Brusick et al. (1991)

MUTAGENICITY STUDIES ON ROTENONE

FINAL REPORT

SUBMITTED TO:

U.S. FISH AND WILDLIFE SERVICE
NATIONAL FISHERY RESEARCH LABORATORY
P.O. BOX 818
LaCROSSE, WISCONSIN 54601

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MD 20795

LBI PROJECT NO. 22063

REVISED 6/24/81

Litton BIONETICS

TABLE OF CONTENTS

I. INTRODUCTION

A. Study Objectives

The goals of this evaluation were to establish the genetic properties of Rotenone in a series of tests which include eukaryotic microbial tests and *in vivo* studies in mice. The following tests were conducted:

- Reverse mutation in haploid yeast strains S138 (frameshift) and S211 (base substitution). The tests were conducted both with and without microsomal activation.
- Mitotic recombination in diploid yeast strain D5. This test was conducted both with and without microsomal activation.
- Mitotic gene conversion in diploid yeast strain D4.
- Somatic cell point mutation analysis in mouse embryonic melamocyte cells *in vivo*.

The data from these studies provides a data base for evaluation of the genotoxicity of Rotenone.

B. Test Compound Description

A sample of 50 gms of Rotenone was received from USDI Fish and Wildlife Service. The sample was purified by Aldrich Chemical Company and indicated to be greater than 97% pure. The Aldrich Chemical Co., Analytical Data Sheet as well as Litton Bionetics, Inc. chemical analysis for stability are attached in Appendix A.

The sample (Batch No. 100287) was received 10/20/80 as two 25 gm bottles. The bottles contained a white powder. Analysis of this material for identity, purity was provided by the manufacturer. Stability in the solvent was determined by LBI Department of Chemistry (see Appendix A).

C. Study Personnel

Study Director:	David Brusick, Ph.D.
Chemist:	Harry Paulin, M.S.
Microbial Geneticist:	D.R. Jagannath, Ph.D.
Senior Microbial Genetics Technician:	Cynthia Rabenold, B.S.
Senior Animal Technician:	Joan McGowan, B.S.

D. Study Dates

Yeast Studies	Initiation	Completion
Mitotic Recombination	01/27/81	02/09/81
Mitotic Gene Conversion	01/27/81	02/09/81
Reverse Mutation	02/06/81	03/16/81

Mouse Studies	Initiation	Completion
Trial 1	11/25/80	01/17/81
Trial 2	01/14/81	03/04/81

II. YEAST STUDIES

A. Introduction

Three types of studies were conducted using haploid and diploid yeast Saccharomyces cervisiae. The two primary studies consisted of reverse mutation analysis in haploid yeast strains and the mitotic recombination assay in a diploid yeast. The strains used in the reverse mutation test respond to mutagens of the two basic mechanistic classes, frameshift (S138) and base pair substitution (S211). The mutation studies were conducted as plate assays employing nonactivation and activation test conditions. It should be noted that activation plate assays with yeast do not typically employ positive activation-dependent controls since compounds giving reproducible responses are not known. We have used compounds such as dimethylnitrosamine or 2-aminoanthracene since these compounds do work occasionally; however, the adequacy of the S9 mix is based on other quality control procedures and not on the response in the plate assay. The only positive controls employed for yeast plate technique[5] are nonactivation controls.

Mitotic crossing-over can be assessed in two ways. One is to measure reciprocal exchanges where both cross-over products can be detected. This is the ideal situation and yeast strain D5 satisfies the conditions of this assessment. The other method of assessment is to measure nonreciprocal recombination (mitotic gene conversion). Nonreciprocal events can be detected either by strain D5 or by strain D4. Strain D5 assays are conducted as suspension assays and as such can be conducted with an adequate S9 activation positive control agent.

Studies with D4 were conducted as plate tests and have the same inherent problems with S9 activation as the reverse mutation studies. Again, the adequacy of the S9 to activate 2-anthramine was established in advance of the tests.

Gene conversion measured in D4 is a nonreciprocal recombination event and is assessed at the tryptophan locus in that strain. This study was not required but was conducted for confirmation of strain D5 observations.

The toxicity of Rotenone to each of the three types of yeast strains was established in preliminary tests and is shown in Figure 1. Results of the yeast tests are described in each of the following subsections.

The significance of induced mitotic crossing-over rests with the resultant expression of recessive genes. The homozygousity of heterozygous alleles provides an opportunity for the production of mutation-like phenomena in somatic cells. While mitotic crossing-over is routinely assessed in fungal cells, it is believed to occur in mammalian cells as well.

