42	41	25	28	120
	-	100	26	32	21	26	117
	-	500	35	35	20	31	96
	-	1000	13	48	10	19	43
	+	0.5	9	16		19	92
	+	1.0	17	20	25	38	84
	+	5	6	26	39	21	107
	+	10	16	22	36	29	143
	+	50	15	18	30	27	118
	+	100	14	23	23	26	148
	+	500	15	25	25	27	81
	+	1000	2	14	9	13	80

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-6	-	0.1	11	20	25	27	118
	-	0.5	24	19	10	50	163
	-	1.0	15	17	22	21	117
	-	5	18	19	18	45	168
	-	10	20	22	16	55	152
	-	50	23	18	10	60	150
	-	100	19	24	16	50	155
	-	500	12	16	13	40	89
	+	0.1	12	17	15	17	149
	+	0.5	18	15	15	58	170
	+	1.0	12	19	13	13	125
	+	5	16	16	17	54	174
	+	10	11	15	21	51	176
	+	50	13	13	17	53	166
	+	100	11	13	19	40	151
	+	500	8	6	11	28	67

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-7	-	0.5	10	16	31	28	119
	-	1	21	14	23	24	103
	-	5	9	15	42	42	101
	-	10	9	35	24	24	118
	-	50	10	36	19	19	95
	-	100	6	40	20	15	91
	-	500	8	11	15	14	86
	+	0.5	2	13	27	28	108
	+	1.0	11	17	17	24	104
	+	5	12	19	10	16	123
	+	10	8	23	18	21	122
	+	50	9	19	24	23	110
	+	100	6	18	26	26	110
	+	500	18	20	13	13	103

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-8	-	0.5	18	10	11	14	
	-	1.0	26	12	13	18	97
	-	5	18	10	14	19	102
	-	10	26	10	12	21	99
	-	50	15	13	15	15	90
	-	100	10	13	20	16	87
	-	500	Toxic	13	18	12	99
	+	0.5	5	23	19	13	91
	+	1.0	12	17	22	20	92
	+	5	9	21	15	21	95
	+	10	9	21	12	13	104
	+	50	12	16	16	20	84
	+	100	9	18	18	21	92
	+	500	15	17	19	25	102

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-9	-	0.5	10	11	14	16	133
	-	1.0	27	18	14	19	114
	-	5	13	23	16	7	121
	-	10	22	13	19	21	87
	-	50	23	8	16	18	83
	-	100	20	11	18	17	90
	-	500	28	11	10	17	87
	-	1000	20	13	10	14	97
	+	0.5	14	19		24	132
	+	1.0	14	18	22	25	97
	+	5	6	20	16	20	87
	+	10	15	18	17	23	91
	+	50	17	12	23	19	100
	+	100	13	20	21	22	71
	+	500	15	18	16	19	93
	+	1000	16	20	14	24	90

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-10	-	0.5	24	11	15	10	134
	-	1	33	16	16	16	107
	-	5	33	17	12	16	118
	-	10	30	16	12	15	110
	-	50	29	14	12	19	116
	-	100	22	4	12	12	92
	-	500	Toxic	Toxic	4	Toxic	86
	+	0.5	9	16	12	17	136
	+	1	18	19	14	16	100
	+	5	17	19	18	24	113
	+	10	18	23	13	15	106
	+	50	13	13	19	11	115
	+	100	14	Toxic	19	9	93
	+	500	8	Toxic	Toxic	Toxic	Toxic

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-11	-	0.5	8	21	24	18	178
	-	1	9	19	40	20	137
	-	5	12	19	21	15	172
	-	10	9	19	36	28	147
	-	50	10	21	14	21	147
	-	100	8	Toxic	17	16	96
	-	500	7	Toxic	Toxic	Toxic	Toxic
	+	0.5	8	30	31	40	103
	+	1	12	43	32	30	124
	+	5	8	43	40	36	116
	+	10	8	22	51	28	109
	+	50	11	32	52	34	104
	+	100	10	34	Toxic	Toxic	93
	+	500	Toxic	Toxic	Toxic	Toxic	Toxic

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-12	-	0.5	6	7	24	17	150
	-	1	12	11	28	24	171
	-	5	8	4	27	17	129
	-	10	8	9	32	24	151
	-	50	11	11	22	8	103
	-	100	Toxic	Toxic	9	Toxic	88
	+	0.5	5	16	37	42	118
	+	1	3	21	33	29	96
	+	5	5	12	21	27	110
	+	10	5	16	19	29	105
	+	50	4	11	17	27	80
	+	100	Toxic	7	19	13	Toxic

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-13	-	0.5	7	20	10	20	138
	-	1	11	15	20	13	122
	-	5	13	20	20	16	131
	-	10	9	18	21	20	128
	-	50	15	11	12	9	161
	-	100	10	10	10	15	87
	+	0.5	3	23	21	17	118
	+	1	4	22	30	18	131
	+	5	2	20	27	17	148
	+	10	5	24	28	18	128
	+	50	2	29	19	16	117
	+	100	7	19	10	12	83

