CENTRAL RESEARCH LIBRARY
DOCUMENT COLLECTION

OAK RIDGE NATIONAL LABORATORY
operated by
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
for the
U.S. ATOMIC ENERGY COMMISSION

LOCKHEED MARTIN ENERGY RESEARCH LIBRARIES
3 4456 0514659 8

ORNL-TM-3603

cy. 88

TESTING FOR THE MUTAGENICITY OF IRRADIATED STRAWBERRIES FED TO RATS IN A HOST-MEDIATED ASSAY WITH NEUROSPORA AS INDICATOR ORGANISM

H. V. Malling, F. J. de Serres, T. Mitchell and P. Nees

OAK RIDGE NATIONAL LABORATORY
CENTRAL RESEARCH LIBRARY
DOCUMENT COLLECTION
LIBRARY LOAN COPY
DO NOT TRANSFER TO ANOTHER PERSON
If you wish someone else to see this document, send in name with document and the library will arrange a loan.
UCN-7969
(3 3-67)

NOTICE This document contains information of a preliminary nature and was prepared primarily for internal use at the Oak Ridge National Laboratory. It is subject to revision or correction and therefore does not represent a final report.

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

ORNL-TM-3603

Contract No. W-7405-eng-26

TESTING FOR THE MUTAGENICITY OF IRRADIATED STRAWBERRIES FED TO RATS IN A HOST-MEDIATED ASSAY WITH NEUROSPORA AS INDICATOR ORGANISM

H. V. Malling, F. J. de Serres, and T. Mitchell

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

and

P. Nees

Wisconsin Alumni Research Foundation, Madison, Wisconsin 53701

OCTOBER 1971

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37830
operated by
UNION CARBIDE CORPORATION
for the
U.S. ATOMIC ENERGY COMMISSION

LOCKHEED MARTIN ENERGY RESEARCH LIBRARIES
3 4456 0514659 8

1. INTRODUCTION

Irradiation of fresh foods for human consumption is potentially useful for prolonging shelf life, for sterilization to reduce or eliminate the need for refrigeration (Conner and Wyman 1965), and for killing insect larvae. For these purposes, fruit is usually irradiated with about 100 — 300 krads, which is lower than the doses usually used for total sterilization (2 mrads). Before such fruit is used for human consumption, it is important that genetic tests be performed to evaluate its mutagenicity, because the treatment could produce mutagenic chemicals from materials in the food. Indeed, formation of mutagenic products by irradiation of organic substances has been reported (Chopra et al., 1963; Holsten et al., 1965; Shaw and Hayes, 1966).

In genetic testing, one can distinguish between two kinds of damage: the induction of chromosome aberrations and the induction of gene mutations. Chromosome damage is relatively easy to score in mammalian systems, e.g. human lymphocytes or any other mammalian tissue, both in vitro and in vivo; but it is difficult and time-consuming to score for gene mutations in any mammalian system (Russell, 1951, 1967). There are a number of microbial systems, however, in which it is possible to score for gene mutations quantitatively (de Serres and Osterbind, 1962; Ames, 1971). Unfortunately, when these microbial systems are used for in vitro tests, the metabolic products formed in mammals — which may be mutagenic — are not always present.

An ingenious approach to this problem has been taken by Drs. M. G. Gabridge and Marvin S. Legator (1969) of the Food and Drug Administration, using the "host-mediated" assay system. In this system an indicator organism is injected into the peritoneal cavity of a rodent, and then the animal is treated with the chemical to be checked for mutagenic activity. The indicator organism is then removed from the test animal and tested for mutations induced during its period of incubation in the mammalian host. Gabridge and Legator have used the induction of reverse mutations of histidine-requiring mutants of Salmonella typhimurium as an indicator system. Because this assay will pick up only certain classes of genetic alterations, it does not yield precise data on the frequency of induction of all types of mutations. In evaluating any mutagen for its relative hazard to man, it is important to know whether it is weak, moderate, or potent with regard to the induction of forward gene mutations.

A more direct assay for the induction of gene mutations is possible with the ad-3 test system in a two-component heterokaryon of Neurospora crassa (de Serres and Osterbind, 1962; Webber and de Serres, 1965). A mutation in either of two different genes (ad-3A, ad-3B) which code sequential steps in the adenine biosynthetic pathway results in a requirement for adenine and in the accumulation of a purple pigment in the vacuoles of the mycelium. In this system, gene mutations can result from point mutation as well as chromosome deletion. The use of Neurospora

as the indicator organism has another distinct advantage (Malling and Cosgrove, 1970); namely, it can be incubated up to 18 hours in the host animal, in contrast to the bacterial system, which is incubated for only 2—3 hours. In feeding experiments it is important to incubate the indicator organism for as long as possible, so that any labile mutagens (that may be present in the animal only immediately after feeding) can be detected, as well as any mutagens that may be produced by metabolism throughout the entire treatment period. It is also important to have positive controls in such an experiment; in the experiments with Neurospora we have used mutagenic treatment with methylmethanesulfonate (MMS) for this purpose. We have found a significant increase in the ad-3 forward-mutation frequency in Neurospora conidia injected into MMS-treated rats when the MMS was either injected into the tail vein or introduced into the stomach by force-feeding. The doses of MMS used were so low that in no case did the animals die during the 18-hour period of incubation with the indicator organism as a result of the MMS treatment.