Figure 1

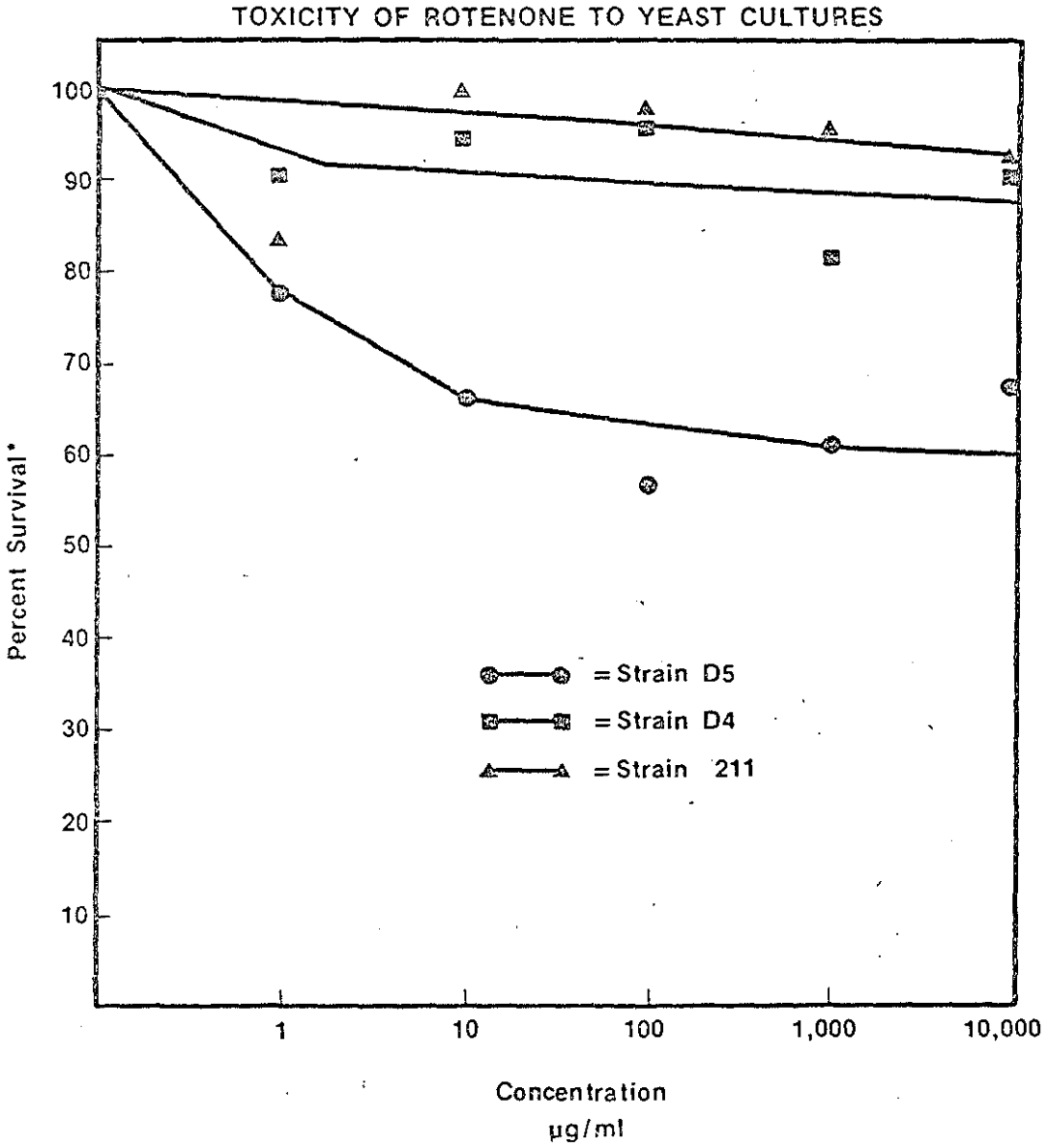


*Plate counts at 10^{-5} and 10^{-6} dilutions of treated cultures. Rotenone was prepared in ethanol as a solvent.

B. MITOTIC RECOMBINATION ASSAY WITH THE YEAST STRAIN D_5

1. OBJECTIVE

The objective of this study was to evaluate a test article for its genetic activity in the yeast strain D_5 with and without a mammalian S9 activation system.

2. RATIONALE

The D_5 indicator strain is a diploid eukaryotic yeast which measures the induction of mitotic recombination. Normally, recombination is measured in meiotic cells, but under defined conditions it can be detected in mitotically dividing cells. The induction of recombination in mitotically dividing cells is measured by the production of homozygous alleles from heterozygous alleles. In the case of strain D_5, the alterations are detected by pigment production in the yeast cells (Zimmermann, 1973). Both nonactivation and activation studies have been reported with D_5 (Brusick and Andrews, 1975). This situation is of considerable importance as most yeast systems do not readily adapt to activation test conditions.

3. MATERIALS

A. Indicator Microorganisms

The Saccharomyces cerevisiae strain D_5 used in this assay was derived from a stock obtained from Dr. F.K. Zimmermann, Technische Hochschule, Darmstadt, German Federal Republic. This strain is diploid and heteroallelic at ade_{2-40} and ade_{2-119} loci.

B. Media

Stocks of yeast strain D_5 are maintained as single colony isolates at 4°C on plates of yeast complete medium. Working stock suspensions of the strain were obtained from stationary phase cultures. A single colony isolate was suspended in saline and an aliquot of the suspension was spread onto yeast complete media plates. The plates were incubated at 30°C for 3-5 days. The lawns were collected into a suspension using phosphate buffer, pH 7.4. An aliquot from this suspension was used in the assays.

3. MATERIALS (Continued)

C. Activation System

(1) S9 homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et al.[1]) was purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

(2) S9 mix

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Organ homogenate from rat liver (S9 fraction)	100 µliters

4. EXPERIMENTAL DESIGN

A. Dosage Selection

The test was run at a minimum of five concentrations. The dose levels were selected on the basis of preliminary toxicity testing (Figure 7).

B. Mutagenicity Testing

The procedure used was a modification of the paper published by Brusick and Andrews[2] and was performed as follows:

(1) Nonactivation assay

To a sterile 17 x 100 mm (or 13 x 100) test tube the following were added:

(a) 0.05 ml of a solution of the test chemical to give appropriate dose.

(b) 0.1 ml - 0.2 ml of indicator organism/s.

4. EXPERIMENTAL DESIGN (Continued)

(c) 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was incubated at 30°C on a rotary shaker for approximately 3 hours. Samples were then removed, diluted in 0.15M saline and plated onto yeast complete medium. All plates were incubated at 30°C for approximately four days. The plates were then refrigerated 1-3 days to intensify the color of the pigmented colonies. The plates were screened for pigmented colonies and sectors using a dissecting microscope with variable magnification. At least 20,000 colonies were screened for cross-over events.

(2) Activation assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details are similar to the procedure for nonactivation assays.

C. Control Compounds

A negative control consisting of the solvent used for the test material was performed in all cases. For negative controls, step "a" of Nonactivation Assays is replaced by 0.05 ml of the solvent. The solvent used to prepare the stock solution of the test material was ethanol. All dilutions of the test material were made using this solvent. The volume of solvent used was equal to the maximum volume used to give the appropriate dose.

A positive control compound known to induce recombination was used in the assays. The concentration and specificity of the compound is given in the following table:

Assay	Chemical	Concentration (µl/ml)
Nonactivation	Ethylmethanesulfonate (EMS)	10.0
Activation	Dimethylnitrosamine (DMN)	100.0

5. RESULTS

The yeast strain D_5 is a diploid strain of Saccharomyces cerevisiae with the genotype $\frac{+ \quad ade_{2-40}}{ade_{2-119} \quad +}$ and is used for studying the genetic events such as reciprocal and nonreciprocal mitotic recombination. The complementing adenine, ade_{-2} markers, ade_{2-40} and ade_{2-119} are used for monitoring the recombinational events in the strain D_5.

Though the reciprocal recombination which involves the exchange of genetic material between two non-sister chromatids during mitosis is a non-mutational genetic event, it results in the homozygocity of the recessive genes. This homozygocity of the recessive genes could bring about deleterious phenotypes which is an indication of chromosomal and hence DNA alteration. The phenotypic expression (or the events) of reciprocal recombination in yeast strain D_5 are the Red-Pink cells representing the homozygocity of the two recessive ade_{-2} alleles; ade_{2-40} and ade_{2-119}. The nonreciprocal recombination which is also known as gene conversion is again a non-mutational genetic event and can occur in dividing or resting cells. At the two-strand stage of nondividing cells of this diploid strain, the non-reciprocal recombination forms red or pink colonies and at the four-strand replicative stage during cell division, gene conversion brings about red-white or pink-white sectored colonies.

These recombinogenic events that occur in mitosis are rare. Nonetheless, when they do occur they could lead to deleterious effects. The results could be considered as "positive" if the total events in a test are equal to or greater than 2.0 times the spontaneous events. An accompanying dose-related effect is also necessary to give confidence to the increase.

The yeast D_5 results are shown in Tables 1 and 2. Table 1 shows nonactivation treatment test results. Rotenone was not toxic to yeast cells even at levels of 10 mg/ml. The responses in Table 1 did not indicate any recombinogenic activity for Rotenone under the condition of the test. All test values were not different from the solvent control value.

Two activation runs are shown in Table 2. The first test appeared to be negative based on internal consistency over the five test levels, but in this run the negative control plates failed to give good growth; therefore, the test was repeated. The second run again did not show any recombinogenic activity. The positive control was slightly low (4X spontaneous) in this run but was adequate.

The interpretation of the data in Tables 1 and 2 is that Rotenone does not induce reciprocal mitotic recombination in yeast strain D_5 either with or without S9 mix.

A third study was conducted but the population plates for scoring were too sparse (<20,000 total) to use and the data were rejected.