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-14	-	1	16	21	24	22	143
	-	10	12	18	32	33	143
	-	50	19	20	28	18	111
	-	100	11	15	22	32	125
	-	500	Toxic	Toxic	Toxic	Toxic	Toxic
	+	1	11	46	27	20	121
	+	10	11	24	25	20	124
	+	50	5	37	27	22	92
	+	100	9	39	22	13	87
	+	500	Toxic	Toxic	Toxic	18	Toxic

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-15	-	1	42	25	43	37	103
	-	10	25	85	37	41	94
	-	50	28	67	36	43	112
	-	100	46	80	30	42	109
	-	500	41	61	22	32	81
	-	1000	41	63	14	32	85
	-	5000	32	33	18	22	97
	+	1	16	20	22	20	98
	+	10	14	36	31	21	125
	+	50	8	35	38	43	90
	+	100	11	30	48	32	121
	+	500	7	39	54	38	102
	+	1000	9	36	53	43	91
	+	5000	11	33	46	48	86

Table 1 (Continued)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-16	-	0.25	13	21	31	23	102
	-	0.5	9	24	20	36	101
	-	1.0	14	25	24	32	106
	-	10	6	31	28	26	90
	-	50	8	20	19	25	90
	-	100	1	21	13	24	71
	-	500	Toxic	Toxic	Toxic	Toxic	Toxic
	+	0.25	7	22	33	20	103
	+	0.5	6	15	26	17	103
	+	1	9	17	23	29	124
	+	10	10	21	38	22	105
	+	50	6	22	9	25	82
	+	100	5	25	13	34	91
	+	500	Toxic	Toxic	Toxic	Toxic	Toxic

Table 1 (Concluded)

Compound	Metabolic Activation	µg of Compound Added per Plate	Average Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
S-17	-	0.5	20	14	13	14	83
	-	1.0	22	10	10	8	66
	-	5	10	19	19	9	76
	-	10	9	15	13	7	77
	-	50	12	Toxic	12	Toxic	Toxic
	-	100	Toxic	Toxic	Toxic	Toxic	Toxic
	+	0.5	17	11	12	19	79
	+	1.0	12	19	17	10	79
	+	5	10	19	22	14	96
	+	10	14	12	15	19	57
	+	50	12	Toxic	Toxic	Toxic	55
	+	100	Toxic	Toxic	Toxic	Toxic	Toxic

Table 2

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM--STRAIN TA100

Compound	Metabolic Activation	µg of Compound Added per Plate	Average TA100 Revertants per Plate
Negative Control			138
			145
S-1	-	1000	134
	-	1500	117
	-	2000	125
	-	2500	109
	-	3000	95
	-	3500	109
	-	4000	124
	+	1000	148
	+	1500	166
	+	2000	144
	+	2500	153
	+	3000	169
	+	3500	153
	+	4000	148

Table 3

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE D3

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		6.7	100	6	9.0
		+		4.5	100	5	11.1
	S-2	-	0.01	6.5	97	15	23.1
		+	0.01	4.0	89	13	32.5
		-	0.1	Toxic			
		+	0.1	Toxic			
2	Negative Control	-		7.8	100	10	12.8
		+		6.0	100	4	6.7
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.05	4.1	53	1478	3605
		+	0.05	5.2	87	1286	2473
	S-2	-	0.01	6.5	83	22	33.8
		+	0.01	6.7	112	24	35.8
3	Negative Control	-		5.3	100	3	5.7
		+		5.8	100	5	8.6
	Positive Control 1,2,3,4 Diepoxy-butane	-	.04	5.9	111	761	1290
		+	.04	4.5	78	663	1473
	S-2	-	.01	4.1	77	9	22.0
		+	.01	4.9	84	4	8.2
		-	.02	2.2	42	15	68.2
		+	.02	2.5	43	20	80.0
		-	.03	Toxic			
		+	.03	Toxic			

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^{5} Survivors
1	Negative Control	-		6.7	100	6	9.0
		+		4.5	100	5	11.1
	S-3	-	0.01	7.0	104	13	18.6
		+	0.01	6.7	149	15	22.4
		-	0.1	7.8	116	8	10.3
		+	0.1	5.4	120	15	27.8
		-	0.5	Toxic			
		+	0.5	Toxic			
2	Negative Control	-		7.8	100	10	12.8
		+		6.0	100	4	6.7
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.05	4.1	53	1478	3605
		+	0.05	5.2	87	1286	2473
	S-3	-	0.1	8.0	103	7	8.8
		+	0.1	7.8	130	9	11.5
3	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-3	-	0.2	6.6	78	0	
		+	0.2	1.5	21	4	26.7