2. MATERIALS AND METHODS

Neurospora strains.—All purple adenine (ad-3) mutants were isolated by the direct method (de Serres and Kølmark, 1958) in the genetically marked two-component heterokaryon (strain No. 12) of N. crassa (Webber and de Serres, 1965). The techniques involved in such forward-mutation experiments were recently summarized by de Serres and Malling (1971).

Preparation of cultures and conidial suspensions. — The procedure for preparing the conidial culture of the heterokaryon strain No. 12 and harvesting the conidia has been described in detail by Webber and de Serres (1965). In brief, the conidial cultures were grown from single-colony isolates of heterokaryotic colonies after the conidia were plated from a silica-gel stock culture onto minimal medium. A total of 125 conidial cultures were prepared in flasks. Just before harvesting, the conidial cultures were divided arbitrarily into five groups (I—V), with 25 flasks in each group. Conidia from these groups were used to prepare five separate suspensions, so that each experiment consisted of five replicates. This procedure gave greater statistical reliability and guarded against the possibility that a high spontaneous frequency of ad-3 mutations in one of the 125 cultures might make it necessary to discard the total experiment.

The conidia were harvested by suspension in 10-12 ml of ice-cold Hanks Balanced Salt Solution (HBSS). To remove lumps of conidia and mycelial fragments, the suspensions from the five groups were filtered through platinum filters. Then they were rinsed three times by centrifugation in HBSS, and their densities were determined by hemacytometer counts. To remove soluble proteins from the conidia the suspensions were stored 5 hours in the refrigerator and then rinsed three more times by centrifugation with HBSS. Each of the five suspensions was divided into two aliquots with 6×10^8 and 2×10^7 conidia per ml, respectively. These were placed in serum vials with rubber stoppers and stored at ice-water temperature in an insulated carrying box for transportation to the Wisconsin Alumni Research Foundation (WARF) in Madison, Wisconsin.

Packaging of conidial suspensions for transportation. — The transportation equipment consisted of an altered suitcase (case), fitted with a tap at the bottom, and a stainless steel box with a lid (box). The box was lined with cotton and precooled before the conidial suspensions in serum vials were placed in it. Then it was enclosed in a plastic bag and placed in the case. Crushed ice was packed around the plastic bag. During transport the ice supply was replenished as needed, and excess water was drained through the tap at the bottom of the case. Upon arrival at WARF the case was placed at 2-6°C.

Injection of rats. — Each rat was injected with 1 ml of suspension from an aliquot containing 6×10^8 conidia per ml. Before injection a few drops of 70% ethanol solution were placed at the site of injection (located on the abdomen close to the left hind leg). The needle was inserted through the skin and through the abdominal wall to approximately half its length, and it was ascertained that it could move easily while still at the injection site to insure that the conidia would be inoculated into the peritoneal cavity. After injection the animals were returned to their cages, where they were left for 18 hours before the conidia were removed. Five rats from each of seven feeding groups (Table 1) were injected with 1 ml of conidial suspension each. The rats were numbered according to the scheme presented in Table 1.

Recovery of conidia from rats. — When the time came to recover the conidia, the rats were transported from the WARF Institute to the WARF special projects laboratory. Each rat was suffocated with CO_2, the jugular vein was cut, and the

rat was thoroughly bled. It was then placed on a dissecting tray, and the entire peritoneal cavity was exposed. The Neurospora conidia are orange and so were easily recognized in the peritoneal cavity. The majority were located around the pancreas beside the stomach in tiny clusters or lumps, and some were found in the intestinal region. (It was necessary to move the intestines around to check for hidden clusters.) The clusters of conidia were removed with sterile tweezers and collected in a 20- X 150-mm test tube containing 4 ml of HBSS. All suspensions were then placed in an ice bath and maintained at that temperature. A glass tube with a teflon homogenizer adapted to a motor was used to homogenize the clusters. When a uniform suspension of conidia was achieved, an additional 6 ml of HBSS was added to the suspensions, and the density was determined with a hemacytometer. The conidial suspensions were then packed in the boxes and maintained at ice-water temperature, to be returned to Oak Ridge.

Upon arrival at ORNL the conidial suspensions were first treated for 1 hour at room temperature with a penicillin (P, 100 units per ml) and streptomycin (S, 0.1 mg per ml) solution to kill any bacterial contaminants. One-tenth milliliter of a 10X PS-stock solution was added for each milliliter of conidial suspension.