TABLE 1

RESULTS OF NONACTIVATION TESTS OF ROTENONE IN YEAST D_5 MITOTIC RECOMBINATION

Treatment[a]	Concentration	Population Scored[b]	Cross-over Events			Events Per 10^3 Survivors
			Reciprocal[c]	Nonreciprocal[d]	Total	
Ethanol Control	100 µl/ml	58,800	2	11	13	0.22
Ethylmethane Sulfonate		37,100	92	603	695	18.73
Rotenone	1 µg/ml	45,800	2	14	16	0.35
Rotenone	10 µg/ml	39,100	1	14	15	0.38
Rotenone	100 µg/ml	42,600	1	20	21	0.49
Rotenone	1,000 µg/ml	54,500	1	11	12	0.22
Rotenone	10,000 µg/ml	38,800	1	9	10	0.26

[a] Solvent employed was ethanol.

[b] Total colonies screened per treatment level for the presence of pigmented areas.

[c] Red/Pink sectors or colonies.

[d] Red/White)
Pink/White)
Complete Pink) Colonies
Complete Red)

TABLE 2

RESULTS OF S9 ACTIVATION TESTS OF ROTENONE IN YEAST D_5 MITOTIC RECOMBINATION

Treatment[a]	Concentration	Population Scored[b]	Cross-over Events			Events Per 10^3 Survivors
			Reciprocal[c]	Nonreciprocal[d]	Total	
Trial 1						
Ethanol Control	100 µl/ml	No Growth	-	-	-	-
Dimethylnitrosamine		2,800	6	85	91	32.50
Rotenone	1 µg/ml	171,200	3	12	15	0.09
Rotenone	10 µg/ml	42,900	2	27	29	0.67
Rotenone	100 µg/ml	55,200	0	22	22	0.39
Rotenone	1,000 µg/ml	47,500	1	15	16	0.33
Rotenone	10,000 µg/ml	40,900	3	15	18	0.44
Trial 2						
Ethanol Control	100 µl/ml	113,300	0	21	21	0.19
Dimethylnitrosamine		94,200	4	71	75	0.80
Rotenone	1 µg/ml	144,300	0	12	12	0.08
Rotenone	10 µg/ml	116,000	1	13	14	0.13
Rotenone	100 µg/ml	109,300	0	26	26	0.24
Rotenone	1,000 µg/ml	116,700	0	10	10	0.09
Rotenone	10,000 µg/ml	123,300	0	22	22	0.18

[a] Ethanol was employed as the solvent.
[b] Total colonies screened per treatment level for the presence of pigmented areas.
[c] Red/Pink sectors or colonies.
[d] Red/White)
Pink/White)
Complete Pink) Colonies
Complete Red)

Test dates:	Initiation	Completion
Trial 1	1/27/81	2/10/81
Trial 2	2/16/81	2/24/81

REFERENCES

1. Ames, B.N., McCann, J. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res., 31:347-364, 1975.

2. Brusick, D., and Andrews, H.: Comparison of the genetic activity of dimethylnitrosamine, ethyl methanesulfonate, 2-acetyl-aminofluorene and ICR-170 in Saccharomyces cerevisiae strains D_3, D_4 and D_5 using in vitro assays and without metabolic activation. Mutation Res., 26:491-500, 1974.

3. Zimmermann, F.K.: A yeast strain for the visual screening for the two reciprocal products of mitotic crossing over. Mutation Res., 21:263-269, 1973.

C. MITOTIC GENE CONVERSION IN SACCHAROMYCES CEREVISIAE STRAIN D_4

1. OBJECTIVE

The objective of this study was to evaluate a test article for its genetic activity in the yeast strain D_4.

2. RATIONALE

D_4 is a diploid strain of Saccharomyces cerevisiae, heteroallelic at the adenine 2 and tryptophan 5 loci. These alleles are stable and show low frequencies of revertibility. Both heteroallelic loci result in nutritional deficiencies (noncomplementing) prohibiting the cells from growing on either minimal or single supplemented media. Mitotic gene conversion is a nonreciprocal event, probably involving alteration of a small number of nucleotide pairs within a single gene, that can generate a wild-type allele at either of the heteroallelic sites. This results in the expression of a functional gene and the loss of the nutritional requirement.

3. MATERIALS

A. Indicator Organism

The Saccharomyces cerevisiae strain D_4 used in this assay was obtained from Dr. F.K. Zimmermann, Technische Hochschule, Darmstadt, German Federal Republic. This strain is diploid and is heteroallelic at ade_2 and trp_5 loci[1].

B. Media

Stocks of yeast strain D_4 are maintained at 4°C on slants of yeast complete medium. Working stock suspensions of the strain are obtained from stationary phase cultures grown at 30°C in yeast complete broth[2]. The selective media consisted of yeast minimal medium[3] supplemented with 30 µg/ml adenine. The overlay agar consisted of 0.6% purified agar with 0.1M Nacl and a trace amount trytophan which is a modification of the method described by Ames et al.[4]

C. Activation System

(1) S9 homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et al.[4]) was purchased from Bionetics Laboratory Products,

3. MATERIALS (Continued)

Litton Bionetics, Inc. and used in this assay. This S9 was pretested for activity with known bacterial mutagens since little or no response was expected with the yeast positive controls under activation conditions based on previous experience.

(2) S9 mix

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Liver homogenate from rat liver (S9 fraction)	100 µliters

4. EXPERIMENTAL DESIGN

A. Dosage Selection

All tests were run at a minimum of four concentrations which were based on preliminary toxicity tests (Figure 1).

B. Mutagenicity Testing

The procedure used was a modification of the paper published by Zimmermann[5] and was performed as follows:

(1) Nonactivation assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath, the following was added in order:

(a) 2.00 ml of 0.6% agar containing 0.1 M NaCl and a trace of tryptophan.

(b) 0.05 ml of a solution of the test chemical to give the appropriate dose.

(c) 0.1 ml - 0.2 ml of indicator organism/s.

(d) 0.50 ml of 0.2M phosphate buffer, pH 7.4.

4. EXPERIMENTAL DESIGN (Continued)

This mixture is swirled gently and then poured into minimal agar plates (see 3B, Media). After the top agar has set, the plates are incubated at 30°C for approximately 4 days. The number of tryptophan convertant colonies growing in the plates were counted and recorded.

(2) Activation assay (optional)

The activation assay was run concurrently with the nonactivation assay but generally does not show an effect with activation dependent agents. The only difference was the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

C. Control Compounds

A negative control consisting of the solvent used for the test material was performed in all cases. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The solvent used to prepare the stock solution of the test material was ethanol. All dilutions of the test material were made using this solvent. The volume of solvent used was equal to the maximum volume used in any of the treatment levels.

Positive control compounds which may be used in the assays are as follows:

Assay	Chemical	Solvent	Concentration per plate
Nonactivation	Ethylmethane sulfonate	None	10 µl
Activation*	2-anthramine (ANTH)	Dimethyl-sulfoxide	2.5 µg
(or) Activation*	Dimethylnitrosame	None	50 µl

*The positive control response is historically inconsistent.

5. RESULTS

The results of the D_4 analysis are shown in Table 3. This assay was included to confirm the findings of the D_5 study.

5. RESULTS (Continued)

Strain D_5 detects not only reciprocal recombination but other observed events such as white colonies with either red or pink sectors and whole red or whole pink colonies, all of which believed to result from nonreciprocal events (Zimmermann, 1973).

The D_4 strain measures only mitotic gene conversion (nonreciprocal events) and the Table 3 responses confirm the D_5 observations that Rotenone does not induce nonreciprocal mitotic crossing over (i.e., mitotic gene conversion).