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		7.8	100	10	12.8
		+		6.0	100	4	6.7
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.05	4.1	53	1478	3605
		+	0.05	5.2	87	1286	2473
	S-4	-	0.01	5.5	71	5	9.1
		+	0.01	5.3	88	8	15.1
		-	0.1	3.9	50	10	25.6
		+	0.1	3.8	63	12	31.6
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-4	-	0.1	0.5	6	5	100
		+	0.1	2.0	27	7	35.0
3	Netagive Control	-		5.3	100	3	5.7
		+		5.8	100	5	8.6
	Positive Control 1,2,3,4 Diepoxy-butane	-	.04	5.9	111	761	1290
		+	.04	4.5	78	663	1473
	S-4	-	.02	2.6	49	1	3.8
		+	.02	5.9	102	5	8.5
		-	.04	5.6	106	5	8.9
		+	.04	4.8	83	6	12.5
		-	.06	3.9	74	9	23.0
		+	.06	4.5	78	Contaminated	
		-	.08	3.6	68	4	11.1
		+	.08	3.6	62	4	11.1
		-	.10	2.2	42	5	22.7
		+	.10	3.1	53	5	16.1

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^{7} Survivors
1	Negative Control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-5	-	0.01	6.7	118	3	4.5
		+	0.01	7.4	168	5	6.8
		-	0.1	6.6	116	0	
		+	0.1	3.7	84	5	13.5
		-	0.5	Toxic			
		+	0.5	Toxic			
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-5	-	0.01	6.8	80	1	1.5
		+	0.01	7.5	104	2	2.7
		-	0.1	7.2	85	6	8.3
		+	0.1	7.0	97	2	2.9
3	Negative Control	-		5.3	100	3	5.7
		+		5.8	100	5	8.6
	Positive Control 1,2,3,4 Diepoxybutane	-	.04	5.9	111	761	1290
		+	.04	4.5	78	663	1473
	S-5	-	0.1	5.5	104	7	12.7
		+	0.1	4.8	83	5	10.4
		-	0.2	4.5	85	3	6.7
		+	0.2	5.7	98	2	3.5
		-	0.3	2.7	51	2	7.4
		+	0.3	3.8	66	2	5.3
		-	0.4	4.5	85	4	8.9
		+	0.4	4.3	74	7	16.3
		-	0.5	.3	6	0	--
		+	0.5	2.9	50	6	20.7

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml (x 10^{-7})	Percent Survivors	Mitotic Recombinants per ml (x 10^{-3})	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		7.8	100	10	12.8
		+		6.0	100	4	6.7
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.05	4.1	53	1478	3605
		+	0.05	5.2	87	1286	2473
	S-6	-	0.1	.9	12	1	11.1
		+	0.1	2.3	38	1	4.3
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-6	-	0.05	.2	2	3	
		+	0.05	.4	6	3	
3	Negative Control	-		5.4	100	2	3.7
		+		4.6	100	4	8.7
	S-6	-	0.03	1.2	22	5	41.7
		+	0.03	2.4	52	3	12.5
4	Negative Control	-		10.1	100	8	7.9
		+		9.75	100	15	15.4
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.04	9.0	90	1712	1902
		+	0.04	8.2	84	1071	1306
	S-6	-	0.01	9.2	91	7	7.6
		+	0.01	11.0	112	12	10.9
		-	0.02	12.5	124	15	12.0
		+	0.02	14.3	147	12	8.4
		-	0.03	15.5	153	12	9.7
		+	0.03	12.6	129	16	12.7
		-	0.04	6.6	65	8	12.1
		+	0.04	1.6	16	3	18.8

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-7	-	0.01	6.6	116	8	12.1
		+	0.01	3.7	84	8	21.6
		-	0.1	7.1	125	3	4.2
		+	0.1	6.6	150	3	4.5
		-	0.5	Toxic			
		+	0.5	Toxic			
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-7	-	0.01	11.8	139	4	3.4
		+	0.01	8.4	117	4	4.8
		-	0.1	9.6	113	5	5.2
		+	0.1	7.4	102	5	6.8
3	Negative Control	-		10.1	100	8	7.9
		+		9.75	100	15	15.4
	Positive Control 1,2,3,4 Diepoxybutane	-	0.04	9.0	90	1712	1902
		+	0.04	8.2	84	1071	1306
	S-7	-	0.1	8.5	84	5	5.9
		+	0.1	9.8	101	8	8.2
		-	0.2	8.4	83	4	4.8
		+	0.2	11.5	118	13	11.3
		-	0.3	Toxic			
		+	0.3	10.8	110	16	14.8
		-	0.4	Toxic			
		+	0.4	Toxic			

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		7.8	100	10	12.8
		+		6.0	100	4	6.7
	Positive Control 1,2,3,4 Diepoxybutane	-	0.05	4.1	53	1478	3605
		+	0.05	5.2	87	1286	2473
	S-8	-	0.1	7.0	90	8	11.4
		+	0.1	6.3	105	3	4.8
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-8	-	0.2	5.1	60	11	21.6
		+	0.2	4.2	58	7	16.7
3	Negative Control	-		10.1	100	8	7.9
		+		9.75	100	15	15.4
	Positive Control 1,2,3,4 Diepoxybutane	-	0.04	9.0	90	1712	1902
		+	0.04	8.2	84	1071	1306
	S-8	-	0.1	9.9	98	6	6.1
		+	0.1	5.7	58	6	10.5
		-	0.2	11.9	118	6	5.0
		+	0.2	11.4	117	8	7.0
		-	0.3	10.0	100	8	8.0
		+	0.3	7.9	81	12	15.2
		-	0.4	8.8	87	10	11.4
		+	0.4	6.3	65	5	7.9