Inoculation of jugs with rat samples.—In the first experiment (Expt 1) each jug was inoculated with 1.5×10^7 conidia. This inoculum produces $\sim 10^6$ colonies per jug, assuming 40% survival. However, the survival was higher than expected (see Table 6). In Expt 2, 60% survival was anticipated, and each jug was inoculated with 1×10^7 conidia. In both experiments, two jugs were inoculated from each rat sample.

In vitro controls.—The serum vials with 2×10^7 conidia per ml were used as in vitro controls. One vial from each of the five conidial suspension groups was kept at 0-4°C. A second vial from each group was placed at 37°C at the time when the conidial suspensions were injected into the rats; at the time of recovery of the conidia from the rats, these vials were returned to ice-water temperatures. Both series of controls were treated with the PS solution, as were the rat samples. Four jugs were inoculated from each of the five conidial suspension groups of the in vitro 4°C control, and two jugs were inoculated from each group of the in vitro 37° C control, with a total of 6×10^6 conidia per jug.

Detection of ad-3 mutations.—The techniques for detecting ad-3 mutants and for making stock cultures have been summarized by de Serres and Malling (1971)

Host-mediated assay with MMS (positive control).—The design of the experiments for the test of the mutagenic activity of strawberry-containing food in the host-mediated assay was followed exactly in the experiments with MMS. Two different routes of administration of MMS to the rats were used: injection into the tail vein and force-feeding. For injection of MMS into the tail vein, the mutagens were dissolved in HBSS and injected 8 hours after the injection of the conidia into the peritoneal cavity. Ten hours later the conidia were recovered and left in HBSS for the same number of hours as the conidial samples from WARF, before inoculation into jugs for genetic analysis. In the force-feeding experiment MMS was dissolved in HBSS. Intubation needles were used to force-feed the animals twice with 1 ml of the MMS solution. The first feeding was done 8 hours and the second 13 hours after the injection of the conidia into the peritoneal cavity.

Rat strain.—In all experiments Sprague-Dawley rats were used. The rats in the strawberry experiments at WARF came from Charles River. Those used in the MMS experiments at ORNL were bred at the Biology Division's own animal facilities.

Preparation of the strawberries.—Irradiation, preparation, and freezing or freeze-drying of the fresh strawberries used in these experiments has been described in the report, "Chronic Toxicity Studies on Irradiated Strawberries," final report of AEC Contract No. AT(11-1)-1722(1971). It should be emphasized that this handling differs drastically from the way irradiated strawberries would be used for human consumption.

Feeding protocol for rats.—The amount of frozen or freeze-dried (dried) strawberries mixed into the rat food and the handling of the rats were described in a petition from the Atomic Energy Commission to the Food and Drug Administration. In brief, the strawberry diet contained either 35% dried strawberries based on dry weight or 25% frozen strawberries based on wet weight.

3. RESULTS

Positive controls in the host-mediated assay with MMS.—In screening programs dealing with testing of chemicals for adverse biological effects, it is important to know the sensitivity of the testing system. When the host-mediated assay is used for evaluation of mutagenesis of a chemical with unknown modes of action, the testing system must be checked for its sensitivity with a known mutagen.

MMS was chosen because its mutagenic activity has been studied in many systems, such as dominant lethals in mice and specific-locus mutations in mice, many fungi, bacteria, and phage systems, and it is known to react with DNA to a high degree.

In the first experiment in Table 2, MMS was tested for mutagenicity in Neurospora in 6-week-old rats; simultaneously, it was tested for mutagenicity in vitro in HBSS at 37°C. MMS appears to be, if anything, only slightly more mutagenic in the host-mediated assay than in vitro, but the difference is not significant. The 6-week-old rats were approximately the same age as the rats which were fed only 4 days with food containing strawberries.

MMS also induced mutations in Neurospora in 8- to 9-month-old rats (Table 3). These rats were approximately the same age as the rats which were fed for 9 months with the food containing strawberries.

The best positive control for feeding experiments is to administer the mutagen by force-feeding. In this case, too, we found that MMS was an effective mutagen, producing ad-3 mutations in Neurospora conidia incubated in the peritoneal cavity of rats during the feeding period (Table 4).

In none of these three experiments were there any deaths of the rats during the 18-hour incubation period of the conidia.

Host-mediated assay in rats fed with food containing irradiated strawberries.— Seven groups of rats were set up with the different diets given in Table 1. Rats will not eat food that contains too large an amount of frozen strawberries. In order to expose the rats to more strawberries, some of the irradiated fruit was dried and

then mixed with the food. It is important to note that in both cases fresh strawberries were irradiated and then frozen or freeze-dried.