TABLE 3

RESULTS OF MITOTIC GENE CONVERSION STUDIES WITH ROTENONE IN YEAST D_4

Treatment	Concentration/Plate	Tryptophane Convertants Per Plate
Nonactivation		
Solvent Control	—[a]	108
Positive Control	—[b]	1,103
Rotenone	1	90
Rotenone	10	103
Rotenone	100	116
Rotenone	500	118
Rotenone	1,000	111
Rotenone	2,500	114
Rotenone	5,000	118
Rotenone	10,000	98
Activation		
Solvent Control	—[a]	127
Positive Control	—[b]	139*
Rotenone	1	124
Rotenone	10	126
Rotenone	100	103
Rotenone	500	106
Rotenone	1,000	108
Rotenone	2,500	119
Rotenone	5,000	111
Rotenone	10,000	107

[a] Ethanol
[b] Nonactivation = EMS
Activation = 2-Anthramine

*See Section 4 of text for explanation.

REFERENCES

1. Zimmermann, F.K., and Schwaier, R.: Induction of mitotic gene conversion with nitrous acid, 1-methyl-3-nitro-1-nitrosoguanidine and other alkylating agents in Saccharomyces cerevisiae. Mol. Gen. Genet., 100:63-76, 1967.

2. Brusick, D.: The mutagenic activity of ICR-170 in Saccharomyces cerevisiae, Mutation Res., 10:11-19, 1970.

3. Magni, G. and von Borsrel, R.C.: Different rates of spontaneous mutation during mitosis and meiosis in yeast. Genetics, 47:1097-1108, 1962.

4. Ames, B.N., McCann, J. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with Salmonella/mammalian microsome mutagenicity test. Mutation Res., 31:347-364, 1975.

5. Zimmermann, F.K.: A yeast strain for the visual screening for the two reciprocal products of mitotic crossing over. Mutation Res., 21:263-269, 1973.

D. REVERSE MUTATION INDUCTION IN SACCHAROMYCES CEREVISIAE STRAINS

1. OBJECTIVE

The objective of this study was to evaluate a test material for its genetic activity in an assay using two haploid yeast strains, a frameshift strain and a base-pair substitution mutant with and without the addition of metabolic activation preparations.

2. RATIONALE

In this system two methionine auxotrophs are used, Saccharomyces cerevisiae strains S138, a frameshift mutant and S211, a base-pair substitution mutant. The mutations in these strains are measured by their revertability to methionine prototrophy and form colonies on synthetic minimal medium.

3. MATERIALS

A. Indicator Organisms

The Saccharomyces cervisiae strains S138 and S211c used in this assay were derived by Pittman and Brusick, (1971) from the prototropic haploid strain S288c obtained from Dr. R.K. Mortimer, University of California, Berkeley, USA.

B. Media

Stocks of the yeast strains S138 and S211 are maintained as isolates at 4°C on plates containing yeast complete media. Working stock suspensions of the strain were obtained from stationary phase cultures grown at 30°C in yeast extract peptone.[4] The selective media consisted of yeast minimal medium.[3] The overlay agar consisted of 0.6% purified agar with 0.1M NaCl which is a modification of the method described by Ames et al.[5]

C. Activation System

(1) S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et al.[5]) was purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

3. MATERIALS (Continued)

(2) S9 Mix

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Liver homogenate from rat liver (S9 fraction)	100 µliters

4. EXPERIMENTAL DESIGN

A. Dosage Selection

All tests were run at a minimum of four concentrations which were based on preliminary toxicity tests (Figure 1).

B. Mutagenicity Testing

The procedure used is a modification of the paper published by Brusick and Mayer[1] and was performed as follows:

(1) Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath, the following is added in order:

(a) 2.00 ml of 0.6% agar containing 0.1 M NaCl and 0.02% nutrient broth.

(b) 0.05 ml of a solution of test chemical to give the appropriate dose.

(c) 0.1 ml - 0.2 ml of indicator organism/s

(d) 0.50 ml of 0.2M phosphate buffer, pH 7.4

This mixture is swirled gently and then poured into minimal agar plates (see 3B, Media). After the top agar has set, the plates were incubated at 30°C for 4-7 days. The number of methionine revertant colonies growing in the plates is counted and recorded.

4. EXPERIMENTAL DESIGN (Continued)

(2) Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference is the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which is added in nonactivation assays and a preincubation period of 3 hours. All other details are similar to the procedure for nonactivation assays.

C. Control Compounds

A negative control consisting of the solvent used for the test material was performed in all cases. For negative controls, step 'b' of Nonactvation Assays is replaced by 0.05 ml of the solvent. The solvent used to prepare the stock solution of the test material was ethanol. All dilutions of the test material were made using this solvent. The volume of solvent used is equal to the maximum amount of solvent used in any treatment level.

Positive control compounds they may be used in the assays are as follows:

Assay	Chemical	Solvent	Concentration per plate	Yeast Strains
Nonactivation	Quinacrine Mustard	Dimethyl-sulfoxide	50 µg	S138
	Ethylmethane .sulfonate	None	10 µl	S211
Activation	2-Anthramine	Dimethyl-sulfoxide	50 µg	S211

5. RESULTS

The results of the reverse mutation runs are shown in Table 4. Reverse mutation was assessed in base-pair substitution (S211) and frameshift (S138) strains over 8 concentrations. None of the data indicates that Rotenone is mutagenic in this organism.

The activation runs, without preincubation, were negative. This is typical of yeast in that the only positive responses from activation-dependent chemicals have been with suspension testing. The fact

TABLE 4

RESULTS OF REVERSE MUTATION TESTS WITH ROTENONE IN BASE PAIR SUBSTITUTIONS AND FRAMESHIFT MUTANTS

Treatment	Concentration/Plate	Reverse Mutation Per Plate			
		S211	S138		
		Trial 3	Trial 1	Trial 2	Trial 3
Nonactivation					
Solvent Control	—[a]	14	4	3	2
Positive Control	—[b]	1030	136	169	302
Rotenone	1 µg	14	2	1	3
Rotenone	10 µg	13	1	1	4
Rotenone	100 µg	16	5	2	3
Rotenone	500 µg	15	1	1	2
Rotenone	1,000 µg	15	3	2	9
Rotenone	2,500 µg	10	2	1	3
Rotenone	5,000 µg	9	3	3	3
Rotenone	10,000 µg	17	5	0	5
Activation					
Solvent Control	—[a]	15			3
Positive Control	—[c]	25*			4*
Rotenone	1 µg	22			7
Rotenone	10 µg	12			5
Rotenone	100 µg	17			2
Rotenone	500 µg	11			4
Rotenone	1,000 µg	15			4
Rotenone	2,500 µg	13			4
Rotenone	5,000 µg	14			7
Rotenone	10,000 µg	15			6

[a]Ethanol was used as the solvent

[b]S211 = Ethylmethane Sulfonate
S138 = Quinacrine mustard

[c]S211 = Dimethylnitrosamine

(Trials 1 and 2 were conducted only using S138 and nonactivation.)

*2-anthramine at 50 µg/ml without preincubation

5. RESULTS (Continued)

that the 2-anthramine was negative is not a reflection of the inability of the activation system to biotransform 2-anthramine to a mutagen. The same batch of S9 was shown in preliminary testing to produce mutagenic metabolites from 2-anthramine in bacteria. The difference appears to be in the sensitivity of yeast to the active metabolites.