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml (x 10^{-7})	Percent Survivors	Mitotic Recombinants per ml (x 10^{-3})	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-9	-	0.01	7.0	123	1	1.4
		+	0.01	6.6	150	13	19.7
		-	0.1	4.9	86	18	36.7
		+	0.1	4.8	109	5	10.4
		-	0.5	7.3	128	8	11.0
		+	0.5	5.5	125	1	1.8
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-9	-	0.01	8.4	98	1	1.2
		+	0.01	7.0	97	2	2.9
		-	0.1	5.0	59	3	6.0
		+	0.1	6.3	88	3	5.0
3	Negative Control	-		8.2	100	5	6.1
		+		7.3	100	5	6.8
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.04	9.3	113	950	1021
		+	0.04	5.5	75	1056	1920
	S-9	-	0.2	6.5	79	8	12.3
		+	0.2	4.7	64	8	17.0
		-	0.4	7.5	91	3	4.0
		+	0.4	6.5	89	3	4.6
		-	0.6	5.7	70	3	5.3
		+	0.6	5.2	71	8	15.4
		-	0.8	7.5	91	7	9.3
		+	0.8	7.4	101	8	10.8
		-	1.0	8.7	106	10	11.5
		+	1.0	6.1	84	7	11.5

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml (x 10^{-7})	Percent Survivors	Mitotic Recombinants per ml (x 10^{-3})	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-10	-	0.01	4.8	84	5	10.4
		+	0.01	3.2	73	3	9.4
		-	0.1	Toxic			
		+	0.1	Toxic			
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-10	-	.01	6.0	71	6	10.0
		+	.01	7.2	100	7	9.7
		-	0.1	Toxic			
		+	0.1	Toxic			
3	Negative Control	-		8.2	100	5	6.1
		+		7.3	100	5	6.8
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.04	9.3	113	950	1021
		+	0.04	5.5	75	1056	1920
	S-10	-	0.01	7.0	85	4	5.7
		+	0.01	7.7	105	1	1.3
		-	0.02	3.0	37	8	26.7
		+	0.02	4.0	55	9	22.5
		-	0.03	.9	11	4	44.4
		+	0.03	3.1	42	3	9.7
		-	0.04	.6	7	8	133.0
		+	0.04	1.3	18	5	38.5
		-	0.05	Toxic			
		+	0.05	Toxic			

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors
1	**Negative Control**	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-11	-	0.01	2.7	47	3	11.1
		+	0.01	1.2	27	1	8.3
		-	0.1	Toxic			
		+	0.1	Toxic			
2	**Negative Control**	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-11	-	0.01	2.7	32	4	14.8
		+	0.01	2.7	38	2	7.4

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-12	-	0.01	1.7	30	13	76.5
		+	0.01	1.3	30	5	38.5
		-	0.1	Toxic			
		+	0.1	Toxic			
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-12	-	0.01	2.4	28	2	8.3
		+	0.01	4.1	57	4	9.8
		-	0.1	.2	2	Toxic	
		+	0.1	.7	10	Toxic	
3	Negative Control	-		8.2	100	5	6.1
		+		7.3	100	5	6.8
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.04	9.3	113	950	1021
		+	0.04	5.5	75	1056	1920
	S-12	-	0.002	8.6	105	7	8.1
		+	0.002	8.5	116	10	1.2
		-	0.004	7.7	94	8	10.4
		+	0.004	5.6	77	5	8.9
		-	0.006	5.5	67	7	12.7
		+	0.006	6.4	88	6	9.4
		-	0.008	5.1	62	2	3.9
		+	0.008	5.0	68	6	12.0
		-	0.01	4.9	60	5	10.2
		+	0.01	6.8	93	10	14.7

Table 3 (Continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml (x 10^{-7})	Percent Survivors	Mitotic Recombinants per ml (x 10^{-3})	Mitotic Recombinants per 10^5 Survivors
1	**Negative Control**	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-13	-	0.01	6.9	121	5	7.2
		+	0.01	4.4	100	3	6.8
		-	0.1	Toxic			
		+	0.1	Toxic			
2	**Negative Control**	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-13	-	0.01	6.3	74	3	4.8
		+	0.01	4.9	68	3	6.1
		-	0.1	Toxic			
		+	0.1	Toxic			
3	**Negative Control**	-		5.4	100	2	3.7
		+		4.6	100	4	8.7
	S-13	-	0.02	1.9	35	2	10.5
		+	0.02	1.9	41	3	15.8
4	**Negative Control**	-		8.2	100	5	6.1
		+		7.3	100	5	6.8
	Positive Control 1,2,3,4 Diepoxybutane	-	0.04	9.3	113	950	1021
		+	0.04	5.5	75	1056	1920
	S-13	-	0.01	7.8	95	2	2.6
		+	0.01	7.3	100	2	2.7
		-	0.02	8.8	107	4	4.5
		+	0.02	7.2	99	2	2.8