Two parallel lines of the seven rat groups were set up for feeding; one line was fed for 4 days and the other for 9 months before Neurospora conidia were injected. Since rats usually eat during the night hours, the conidia were injected at 10 P.M. and left in the peritoneal cavity for 18 hours. This procedure insures the exposure of the Neurospora conidia to any short-lived mutagenic metabolic breakdown products that might be formed immediately following feeding and digestion of irradiated strawberries. Conidia incubated in the peritoneal cavity of rats fed for 4 days on a diet containing frozen or freeze-dried irradiated strawberries did not show any increase in the frequency of recovered ad-3 mutations over the frequency of recovered ad-3 mutations among conidia incubated in rats on normal food (Table 6). A similar result was obtained with rats fed with frozen or freeze-dried irradiated strawberries for 9 months before the injection of the conidia (Table 7).

In Tables 8 and 9 the frequencies of ad-3 mutation obtained with a 4-day feeding period and a 9-month feeding period are given. In Table 10 the distribution of the number of ad-3 mutants obtained with each suspension is shown. These data have been used in the statistical analysis below.

The analysis of variance is shown in Table 11. The result of the F-test for differences among diets gave the same mutation rate. Our estimate of this overall mutation rate is 0.616 mutants per 10^6 survivors, with a standard error of 0.090.

(This standard error does not include variation from experiment to experiment, nor from one suspension to another. Thus, our 95% confidence interval [0.436, 0.796] for the true mutation rate pertains to these two particular experiments and to the particular suspensions used.)

Figure 1 shows 95% confidence intervals for differences of interest. These differences are given in terms of number of mutants per 10^6 survivors. Note that treatment groups have been combined in many cases. For example, the difference "strawberries-normal" represents the average mutation rate of all six groups associated with a strawberry diet minus the mutation rate associated with the normal diet. Similarly, the difference "irradiated strawberries (300 krads)-irradiated strawberries (150 krads)" represents the average mutation rate of both groups associated with a diet of strawberries irradiated at 300 krads minus the average mutation rate of both groups associated with a diet of strawberries irradiated at 150 krads. All other differences shown in Figure 1 may be interpreted in the same way. If a 95% confidence interval falls to the right of 0, we may conclude that, at this level of confidence, the corresponding difference is positive. If the 95% confidence interval falls to the left of 0, we may conclude that the corresponding difference is negative, again with 95% confidence. In Figure 1, however, all confidence intervals cover the value 0, so we cannot declare any of these differences "significant," especially in light of the result of the F-test in Table 11. It is interesting, however, to note those intervals which nearly avoid covering 0. If greater precision is desired, these confidence intervals can be narrowed with further repetitions of the same experiment.

An examination of the "residuals" (the differences between the observed data and the "predicted values" obtained by the analysis) indicate two data points with unusually high mutation rates, which could not be explained in terms of the diet or the suspension. These two points are indicated by asterisks in Table 10. For our own interest, a second analysis was performed in which these two "outliers" were removed. Briefly, the results were:

1. The F-statistic for differences among treatments, although nearly twice as large as before, was still not significant, even at the 0.25 level.
2. The confidence intervals of Figure 1 were reduced to about 75% of their original width. However, two of the confidence intervals failed to cover 0, namely, the difference between the freeze-dried unirradiated strawberries (both frozen and freeze-dried) and normal food. Both these confidence intervals were found to be just slightly to the right of 0.

This second analysis on the frequency of mutants which could be made homokaryotic (in stock) is in complete agreement with the previous conclusion that feeding with irradiated strawberries did not increase the frequency of ad-3 mutations over feeding with nonirradiated strawberries. In general, there is insufficient evidence to declare to significant difference among diets. If such differences do exist, they most likely do not exceed one mutant per 10^6 survivors. (The spontaneous mutation rate, observed in the two in vitro controls, is estimated to be 0.49 mutants per 10^6 survivors.)

4. DISCUSSION

In most of the published literature reporting studies of adverse biological effects of irradiated food or organic material, the radiation used has been X-rays from therapeutic X-ray machines or, as in the present case, γ-rays from a ^{60}Co source. There is no possibility of introduction of radioactive material into the food with such sources. Therefore, adverse effects, when formed, were attributed to the formation of toxic radiolytic breakdown products from the irradiated material.

Many different types of radiolytic products are formed during irradiation of glucose and sucrose, two of the most common carbohydrates found in food, and only relatively few have been identified. The spectrum of breakdown products formed by irradiation is likely to vary with the type of food, irradiation source, dose, dose rate, and many other experimental conditions. Genetic tests for mutagenicity of irradiated organic material in both lower and higher organisms have given both negative and positive results (for review see Scarascia-Mugnozza et al., 1965). The discrepancies between results may be explained on the basis of the variations above, or of the testing systems for mutagenicity. There is little doubt that under certain experimental conditions compounds are formed which possess mutagenic activity. The most important question is whether man can detoxify these compounds.