The conclusions drawn are that Rotenone was not mutagenic in this assay using the standard plate assay method.

REFERENCES

1. Brusick, D.J. and Mayer, V.N.: New developments in mutagenicity screening techniques with yeast. Environmental Health Perspectives, 6:83-96, 1973.

2. Pittman, D. and Brusick, D.J.: Detection of presumptive basepair substitution and frameshift mutations in Saccharomyces cerevisiae. Molec. Gen. Genetics, III:352-356, 1971.

3. Magni, G. and von Borstel, R.C.: Different rates of spontaneous mutation during mitosis and meiosis in yeast. Genetics, 47:1097-1108, 1962.

4. Zimmermann, F.K.: A yeast strain for visual screening for the two reciprocal products of mitotic crossing over. Mutation Res., 21:263-269, 1973.

5. Ames, B.N., McCann, J. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with Salmonella/mammalian microsome mutagenicity test. Mutation Res., 31:347-364, 1975.

III. RODENT IN VIVO SOMATIC MUTATION ASSAY

A. Introduction

Rotenone was tested for its ability to induce somatic mutation in mouse embryo melanocyte cells *in vivo*.

The procedure described here is designed to detect specific-locus somatic mutations in mice (Russell and Major[4]; Fahrig[1]; Russell[3]). Genetic effects such as point mutations, deletions of various amount of chromosomal material, and somatic crossing-over in melanocyte precursor cells measured are in developing embryos that are heterozygous at five specific coat color loci (see Figure A). Melanocyte precursor cells carrying a mutation at any wild-type allele of the five coat color loci will develop into clones of "mutant" melanocytes. Such clones can be readily recognized as coat color mosaic patches on heterozygous mice. Since each developing embryo contains approximately 150 to 200 melanocyte precursor cells, a relative small number of animals is required to provide reliable data in this *in vivo* somatic mutation assay.

The procedure involved treating females carrying 9-to 12-day embryos that were heterozygous at specific coat color loci and subsequently examining the young, after birth, for any mosaic patches (i.e., clones of mutant cells) in differentiated fur. These spots could result from a number of mechanisms, i.e., point mutations, chromosomal deletions, whole chromosomes loss (caused by nondisjunction and other mechanisms) as well as mitotic crossing-over.

B. Study Designs

1. Animals

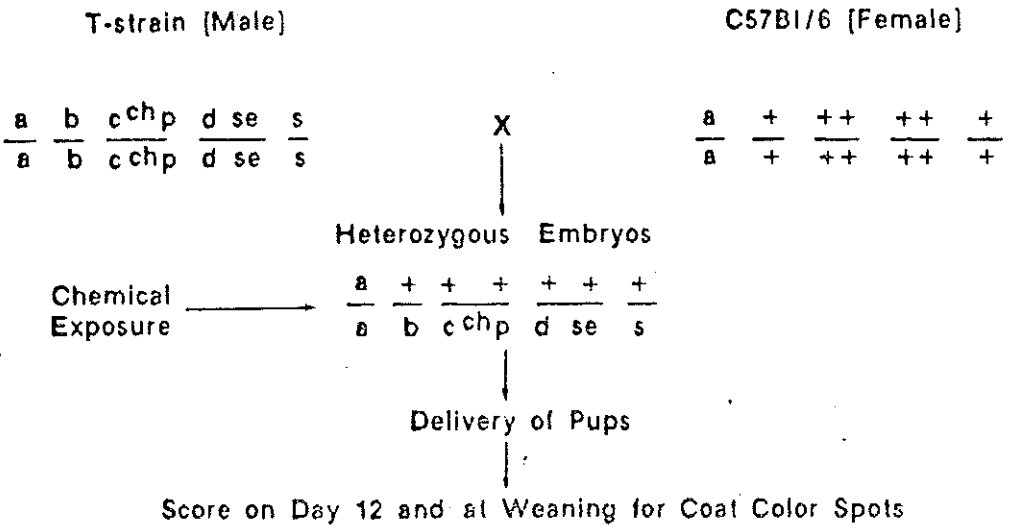
The T-strain of mice used for this assay was originally obtained from Dr. L. Russell at Oak Ridge National Laboratory, Tennessee and has been maintained as a breeding colony at Litton Bionetics, Inc. (LBI). The C57B1/6J female mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. Genotypes of the T-strain males, the C57B1/6J females and their progeny are shown in Figure 1. The basic mating protocol employed in this assay is also presented in this figure.

2. Control Articles

Ethylnitrosourea was used as the positive control article and was administered at 50 mg/kg via a single intraperitoneal (IP) injection. DMSO or corn oil was used as the negative control vehicle and was administered via oral gavage concurrently with the test article.

FIGURE 1

DESCRIPTION OF THE BASIC PROTOCOL FOR MEASURING THE INDUCTION OF SOMATIC IN VIVO MUTATION



Description of Alleles

a = non-agouti
b = brown
c^{ch} = chinchilla
p = pink eyed dilution
d = dilute
se = short ear
s = pibald

C. Experimental Design

1. Animal Husbandry

T-strain mice were individually housed. Female mice were group housed up to 15 per cage during quarantine period, and up to 2 per cage during post-treatment gestation. Certified Purina Laboratory Chow of approximately 6 and 9% fat were given to the T-strain males and the C57B1/6J females, respectively. Light was provided on a 12-hour light/dark cycle.

Plugged females were assigned to study groups at random according to LBI Standard Operating Procedures (SOP) and were identified by ear tags. Control and treatment groups were identified by cage cards.

Sanitary cages and bedding were used throughout. Personnel handling animals or working within the animal facilities were required to wear suitable protective garments. When appropriate, individuals with respiratory or other overt infections were excluded from the animal facilities.

2. Mating Scheme

During the mating period, each T-strain male was mated to two C57B1/6J females. The females were checked for copulation plugs daily. Plugged females were ear-tagged and randomly assigned to one of the control and treatment groups. The mating period was continued until at least fifty plugged females were obtained for each of the treatment groups and the control groups. This proceudre was used for each of the two trials.

3. Dose Selection

Dose selection was based upon toxicity information developed prior to the initiation of testing. No oral acute LD50 by gavage was available. Rotenone was administered to mice by oral gavage in both corn oil (as a suspension) and DMSO (as a solution). The LD50 values obtained were markedly different. Rotenone prepared in corn oil was essentially non-toxic producing death in 1 of 4 female mice after 14 days following a single dose of 1000 mg/kg and no deaths at 330 mg/kg. Rotenone prepared in DMSO produced death in 6 of 6 female mice dosed acutely with 330 mg/kg, 100 mg/kg and 33 mg/kg. The DMSO LD50 was near 3 mg/kg; however, the data generated in these studies was not amenable to exact calculation of an LD50 using the methods stated in the protocol.

It was decided to use the DMSO solution of Rotenone for the somatic mutation test. The reason being that the test article went into solution and could be given at levels showing some degree of toxicity. The dose levels selected were 1.0, 0.17 and 0.05 mg/kg/day. Later a level of 0.5 mg/kg/day was included. A single experiment at 1000 mg/kg/ day for 4 days was run using the corn oil suspension for testing perspective with the corn oil suspension. This single dose level was run to ensure that no unusual findings would be missed by using a suspension of the Rotenone. The DMSO and corn control was considered adequate to cover this single level test.

Table 5 provides a summary of dosing information for this study.