Table 3 (Concluded)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Mitotic Recombinants per ml ($\times 10^{-3}$)	Mitotic Recombinants per 10^5 Survivors
1	Negative Control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	S-14	-	0.01	1.1	19	3	27.3
		+	0.01	1.1	25	3	27.3
		-	0.1	Toxic			
		+	0.1	Toxic			
2	Negative Control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	S-14	-	.01	4.5	53	8	17.8
		+	.01	4.1	57	3	7.3
		-	.1	.5	6	2	
		+	.1	.8	11	2	
3	Negative Control	-		5.4	100	2	3.7
		+		4.6	100	4	8.7
	S-14	-	.005	1.3	24	2	15.4
		+	.005	1.8	39	1	5.6
4	Negative Control	-		8.2	100	5	6.1
		+		7.3	100	5	6.8
	Positive Control 1,2,3,4 Diepoxy-butane	-	0.04	9.3	113	950	1021
		+	0.04	5.5	75	1056	1920
	S-14	-	0.01	8.2	100	9	11.0
		+	0.01	5.5	75	5	9.1
		-	0.05	2.5	30	4	16.0
		+	0.05	3.4	47	7	20.6

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Final Report

IN VITRO MICROBIOLOGICAL MUTAGENICITY STUDIES OF DOW CHEMICAL COMPANY COMPOUNDS

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SUMMARY

SRI examined 17 Dow Chemical Company compounds for mutagenicity in five strains of Salmonella typhimurium and for mitotic recombination in Saccharomyces cerevisiae D3. The results of the Salmonella tests for all compounds and the S. cerevisiae tests for 14 compounds were presented in an interim report submitted in January. Four compounds required further testing: S-1, S-15, S-16, and S-17 were retested in the S. cerevisiae assay procedure, and the results are reported here.

S-1 and S-15 only marginally increased mitotic recombination, and S-16 and S-17 did not increase mitotic recombination at all.

INTRODUCTION

SRI examined 17 Dow Chemical Company compounds for mutagenicity in five strains of Salmonella typhimurium (TA1535, TA1537, TA1538, TA98, TA100) and for mitotic recombination in Saccharomyces cerevisiae D3. The results of all the Salmonella tests and of 14 of the 17 Saccharomyces tests were reported in January 1976.

SRI retested four compounds for mitotic recombination with S. cerevisiae D3. An Aroclor 1254-stimulated, rat-liver-homogenate metabolic activation system was included in this assay procedure to provide metabolic steps that the yeast are either incapable of conducting or that they do not carry out under the assay conditions. The purpose of this study was to determine whether the compounds caused genetic mutation in microorganisms.

The assay procedure with S. typhimurium has been proven to be 85 to 90% accurate in detecting carcinogens as mutagens, and it has about the same accuracy in identifying chemicals that are not carcinogenic.[1] The assay procedure with S. cerevisiae is about 60% accurate in detecting carcinogens as agents that increase mitotic recombination.[2] The combination of these two assay procedures significantly enhances the probability of detecting potentially hazardous chemicals. However, because the test systems are not 100% accurate, neither a positive nor a negative response proves that a chemical is hazardous or nonhazardous to man.

METHODS

Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98 and TA100

The S. typhimurium strains used at SRI were obtained from Dr. Bruce Ames of the University of California at Berkeley.[3-5] All are histidine auxotrophs (*his*$^-$) by virtue of mutations in the histidine operon. In addition to the mutations in the histidine operon, five of the indicator strains have mutations in the lipopolysaccharide coat (*rfa*$^-$) and deletions that cover a gene involved in the repair of uv damage (*uvrB*$^-$). The *rfa*$^-$ mutation makes the strains more permeable to large molecules, thereby increasing their sensitivity to these molecules. The *uvrB*$^-$ mutation decreases repair of some types of chemically damaged DNA and thereby enhances sensitivity to some mutagenic chemicals. Strain TA1535 is reverted to histidine prototrophy (*his*$^+$) by many mutagens that cause base-pair substitutions. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. TA1537 is more sensitive than TA1538 to mutation by some acridines and benzanthracenes, but the difference is quantitative rather than qualitative. TA100 is derived from TA1535 by the introduction of the R factor plasmid pKM101.[6] The introduction of this plasmid, which confers ampicillin resistance to the strain, greatly enhances the sensitivity of the strain to some base-pair substitution mutagens. We have shown that mutagens such as benzyl chloride and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (known as AF2) can be detected in plate assays by TA100 but not by TA1535. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens--e.g., ICR-191, benzo(a)pyrene, alfatoxin B_1, and 7,12-dimethylbenz(a)-anthracene. TA98 is derived from TA1538 by the addition of the same plasmid, and the plasmid makes this strain more sensitive to some mutagens.