The present experiments with the host-mediated assay showed no increase in the frequency of ad-3 mutations among heterokaryotic conidia incubated in the

peritoneal cavity of rats during feeding with food containing irradiated strawberries over nonirradiated controls. Thus this assay provides no evidence that irradiated strawberries are mutagenic.

It is important to remember that the strawberries used in the present experiments with rats were processed by a protocol which differs from that proposed for processing strawberries for human consumption. In the latter case fresh strawberries are to be irradiated and then consumed within a few days or weeks after irradiation.

Since only fresh strawberries which were first irradiated and then frozen or freeze-dried were used, we cannot exclude the possibility of the formation, during irradiation, of some toxic (mutagenic), labile compound(s) which was effectively removed during the freeze-drying operation or during the storage period while the strawberries were frozen.

Acknowledgements.—The authors wish to thank Dr. R. F. Kimball and Dr. E. H. Y. Chu for their critical reviews of this manuscript, Mrs. Diana Smith for her help in preparing the statistical analysis, and Mr. David Carroll, Mr. Earl Gourley, Mr. Ernie Smith, and Mr. John Wassom for their valuable technical assistance.

This research was sponsored by the Atomic Energy Commission under contract with the Union Carbide Corporation.

5. REFERENCES

1. Ames, Bruce N. The detection of chemical mutagens with enteric bacteria. In Environmental Mutagens: Prinicples and Methods for Their Detection. (A. Hollaender, Ed.), Plenum Press, New York, 1971.

2. Chopra, V. L., A. T. Natarajan, and M. S. Swaminathan. Cytological effects observed in plant materials grown on irradiated fruit juices. Radiat. Bot. 3 (1963) 1-6.

3. Connor, J. T., and T. G. Wyman. Current Status and Commercial Prospects for Radiation Preservation of Food. U. S. Department of Commerce, U.S. Atomic Energy Commission/AT (49-11)-2524, 1965.

4. de Serres, F. J., and R. S. Osterbind. Estimation of the relative frequencies of X-ray-induced viable and recessive lethal mutations in the ad-3 region of Neurospora crassa. Genetics 47 (1962) 793-796.

5. de Serres, F. J., and H. G. Kølmark. A direct method for determination of forward-mutation rates in Neurospora crassa. Nature 182 (1958) 1249-1250.

6. de Serres, F. J., and H. V. Malling. Measurement of recessive lethal damage over the entire genome and at two specific loci in the ad-3 region of a two-component heterokaryon of Neurospora crassa. In Chemical Mutagens: Principles and Methods for Their Detection. (A. Hollaender, Ed.), Plenum Press, New York, 1971.

7. Gabridge, M. G., and M. S. Legator. A host-mediated assay for the detection of mutagenic compounds. Proc. Soc. Exp. Biol. Med. 130 (1969) 831.

8. Holsten, R. D., M. Sugii and F. C. Steward. Direct and indirect effect of radiation on plant cells: their relation to growth and growth induction. Nature 208 (1965) 850-856.

9. Malling, H. V., and G. E. Cosgrove. The internal level of mutagens in animals II. In Chemical Mutagenesis in Mammals and Man, pp. 271-278. (F. Vogel and G. Röhrborn, eds.), Springer-Verlag, Berlin, 1970.

10. Russell, W. L. X-ray induced mutations in mice. Cold Spring Harbor Symp. Quant. Biol. 16 (1951) 327-366.

11. Russell, W. L. Repair mechanisms in radiation mutation induction in the mouse. Brookhaven Symp. Biol. 20 (1967) 179-189.

12. Shaw, M., and E. Hayes. Effects of irradiated sucrose on the chromosomes of human lymphocytes in vitro. Nature 212 (1966) 1254-1256.

13. Scarascia-Mugnozza, G. T., and A. T. Natarajan and L. Ehrenberg. On genetic effects produced by irradiated food and food components. European Nuclear Energy Agency, SEN/IR(65)15, Paris, 1965. 17 pp.

14. Webber, B. B., and F. J. de Serres. Induction kinetics and genetic analysis of X-ray-induced mutations in the ad-3 region of Neurospora crassa. Proc. Nat. Acad. Sci. U.S.A. 53 (1965) 430-437.