4. Route of Administration and Treatment

The initial run was conducted with Rotenone prepared in DMSO. The solution was administered by gavage to plugged female C57B1/6 mice on days 8, 9, 10 and 11 of gestation. The second trial was conducted essentially the same as the first except that the DMSO was diluted to 50% with corn oil to reduce the total volume of DMSO given to the mice. Solvent controls were conducted with each run. Ethylnitrosourea (ENU) dissolved in 0.9% saline was administered at 50 mg/kg (IP) on day 10 of gestation (see Table 5). The single 1000 mg/kg dose in corn oil was also administered by gavage.

Toxic signs and mortality were recorded during the dosing period and are recorded in Table 6.

5. Spot Observation

All exposed embryos were allowed to develop to birth. At birth, the litter size was determined, and external morphologies of the pups were examined for any gross abnormalities. The newborns were scored for spots on day 12 and again at the time of weaning. No additional spots were noted at the second scoring. White spots near the ventral midline (white midventral spots) were recorded separately from all other coat color spots because they represent melanocyte toxicity and not mutation induction (Russell[3]). Animals with mutant spots were scored and the pelts saved. Frequencies of coat color spots in the treated groups were compared against the negative control according to Fisher's exact test to determine any significant increase. Generally, the increases over the background that are statistically significant at the 5% level and a dose-response relationship are considered positive response.

E. Results

The results of the tests are detailed on Tables 7 and 8. Table 7 shows the two runs separately and Table 8 shows the combined study. There were no indications of Rotenone-induced somatic mutation induction.

Some observations should be noted concerning the test results.

- There appeared to be some depression of fertility and or embryonic development both from the DMSO and from the Rotenone. The two groups of animals with the highest percent fertility were the two positive control groups which received neither DMSO or Rotenone. The 30-40% fertility is more typical for the C57B1/6xT cross in our experience.

- Rotenone while toxic to the pregnant female mice, did not produce significant increases in midventral white spots which is an indication of embryo melanocyte toxicity; the only exception to this generalization was the single high

TABLE 5

DOSING INFORMATION

Sponsor U.S. Fish & Wildlife Services | Study ID Somatic Mutation | Initiation Date 11/25/80* 01/14/81†

Project No. 22063 | Strain/Species C57BL/6 Mice Sex ♀ | Termination Date 01/17/81* 03/04/81†

Compound Rotenone | Breeder Charles River | Location Kensington, MD

Assay No. 5410 | Purchase Order No. 100729* and 101739† | Room Nos. 11x (dosed W5b)

Treatment	Vehicle	Dosage[b] [=mg/kg]	Number of Administrations	Route of Administration	Volume/ Animal [ml]	Total Applied Dose [mg/kg]	Animal[c] Numbers
Dimethyl Sulfoxide (DMSO)	NA	NA	4	PO	0.1	NA	1223-1313*
Corn Oil & DMSO	NA	NA	4	PO	0.1	NA	2739-2788†
Rotenone	DMSO	0.05	4	PO	0.1	0.20	1323-1413*
Rotenone	DMSO & Corn Oil	0.05	4	PO	0.1	0.20	2789-2838†
Rotenone	DMSO	0.17	4	PO	0.1	0.68	1423-1512*
Rotenone	DMSO & Corn Oil	0.17	4	PO	0.1	0.68	2839-2863†
Rotenone	DMSO	0.5	4	PO	0.1	2.00	1523-1612*
Rotenone	DMSO	1.0	4	PO	0.1	4.00	1314-1322* 1414-1422* 1513-1522* 1613-1622* 1713-1722*
Rotenone	DMSO & Corn Oil	1.0	4	PO	0.1	4.00	2684-2913†

[a] Plus two untagged mice used to compensate for mice that died during dosing.

[b] Dosage based on LD_{50} determination.

[c] Mice found dead during dosing. See Table 6.

TABLE 5 (Continued)

DOSING INFORMATION

Treatment	Vehicle	Dosage[b] [=mg/kg]	Number of Administrations	Route of Administration	Volume/ Animal [ml]	Total Applied Dose [mg/kg]	Animal[c] Numbers
Rotenone	Corn Oil	1000	4	PO	0.2	4000	2939-2988†
Ethylnitrosurea	0.9% Saline	50	1	IP	0.1	50	1623-1712* 2914-2938†

[b]Dosage based on LD_{50} determination.
[c]Mice found dead during dosing. See Table 6.

dose of Rotenone (1000 mg/kg) in corn oil. We have no explanation for this melanocyte toxic response.

- Because of the fertilitly depression of Rotenone and DMSO, the total number of living pups that could be scored was lower than anticipated. However, even with this problem, the results were clearly negative based on the control frequencies recorded for this study.

- A suspension of Rotenone in corn oil given at a level of 1000 mg/kg/day did not show any increase in recessive spots.

The toxicity and mutation frequencies are summarized in tabular and graphic form in Table 9 and Figure 2.

No other unusual findings were noted in the study and the data are interpreted as negative.

TABLE 6

A. Summary of Female Mice Found Dead During Treatment

Treatment Level	Animal Nos.
Negative Control[a]	1223, 1233, 1238, 1300, 1308
0.05 mg/kg DMSO	1326, 1345
0.05 mg/kg Corn Oil and DMSO	2802
0.17 mg/kg DMSO	1442, 1445, 1447, 1476, 1496, 1511, 1512
0.17 mg/kg Corn Oil and DMSO	2857
0.5 mg/kg DMSO	1560, 1567, 1572, 1574, 1590, 1591, 1599, 1603, 1605, 1609, 1610
1.0 mg/kg DMSO	1315, 1316, 1321, 1514, 1515, 1614, 1617, 1718
1.0 mg/kg Corn Oil and DMSO	2871
1000 mg/kg Corn Oil	2939, 2940, 2942, 2944, 2945, 2951, 2952, 2956, 2957, 2984, 2988

B. Toxic Signs Noted

No toxic signs were noted in the lower dose level of studies using DMSO as the vehicle (0.05 and 0.17 mg/kg). Several animals in the 0.5 mg/kg dose group were noted as appearing lethargic and clammy after dosing. No specific effects were otherwise recorded for the group. At 1 mg/kg/day several animals died following convulsions and others again appeared lethargic and clammy following dosing.

[a]DMSO only.