All the indicator strains are stored at -80°C. For each experiment, an inoculum from frozen stock cultures is grown overnight at 37°C in a nutrient broth consisting of 1% tryptone and 0.5% yeast extract. After stationary overnight growth, the cultures are shaken for 3 to 4 hours to ensure optimal growth. Each culture is checked for sensitivity to crystal violet. The presence of the rfa$^-$ mutation makes the indicator strains sensitive to this dye, whereas the parent strain, rfa$^+$, is not sensitive to the dye. However, the mutation is reversible, leading to the accumulation of rfa$^+$ cells in the culture. Therefore, the cells must be tested routinely to ensure their sensitivity to crystal violet. Each culture also is tested by specific mutagens known to revert each test strain (positive controls).

To a sterile 13 x 100 mm test tube placed in a 43°C heating block, we add in the following order:

Assays in agar

(1) 2 ml of 0.6% agar*

(2) 0.1 ml of indicator organisms

(3) 0.5 ml of metabolic activation mixture (optional)

(4) Up to 100 µl of a solution of the test chemical.

For negative controls, we use steps (1), (2), and (3) (optional) and 100 µl of the solvent used for the test chemical.

This mixture is stirred gently and then poured onto minimal agar plates.** After the soft agar has set, the plates are incubated at 37°C for 2 days. The number of his$^+$ revertants (colonies that grow on plates lacking a sufficient amount of histidine to support colony formation) are counted and recorded. Some of the revertants are routinely tested to confirm that they are his$^+$, require biotin, and are sensitive to crystal violet (rfa$^-$).

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* 0.6% agar contains 0.05 mM histidine and 0.05 mM biotin.

** Minimal agar plates consist of 15 g of agar, 20 g of glucose, 0.2 g of $MgSO_4.7\ H_2O$, 2 g of citric acid monohydrate, 10 g of K_2HPO_4, and 3.5 g of $NaHNH_4PO_4.H_2O$ per liter.

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid heterozygous for a mutation in an adenine-metabolizing enzyme.[7] Cells homozygous for this mutation produce a red dye when grown on medium containing adenine. Adenine-requiring homozygotes can be generated from the heterozygotes by mitotic recombination. Many mutagens increase the frequency of mitotic recombination. Mitotic recombination is indicated by the development of colonies with red pigmentation, and the degree of conversion to this pigmented colony indicates the mutagenicity of a compound or its metabolite.[8]

The Saccharomyces test strain from the liquid nitrogen is grown overnight at 30°C with aeration of 1.0% tryptone and 0.5% yeast extract. The cells are washed twice in 0.067M PO_4 buffer (pH 7.4) and resuspended in the same buffer at a concentration of 10^8 cells/ml.

The in vitro yeast mitotic recombination assay in suspension consists of 5 x 10^7 washed, stationary-phase yeast cells in 1 ml of 0.067M PO_4 buffer (pH 7.4) and 50 mg/ml of the test chemical (or a fraction of the concentration required to give 50% killing). The suspension is incubated at 30° for 4 hours. After incubation, the sample is diluted serially in sterile saline and plated on tryptone-yeast agar plates. Plates of a 10^{-3} dilution are incubated for 2 days at 30°C, followed by 2 days at 4°C to enhance the development of the red pigment indicative of adenine-negative homozygosity. To detect red colonies or red sectors, we scan the plates with a dissecting microscope at 10 x magnification. Plates of a 10^{-5} dilution are incubated for 2 days at 30°C for determination of the total number of colony-forming units.

The in vitro yeast mitotic recombination assay in suspension with metabolic activation is conducted as above with the addition of the metabolic activation system to the incubation mixture.

Metabolic Activation--Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic mutagens (e.g., dimethylnitrosamine) are inactive unless they are converted to their active form by being metabolized. Ames

has described the metabolic activation systems we use.[10] Adult male mice are given a single 500-mg/kg intraperitoneal injection of a polychlorinated biphenyl (Aroclor 1254).[10] Four days after the injection, the animals' food is removed. On the fifth day, the mice are killed.

The livers are removed aseptically and placed in preweighed, sterile glass beakers. The organ weight is determined, and all subsequent operations to the metabolic activation step are conducted in an ice bath. The organ is washed in an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl and homogenized with a potter-elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant is removed and stored in liquid nitrogen. To the postmitochondrial supernate are added $MgCl_2$, KCl, glucose-6-phosphate, TPN, and sodium phosphate (pH 7.4).

RESULTS AND DISCUSSION

Tables 1 through 4 present the results of the microbiological assays of each compound for mitotic recombination in S. cerevisiae D3. We tested each compound once to determine the maximum dose tolerable to the microorganism and at least twice more to accurately determine the effects of the chemical on mitotic recombination. A positive response is indicated by a more than threefold increase in the absolute number of mitotic recombinants per ml as well as in the relative number of mitotic recombinants per 10^5 survivors.