Table 1. Injection scheme and identification numbers of rats on varying diets

Diet	Suspension injected				
	I	II	III	IV	V
Normal food	1	2	3	4	5
Normal food plus nonirradiated frozen strawberries	6	7	8	9	10
150-krad-irradiated frozen strawberries	11	12	13	14	15
300-krad-irradiated frozen strawberries	16	17	18	19	20
nonirradiated dried strawberries	21	22	23	24	25
150-krad-irradiated dried strawberries	26	27	28	29	30
300-krad-irradiated dried strawberries	31	32	33	34	35

Table 2. Frequency of ad-3 mutations among heterokaryotic conidia of N. crassa incubated in HBSS at 37° C (controls) and in the peritoneal cavity of 6-week-old rats during treatment with 100 mg/kg MMS injected into the tail vein

Incubation	MMS (mg/kg)*	Survival (%)	ad-3 mutants (no.)	ad-3 mutations (per 10^6 survivors)	95% Confidence limit	
					Lower limit	Upper limit
In HBSS (in vitro)	0	100	2	0.4	—	—
	100	77.0	77	24.1	18.8	29.6
In rats (in vivo)	0	65.6	3	0.8	—	—
	100	56.0	170	35.6	30.1	41.2

*Expressed either on the basis of the amount of HBSS or the weight of the rats.

Table 3. Frequency of ad-3 mutations among heterokaryotic conidia of N. crassa incubated in the peritoneal cavity of 6-month-old rats during their treatment with 150 mg/kg MMS

Rats	Survival (%)	ad-3 mutations (no.)	ad-3 mutations (per 10^6 survivors)	95% Confidence limit	
				Lower limit	Upper limit
Control	100	3	0.3	0.07	0.70
Treated	96	337	37.8	33.7	41.8

Table 4. Frequency of ad-3 mutations among heterokaryotic conidia of N. crassa incubated in the peritoneal cavity of 10- to 14-week-old rats during force-feeding of the rats with various doses of MMS

Total dose of MMS (mg/kg)	Survival (%)	ad-3 mutations (no.)	ad-3 mutations (per 10^6 survivors)	95% Confidence limit	
				Lower limit	Upper limit
0	100	4	0.57	0.19	1.36
50	113	2	0.51	—	—
100	74.6	21	2.26	1.38	3.42
150	86.5	49	4.88	3.59	6.32
200	82.2	95	10.94	8.73	13.23

Table 5. Number of ad-3 mutations in the in vitro controls in each of the conidial suspensions used in the two strawberry-feeding experiments

Expt.	Temp. (° C)	No. of ad-3 mutations in each suspension					No. of background colonies from each suspension ($\times 10^6$)				
		I	II	III	IV	V	I	II	III	IV	V
1	4	0	2	4	2	4	3.85	4.06	3.38	2.68	3.90
	37	0	1	1	2	1	1.22	1.37	1.19	1.33	1.41
2	4	1	2	0	2	1	2.02	2.78	2.60	2.10	3.05
	37	0	0	0	1	0	0.85	1.35	1.15	1.26	1.27

Table 6. Frequency of ad-3 mutations among heterokaryotic conidia of N. crassa incubated in the peritoneal cavity of rats on a 4-day diet containing nonirradiated and irradiated strawberries

Incubation	Survival (%)	ad-3 mutations (no.)	ad-3 mutations (per 10^6 survivors)	95% Confidence limit	
				Lower limit	Upper limit
Control (4° C HBSS)	100.0	12	0.671	0.373	1.137
Control (37° C HBSS)	69.3	5	0.767	0.302	1.714
In rats on normal food	51.9	8	0.484	0.199	0.903
In rats on normal food plus					
nonirradiated frozen strawberries	53.0	6	0.476	0.207	1.016
nonirradiated freeze-dried strawberries	53.1	8	0.640	0.263	1.194
150-krad-irradiated frozen strawberries	68.0	2	0.208	0.369	0.695
300-krad-irradiated frozen strawberries	77.0	11	0.607	0.293	1.051
150-krad-irradiated freeze-dried strawberries	63.7	10	0.666	0.354	1.175
300-krad-irradiated freeze-dried strawberries	60.7	14	1.089	0.630	1.785

Table 7. Frequency of ad-3 mutations among heterokaryotic conidia of N. crassa incubated in the peritoneal cavity of rats on a 9-month diet containing nonirradiated and irradiated strawberries

Incubation	Survival (%)	ad-3 mutations (no.)	ad-3 mutations (per 10^6 survivors)	95% Confidence limit	
				Lower limit	Upper limit
Control (4° C HBSS)	100	6	0.478	0.208	1.021
Control (37° C HBSS)	93.9	1	0.170	0.008	0.903
In rats on normal food	95.1	3	0.301	0.082	0.814
In rats on normal food plus nonirradiated frozen strawberries	92.8	8	0.823	0.338	1.535
nonirradiated freeze-dried strawberries	71.4	7	1.171	0.549	2.304
150-krad-irradiated frozen strawberries	83.6	6	1.714	0.746	3.662
300-krad-irradiated frozen strawberries	89.0	7	0.752	0.353	1.479
150-krad-irradiated freeze-dried strawberries	81.5	9	1.319	0.653	2.458
300-krad-irradiated freeze-dried strawberries	82.3	10	1.161	0.618	2.048