TABLE 7

SUMMARY OF TWO RUNS OF THE MOUSE SOMATIC MUTATION TEST FOR ROTENONE

Treatment	Applied Dose [mg/kg]	Pregnant♀/Mated ♀ [%]	Number of Animals			Observed Coat Color Spots	
			Born[ALS][a]	Surviving to 12 Days[%]	Abnormal Morphology	White Midventral[%]	RS[%][b]
DMSO (dimethyl sulfoxide)	NA	10/91 [11%]	47 [4.7]	29 [61.7%]	♀ with umbilical mass	0 [0%]	0 [0%]
Corn Oil & DMSO	NA	11/50 [22%]	64 [7.1]	55 [85.9%]	0	0 [0%]	3 [5.5%]
Rotenone (DMSO)	0.05	11/91 [12.1%]	44 [4.0]	36 [81.8%]	0	2 [5.6%]	1 [2.8%]
Rotenone (Corn Oil & DMSO)	0.05	16/50 [32%]	103 [6.4]	71 [68.9%]	♂ with umbilical mass ♂ with bent tail	1 [1.4%]	3 [4.2%]
Rotenone (DMSO)	0.17	19/90 [21%]	90 [4.7]	76 [84.4%]	0	0 [0%]	3 [3.9%]
Rotenone (Corn Oil & DMSO)	0.17	4/25 [16%]	24 [8.0]	22 [91.7%]	0	0 [0%]	0 [0%]
Rotenone (DMSO)	0.50	9/90 [10%]	48 [5.3]	39 [81.3%]	0	0 [0%]	0 [0%]
Rotenone (DMSO)	1.0	6/50+ [12%]	25 [4.2]	13 [52.0%]	0	0 [0%]	0 [0%]
Rotenone (Corn Oil & DMSO)	1.0	13/49+ [26.5%]	65 [7.2]	44 [67.7%]	0	0 [0%]	2 [4.5%]
Rotenone (Corn Oil)	1000.0	9/50 [18%]	50 [6.3]	49 [98.0%]	0	7 [14.3%]	1 [2.0%]
Ethylnitrosurea (ENU)[1]	50.0	28/90 [31%]	113 [4.3]	89 [78.8%]	0	22 [24.7%]	21 [23.4%]
Ethylnitrosurea (ENU)[2]	50.0	8/25 [32%]	61 [7.6]	52 [85.2%]	0	5 [9.6%]	5 [9.6%]

[a]Average litter size.
[b]Recessive coat spot (somatic mutation). Animals were examined for spots twice and the results were identical for each scori
[1]Test 11/25/80 - 1/17/81.
[2]Test 1/14/81 - 3/4/81.
+One ♀ missing from cage after last dose.

TABLE 8

SUMMARY OF THE MOUSE SOMATIC MUTATION TEST FOR ROTENONE

Treatment	Applied Dose [mg/kg]	Pregnant♀/Mated ♀ [%]	Number of Animals Born[ALS][a]	Number of Animals Surviving to 12 Days[%]	Number of Animals Abnormal Morphology	Observed Coat Color Spots White Midventral[%]	Observed Coat Color Spots RS[%][b]
Solvent Negative Control	NA	21/141 [14.9]	111 [5.8]	84 [75.7]	♀ with umbilical mass	0 [0]	3 [3.6]†
Rotenone	0.05	27/141 [19.1]	147 [5.4]	107 [72.8]	♂ with umbilical mass ♂ with bent tail	3 [2.8]	4 [3.7]
Rotenone	0.17	23/115 [20.0]	114 [5.2]	98 [86.0]	0	0 [0]	3 [3.9]
Rotenone	0.50	9/90 [10.0]	48 [5.3]	39 [81.3]	0	0 [0]	0 [0]
Rotenone	1.0	19/99 [19.2]+	90 [6.0]	57 [63.3]	0	0 [0]	2 [4.5]
Rotenone	1000.0*	9/50 [18.0]	50 [6.3]	49 [98.0]	0	7 [14.3]	1 [2.0]
Ethylnitrosurea	50.0	36/115 [31.3]	174 [5.1]	141 [81.0]	0	27 [19.1]	26 [18.4]

[a]ALS = Average litter size.
[c]RS = Recessive spot somatic mutation.
+One ♀ missing from cage after last dose.
*A single dose level using corn oil as the vehicle. (See page 26 for a discussion).
†One animal with whole body mosaic black and white coat color not included in the number of mice with spots.

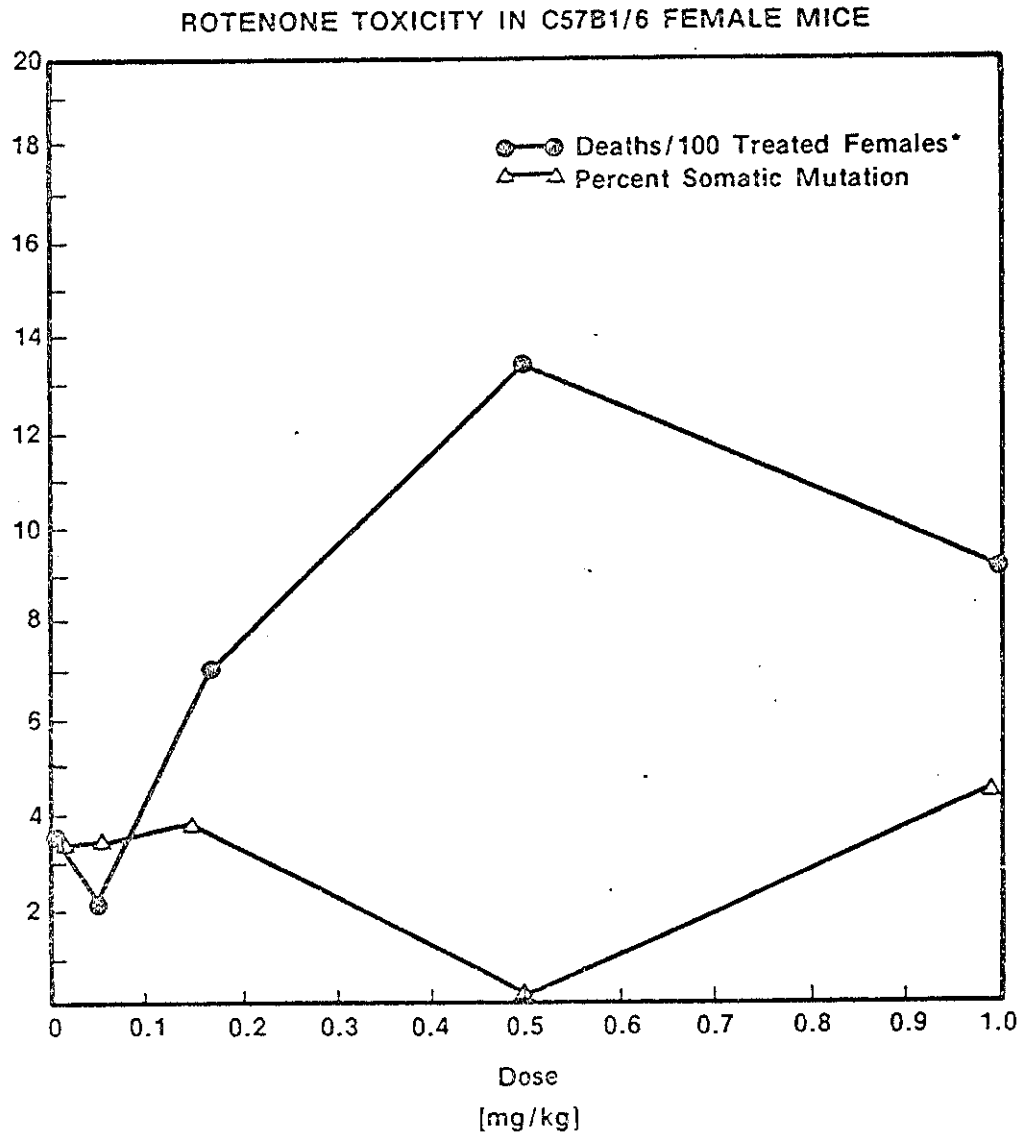
TABLE 9

SUMMARY OF TOXICITY AND GENOTOXICITY OF ROTENONE IN FEMALE C57B1/6 MICE

Treatment	No. Females Mated*	Deaths During Treatment [%]*	Coat Color Spots	
			MVC [%]	Other [%]
DMSO Controls	141	5 [3.5]	0 [0.0]	3 [3.6]
Rotenone 0.05 mg/kg	141	3 [2.1]	3 [2.8]	4 [3.7]
0.17 mg/kg	115	8 [7.0]	0 [0.0]	3 [3.9]
0.50 mg/kg	90	11 [12.2]	0 [0.0]	0 [0.0]
1.00 mg/kg	99	9 [9.1]	0 [0.0]	2 [4.5]
Ethylnitrosurea 50 mg/kg	115	0 [0.0]	27 [19.1]**	26 [18.4]**

*Includes data from both experiments run at each level.
**Significant from the control at $p \leq 0.01$ using the Fishers Exact Test.