Compounds S-1 and S-15 caused an increase in the mitotic recombination frequency that was twofold greater than the normal, expected frequency. However, in repeated testing, neither compound gave a strong positive response and neither compound was mutagenic in the previous Salmonella assay. Therefore, we do not believe S-1 and S-15 are mutagens by these procedures. Because compounds S-16 and S-17 did not increase mitotic recombination in any of these assays, we do not believe these compounds are mutagens by these procedures.

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Table 1

IN VITRO ASSAYS OF COMPOUND S-1 WITH SACCHAROMYCES CEREVISIAE D3

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells/ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
1	Negative control	-		6.7	100	6	9.0
		+		4.5	100	5	11.1
	S-1	-	0.01	7.6	97	10	13.1
		+	0.01	6.4	123	8	12.5
		-	0.1	7.1	106	13	18.3
		+	0.1	5.3	118	15	28.3
		-	0.5	4.8	72	8	16.7
		+	0.5	6.2	138	18	29.0
2	Negative control	-		7.8	100	10	12.8
		+		6.0	100	4	6.7
	Positive control 1,2,3,4-Diepoxybutane	-	0.05	4.1	53	1478	3605
		+	0.05	5.2	87	1286	2473
	S-1	-	0.1	8.3	106	15	18.1
		+	0.1	7.1	118	16	22.5
		-	0.5	Toxic			
		+	0.5	Toxic			

Table 1 (concluded)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells/ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
3	Negative control	-		5.3	100	4	7.5
		+		3.6	100	2	5.6
	Positive control 1,2,3,4-Diepoxybutane	-	0.04	3.4	64	236	694
		+	0.04	4.4	122	225	511
	S-1	-	0.1	4.9	92	1	2.0
		+	0.1	3.9	108	1	2.6
		-	0.2	5.3	100	4	7.5
		+	0.2	3.2	89	2	6.3
		-	0.3	4.0	75	2	5.0
		+	0.3	3.4	94	1	2.9
		-	0.4	3.8	72	4	10.5
		+	0.4	2.9	81	2	6.9
		-	0.5	2.6	49	5	19.2
		+	0.5	3.4	94	5	14.7
4	Negative control	-		6.3	100	10	15.9
		+		6.0	100	8	13.3
	Positive control 1,2,3,4-Diepoxybutane	-	0.04	4.5	71	1125	2500
		+	0.04	4.3	72	818	1902
	S-1	-	0.1	6.0	95	7	11.7
		+	0.1	5.1	85	4	7.8
		-	0.2	7.0	111	4	5.7
		+	0.2	5.3	88	6	11.3
		-	0.3	6.0	95	7	11.7
		+	0.3	4.9	82	10	20.4
		-	0.4	4.5	71	14	31.1
		+	0.4	5.3	88	10	18.9
		-	0.5	3.6	57	12	33.3
		+	0.5	4.2	72	12	28.6

Table 2

IN VITRO ASSAYS OF COMPOUND S-15 WITH SACCHAROMYCES CEREVISIAE D3

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells/ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
1	Negative control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	Positive control 1,2,3,4-Diepoxybutane	-	0.05	.8	14	514	
		+	0.05	.3	3	94	
	S-15	-	0.01	4.7	82	3	6.4
		+	0.01	6.4	145	10	15.6
		-	0.1	Toxic			
		+	0.1	Toxic			
2	Negative control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	Positive control 1,2,3,4-Diepoxybutane	-	0.05	.3	4	152	
		+	0.05	1.6	22	566	3537
	S-15	-	0.01	6.2	73	4	6.5
		+	0.01	6.8	94	2	2.9
		-	0.1	3.3	38	13	39.4
		+	0.1	3.7	51	10	27.0

Table 2 (concluded)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
3	Negative control	-		5.3	100	4	7.5
		+		3.6	100	2	5.6
	Positive control 1,2,3,4-Diepoxybutane	-	0.04	3.4	64	236	694
		+	0.04	4.4	122	225	511
	S-15	-	0.02	4.8	91	4	8.3
		+	0.02	5.3	147	1	1.9
		-	0.06	5.7	108	3	5.3
		+	0.06	5.3	147	4	7.5
		-	0.08	4.2	79	1	2.4
		+	0.08	4.9	136	1	2.0
		-	0.10	4.9	92	4	8.2
		+	0.10	4.3	119	4	9.3
4	Negative control	-		4.8	100	3	6.3
		+		4.6	100	6	13.0
	Positive control 1,2,3,4-Diepoxybutane	-	0.04	6.6	138	583	883
		+	0.04	5.5	120	1198	2178
	S-15	-	0.02	6.6	138	4	6.1
		+	0.02	4.6	100	2	4.3
		-	0.06	5.6	117	3	5.4
		+	0.06	4.8	104	7	14.6
		-	0.08	5.4	113	6	11.1
		+	0.08	5.7	124	5	8.3
		-	0.10	5.7	119	2	3.5
		+	0.10	4.5	98	12	26.7