Table 8. Total number of background colonies and number of mutants in stock in aliquots from samples of conidia incubated in rats on a 4-day diet containing nonirradiated and irradiated strawberries

Incubation	Background colonies ($\times 10^6$)	ad-3 mutants in stock (no.)	ad-3 mutations in stock (per 10^6 survivors)
Control (4° C HBSS)	17.882	11	0.62
Control (37° C HBSS)	6.521	3	0.46
In rats on normal food	12.219	5	0.41
In rats on normal food plus			
nonirradiated frozen strawberries	9.971	6	0.60
nonirradiated freeze-dried strawberries	12.502	7	0.56
150-krad-irradiated frozen strawberries	9.613	2	0.21
300-krad-irradiated frozen strawberries	18.122	8	0.44
150-krad-irradiated freeze-dried strawberries	15.006	8	0.53
300-krad-irradiated freeze-dried strawberries	12.852	8	0.62

Table 9. Total number of background colonies and number of mutants in stock in aliquots from samples of conidia incubated in rats on a 9-month diet containing nonirradiated and irradiated strawberries

Incubation	Background colonies ($X 10^6$)	ad-3 mutants in stock (no.)	ad-3 mutations in stock (per 10^6 survivors)
Control (4° C HBSS)	12.55	6	0.48
Control (37° C HBSS)	5.891	1	0.17
In rats on normal food	9.947	3	0.30
In rats on normal food plus nonirradiated frozen strawberries	9.716	6	0.62
nonirradiated freeze-dried strawberries	5.973	5	0.84
150-krad-irradiated frozen strawberries	3.499	5	1.43
300-krad-irradiated frozen strawberries	9.306	4	0.43
150-krad-irradiated freeze-dried strawberries	6.820	5	0.73
300-krad-irradiated freeze-dried strawberries	8.610	8	0.93

Table 10. ad-3 Mutants in stock (per 10^6 survivors)

Strawberries in diet	Suspension				
	I	II	III	IV	V
a. 4-Day diet					
None (normal food)	0	0.273	0.465	0	0.484
Frozen (0 krad)	X*	1.014	0	0.379	0.741
Frozen (150-krad)	X	X	0	0.294	0.390
Frozen (300-krad)	0	0.310	0.617	0.588	0.663
Freeze-dried (0 krad)	0.680	0	0	1.217	0.902
Freeze-dried (150-krad)	0.851	0.541	0	0	1.005
Freeze-dried (300-krad)	1.377	0	0	0.405	0.883
b. 9-Month diet					
None (normal food)	0	0.402	0	0	1.159
Frozen (0 krad)	1.483	0.441	0	1.330	0
Frozen (150-krad)	0.596	2.197†	X	X	X
Frozen (300-krad)	0	0.491	0	1.359	0.537
Freeze-dried (0 krad)	2.269	0	0	1.476	X
Freeze-dried (150-krad)	0	0.643	3.043†	0	X
Freeze-dried (300-krad)	0.498	0.664	0.716	1.048	1.676

* X = missing observation.

† May be "outlier."

Table 11. Analysis of variance for data in Table 10

Source	Sum of squares	Degrees of freedom	Mean square
Mean	21.028	1	
Between experiments (short- and long-term feeding)	1.341	1	
Between suspensions within experiments	1.534	8	
Between diets	1.871	6	0.312
Residual	20.932	46	0.455
Total	46.706	62	

Test for treatment differences: $F_{6,46} = 0.312/0.455 = 0.686$. Since $F_{6,50} = 0.903$ for $P > 0.50$; then the F value for $F_{6,46}$ corresponds to a $P > 0.50$.