Figure 2



*Deaths during exposure period.

REFERENCES

1. Fahrig, R.: A mammalian spot test: Induction of genetic alterations in pigment cells of mouse embryos with X-rays and chemical mutagens. Mol. Gen. Genetics, 138:309-314, 1975.

2. Finney, D.J.: Probit Analysis. Cambridge University. Press, 1971.

3. Russell, L.B.: Validation of the in vivo somatic mutation method in the mouse as a prescreen for germinal point mutations. Arch. Toxicol., 38:75-85, 1977.

4. Russell, L.B. and Major, M.H.: Radiation-induced presumed somatic mutations in the house mouse. Genetics, 42:161-175, 1975.

IV. SUMMARY ANALYSIS

A. Introduction

The wide use of rotenone as an environmental pesticide has generated a need to evaluate this material for its genotoxic effects. The selection of eukaryotic microbial assay systems and an in vivo mammalian test model permitted the evaluation to cover a wide range of genetic endpoints including mutation, somatic crossing-over and cytotoxicity.

B. Microbial Responses

Three distinct endpoints were studied in strains of yeast Saccharomyces cerevisiae. Rotenone solubilized in ethanol was not found to be highly toxic up to 10,000 µg/ml or plate. It was also inactive in tests designed to measure mutation, mitotic recombination and mitotic gene conversion. Tests employing microorganisms employed both direct and S9 activated exposure conditions. While S9 activation of chemicals in yeasts tests is not common, the results tended to preclude formation of a genetically active metabolite.

C. In Vivo Mammalian Responses

The mouse coat color spot test for somatic mutation is considered to detect chromosomal loss and mitotic crossing-over as well as specific locus somatic mutation. Rotenone was not found to induce significant increases of coat spots in mice rate concentrations up to 1 mg/kg (prepared in DMSO) or at a single dose of 1000 mg/kg (prepared in corn oil). The only finding of possible significance was an increase in midventral white spots (indicators of melanocyte toxicity not mutation) in the 1000 mg/kg Rotenone exposure. The interpretation of this observation would require additional testing, but the spots cannot be considered a consequence of genotoxic events.

D. Assessment

Rotenone was not active in either microbial tests for mutation or mitotic recombination and failed to produce evidence of somatic mutation in the mouse spot test.

APPENDIX A

CHEMICAL INFORMATION FOR THE ROTENONE SAMPLE EMPLOYED IN THIS STUDY

United States Department of the Interior
FISH AND WILDLIFE SERVICE
National Fishery Research Laboratory
P.O. Box 818
La Crosse, Wisconsin 54601

IN REPLY REFER TO:

October 14, 1980

Dr. Sky Benson
Genetics Department
Litton Bionetics
5516 Nicholson Lane
Kensington, Maryland 20795

Dear Dr. Benson:

We are sending you two 25g bottles of purified rotenone for the mutagenicity tests. The rotenone was purified by Aldrich Chemical Company. Our analysis of this material indicates greater than 97% purity.

Sincerely yours,

John L. Allen
Chemist

JLA:bl

5-4/1C

Chemists Helping Chemists in Research and Industry

aldrich chemical company, inc.

ANALYTICAL DATA

Date November 12, 1980

Our: R200-1 Rotenone, 97%

Batch No.: 100287

Analytical Results:

Appearance	white powder		
m.p.	165-167° C.	b.p.	
n_D^{20}		$[\alpha]_D^{22}$	-231.0° (c=2% in Benzene)

Spectral Data:

I.R. Conforms to structure and standard as illustrated on page 794 D of the "Second Edition" of "The Aldrich Library of Infrared Spectra".

U.V.

N.M.R.

Assay: TLC: 1 trace impurity

V.P.C.

Titration

Other:

CJP/cw

Charles J. Pouchert,
Vice President-Quality Contro

P.O. Box 355 Milwaukee, Wisconsin 53201 USA Telephone (414) 273-3850 Cable Aldrichem TWX 910-262-3052 Telex 26-

Stability of Rotenone Preparation

1. Method and Results

An attempt was made to determine the stability of the rotenone preparation used in this study by scanning ultraviolet spectrophotometry.

Calibration was performed using a rotenone reference standard (lot No. 578-R5P-1424; 97.3% pure) supplied by the Penick Corporation. A reference solution containing 1.46 ppm rotenone (corrected for purity) was prepared using 1.6% v/v dimethylsulfoxide (DMSO) in ethanol. The DMSO used was the same lot (Sigma #59C-0490) as used to formulate the test material for mutagenic bioassay.

A reagent control consisting of 1.6% v/v DMSO in ethanol was also prepared.

The test formulation (ID #5410) was labelled as containing 0.096 mg/ml rotenone per mililiter of DMSO. An aliquot of the formulation was taken immediately after preparation and diluted with ethanol so that the final concentration of DMSO was also 1.6% v/v in ethanol; the calculated concentration of rotenone was 1.54 ppm.

Spectrophotometric analysis was performed with a Beckman Model 3600 recording spectrophotometer, scanning in 1 cm path length cuvettes from 340 to 280 nm. All scans were made against the reagent control in the reference cell. Scans were obtained for the reagent control, the rotenone reference solution and the diluted test formulation. For each scan the absorbances at 304, 294 and 284 nm were measured. The absorbances found for the reference solution and the test formulation were corrected for the absorbances of the reagent control.

The absorbance factor (F) was calculated for the reference solution and the test formulation from the following expression:

$$F = [2A_{294nm} - (A_{284nm} + A_{304nm})]$$

where A refers to the net absorbance at the indicated wavelength.

The amount of rotenone found in the test formulation was determined from:

$$\text{mg rotenone/ml} = C \times \frac{F_u}{F_s} \times D$$

where C = concentration of reference solution (1.46 ppm)

F_u = absorbance factor of test formulation

F_S = absorbance factor of reference solution

D = factor correcting for dilution of test formulation

The value obtained for the test formulation was 0.0608 mg/ml, or 63.3% of the labelled concentration.

2. Discussion

The result obtained by analysis of the test formulation is extremely questionable. The absorbance values obtained for the reference standard and the test formulation were extremely low (<0.1 optical density units), while the absorbance values of the reagent control were significant (as much as 41% of the standard or test formulation absorbance. Therefore, any small absolute variation in background absorbance from the DMSO in the solution causes a large variation in the net result. Moreover, examination of the scans showed that there was little differentiation of maxima and minima in the reference spectrum at this level of concentration. Decreasing the dilution volume did not seem feasible, since the absorbance of DMSO itself was approaching a cut-off value below 300 nm.

In short, the lack of sensitivity and extreme variability of the procedure made it impossible to obtain data with any suitable degree of precision and/or accuracy. Because of the almost universal miscibility of DMSO with other solvents, any attempts to isolate rotenone from the sample matrix was beyond the scope of the study.