Table 3

IN VITRO ASSAYS OF COMPOUND S-16 WITH SACCHAROMYCES CEREVISIAE D3

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells/ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
1	Negative control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	Positive control	-	0.05	0.8	14	514	
	1,2,3,4-Diepoxybutane	+	0.05	0.3	3	94	
	S-16	-	0.01	1.2	21	10	83
		+	0.01	2.3	52	3	13.0
		-	0.1	Toxic			
		+	0.1	Toxic			
2	Negative control	-		3.5	100	7	8.2
		+		7.2	100	5	6.9
	Positive control	-	0.05	0.3	4	152	
	1,2,3,4-Diepoxybutane	+	0.05	1.6	22	566	3537
	S-16	-	0.01	3.5	41	10	28.6
		+	0.01	3.8	53	10	26.3
		-	0.1	Toxic			
		+	0.1	Toxic			

Table 3 (concluded)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells/ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
3	Negative control	-		5.3	100	4	7.5
		+		3.6	100	2	5.6
	Positive control	-	0.04	3.4	64	236	694
	1,2,3,4-Diepoxybutane	+	0.04	4.4	122	225	511
	S-16	-	0.004	5.3	100	2	3.8
		+	0.004	4.5	125	1	2.2
		-	0.008	3.9	74	3	7.7
		+	0.008	3.4	64	3	8.8
		-	0.01	3.3	92	3	9.0
		+	0.01	2.5	47	1	4.0
		-	0.02	3.8	72	2	5.3
		+	0.02	3.0	83	4	13.3

Table 4

IN VITRO ASSAYS OF COMPOUND S-17 WITH SACCHAROMYCES CEREVISIAE D3

Exper. No.	Compound	Metabolic Activation	Concentration (x/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
1	Negative control	-		5.7	100	4	7.0
		+		4.4	100	3	6.8
	Positive control	-	0.05	0.8	14	514	
	1,2,3,4-Diepoxybutane	+	0.05	0.3	7	94	
	S-17	-	0.01	3.6	63	1	2.8
		+	0.01	1.7	38	8	47.1
		-	0.1	Toxic			
		+	0.1	Toxic			
2	Negative control	-		8.5	100	7	8.2
		+		7.2	100	5	6.9
	Positive control	-	0.05	0.3	4	152	
	1,2,3,4-Diepoxybutane	+	0.05	1.6	22	566	3537
	S-17	-	0.01	5.9	69	18	30.5
		+	0.01	5.5	76	4	7.3

Table 4 (concluded)

Exper. No.	Compound	Metabolic Activation	Concentration (x/v or v/v)	Surviving Cells per ml ($\times 10^{-7}$)	Percent Survivors	Recombinants per ml ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
3	Negative control	-		5.3	100	4	7.5
		+		3.6	100	2	5.6
	Positive control 1,2,3,4-Diepoxybutane	-	0.04	3.4	64	236	694
		+	0.04	4.4	122	225	511
	S-17	-	0.006	4.8	91	5	10.4
		+	0.006	4.2	117	1	2.4
		-	0.010	4.6	87	1	2.2
		+	0.010	3.5	97	1	2.9
		-	0.020	6.3	119	3	4.8
		+	0.020	4.7	131	5	10.6
		-	0.03	4.7	89	3	6.4
		+	0.03	4.0	111	5	12.5

Stanford Research Inst.
SRI Project No. LSC-4378-1

Activation System: Aroclor 1254 stimulated mouse liver

Test Strain:
Bacterial-Salmonella typhimurium — TA98, TA100, TA1535, TA1537, TA1538
Yeast-Saccharomyces cerevisiae — D3

Submitted by: L. W. Rampy

	Bact-non-act.	Bact-act.	Yeast-non-act.	Yeast-act.	
S-1	-	-	(+)	(+)	Acrylonitrile From tox. study
S-2	-	-	+	+	Vinylbenzyl chloride Lot #06174
S-3	-	-	-	-	Benzene Bay City Pipeline, 5/28/75
S-4	-	-	(+)	(+)	Styrene tars LUWA, Styrene finishing still tar column, 580 Bldg.
S-5	-	-	(+)	(+)	Chloroacetic Acid Batch 145 (1501), 5/28/75
S-6	-	-	-	-	Styrene From inhal. tox. study (5 ppm TBC), 6/75
S-7	-	-	-	-	Chloroacetyl chloride Lot 05285-5; Batch 3105 tank, 5/28/75
S-8	-	-	-	-	50/50 Polyethylbenzene/ DOWFROTH* Ref. #408-3-5-1
S-9	-	-	-	-	Decabromodiphenyl Oxide Lot #09014-317
S-10	-	-	(+)	(+)	Perchloroethylene (DOWPER* formulation, W. Dengler, H. Farber, 6/75)
S-11	-	-	-	-	Divinyl Benzene Lot #05135
S-12	-	-	-	-	Pentachlorophenol (tars) 6/12/75

+ = positiv (all strains unless otherwise indicat d)
- = negativ (all strains unless otherwise indicat d)
(+) = marginally positive (all strains unless otherwise indicated)
(-) = marginally negative (all strains unless otherwis indicated)

5-4-76