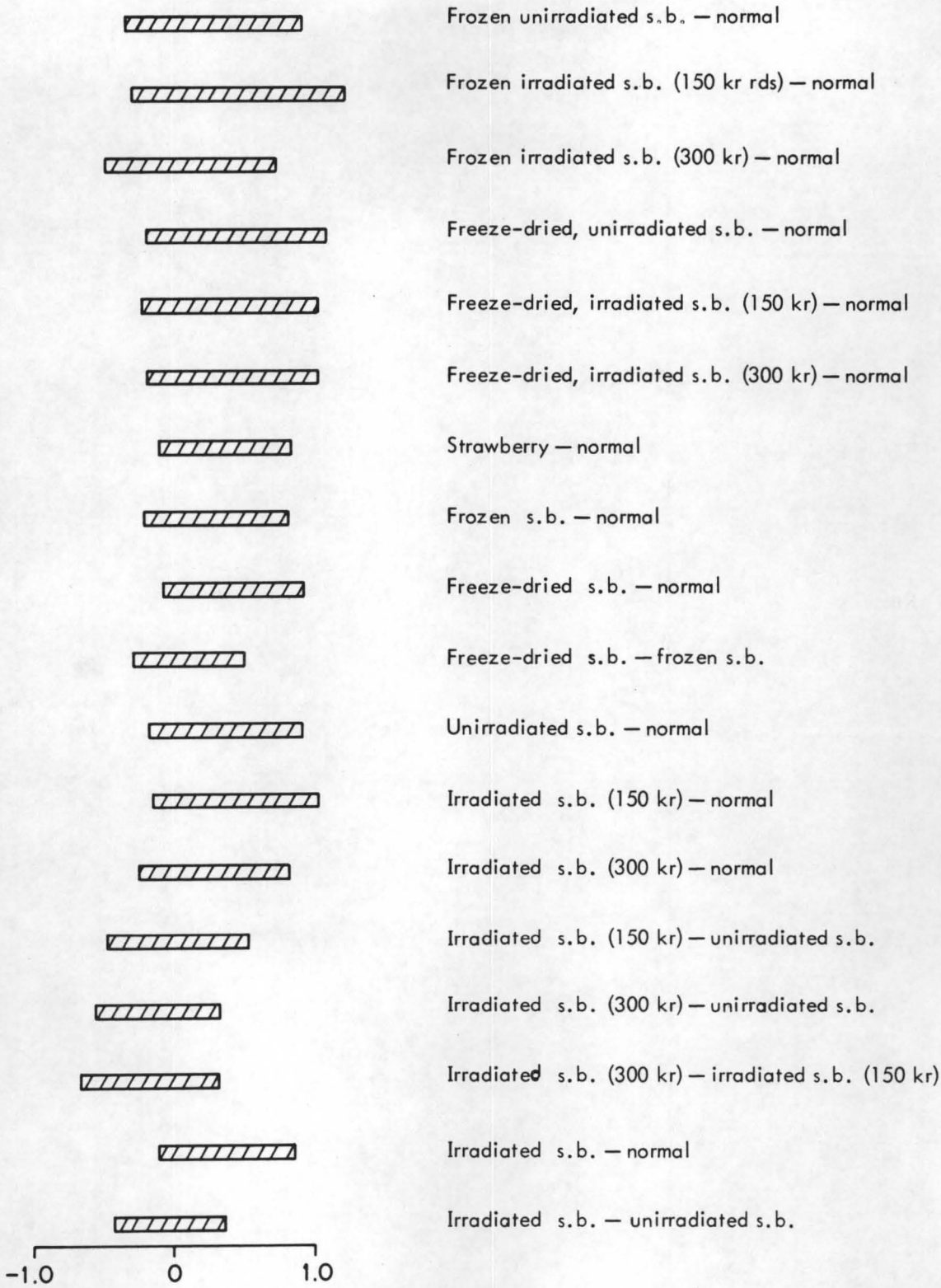

Fig. 1. 95% Confidence intervals for differences of interest (mutants per 10^6 survivors).

INTERNAL DISTRIBUTION

1. H. I. Adler
2. W. E. Barnett
3. J. G. Brewen
4. S. F. Carson
5. E. H. Y. Chu
6. F. L. Culler
7. F. J. de Serres
8. W. M. Generoso
9. A. Hollaender
10-19. J. L. Liverman
20-69. H. V. Malling
70-74. T. Mitchell
75. K. Z. Morgan
76. W. L. Russell
77-86. Biology Division Editorial Office
87. Biology Library
88-89. Central Research Library
90. ORNL – Y-12 Technical Library Document Reference Section
91-95. Laboratory Records Department
96. Laboratory Records, ORNL RC
97. ORNL Patent Office
98. Environmental Mutagen Information Center

EXTERNAL DISTRIBUTION

99-103. Dr. N. Barr, Division of Research, U. S. Atomic Energy Commission, Washington, D.C.
104. Dr. C. W. Edington, Chief, Biology Branch, Division of Biology and Medicine, U. S. Atomic Energy Commission, Washington, D.C.
105. Dr. Charles R. Fisher, Life Science Division, IAEA, Kaerntnerring 11, P. O. Box 590, A-1011 Vienna, Austria.
106. Dr. R. F. Kimball, Biology Branch, Division of Biology and Medicine, U. S. Atomic Energy Commission, Washington, D.C.
107-111. Dr. P. O. Nees, Wisconsin Alumna Research Foundation, P. O. Box 2037, Madison, Wisconsin 53701.
112. Dr. J. R. Totter, Director, Division of Biology and Medicine, U. S. Atomic Energy Commission, Washington, D. C.
113-122. Dr. Murray Schulman, Biology Branch, U. S. Atomic Energy Commission, Washington, D.C. 20545.
123. Dr. Arnold H. Sparrow, Biology Department, Brookhaven National Laboratory, Upton, Long Island, New York.
124-125. Division of Technical Information Extension.
126. Laboratory and University Division, AEC, ORO.